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1 **Short title:** Role of plasma membrane aquaporins in maize

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6 **Modification of the expression of the aquaporin ZmPIP2;5 affects water**
7 **relations and plant growth**

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32 **One-sentence summary:** Reverse genetic approaches demonstrate that the maize plasma
33 membrane PIP2;5 aquaporin plays a role in controlling root radial water movement, leaf
34 hydraulic conductivity, and plant growth.

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37 **Author contributions:** L.D., T.M., H.N., B.P., and F.C. designed the experiments. L.D., T.M.,
38 H.N., A.M., B.P., and S.A. performed the experiments. M.V.L. supervised the maize
39 transformation. L.D., T.M., V.C., H.N., A.M., B.P., S.A., F.T., X.D., M.V.L., and F.C. analyzed
40 the data. L.D. and F.C. wrote the manuscript. All the authors contributed to the discussion and
41 revision of the manuscript.

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53 **Abstract**

54

55 In maize (*Zea mays*), the plasma membrane intrinsic protein PIP2;5 is the most highly
56 expressed aquaporin in roots. Here, we investigated how deregulation of PIP2;5 expression
57 affects water relations and growth using maize overexpressing (OE; B104 inbred) or knockout
58 (KO; W22 inbred) lines. The hydraulic conductivity of the cortex cells of roots grown
59 hydroponically was higher and lower in PIP2;5 OE and *pip2;5* KO lines, respectively,
60 compared with their corresponding wild-type (WT) plants. While whole root conductivity
61 decreased in the KO lines compared to the WT, no difference was observed in OE plants. This
62 paradox was interpreted using the MECHA hydraulic model, which computes the radial flow
63 of water within root sections. The model hints that the plasma membrane permeability of the
64 cells is not radially uniform but PIP2;5 may be saturated in cell layers with apoplastic barriers,
65 i.e. the endodermis and exodermis, suggesting the presence of post-translational mechanisms
66 controlling the abundance of PIP in the plasma membrane in these cells. At the leaf level,
67 where the *PIP2;5* gene is lowly expressed in WT plants, the hydraulic conductance was
68 higher in the PIP2;5 OE lines compared with the WT plants, whereas no difference was
69 observed in the *pip2;5* KO lines. The temporal trend of leaf elongation rate used as a proxy of
70 that of xylem water potential was faster in PIP2;5 OE plants upon mild stress, but not in
71 well-watered condition, demonstrating that PIP2;5 may play a beneficial role for plant growth
72 under specific conditions.

73

74

75 Introduction

76
77 Aquaporins, belonging to the plasma membrane intrinsic protein (PIP) subfamily, are
78 major actors controlling membrane water permeability. The physiological functions of PIPs
79 are straightforward at the cell level (Hachez et al., 2006; Hachez et al., 2008; Hachez et al.,
80 2012; Heinen et al., 2014), but are considerably more complex at the organ and whole-plant
81 levels. For example, overexpression or silencing of PIP aquaporins has contrasting effects on
82 root hydraulics (Siefritz et al., 2002; Hachez et al., 2006; Postaire et al., 2010; Sade et al.,
83 2010), root development (Péret et al., 2012), leaf hydraulic conductance (K_{leaf}) (Prado and
84 Maurel, 2013; Sade et al., 2014), stomatal movement (Grondin et al., 2015; Wang et al., 2016;
85 Rodrigues et al., 2017), and transpiration (T_r) (Maurel et al., 2016). This is largely because the
86 transcellular path, in which water crosses the cell membranes mainly through aquaporins
87 (Steudle and Peterson, 1998; Steudle, 2000) occurs simultaneously together with other two
88 pathways, namely the apoplastic path, in which water flow goes through the cell wall, and the
89 symplastic path, in which water moves through the plasmodesmata. The overall root hydraulic
90 conductivity (L_{pr}) is the integration of conductivity from the three pathways; their
91 contribution to the L_{pr} varies according to root anatomy development and environmental
92 factors (drought, high salinity, nutrient availability, and anoxia, etc.). To better understand the
93 complexity of root radial hydraulic conductivity and integrate the multiple variables,
94 mathematical models have been developed (Steudle and Peterson, 1998; Zwieniecki et al.,
95 2002; Foster and Miklavcic, 2017; Couvreur et al., 2018). Among them, the 'MECHA' model
96 (Couvreur et al., 2018) predicts the root radial hydraulic conductivity, based on the detailed
97 radial anatomy of the root and the distribution of the cell wall hydraulic conductivity, the cell
98 plasma membrane permeability, the hydraulic conductance, the frequency of plasmodesmata,
99 and the membrane reflection coefficients. MECHA is therefore appropriate to address
100 subcellular hydraulics and its impact on the radial transport of water.

101 Here, we analyzed the effects of the overexpression or silencing of the maize (*Zea mays*)
102 PIP2;5 at both cellular and whole-plant levels. The *PIP2;5* gene encodes an active water
103 channel (Fetter et al., 2004), is the most expressed *PIP* gene in the primary root (Hachez et al.,
104 2006), and shows a polarized localization at the plasma membrane side facing the external
105 medium, supporting a function in root water uptake. Another clue for the involvement of
106 PIP2;5 in radial water movement is its high expression in cell types with Casparian strips
107 (exodermis and endodermis), places where water has to enter the symplast to continue its flow
108 to the xylem vessels (Hachez et al., 2012). In roots, the expression of *PIP2;5* mRNA and its
109 protein abundance are modulated by diurnal and circadian rhythm, osmotic stress, and growth
110 conditions (aeroponic and hydroponic) (Hachez et al., 2012; Caldeira et al., 2014). In addition,
111 PIP2;5 proteins are more or less abundant in maize lines overexpressing or silenced for an
112 ABA biosynthesis gene, respectively (Parent et al., 2009). The *PIP2;5* gene is weakly
113 expressed in leaves, with a maximum of expression at the end of the elongation zone and the
114 zone where the leaf emerges from the sheath and where lignification of the metaxylem is
115 observed (Hachez et al., 2008). Altogether, these data suggest that PIP2;5 plays important
116 roles in regulating water relations in maize, but genetic approaches to further understand its
117 physiological function are missing.

118 We first characterized PIP2;5 overexpressing (OE) and *pip2;5* knockout (KO) lines at the

119 molecular and cellular levels and addressed the question of the effects of the manipulation of
120 PIP2;5 expression at the plant level by collectively examining the hydraulic conductance in
121 roots and leaves and the time course of leaf elongation rate, considered here as a way to
122 indirectly assess the changes in xylem water potential, following fluctuations of the
123 evaporative demand (Caldeira et al., 2014; Caldeira et al., 2014). While the cell hydraulic
124 conductivity was affected by the deregulation of PIP2;5 expression, the contrasting results at
125 the organ levels suggest that upscaling requires a modeling approach to decipher the dataset
126 presented here.

127

128 Results

129 Generation, Isolation, and Molecular Characterization of Maize Lines Deregulated in 130 PIP2;5 Expression

131 To determine the role of PIP2;5 aquaporin in maize, we first prepared a genetic construct
132 aiming at constitutively overexpressing the *PIP2;5* gene under the control of the *p35S*
133 promoter (Fig. 1A), and performed an *Agrobacterium*-mediated transformation of the inbred
134 B104. Two independent *PIP2;5* overexpressing lines (PIP2;5 OE-4 and OE-13) with high
135 PIP2;5 protein content from Western blotting analysis (see below) were selected for further
136 molecular characterization. In addition, we obtained from the “Maize Genetic COOP Center”
137 (<http://maizecoop.cropsci.uiuc.edu/>) a putative *pip2;5* knockout (*pip2;5* KO) W22 inbred line
138 (UFMu00767) containing a *Mu* transposon in the *PIP2;5* gene (Fig. 1B). The presence and
139 the site of insertion of the *Mu* transposon in *PIP2;5* gene were determined by
140 PCR-amplification of genomic DNA using *PIP2;5* and *Mu* specific primers; this showed that
141 the *Mu* transposon was inserted in the second intron of the *PIP2;5* gene (Fig. 1B).

142 To confirm the overexpression and downregulation of *PIP2;5* in the maize lines,
143 microsomal fractions were prepared from roots and leaves, and PIP2;5 was immunodetected
144 using specific antibodies (Hachez et al., 2006). In roots, where the endogenous *PIP2;5* gene is
145 the highest expressed *PIP* gene, a 17% and 141% increase in PIP2;5 protein abundance in the
146 PIP2;5 OE-4 and OE-13 lines, respectively, were observed when compared with the
147 non-transgenic segregating siblings (B104, named afterwards WT-B104) (Fig. 1C). In leaves,
148 where the endogenous *PIP2;5* is lowly expressed, more than 10-fold higher PIP2;5 protein
149 abundance was detected in the PIP2;5 OE-4 and OE-13 lines compared with the WT-B104
150 plants (Fig. 1D). On the other hand, the PIP2;5 protein level was ninefold lower in the roots of
151 *pip2;5* KO line than in roots of non-transgenic segregating siblings (W22, named afterwards
152 WT-W22) (Fig. 1C). No significant difference in PIP2;5 signal intensity was found between
153 *pip2;5* KO and WT-W22 leaves (Fig. 1D), but the PIP2;5 signals in leaves were hardly
154 detectable and only observed after a very long exposure.

155 We also analyzed the *PIP2;5* mRNA levels in roots and leaves by RT-qPCR. In the OE
156 plants, while no difference in *PIP2;5* endogenous mRNA levels were observed, a high *PIP2;5*
157 transgene mRNA signal was detected using a *PIP2;5*-specific forward primer and a construct
158 linker-specific reverse primer (Supplemental Fig. S1, A-D). No amplification was detected
159 with this pair of primers in the WT-B104 plants. In *pip2;5* KO plants, we observed 2175-fold
160 and fivefold lower mRNA level in roots (Supplemental Fig. S1E) and leaves (Supplemental
161 Fig. S1F), respectively, than in the WT-W22 plants. To investigate the reason why a very faint
162 protein signal was still observed in the leaf extract by immunodetection, we performed

163 RT-qPCR with primers flanking the Mu insertion site and detected a weak signal
164 (Supplemental Fig. S2), suggesting that the *pip2;5* KO plants are not a complete
165 loss-of-function line.

