

1 **Aquaporins are main contributors of root hydraulic conductivity in pearl**

2 **millet [*Pennisetum glaucum* (L) R. Br.]**

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24 Abstract

25 Pearl millet is a key cereal for food security in arid and semi-arid regions but its yield is
26 increasingly threatened by water stress. Physiological mechanisms consisting in saving water
27 or increasing water use efficiency can alleviate that stress. Aquaporins (AQP) are water
28 channels contributing to plant hydraulic balance that are supposedly involved in these
29 mechanisms by mediating root water transport. However, AQP remain largely
30 uncharacterized in pearl millet. Here, we studied AQP function in root water transport in two
31 pearl millet lines contrasting for water use efficiency (WUE). We observed that these lines
32 were also contrasting for root hydraulic conductivity (Lpr) and AQP contribution to Lpr, the
33 line with lower WUE showing significantly higher AQP contribution to Lpr. To investigate the
34 AQP isoforms contributing to Lpr, we developed genomic approaches to first identify the
35 entire AQP family in pearl millet and second study the plasma membrane intrinsic proteins
36 (PIP) gene expression profile. We identified and annotated 33 AQP genes in pearl millet among
37 which ten encoded PIP isoforms. *PgPIP1-3* and *PgPIP1-4* were significantly more expressed in
38 the line showing lower WUE, higher Lpr and higher AQP contribution to Lpr. Overall, our study
39 suggests that AQP from the PIP1 family are the main contributor of Lpr in pearl millet and are
40 possibly associated to whole plant water use mechanisms. This study paves the way for further
41 investigations on AQP functions in pearl millet hydraulics and adaptation to environmental
42 stresses.

43

44 **Key-words:** pearl millet, aquaporin, water, roots.

45

46 The newly sequenced nucleotide sequences reported in this article have been submitted to
47 GenBank under the submission number 2333840 (TPA grp467567). Assignment of GenBank
48 accession number is in process.

49

50 **Introduction**

51 Plant hydraulics depends on soil water capture by roots, transport to the leaves and diffusion
52 as vapor from the stomatal cavity to the atmosphere. In this plant hydraulic continuum, the
53 radial water transport from the soil solution to the xylem vessels uses two paths: the
54 apoplastic path where water can flow along the cell wall structures, and the cell to cell path
55 where water can flow across cell membranes (transcellular) or along cytoplasmic continuities
56 formed by plasmodesmata (symplastic) [1]. Extracellular hydrophobic barriers of lignin and
57 suberin located in the endodermis are thought to restrict diffusion of water along the
58 apoplastic path [2]. In the presence of such barriers, the cell to cell path is favored by water
59 channels present in cell membranes called aquaporins [3].

60 Aquaporins (AQP) are present throughout the living kingdom at the exception of
61 thermophilic Archaea and intracellular bacteria [4]. They belong to the Major Intrinsic Proteins
62 (MIP) superfamily and are characterized by six transmembrane domains and two highly
63 conserved Asn-Pro-Ala (NPA) motifs [5]. Other typical signatures of AQP consist in selectivity
64 filters domains structuring the pore and composed of the aromatic/arginine (ar/R) motifs and
65 the Froger's residues [6,7]. In higher plants, AQP isoforms fall into five families comprising the
66 Plasma membrane Intrinsic Proteins (PIP), the Tonoplast Intrinsic Proteins (TIP), the
67 Nodulin26-like Intrinsic Proteins (NIP), the Small Intrinsic Proteins (SIP) and the
68 uncharacterized (X) Intrinsic Proteins (XIP) [8]. Although plant AQP have been localized
69 throughout the cell secretory system, PIP, NIP and XIP are preferential residents of the plasma
70 membranes while TIP accumulate in the tonoplast and SIP in the endoplasmic reticulum [9–
71 11]. Functional studies combined with modelling approaches demonstrated that, more than
72 being permeable to water, AQP possess a wide range of selectivity profiles [12,13]. Some PIP

73 isoforms are thereby permeable to H₂O₂ and CO₂, some TIP isoforms to NH₃ and urea, and
74 some NIP to small organic solutes or mineral nutrients [14–18]. AQP possess wide range of
75 physiological functions and have now been identified in a number of crop species such as rice,
76 maize, tomato, cotton, sorghum, *Setaria italica*, watermelon or *Cannabis sativa* [19–24].