166 To investigate whether the expression of other PIPs was affected by the deregulation of
167 *PIP2;5* gene in both roots and leaves, RT-qPCR and Western blotting were performed. No
168 significant difference in the mRNA levels of most *PIPs* was observed between *PIP2;5* OE
169 lines or *pip2;5* KO and their corresponding WT lines in both roots and leaves (Supplemental
170 Fig. S1). Similarly, at the protein level, no significant difference in PIP1;2 and PIP2;1/2;2 was
171 observed between the OE or KO lines and their respective WT plants (Supplemental Fig. S3).

172 Because we used two different maize genetic backgrounds in this work, we checked that
173 the expression pattern of *PIP2;5* was similar in the W22 and B73 lines (Hachez et al., 2006,
174 2008 and 2012). Similar to the previous results obtained in the B73, an intense signal of
175 *PIP2;5* was immunodetected in the exo- and endodermis cells of W22 root, where the lignin
176 and suberin are deposited (Supplemental Fig. S4, A and B). *PIP2;5* was also the most highly
177 expressed *PIP* gene in W22 roots and was lowly expressed in leaves (Supplemental Fig. S4C).

178

179 **Altered Hydraulic Conductance in the *PIP2;5* Deregulated Plants**

180 We first investigated the effect of *PIP2;5* deregulation on the hydraulic conductivity of the
181 root cortex cell (L_{pc}), measured with a cell pressure probe. The half-time of water exchange
182 ($T_{1/2}$) across the membrane of root cortex cells was 1.9 to 1.8 times shorter (faster water flow)
183 in the two *PIP2;5* OE lines than in WT-B104, whereas $T_{1/2}$ was 2.8 times longer (slower water
184 flow) in the *pip2;5* KO line with respect to the WT-W22 (Table 1). As a result, the L_{pc} was 69%
185 and 67% higher in *PIP2;5* OE-4 and OE-13 lines, respectively (Fig. 2, A and B). In contrast,
186 the L_{pc} decreased by 63% in the *pip2;5* KO line compared with the WT-W22 (Fig. 2C). The
187 turgor pressure and the cell elastic modulus (ϵ) were not affected by the deregulation of
188 *PIP2;5* expression (Table 1), with the exception of a higher $\epsilon_{corrected}$ in *PIP2;5* OE-13 lines than
189 in WT-B104 plants. Besides, a bigger cell volume was also measured in *PIP2;5* OE-13 lines
190 than in WT-B104 plants, suggesting that *PIP2;5* overexpression in this line has affected the
191 root cell expansion. Consistently, the membrane water permeability of leaf mesophyll
192 protoplasts (P_{os}) was significantly higher in both *PIP2;5* OE lines than in WT-B104 plants (Fig.
193 2, D and E). In comparison with WT-B104, an 85% and 60% increase in P_{os} was observed in
194 *PIP2;5* OE-4 and *PIP2;5* OE-13 lines, respectively. On the other hand, no difference in P_{os}
195 was observed between the WT-W22 and *pip2;5* KO lines (Fig. 2F), due to the fact that *PIP2;5*
196 is barely expressed in WT leaves. It is worth mentioning that the L_{pc} and P_{os} mean values were
197 higher in WT-W22 than in WT-B104, suggesting that both inbred lines have different intrinsic
198 membrane permeabilities.

199 Consistent with the results at the cell level, the leaf hydraulic conductance (K_{leaf})
200 measured with a hydraulic conductance flow meter (HCFM), increased by 58 % and 171 % in
201 the *PIP2;5* OE-4 and OE-13 lines, respectively, when compared with the K_{leaf} of WT-B104
202 plants (Fig. 3, D and E), whereas no significant difference in K_{leaf} was found between *pip2;5*
203 KO plants and WT-W22 plants (Fig. 3F). However, at the root level, the increase in L_{pc} did
204 not result in a significant difference in the whole root conductance (L_{pr}) between the
205 WT-B104 and the *PIP2;5* OE lines (Fig. 3, A and B). Conversely, L_{pr} was significantly lower
206 in the *pip2;5* KO line than in the WT-W22 (Fig. 3C).

207 The lack of correlation between cortex L_{pc} and L_{pr} in PIP2;5 OE plants is not a
208 straightforward result to decipher. There are two reasons for this: (1) There are multiple
209 hydraulic media in series across the root radius (including cell walls, membranes, and
210 plasmodesmata, whose hydraulic conductivity may vary in each cell layer). L_{pr} is mostly
211 sensitive to the hydraulic conductivity of media that limit water flow the most (e.g. at
212 gatekeeper cell layers, i.e. endodermis and exodermis, where water flow through cell walls is
213 limited by apoplastic barriers). (2) Root hydraulic media are arranged both in series and
214 parallel, so that water pathways may bypass some of the most limiting media (e.g. at
215 gatekeeper cells, water may bypass cell walls by flowing through membranes, and a fraction
216 of water flow also bypasses gatekeeper cell membranes by using a symplastic path). Hence,
217 the quantitative modeling tool MECHA (Couvreur et al., 2018) with subcellular resolution of
218 water flow was needed to statistically validate the significance of hypotheses possibly
219 explaining the lack of correlation between cortex L_{pc} and L_{pr} between WT and PIP2;5 OE
220 plants. Hypothesis A considered that plasma membrane L_{pc} is uniform across cell layers (Fig.
221 4A), while hypothesis B considered that PIP2;5 is already “saturated” in WT gatekeeper cells
222 (their L_{pc} equals that measured in the cortex of PIP2;5 OE lines) and “unsaturated” in other
223 cell layers (their L_{pc} equals that measured in the WT cortex) (Fig. 4E). Three values of cell
224 wall hydraulic conductivity (k_{w1-3}), spanning a range from the literature, were considered for
225 each hypothesis. Statistical analysis of results from this combined modeling and experimental
226 approach suggested that, given the observed L_{pc} in WT-B104 and PIP2;5 OE plants, radially
227 uniform patterns of cell membrane permeability may not account for the observed contrast
228 between L_{pr} in WT-B104 and PIP2;5 OE-4 line, regardless of the cell wall hydraulic
229 conductivity k_w (Fig. 4C, $p < 0.01$). The simulations reproduced the observed contrasts
230 between PIP2;5 OE and KO lines (Fig. 4, F-H) at the conditions that k_w was higher than
231 $6.9 \times 10^{-10} \text{ m}^2 \cdot \text{s}^{-1} \cdot \text{MPa}^{-1}$ (k_{w2}), and that the contribution of PIP2;5 to L_{pc} was saturated in the
232 endodermis and exodermis of WT lines (Fig. 4E, $p < 0.05$).

233

234 **Altered Plant Growth in PIP2;5 Deregulated Plants Under Water Deficit Conditions**

235 To further investigate the effect of PIP2;5 deregulation on water relations and plant growth,
236 we examined the time course of leaf elongation rate (LER) over changes in environmental
237 conditions. During the day, while the evaporative demand increases, hydraulic resistances to
238 water transfer can rapidly decrease the leaf water potential (Ψ_{leaf}) and the leaf growth, but it is
239 only observable under suboptimal water conditions (Bouchabké et al., 2006). We therefore
240 measured the leaf water potential and expansion of PIP2;5 OE-4 plants and their WT-B104
241 under well-watered conditions (Fig. 5C, in order to observe putative intrinsic differences of
242 leaf expansion rate) and under moderate water deficit (Fig. 5D, in order to observe the effects
243 of hydraulics).

244 Under moderate water deficit ($\Psi_{soil} = -0.23$ to -0.26 MPa), we measured a higher leaf
245 water potential in the PIP2;5 OE-4 line than in WT-B104 plants, while the difference was not
246 observed under well-watered conditions (Fig. 5B). A significantly faster recovery of LER was
247 observed after the early-morning drop in PIP2;5 OE-4 compared with WT-B104 plants
248 resulting in large differences of LER during the day (Fig. 5D, inset). Overall, the mean LER
249 of PIP2;5 was higher in OE-4 than in WT-B104 over one day (Supplemental Fig. S5, 0.75
250 $\text{mm} \cdot \text{h}^{-1}$ vs $0.40 \text{ mm} \cdot \text{h}^{-1}$). During the night, the average LER of PIP2;5 OE-4 was $1.42 \text{ mm} \cdot \text{h}^{-1}$

251 and it was also significantly higher ($p < 0.001$) than the average LER of WT-B104 (1.05
252 $\text{mm}\cdot\text{h}^{-1}$). As expected, these differences were not observed in well-watered conditions (Fig.
253 5C), indicating that no pleiotropic effects had affected the intrinsic potential leaf expansion
254 rate of OE plants.

255 A faster recovery of LER was correlated with an increase in PIP aquaporin expression,
256 Ψ_{leaf} and L_{pr} (Parent et al., 2009; Caldeira et al., 2014). While no change in L_{pr} was recorded
257 in PIP2;5 OE plants compared with the WT-B104 plants in well-watered condition, we
258 compared the L_{pr} in response to short term osmotic stress (10% w/v polyethylene glycol (PEG)
259 6000; $\Psi = -0.15$ MPa) and observed a higher L_{pr} , but not significantly different, in PIP2;5
260 OE-4 than in WT-B104 plants (Supplemental Fig. S6). These results suggest that the higher
261 root hydraulic conductivity for PIP2;5 OE-4 observed under low osmotic potential translated
262 into differences of leaf water potential and leaf expansion under moderate water deficit, which
263 was not the case under well-watered conditions due to the very low difference of root
264 hydraulic conductivity.

265

266 Discussion

267

268 A better understanding of the functional role of PIP aquaporins in plant water relations is
269 essential to develop crop lines that use water more efficiently and are more tolerant to water
270 deficit. To this aim, we investigated the direct contribution of PIP2;5, the most expressed PIP
271 aquaporin in maize roots, using reverse genetic approaches. Overexpression of *PIP2;5* under
272 the control of the 35S promoter led to a less than two-fold increase in the PIP2;5 protein level
273 in roots, where PIP2;5 is already highly expressed, and an approximately 10-fold increase in
274 leaves, where PIP2;5 is lowly expressed. This difference in PIP2;5 protein abundance
275 according to the organ suggests the existence of post-transcriptional or post-translational
276 regulation mechanisms that prevent an excess of PIP2;5 proteins according to the cell type.
277 Different cellular mechanisms modifying PIP abundance in the plasma membrane have been
278 reported (Chaumont and Tyerman, 2014; Maurel et al., 2015), and involve internalization of
279 PIPs from the plasma membrane for their degradation and/or recycling. Negative feedback of
280 *PIP* gene transcription could not be excluded either, even though it was not detected by
281 RT-qPCR. The decrease in *PIP2;5* gene expression was obtained using the UniformMu
282 transposon mutated line UFMu00767 (McCarty et al., 2005; McCarty et al., 2013; Hunter et
283 al., 2014). In this line, the transposon was inserted in the second intron leading to an
284 important decrease in mRNA and protein levels. However, a very weak signal for PIP2;5
285 protein was still observed in roots, indicating that the line was not a complete knockout as
286 demonstrated by RT-qPCR using primers flanking the Mu insertion site allowing the
287 detection of a weak signal in *pip2;5* KO samples (Supplemental Fig. S2).

288 The cortex cell L_{pc} in intact roots was dependent on PIP2;5 expression levels, with higher
289 and lower values in OE and KO lines, respectively. As no change in the abundance of other
290 PIP aquaporins was detected, this is a direct evidence that PIP2;5 facilitated the water
291 diffusion through the cell membranes. We previously showed a correlation between PIP
292 expression and the L_{pc} in roots. The higher abundance of PIPs, including PIP2;5, during the
293 day than during the night, or after a short (8 h) PEG treatment is correlated with variation in
294 the L_{pc} values (Hachez et al., 2012). Overexpression or knocking out *PIP* genes in other plant

295 species also results in higher or lower L_{pc} . For instance, in *Arabidopsis thaliana pip2;2* KO
296 lines, L_{pc} of the root cortex cells is reduced by ~25% when compared with WT plants (Javot et
297 al., 2003). In contrast, L_{pc} is higher in PIP2;5 overexpressing Arabidopsis lines than in WT
298 under low temperature (Lee et al., 2012).

299 While the L_{pr} of *pip2;5* KO plants was significantly decreased, no increase in L_{pr} was
300 recorded in PIP2;5 OE plants under well-watered conditions, indicating that the abundance of
301 PIP2;5 in the root cell membranes is not always correlated to the L_{pr} . The uncorrelated data
302 between PIP abundance, L_{pc} , and L_{pr} was previously observed in maize plants subjected to a
303 short PEG stress, which induces a higher PIP expression and a higher L_{pc} , but no change in L_{pr}
304 (Hachez et al., 2012). The composite water transport model (Steudle and Peterson, 1998)
305 assumes that L_{pr} is controlled by the hydraulic conductivity of apoplastic and cell-to-cell
306 pathways in parallel. We proposed that radial variations along each pathway critically affect
307 water transport due to the presence of gatekeeper cells at the beginning (epidermis and
308 exodermis) or the end (endodermis) of the radial path of water (Hachez et al., 2012;
309 Chaumont and Tyerman, 2014). While the current cell pressure probe technology did not
310 allow to directly verify this hypothesis, the use of the quantitative modeling framework
311 MECHA (Couvreur et al., 2018) gave us the opportunity to get a better understanding of the
312 mechanisms involved. The statistical comparison of our measured and simulated L_{pr} results
313 largely supports the hypothesis that plasma membrane permeability is not radially uniform in
314 WT (hypothesis A rejected, $p < 0.01$, Fig. 4C) but may be saturated in the endodermis and
315 exodermis (hypothesis B, Fig. 4E), thus following the observed radial aquaporin expression
316 patterns observed by Hachez et al. (2006). This is an important result, as none of the simplest
317 to most sophisticated models of radial water flow account for such heterogeneity, which does
318 affect L_{pr} in WT. On the other hand, knocking-out *PIP2;5* gene expression led to a decrease in
319 L_{pr} suggesting that PIP2;5 is an essential actor facilitating radial water flow and controlling
320 whole root conductivity, as also observed in our simulations. Quantification of the level of
321 active PIP2;5 proteins in the endodermis would be very informative. Indeed, *PIP2;5* gene
322 overexpression in this specific cell type would not lead to an increase in PIP2;5 protein due to
323 the above-mentioned post-translational mechanisms and that a maximum of active PIP2;5
324 (and other PIPs) was already reached. These predictions could be refined by generating maize
325 lines with deregulated PIP expression exclusively in the endodermis or the exodermis.

326 Maize lines expressing *PIP2;5* cDNA under the control of *p35S* promoter showed a much
327 higher increase in PIP2;5 protein abundance in leaves than in roots. Considering that general
328 PIP expression in maize leaves is lower than in roots and that *PIP2;5* is very lowly expressed
329 in leaves (Hachez et al., 2006; Hachez et al., 2008; Heinen et al., 2009), we hypothesize that
330 overexpressing PIP2;5 in this organ was not limited by similar post-transcriptional or
331 post-translational mechanisms observed in roots. Similar to what was observed in root cells,
332 an increase in P_{os} was measured for the leaf mesophyll cells overexpressing PIP2;5. We
333 previously demonstrated that transient expression of PIP2;5 in mesophyll cells increases the
334 water membrane permeability (Besserer et al., 2012). But in contrast to the effect of
335 PIP2;5OE on the whole root conductance, overexpression of PIP2;5 led to a higher K_{leaf}
336 compared to the K_{leaf} of WT-B104 plants (Fig. 3, D and E). A similar result was found, for
337 instance, in tomato plants overexpressing NtAQP1 (Sade et al., 2010). Inversely,
338 downregulation of *PIP1* gene expression in Arabidopsis plants results in a decrease in the

339 mesophyll cell P_{os} and the K_{leaf} (Sade et al., 2014). K_{leaf} and the leaf radial water flow are
340 thought to be mainly controlled by the vascular bundle sheath cells surrounding the veins
341 (Sack and Holbrook, 2006; Shatil-Cohen et al., 2011; Buckley, 2015). These cells can have
342 suberized walls, and are associated with a very low apoplastic flow. Arabidopsis plants, in
343 which *PIP1* genes are specifically silenced in these cells, also exhibit decreased mesophyll
344 and bundle sheath P_{os} and decreased K_{leaf} (Sade et al., 2014). In our work, PIP2;5 OE plants
345 exhibited higher P_{os} of the mesophyll cells and possibly also of the bundle sheath cells,
346 resulting in an enhancement of conductance in bundle sheath, mesophyll cells, and K_{leaf} . The
347 observation that no difference in P_{os} and K_{leaf} was observed in *pip2;5* KO plants was expected
348 since PIP2;5 is lowly expressed in leaf tissues (Hachez et al., 2008). Extending the MECHA
349 model to leaf tissues and cells will be very useful to address these questions related to leaf
350 water relations.

351 LER is controlled by plant hydraulic properties, including leaf and xylem water potential,
352 transpiration and root hydraulic conductivity (Caldeira et al., 2014), and involved hormonal
353 regulation (Nelissen et al., 2012; Avramova et al., 2015; Nelissen et al., 2018). Actually, we
354 considered that short-term variation of LER is a proxy of the change of xylem water potential
355 (Parent et al; 2009; Caldeira et al., 2014a). In a mild water deficit, a faster recovery of LER
356 after the early morning drop was recorded in PIP2;5 OE-4 compared with WT-B104 plants,
357 and a higher LER during the day and night was recorded. This LER response in PIP2;5 OE
358 plants can be correlated with a higher K_{leaf} and is consistent with the effect of K_{leaf} on LER
359 recovery observed in transgenic plants that differ in ABA concentration in the xylem sap,
360 showing differences in L_{pr} (Parent et al., 2009). Interestingly, while WT-B104 and PIP2;5 OE
361 plants had a similar L_{pr} in well-watered conditions, an osmotic stress led to an increased L_{pr} in
362 the OE plants, suggesting that changes in hydraulic conductance (L_{pr} and K_{leaf}) by PIP2;5
363 deregulation at the cell level translated into an overall change in whole plant hydraulic fluxes
364 (and leaf water potential) that affects the LER in mild-stress conditions. The observation that
365 the LER was not affected in well-watered plants implies that molecular mechanisms
366 controlling the overall root hydraulic conductance occur in some cell types resulting in a
367 non-uniform distribution of water permeability (see above) or at specific positions along the
368 root axis (e.g. the connection between root and leaf xylems).

369 In conclusion, deregulation of PIP2;5 aquaporin expression in maize plants highlighted
370 their role in controlling the root and leaf cell water permeability. However, understanding the
371 results obtained at the root level has required a hydraulic model developed at the cell and
372 tissue scales. MECHA allowed us to discard the hypothesis of radially uniform L_{pc} , and
373 suggests that, in well-watered conditions, the gatekeeper cells of WT plants have a saturated
374 membrane permeability. Transposing the model to the aerial part will definitely offer
375 possibilities to better understand the hydraulic properties of tissues and cells in diverse
376 conditions and investigate the role and regulation of aquaporins in specific hydraulic
377 processes. Indeed, we showed that the increase in the hydraulic conductance by
378 overexpressing aquaporin positively affects the LER under moderate stress conditions.

379

380

381

382 **Materials and Methods**

383

384 **Plasmids and genetic construction to generate PIP2;5 overexpressing lines**

385 The cauliflower mosaic virus *p35S* promoter was PCR-amplified from the pMDC43 vector
386 (Curtis and Grossniklaus, 2003) and cloned into the Gateway entry vector pDONR P4-P1R
387 (Invitrogen, Carlsbad, US) following the manufacturer's instructions. The *YFP* cDNA
388 followed by the *tnos* terminator sequence was amplified together from the pH35GY vector
389 (Kubo et al., 2005) and cloned into the pDONR221 vector. Finally, a uracil-excision based
390 cloning cassette (USER) was added downstream of the *p35S* promoter sequence and
391 subsequently cloned into pDONR P2R-P3 (Nour-Eldin et al., 2006; Hebelstrup et al., 2010).
392 These three entry vectors were verified by sequencing and their inserts brought together by
393 LR recombination into the pBb7m34GW backbone vector suitable for maize (*Zea mays*)
394 transformation (Karimi et al., 2013) (<https://gateway.psb.ugent.be>). The latter contains the *bar*
395 selectable gene under the control of the *p35S* promoter that induces resistance to the herbicide
396 bialaphos and allows selecting transformed calli and shoots using phosphinotricin-containing
397 media. The cDNA encoding *ZmPIP2;5* was PCR-amplified using USER primers (forward
398 primer: 5' GGTCTTAAUATGGCCAAGGACATCGAGG 3'; reverse primer: 5'
399 GGCATTAAUCTAGCGGCTGAAGGAGGCA 3') and inserted into the destination vector to
400 obtain the final vector. This plasmid was used to transform the hypervirulent EHA101
401 *Agrobacterium tumefaciens* strain (Hood et al., 1986) by heat shock and subsequently used for
402 transformation of maize immature embryos from B104 inbred line according to the method
403 described by Coussens et al. (2012), in collaboration with the Maize Transformation platform
404 of the Center Plant Systems Biology (VIB-Ghent University, Belgium). Briefly, immature
405 embryos were isolated from the ears 12 to 14 days after fertilization and co-cultivated with
406 EHA101 *A. tumefaciens* strain carrying the plasmid of interest for three days in the dark at
407 21°C. After co-cultivation, immature embryos were cultivated for one week without selection,
408 followed by a four-month period of subculturing on selective media containing increasing
409 amounts of phosphinotricin (starting from 1.5 mg/l to 6 mg/l) to select transformed
410 embryogenic calli in dark conditions at 25°C. After selection, the embryogenic calli were
411 transferred to bigger containers and placed in a growth chamber (24°C, 55 μ E.m⁻².s⁻¹ light
412 intensity, 16h/8h day/night regime) for transgenic T0 shoot/plantlet formation *in vitro*. Eleven
413 rooted transgenic T0 plants selected from independently transformed immature embryos
414 tested positive for the phosphinotricin acetyltransferase (PAT) enzyme (encoded by the *bar*
415 selectable marker gene) using the TraitChek Crop and Grain Test Kit (Strategic Diagnostics,
416 Newark, DE, USA). The presence of the transgenic *ZmPIP2;5* cDNA insertion was also
417 confirmed by PCR amplification of genomic DNA using *p35S* forward primer (5'
418 CCACTATCCTTCGCAAGACCC 3') and *T35S* reverse primer (5'
419 GGTGATTTTTCGGACTCTAGCAT 3'). The transgenic T0 plants were transferred to soil
420 and kept one month in a growth chamber (16h/8h day/night regime at 24 °C, 55 μ mol.m⁻².s⁻¹
421 light intensity, and 55% relative humidity) in 1 L pots for acclimation, and then moved to a
422 greenhouse (26 °C/22 °C, day/night temperature, 300 μ mol.m⁻².s⁻¹ light intensity under
423 16h/8h day/night regime) in 10 L pots until flowering. A backcross with the B104 genotype
424 was performed by either pollinating ears of T0 plants with B104 pollen, or pollinating B104
425 ears with T0 plants pollen. The ears were then harvested 4 weeks after fertilization and dried
426 for several weeks at 25°C before sowing the segregating T1 generation. The T2 generation

11

427 was generated by backcross between B104 and the heterozygous T1 plant in the greenhouse.
428 T1 and T2 generation heterozygous plants segregating for the *ZmPIP2;5* transgene (PIP2;5
429 OE) and non-transgenic siblings (WT-B104) were used in this study. Finally, two independent
430 overexpressing lines (PIP2;5 OE-4 and PIP2;5 OE-13) with high *ZmPIP2;5* protein content in
431 Western blotting analysis were used for all further measurements.

432

433 *pip2;5* KO line

434 The *pip2;5* KO line (UFMu00767, generated from the W22 inbred line, was found in
435 MaizeGDB (<https://maizegdb.org/>) and obtained from Uniform Mu stocks in “Maize Genetic
436 COOP Center” (<http://maizecoop.cropsci.uiuc.edu/>). To confirm the Mu transposon insertion
437 in *PIP2;5* gene, gDNA was extracted from the second leaf of one-week old maize seedling,
438 with the extraction buffer TPS (100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 M KCl). PCR
439 was performed using a Mu-TIR specific forward primer (McCarty et al., 2013) (Mu-Terminal
440 Inverted Repeat, TIR6: 5` AGAGAAGCCAACGCCAWCGCCTCYATTTTCGTC 3`) and
441 *PIP2;5* specific reverse primer (5` CGTCTACACCGTCTTCTCCG 3`) to detect the Mu
442 insertion, and with *PIP2;5* specific primers (Forward: 5` AGGCAGACGATCCCAGCTT 3`,
443 Reverse: 5` CGTCTACACCGTCTTCTCCG 3`) to detect the *PIP2;5* gene. Heterozygous
444 plants were self-pollinated and a segregation ratio of 1:2:1 for WT-W22, heterozygous, and
445 homozygous seeds was obtained. The WT-W22 and homozygous plants were used for the
446 measurements.

447

448 **Growth conditions**

449 Hydroponic culturing was conducted to obtain plant material for the measurements of the cell
450 hydraulic conductivity, root hydraulic conductivity, and leaf hydraulic conductance. Maize
451 seeds were surface sterilized using 2% (w/v) NaClO solution for 5 min, rinsed with distilled
452 water, and placed between two wet tissue papers in a square Petri dish (Greiner Bio-One,
453 Vilvoorde, Belgium). The seeds were put in the dark at 30°C for 72 h. After germination, the
454 seedlings were transferred to a 2 L beaker with 1/2 strength nutrient solution (1.43 mM
455 Ca(NO₃)₂·4H₂O, 0.32 mM KH₂PO₄, 0.35 mM K₂SO₄, 1.65 mM MgSO₄·7H₂O, 9.1 μM
456 MnCl₂·4H₂O, 0.52 μM (NH₄)₆Mo₇O₂₄·4H₂O, 18.5 μM H₃BO₃, 0.15 μM ZnSO₄·7H₂O, 0.16
457 μM CuSO₄·5H₂O, and 35.8 μM Fe-EDTA. The nutrient solution pH was adjusted to 5.5 and
458 replaced every two days. The nutrient solution was aerated with the aid of aquarium diffusers.
459 After one week, the full-strength nutrient solution was used until the end of experiments. The
460 plants were grown in a growth chamber at a 16h/8 h light/dark cycle (25/18°C) and a daytime
461 light intensity of 200 μmol·m⁻²·s⁻¹ at the top of the leaf level.

462 Soil culturing was conducted in the growth chamber or in the greenhouse. Maize seeds
463 were surface sterilized using 2% (w/v) NaClO solution for 5 min, rinsed with distilled water,
464 and placed in Jiffypots® (6 cm diameter), filled with 80% potting soil (DCM, Grobendonk,
465 Belgium) and 20% vermiculite (Agra-vermiculite, Pull Rhenen, Netherland). After two weeks,
466 the seedlings were transferred to a 2 L pots (MCl 17, Pöppelmann, Geluwe, Belgium) filled
467 with the same substrate and vermiculite. After another 4 weeks, the plants were transferred to
468 a 10 L pot (MCl 29).

469

470 **Protein extraction and Western blot**

471 Microsomal membrane fractions were prepared as described by Hachez et al. (2006) with a
472 few modifications. Briefly, leaf (newly expanded leaf) or root (primary root) tissues from
473 one-week old maize seedling were flash-frozen in liquid nitrogen in aluminum foil and
474 grinded with mortar and pestle using 2 ml of extraction buffer (250 mM sorbitol, 50 mM
475 Tris-HCl pH 8, 2 mM EDTA,) containing freshly added 0.6% (w/v) polyvinyl pyrrolidone
476 K30, 1 mM phenylmethanesulfonate, 0.5 mM dithiothreitol, and supplemented with 2 $\mu\text{g}\cdot\text{ml}^{-1}$
477 of protease inhibitors (leupeptin, aprotinin, antipain, pepstatin, and chymostatin). Debris were
478 removed with a first centrifugation at 770g at 4°C for 10 min and the subsequent supernatant
479 was centrifuged at 10,000g at 4°C for 10 min. The supernatant was then centrifuged at
480 54,000g at 4°C for 30 min and the resulting pellet (microsomal fraction) was resuspended in
481 50 μl of suspension buffer (330 mM sucrose, 5 mM KH_2PO_4 , 3 mM KCl, pH 7.8) and
482 sonicated twice for 5 s. Total protein concentration was determined by the Bradford protein
483 assay (Bradford, 1976).

484 Twenty μg (for root) and 30 μg (for leaf) of total proteins were mixed with 6X Laemmli
485 buffer (240 mM Tris-HCl, pH 6.8, 6% w/v SDS, 30% w/v glycerol, 0.05% w/v bromophenol
486 blue) and freshly added 10% w/v dithiothreitol, in a total volume of 45 μl . They were
487 solubilized for 10 min at 60°C were loaded in 12% acrylamide gels (Eurogentec, Seraing,
488 Belgium) for separation by electrophoresis (120V, ~1h). After transfer to a polyvinylidene
489 fluoride membrane (Trans-Blot[®] Turbo[™] Mini PVDF Transfer Packs, Biorad, CA, USA), the
490 proteins were immunodetected using antisera raised against the amino-terminal peptides of
491 ZmPIP1;2, ZmPIP2;1/2;2, and ZmPIP2;5 (Hachez et al., 2006; Hachez et al., 2012). The
492 antibody raised against H^+ -ATPase (PMA) (Morsomme et al., 1996) was used as control to
493 normalize the protein level. The blotting signal was detected using an Amersham imager 600
494 (GE Healthcare, Chicago, USA). The signal was quantified with ImageJ software (National
495 Institutes of Health, <http://rsb.info.nih.gov/ij>) and normalized with the signal of PMA.

497 **RNA extraction and reverse transcription quantitative PCR (RT-qPCR)**

498 One-week old maize seedlings were used for RNA extraction and RT-qPCR. The whole newly
499 expanded leaf and primary root were harvested and immediately frozen in liquid nitrogen, and
500 the samples were stored at -80°C until RNA extraction. RNA extraction was performed with
501 the Spectrum[™] Plant Total RNA Kit (Sigma-Aldrich, Mo, US) according to the
502 manufacturer's instructions. DNase I (Sigma-Aldrich) digestion was performed directly on
503 the column during RNA extraction according to the manufacturer's recommendations. The
504 RNA concentrations and the quality of each sample were measured with a Nanodrop
505 ND-1000 (Isogen Life Science, Utrecht, Netherland), and 1.5 μg of total RNA was used for
506 reverse transcription and the cDNA synthesis was performed with the Moloney Murine
507 Leukemia Virus Reverse Transcriptase (M-MLV RT) kit (Promega, Leiden, Netherland)
508 according to the manufacturer's instructions. RT-qPCR was performed in 96-well plates using
509 a StepOnePlus[™] Real-Time PCR System (Life Technologies, Foster City, CA, USA) in a
510 volume of 20 μl containing 10 μl of RT-qPCR Mastermix Plus for SYBR Green I (Eurogentec,
511 Liège, Belgium), 1 μl of forward and reverse primers (Supplemental Table S1), 1 μl cDNA,
512 and 7 μl DEPC- H_2O . The PCR cycle program was 2 min at 50°C, 10 min at 95°C for DNA
513 polymerase activation, and 40 cycles of 15 s at 95°C and 60 s at 60°C. The $2^{-\Delta\text{Ct}}$ method was
514 used to analyze the relative expression of 6 *ZmPIP1s* (*ZmPIP1;1*, *ZmPIP1;2*, *ZmPIP1;3*,

515 *ZmPIP1;4*, *ZmPIP1;5*, and *ZmPIP1;6*) and 6 *ZmPIP2s* (*ZmPIP2;1*, *ZmPIP2;2*, *ZmPIP2;3*,
516 *ZmPIP2;4*, *ZmPIP2;5*, and *ZmPIP2;6*). Three reference genes, *ACT1*, *EF1- α* , and
517 *polyubiquitin* were used to normalize the expression.

518

519 **Cell pressure probe measurement**

520 One-week old maize seedlings were used for cell pressure probe measurements, according to
521 Hachez et al. (2012) and Volkov et al. (2006). Briefly, the plant and primary root was
522 maintained in a Petri dish containing the nutrient solution and the root region near to the last
523 lateral root (5-7 cm near the root tip) was measured. Cell turgor pressure (P), $T_{1/2}$ (half-time of
524 water across cell membrane), and pressure change value (ΔP) were recorded. These
525 parameters for three to five cells from each plant were measured and three to five plants were
526 analyzed. After the measurements, average values of cell volume and cell surface area were
527 calculated through microscopic analyses of 7-30 cells from the 3rd to 6th cell layer taken at 5-7
528 cm from the root tip. Cell osmotic pressure was checked with micro-osmometer (Advanced,
529 model 3300, Norwood, Massachusetts, USA). In this study, a cell osmotic pressure of 0.88
530 MPa was used for all calculations. Cell elastic modulus (ϵ) and cell hydraulic conductivity
531 (L_{pc}) were calculated according to Volkov et al. (2006).

532

533 **Root hydraulic conductivity (L_{pr}) and leaf hydraulic conductance (K_{leaf}) measurement**

534 Three-week old maize plants growing in phytotron were used for root hydraulic conductivity
535 (L_{pr}) measurements, according to Ding et al. (2015). In the phytotron, the light cycling was
536 from 06:00 to 22:00. In the morning between 09:00~11:00 (measurements beginning three
537 hours after light onset), the primary root was cut and connected to a hydraulic conductance
538 flow meter (HCFM, Decagon Devices, Pullman, WA, USA). The connection was performed as
539 quickly as possible (< 1 or 2 min) to minimize the effect of the root excision on the L_{pr} as
540 previously reported by Vandeleur et al. (2014). Transient method was used to recording the
541 value change of flow rate (F) and applied pressure (Pi), and the slope rate (K_r , $Kg.s^{-1}.MPa^{-1}$)
542 was analyzed between the correlation of F and Pi. For one root, three to five replications were
543 performed for the measurement. After the measurement, the primary root was scanned with
544 Epson perfection V33 scanner (EPSON, Japan) and the root surface area was analyzed with
545 ImageJ (National Institutes of Health, <http://rsb.info.nih.gov/ij>). Then the K_r was normalized
546 with the root surface area for the L_{pr} calculation. For PEG treated plants, 10% (w/v)
547 PEG-6000 was added to the nutrient solution in hydroponic culture, and L_{pr} was measured
548 after 2- and 4-h.

549 Leaf hydraulic conductance (K_{leaf}) was measured between 13:00~15:00 (measurements
550 beginning seven hours after light onset) with HCFM (Tyree et al., 2004; Ferrio et al., 2012)
551 using three-week old maize plants. The newly expanded leaf was cut with the leaf sheath, and
552 coated surrounding a plastic stick covered by UHU@patafix (UHU, Baden, Germany). Then a
553 tape of polytetrafluoroethylene film was wrapped around the leaf sheath. After, the leaf sheath
554 was excised under water and connected with the HCFM. Approximately 0.2 MPa pressure
555 was applied in the system and quasi-steady state method was used to record the flow rate and
556 conductance by every 8 s during 10-30min until the conductance was constant. During the
557 measurement, the leaf was immersed in water to stop transpiration. After the measurement,
558 the leaf was scanned with Epson perfection V33 scanner (EPSON, Japan) and leaf area was

559 analyzed with ImageJ software. The hydraulic conductance was normalized with the leaf area
560 to calculate the K_{leaf} .

561

562 **Protoplast swelling assay**

563 Leaf mesophyll protoplasts were isolated from newly expanded leaves of three-week old
564 maize plants, and swelling assay was performed according to Moshelion et al. (2004) and
565 Shatil-Cohen et al. (2014). The leaf abaxial side was scratched with a glass-paper, and then
566 the leaf was cut into small sections and transferred to the digestion buffer (the scratched side
567 contact with the buffer), including 0.6% (w/v) cellulose R10 (Duchefa Biochemine, Haarlem,
568 Netherland), 0.1% (w/v) pectolyase (Sigma-Aldrich, Mo, US), 0.3% (w/v) Macerozyme R10
569 (Duchefa Biochemine, Haarlem, NL), 5 mg.ml⁻¹ bovine serum albumin, and 5 mg.ml⁻¹
570 polyvinyl pyrrolidone K30. The analysis of the osmotic water permeability coefficient was
571 according to Shatil-Cohen et al. (2014).

572

573 **Leaf elongation rate measurement**

574 Leaf growth was measured in the high throughput phenotyping platform Phenodyn (Sadok et
575 al., 2007) of the LEPSE laboratory in Montpellier
576 (<https://www6.montpellier.inra.fr/lepse/M3P>). Plants were grown in one PVC column, filled
577 with clay balls, which allows to tare columns at 1.2 kg. Then, columns were filled with 4.4 kg
578 of a mix of loam (5-10%) and soil. Plants were daily watered with nutritive solution.
579 Sampling of the newest leaf was carried out at 4-leaf stage and a PCR was made in order to
580 characterize the transgenic PIP2;5 OE plants. After the characterization, three plants were
581 kept in the column, including either three WT-B104 or PIP2;5 OE plants or a mix of them
582 (either two WT-B104 or two PIP2;5 OE) for the LER measurement. Plants were grown under
583 a 14 h light / 10 h dark cycle at 19-26°C (day, mini-maxi)/19-21°C (night, mini-maxi) in
584 greenhouse. Well-watered conditions were kept until plants reached the four leaf
585 developmental stage. Then a progressive water deficit was applied. Each pot was placed on a
586 scale with automated irrigation to impose the targeted soil water potential. LER was measured
587 when the tip of the 6th leaf appeared above the whorl and lasted until the appearance of leaf 8.
588 LER was expressed in thermal time, via equivalent days at 20°C according to Parent et al.
589 (2010). Ψ_{leaf} was measured with a pressure chamber between 12:00-14:00 in the greenhouse
590 with non-expanding leaves.

591

592 **Inference on radial patterns of cell membrane permeability**

593 In order to test hypotheses on the radial pattern of plasma membrane permeability, we
594 simulated how measured cell-scale permeability (L_{pc}) translates into root hydraulic
595 conductivity (L_{pr}) for each pattern, and compared the distributions of simulated and measured
596 L_{pr} .

597 Assuming that the axial resistance to water flow is negligible, the simulation framework
598 MECHA (Couvreur et al., 2018) estimates L_{pr} from root transverse anatomy and subcellular
599 scale hydraulic properties. In order to plug the measured L_{pc} into the model, we partitioned it
600 into its two main components: the plasma membrane hydraulic conductivity (k_{AQP} , including
601 the contribution of aquaporins) and the conductance of plasmodesmata per unit membrane
602 surface (k_{PD}). The latter parameter was assumed to equal $2.4 \times 10^{-7} \text{ m.s}^{-1}.\text{MPa}^{-1}$ following

603 (Couvreur et al., 2018), based on plasmodesmata frequency data from Ma and Peterson (2001)
604 and the low range of plasmodesmata conductance estimated by Bret-Harte and Silk (1994).
605 This value was subtracted from the measured L_{pc} to obtain k_{AQP} . As the value of the cell wall
606 hydraulic conductivity parameter (k_w) is highly uncertain, a range of “low” ($k_{w1} = 6.9 \times 10^{-11}$
607 $m^2 \cdot s^{-1} \cdot MPa^{-1}$), “medium” ($k_{w2} = 6.9 \times 10^{-10} m^2 \cdot s^{-1} \cdot MPa^{-1}$), and “high” ($k_{w3} = 1.4 \times 10^{-8}$
608 $m^2 \cdot s^{-1} \cdot MPa^{-1}$) values were tested. Finally, the hydrophobic wall segments of Casparian strips
609 in the endodermis and exodermis were attributed to null hydraulic conductivity. As
610 experimental observations confirmed that root anatomy only varied slightly between the
611 tested lines, the same geometrical layout was used for all of them, except for the mutant
612 PIP2;5 OE-13 whose cell sizes were multiplied by 1.17, as observed experimentally. The
613 anatomy was representative of a maize primary root (0.9 mm diameter), five centimeters
614 proximal to the tip. For details, see Couvreur et al. (2018). In the future, it will be possible to
615 generate anatomies more representative of each mutant with the tool GRANAR (Heymans et
616 al., 2019).

617 In hypothesis A we assumed that plasma membrane permeability (k_{AQP}) is radially
618 uniform and equal to that measured in cortical cells, in WT, PIP2;5 OE and *pip2;5* KO lines,
619 respectively (Fig. 4A). In hypothesis B we assumed that while k_{AQP} uniformly saturates in the
620 PIP2;5 OE lines, it also saturates in the endodermis and exodermis of the WT (Fig. 4E).
621 Besides, in this hypothesis we also assumed that since PIP2;5 is not the only aquaporin highly
622 expressed in the endodermis and exodermis, these cell layers may retain k_{AQP} values as high
623 as half of the saturated values in the *pip2;5* KO line (Fig. 4E).

624 L_{pr} values were estimated with MECHA for each combination of measured L_{pc} (10 to 14
625 repetitions) by hypothesized radial pattern (2) by WT or PIP2;5 deregulated lines (6) by cell
626 wall hydraulic conductivity value (3). Relative L_{pr} were calculated by dividing the L_{pr} in WT
627 and associated deregulated line by the average L_{pr} of the WT line. A lognormal transformation
628 was applied to the relative L_{pr} in order to correct for the skewness of their distributions in the
629 following statistical analyses. Contrasts between measured and simulated relative L_{pr} in WT
630 and associated deregulated lines were then investigated with ANOVA2 functions in the
631 software SAS (SAS Institute, Inc., Cary, North Carolina). The contrast between measured L_{pr}
632 in WT and PIP2;5 deregulated lines was considered significantly different from the contrast
633 between simulated L_{pr} in WT and deregulated lines starting at a p -value of 0.05.

634

635 **Statistical analysis**

636 Student's t test was applied to determine the significance of differences of average values
637 between the PIP2;5 OE/KO lines and their respective WT plants. In Fig. 4 and 5B, one-way
638 ANOVA with Tukey post test was used to compare leaf water potential between WT-B104
639 and PIP2;5 OE-4 plants under control and water deficit conditions.

640

641 **Accession numbers**

642 All accession numbers of the genes are listed in Supplemental Table S2.

643

644

645 **Acknowledgements**

646 We thank Lucie Bugeia for her help in performing the LER measurement in the *Phenodyn*

647 platform.

648

649 Supplemental Data

650

651 **Supplemental Figure S1.** Levels of PIP transcripts in WT, PIP2;5 OE-4, PIP2;5 OE-13 and
652 *pip2;5* KO lines in roots and leaves.

653

654 **Supplemental Figure S2.** RT-PCR amplification of *PIP2;5*.

655

656 **Supplemental Figure S3.** Comparison of PIP1;2 and PIP2;1/2;2 protein levels in WT-B104
657 and PIP2;5 OE lines or WT-W22 and *pip2;5* KO line

658

659 **Supplemental Figure S4.** Localization and expression of PIP2;5 in W22 line.

660

661 **Supplemental Figure S5.** Mean leaf elongation rate (LER) in the night and day.

662

663 **Supplemental Figure S6.** Comparison of L_{pr} between WT-B104 and PIP2;5 OE-4 line under
664 control and PEG treatments.

665

666 **Supplemental Table S1.** Primers used for the RT-qPCR experiments.

667

668 **Supplemental Table S2.** Accession numbers of the genes.

669

670

671 **Table 1.** Cell pressure probe measurements of maize root cortex cells in WT, PIP2;5 OE lines,
672 and *pip2;5* KO. Root cortical cells from the 3rd to 6th cell layer were punctured in the morning.
673 The values are means \pm SE (n = 10~15 cells from three to five plants). Significant difference
674 (p < 0.05) among the treatments is indicated by different letters. $T_{1/2}$, the half time of water
675 exchange through the cell membrane. ϵ , the cell elastic modulus.

676

	Turgor pressure (MPa)	$T_{1/2}$ (s)	Cell volume (10^{-14} m^3)	Cell surface area (10^{-8} m^2)	$\epsilon_{\text{measured}}$ (MPa)	$\epsilon_{\text{corrected}}$ (MPa)
WT-B104	0.37 \pm 0.01a	1.70 \pm 0.14a	8.38 \pm 1.63a	1.40 \pm 0.17a	1.24 \pm 0.20a	1.80 \pm 0.30a
PIP2;5 OE-4	0.35 \pm 0.01a	0.89 \pm 0.07b	6.16 \pm 1.07a	1.17 \pm 0.12a	0.89 \pm 0.06a	1.74 \pm 0.09a
WT-B104	0.40 \pm 0.01a	1.75 \pm 0.15a	6.46 \pm 0.81b	1.11 \pm 0.09b	0.98 \pm 0.12a	1.43 \pm 0.16b
PIP2;5 OE-13	0.42 \pm 0.01a	0.97 \pm 0.08b	9.47 \pm 0.94a	1.41 \pm 0.10a	1.49 \pm 0.23a	2.74 \pm 0.35a
WT-W22	0.37 \pm 0.01a	0.90 \pm 0.08b	9.60 \pm 0.88a	1.35 \pm 0.09a	1.38 \pm 0.18a	2.74 \pm 0.38a
<i>pip2;5</i> KO	0.34 \pm 0.02a	2.56 \pm 0.11a	8.29 \pm 0.46a	1.21 \pm 0.05a	1.61 \pm 0.12a	2.14 \pm 0.19a

677

678

679 **Figure legends**

680

681 **Figure 1.** PIP2;5 protein levels in the maize lines deregulated in its expression. **A.** Schematic
682 representation of the T-DNA used to overexpress PIP2;5 in the B104 maize line. *p35S*, 35S
683 promoter; YFP, yellow fluorescent proteins; *mos*, terminator of the nopaline gene; *bar*, the
684 gene conferring resistance to bialaphos. **B.** Genomic structure of the *PIP2;5* gene with the
685 position of the Mu transposon insertion in the *pip2;5* KO line (W22 background). The data
686 source of gene position and insertion site is from “Maize B73 RefGen_v3” in MaizeGDB
687 (<https://maizegdb.org/>). The *PIP2;5* exons are in red. **C** and **D.** PIP2;5 protein level in root (**C**)
688 and leaf (**D**) in wild type (WT, indicated by WT-B104 and WT-W22), two PIP2;5
689 overexpressing lines (PIP2;5 OE-4 and OE-13), and *pip2;5* knockout line (*pip2;5* KO). The
690 plants were cultured under hydroponic conditions and the microsomal fractions were
691 extracted from primary roots and leaves of one-week old seedlings. Proteins (20 µg (C) or 30
692 µg (D)) were subjected to Western blotting using antibodies raised against PIP2;5 or PMA
693 (H⁺-ATPases). The PMA signal was used to control the gel loading and normalize the PIP2;5
694 signals (right panels). In the quantification panels, data are expressed as the mean ± SE
695 coming from two independent experiments and each experiment containing two to three
696 plants for each maize line. Significant differences among the treatments are indicated by *
697 (p<0.05), ** (p<0.01), and *** (p<0.001).

698

699 **Figure 2.** Membrane water permeability of root cortex cells and leaf mesophyll cells.
700 Hydraulic conductivity of root cortical cells (L_{pc} , A-C) and the osmotic water permeability
701 coefficient of leaf mesophyll cell protoplasts (P_{os} , D-F) in WT, PIP2;5 OE-4, PIP2;5 OE-13,
702 and *pip2;5* KO line. One- and three-week old plants grown under hydroponic conditions were
703 used for the measurements of L_{pc} and P_{os} , respectively. Individual data dots are shown and
704 data are also expressed as the mean ± SE of 10 to 15 cells from three to five plants for the L_{pc}
705 and more than 30 protoplasts coming from two plants for the P_{os} . Significant differences
706 among the treatments are indicated by * (p<0.05), ** (p<0.01), and *** (p<0.001). ns
707 indicates not significantly different (p>0.05).

708

709 **Figure 3.** Root hydraulic conductivity and leaf hydraulic conductance. Root hydraulic
710 conductivity (L_{pr} , A-C) and the leaf hydraulic conductance (K_{leaf} , D-F) in WT, PIP2;5 OE-4,
711 PIP2;5 OE-13, and *pip2;5* KO line. L_{pr} was measured with two-week old maize seedlings and
712 K_{leaf} of newly expanded leaves was measured with three-week old plants. Individual data dot
713 are shown and data are also expressed as the mean ± SE of roots and leaves from 4 to 6 plants.
714 Significant differences among the treatments are indicated by * (p<0.05) and ** (p<0.01). ns
715 indicates not significantly different (p>0.05).

716

717 **Figure 4.** Contrast analysis between experimental and simulated L_{pr} using the MECHA model.
718 Contrast analysis between measured (Exp) and simulated (k_{w1} , k_{w2} , and k_{w3}) L_{pr} in WT (blue)
719 and PIP2;5 deregulated lines (red: *pip2;5* KO; green: PIP2;5 OE) for hypothesized radial
720 patterns of plasma membrane permeability (k_{AQP}). * and ** for significantly different
721 contrasts between measurements and simulations (p < 0.05 and 0.001; e.g. contrast between
722 WT and OE larger in simulations than experiments in panel B). Circles/bars: 25th, 50th, 75th

723 percentiles; Whiskers: most extreme non-outlier data point.

724

725 **Figure 5.** Daily time courses of environmental conditions, leaf water potential, and leaf
726 elongation rate under well-watered and moderate water deficit treatments. In this experiment,
727 wild type (WT-B104) and PIP2;5 overexpression line (PIP2;5 OE-4) were compared. In A,
728 blue and red traces indicate vapor pressure deficit (VPD) and photosynthetic photon flux
729 density (PPFD), respectively. In B, white and grey filled bars show leaf water potential of
730 WT-B104 and PIP2;5 OE-4, respectively, under control and water deficit conditions. In C and
731 D, blue and red traces show the leaf elongation rate (LER) of WT-B104 and PIP2;5 OE-4,
732 respectively. Gray traces show the daily soil water potential (Ψ_{soil}). Gray backgrounds show
733 the sunset period. In dashed line square, the slope rate is analyzed, and the result is inserted.
734 LER is expressed per unit thermal time ($\text{mm}\cdot\text{h}^{-1}\cdot 20^{\circ}\text{C}$). Error bars indicate standard error ($n=$
735 $3\sim 9$ plants). In B, one-way ANOVA with Tukey post test is used to compare the significant
736 differences between WT-B104 and PIP2;5 OE-4 plants under control and water deficit
737 conditions. Significant differences are indicated by different letters ($p<0.05$). In the insert of D,
738 Student's t-test is used to compare the significant difference between WT-B104 and PIP2;5
739 OE-4 plants. * indicates the significant difference at level of $p<0.05$.

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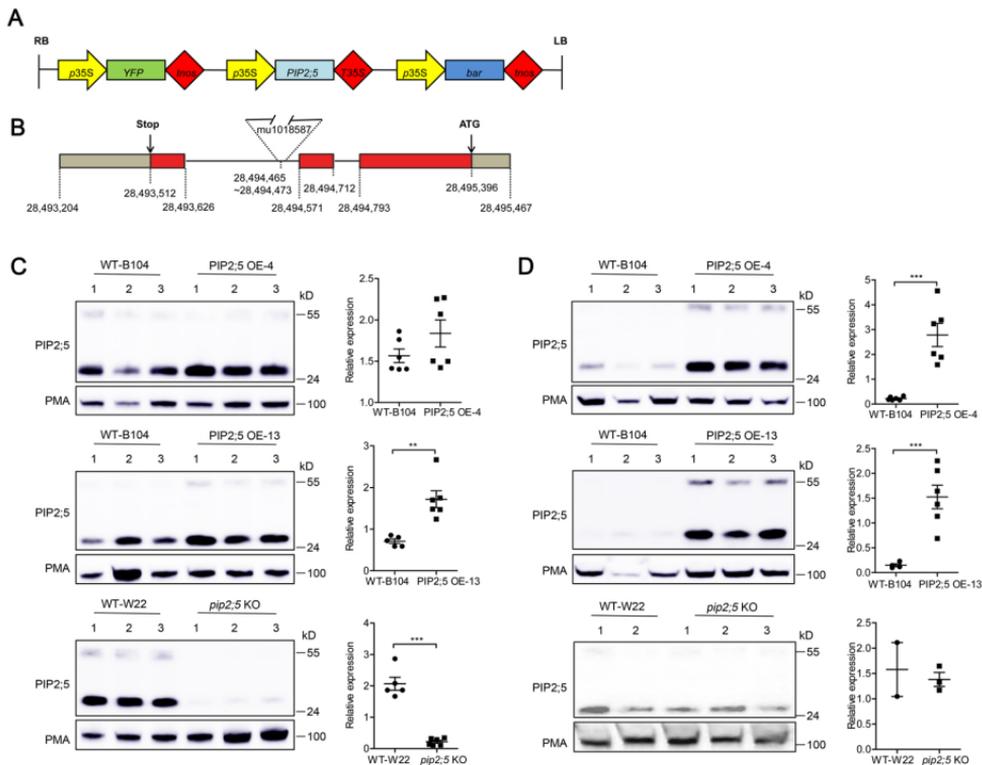


Figure 1. A. Schematic representation of the T-DNA used to overexpress PIP2.5 in the B104 maize line. p35S, 35S promoter; YFP, yellow fluorescent proteins; nos, terminator of the thopalapine gene; bar, the gene conferring resistance to bialaphos. B. Genomic structure of the PIP2.5 gene with the position of the Mu transposon insertion in the *pip2.5* KO line (W22 background). The data source of gene position and insertion site is from "Maize B73 RefGen_v3" in MaizeGDB (<https://maizegdb.org/>). The PIP2.5 exons are in red. C and D. PIP2.5 protein level in root (C) and leaf (D) in wild type (WT, indicated by WT-B104 and WT-W22), two PIP2.5 overexpressing lines (PIP2.5 OE-4 and OE-13) and *pip2.5* knockout line (*pip2.5* KO). The plants were cultured in hydroponic condition and the microsomal fractions were extracted from primary roots and leaves of one week-old seedlings. Proteins (20 μ g (C) or 30 μ g (D)) were subjected to Western blotting using antibodies raised against PIP2.5 or PMA (H-ATPases). The PMA signal was used as a loading control. The relative expression of PIP2.5 in the different panels, data are expressed as the mean \pm SE coming from two independent experiments. The treatments are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

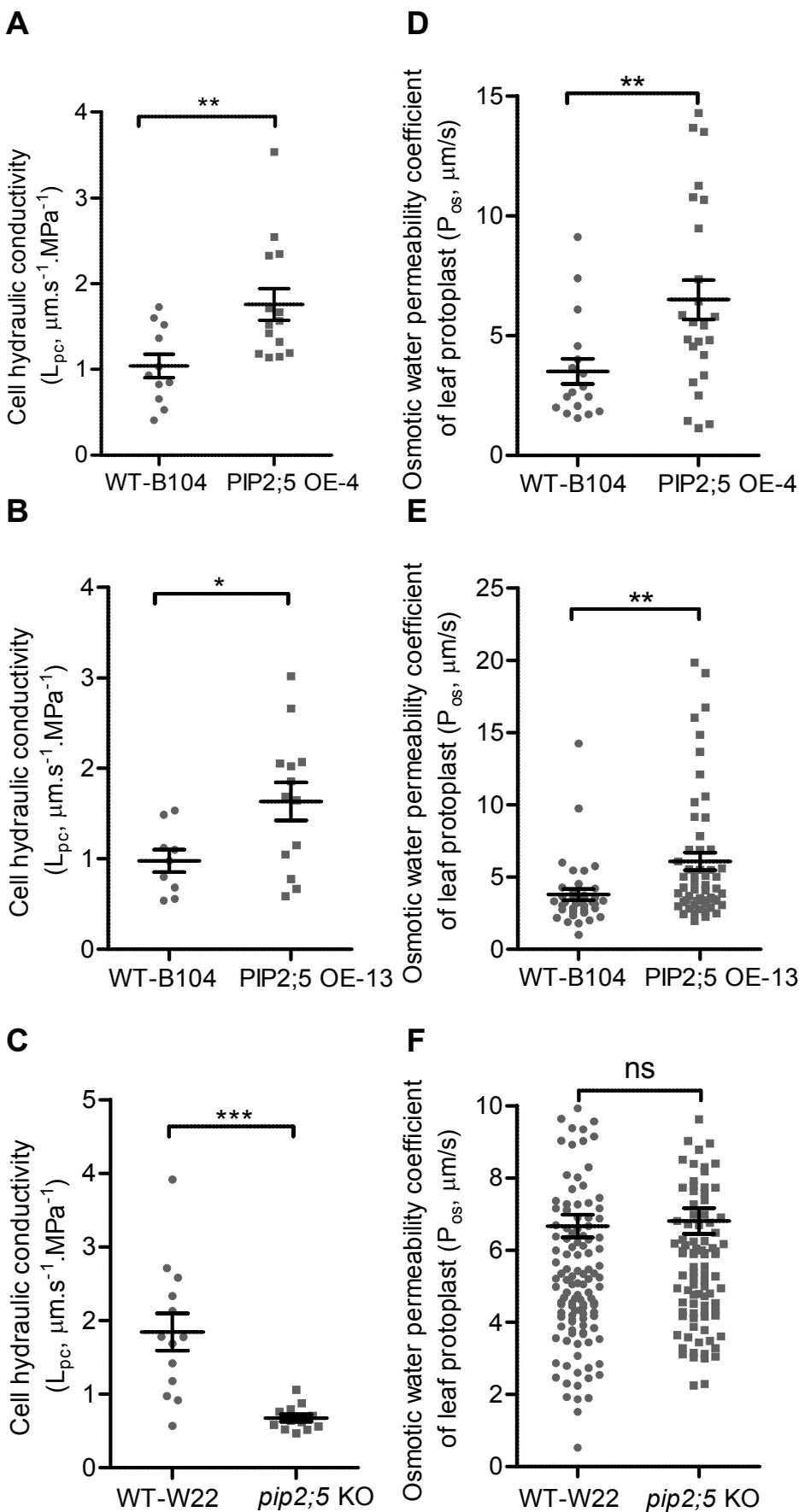


Figure 2. Hydraulic conductivity of root cortical cells (L_{pc} , A-C) and the osmotic water permeability coefficient of leaf mesophyll cells protoplasts (P_{os} , D-F) in WT, PIP2;5 OE-4, PIP2;5 OE-13 and *pip2;5* KO line. One-and-three-week old plants growing in hydroponic condition were used for the measurements of L_{pc} and P_{os} , respectively. Individual data dots are shown and data are also expressed as the mean \pm SE of 10 to 15 cells from three to five plants for the L_{pc} and more than 30 protoplasts coming from two plants for the P_{os} . Significant differences among the treatments are indicated by asterisks (*, **, ***). ns indicates not significantly different ($p < 0.05$).

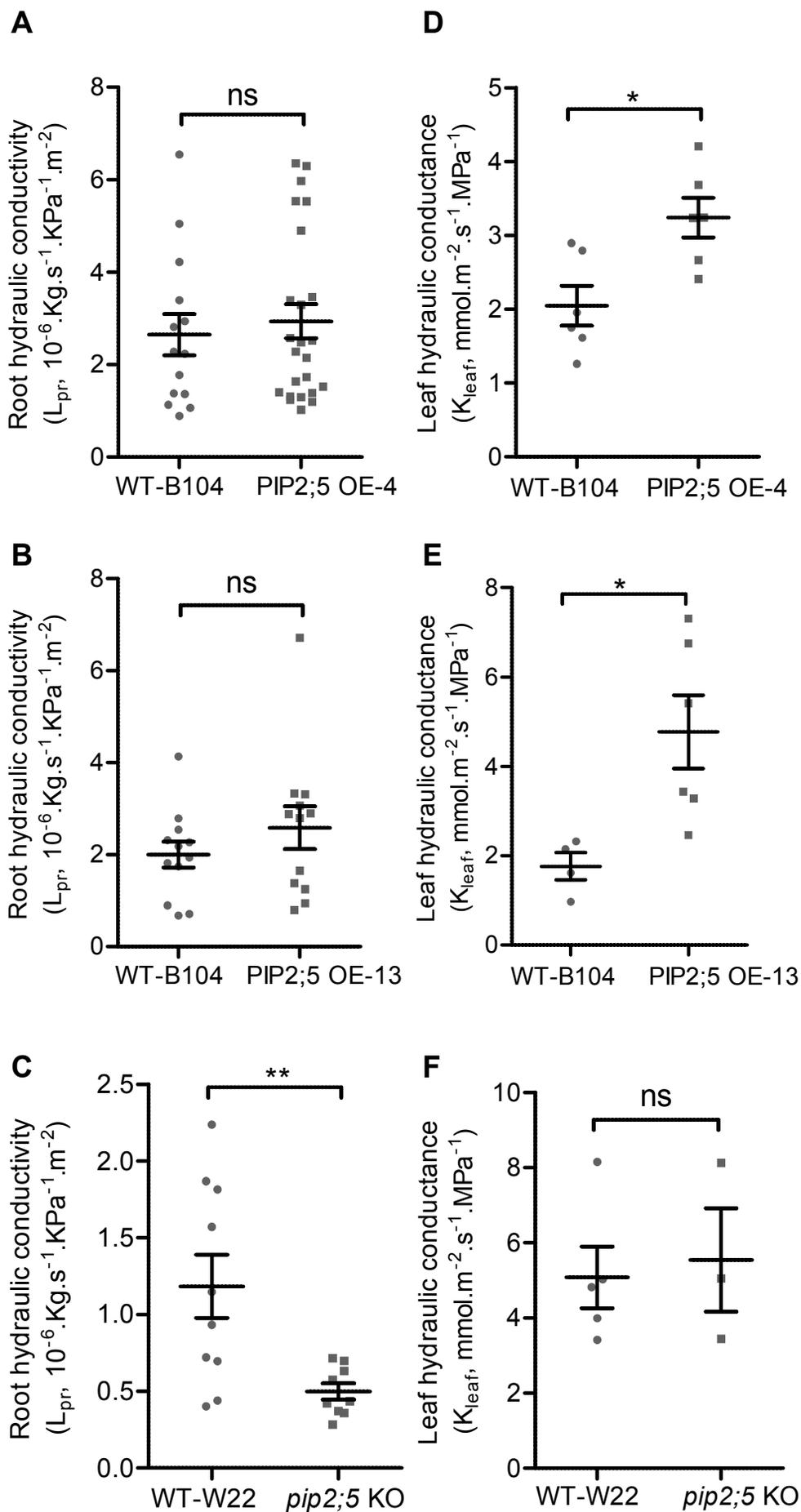


Figure 3. Root hydraulic conductivity (L_{pr} , A-C) and the leaf hydraulic conductance (K_{leaf} , D-F) in WT, PIP2;5 OE-4, PIP2;5 OE-13 and *pip2;5* KO line. L_{pr} was measured with two-week old maize seedlings and K_{leaf} of newly expanded leaf was measured with three-week old plants. Individual data dots are shown and data are also expressed as the mean \pm SE of roots and leaves from 4 to 6 plants. Significant differences between WT and PIP2;5 deregulation plants are indicated by * (p < 0.05) or ns (not significant). Downloaded from www.plantphysiol.org on January 28, 2020. Copyright © 2020 American Society of Plant Biologists. All rights reserved.

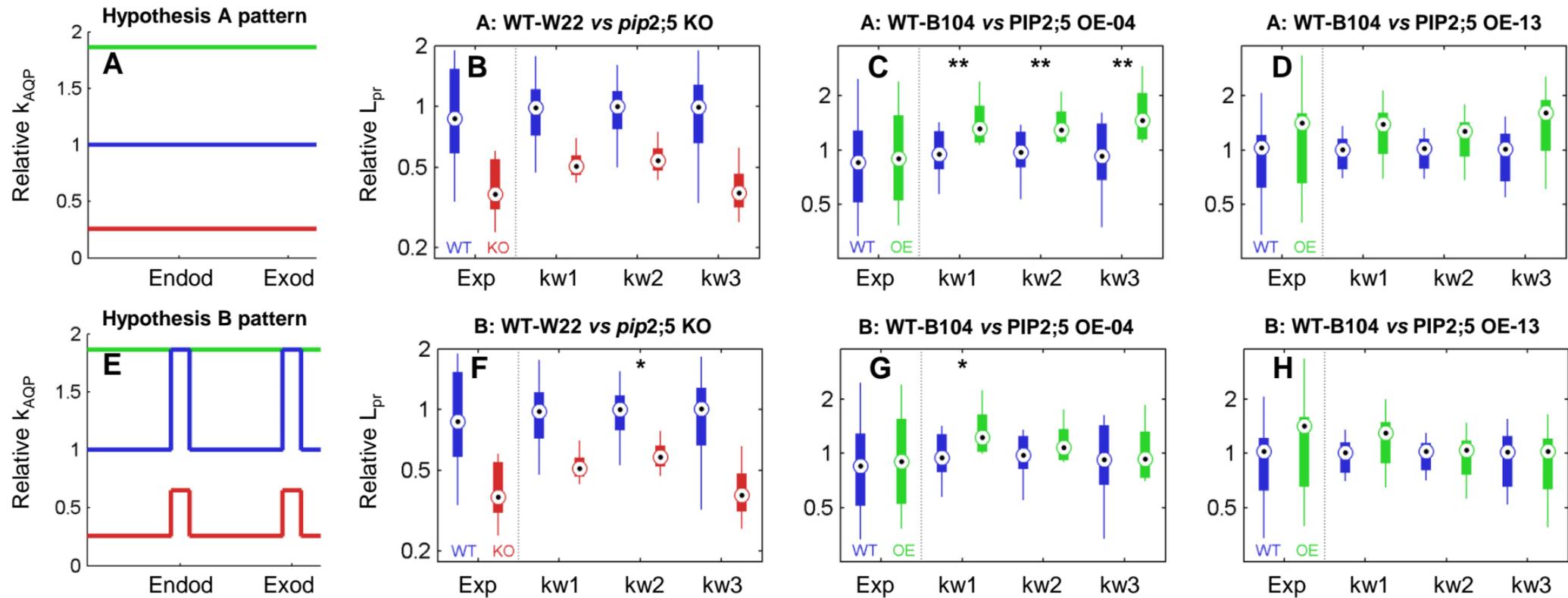


Figure 4. Contrast analysis between measured (Exp) and simulated (k_{w1} , k_{w2} and k_{w3}) L_{pr} in WT (blue) and PIP2;5 deregulated lines (red: *pip2;5* KO; green: PIP2;5 OE) for hypothesized radial patterns of plasmalemma permeability (k_{AQP}). * and ** for significantly different contrasts between measurements and simulations ($p < 0.05$ and 0.001 ; e.g., contrast between WT and OE-larger in simulations than exp. experiments in panel B). Circles/bars: 25th, 50th, 75th percentiles; Whiskers: most extreme non-outlier data point.

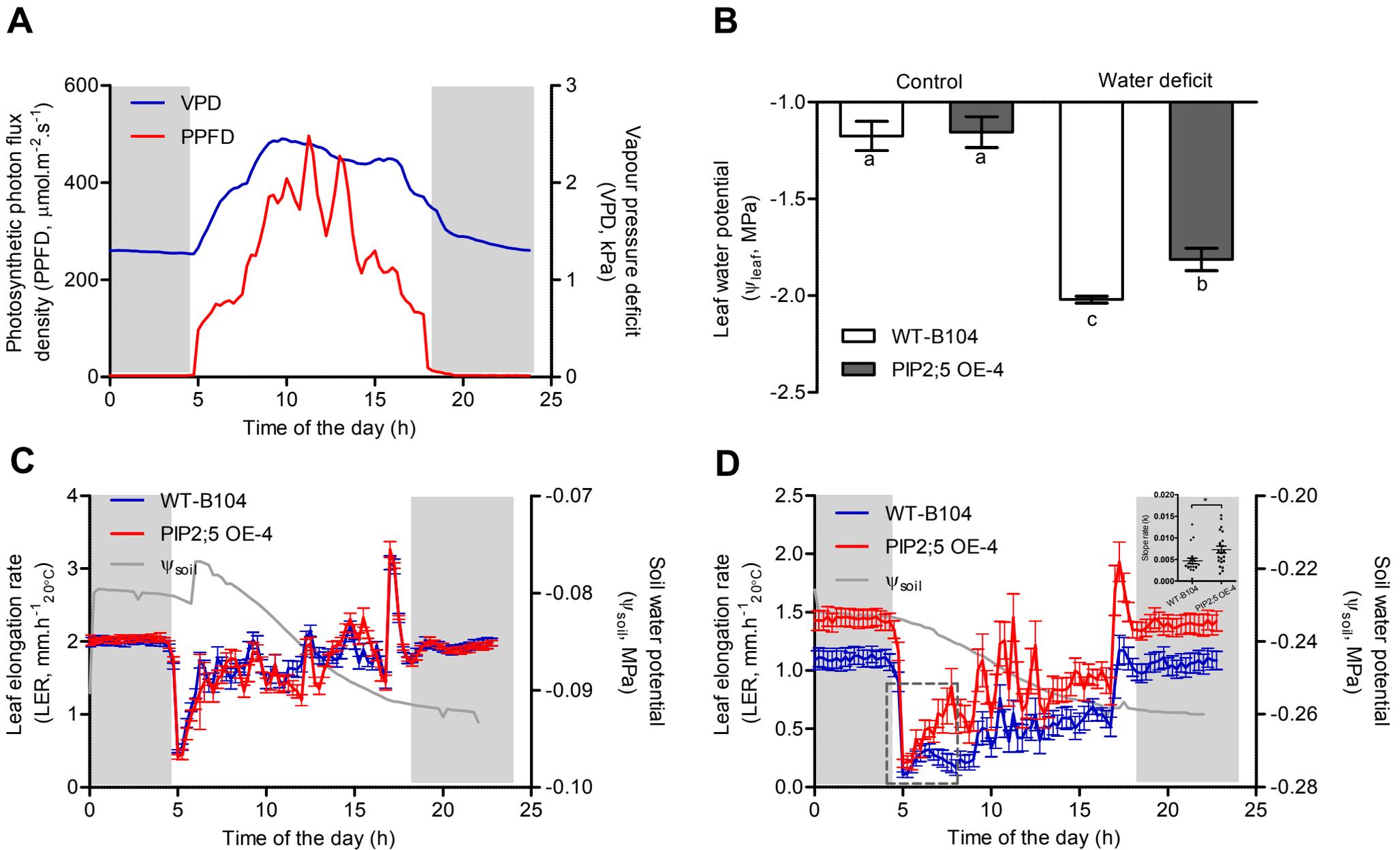


Figure 5. Daily time courses of environmental conditions (A), leaf water potential (B) and leaf elongation rate (LER) under well-watered (C) and moderate water deficit (D) treatments. In this experiment, wild type (WT-B104) and PIP2;5 overexpression line (PIP2;5 OE-4) were compared. In A, blue and red trace shows vapor pressure deficit (VPD) and photosynthetic photon flux density (PPFD), respectively. In B, white and grey filled bars show leaf water potential of WT-B104 and PIP2;5 OE-4, respectively, under control and water deficit conditions. In C and D, blue and red traces show the LER of WT-B104 and PIP2;5 OE-4, respectively. Gray traces show the daily soil water potential (Ψ_{soil}). Gray backgrounds show the sunset period. In dashed line square, the slope rate is analyzed, and the result is inserted. LER is expressed per unit thermal time ($\text{mm}\cdot\text{h}^{-1}\cdot 20^{\circ}\text{C}$). Error bars indicate standard error ($n = 3\text{--}9$ plants). In B, one-way ANOVA with Tukey post test is used to compare the significant differences between WT-B104 and PIP2;5 OE-4 plants under control and water deficit conditions. Significant differences are indicated by letters (a, b, c). In D, Student's t-test is used to compare the significant difference between WT-B104 and PIP2;5 OE-4 plants. * indicates the significant difference at level of $p < 0.05$.

Parsed Citations

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