77 In plants, AQP are involved in water transport in both roots and shoots. In *Arabidopsis*
78 for instance, isoforms AtPIP2-2 and AtPIP1-2 contributes to around 14% and 20% of the root
79 osmotic conductivity and shoot hydraulic conductivity, respectively [25,26]. AQP also have
80 important roles in plant growth, CO₂ fixation, nutrient allocation, reproduction or biotic
81 interactions [8]. In recent years, AQP functions in plant water relations have received more
82 attention as a potential target for crop improvement, particularly to increase tolerance to
83 drought [27–32]. For instance, AQP could regulate root water transport in order to match
84 transpiration and therefore contribute to mitigate disparity between water supply and
85 demand in soybean upon “atmospheric” water stress caused by high evaporative demand
86 [33]. This assumption is supported by the increased AQP expression and root hydraulic
87 conductivity upon transpiration demand in rice and grapevine [34,35]. Furthermore,
88 overexpression of *SiTIP2-2* in tomato increased transpiration rate and was associated with
89 improved fruit yield upon moderate soil water stress [36]. Conversely, in other crops more
90 adapted to hot and dry climates such as pearl millet, lower transpiration under high vapor
91 pressure deficit (VPD) has been proposed to be beneficial for tolerance to “atmospheric”
92 water stress and potentially associated with AQP function in root hydraulics [30,37,38].
93 Therefore, AQP may be involved in different physiological mechanisms that determine the
94 extent of water usage by the plant [39]. In fact, specific isoforms might be involved in different
95 scenarios, calling for a better understanding of AQP family members and their functions in
96 crops [28].

97 Pearl millet [*Pennisetum glaucum* (L) R Br.] is a key cereal for food security in arid and
98 semi-arid regions [40]. However, its yield remain low and is often affected by climate
99 unpredictability (heat waves and dry spells) that are forecast to worsen in future climate
100 change scenarios [41,42]. The recent release of the pearl millet genome has opened new ways
101 for functional genomic-based efforts aiming at improving its yield and tolerance to abiotic
102 stresses [43]. In this study, we evaluated the potential links between AQP function in roots
103 and water use, a drought tolerance-related trait, by measuring AQP contribution to Lpr and
104 AQP genes expression in the roots of two pearl millet inbred lines contrasting for water use
105 efficiency. To have more insights into the AQP isoforms contributing to root hydraulics, we
106 characterized the entire AQP family in pearl millet using a genomic approach.

107

108 **Materials and methods**

109 **Plant material and growth conditions**

110 IP4952 and IP17150, two pearl millet inbred lines that are part of the Pearl Millet inbred
111 Germplasm Association Panel (PMiGAP) were used in this study [43]. The water use efficiency
112 of these two lines was characterized across two lysimeters experiments performed under
113 well-irrigated conditions at the International Crops Research Institute for the Semi-Arid
114 Tropics (ICRISAT, India) according to [44]. These experiments indicated that IP4952 and
115 IP17150 displayed relatively low and high water use efficiency, respectively (2.35 versus 3.72)
116 [45]. These two lines were used for root hydraulic conductivity measurements and AQP
117 expression analyses in which plant were grown in hydroponic conditions into a nethouse at
118 the IRD/ISRA Bel Air research station (Dakar, Senegal; 14.701615 N – 17.425359 W). Plants
119 were germinated in Petri dishes into a chamber at 37°C for two days in the dark. Plants were

120 further exposed to light (37°C, 12h day/night cycle) for one day before being transplanted on
121 top of a black mat covering a 30L container (45x40x24 cm containing 40 plants in total) filled
122 with half strength Hoagland solution [46]. The system allowed roots to strictly develop in the
123 nutrient solution without been exposed to light. Oxygen was provided to the roots through
124 constant bubbling of the solution using an air pump.

125

126 **Root hydraulic conductivity and aquaporin contribution**

127 Root hydraulic conductivity (Lpr) was measured in April-Mai 2019 (17/34°C and 34/100% in
128 minimum/maximum temperature and humidity, respectively) from 9AM to 12PM on 15 days
129 old plants grown in hydroponic conditions. The average temperature, relative humidity and
130 VDP at the time of measurements were $24.4 \pm 0.3^\circ\text{C}$, $75.1 \pm 1.2\%$ and 0.8 ± 0.1 , respectively.
131 Plants were grown sequentially to be analyzed at the same age in a randomized design taking
132 into account the time of measurement. Lpr measurements were performed using a pressure
133 bomb (model 1000, PMS instrument company, USA) according to [47] and [48]. Briefly, plants
134 were inserted into the pressure chamber filled with nutrient solution complemented or not
135 by 2mM of azide (NaN_3). The hypocotyl was carefully threaded through the silicone grommet
136 of the pressure chamber lid while the intact root system was sealed into the chamber. Roots
137 were pressurized with compressed air at 0.4 MPa for 5 min to equilibrate, followed by xylem
138 sap collection at 0.1, 0.2 and 0.3 MPa for 5 min using pre-weighed 2 ml Eppendorf tubes filled
139 with cotton placed on top of the stem. The mass of xylem sap exuded at each pressure was
140 determined by weighing and used to calculate the xylem sap flow (slope of xylem sap weight
141 at each pressure). After the measurements, roots systems were scanned to determine root
142 surface area using WhinRhizo Pro version 2012b (Regent Instruments, Canada). Xylem sap flux

143 was divided by root surface area to calculate Lpr. AQP contribution to Lpr was estimated using

144 relative Lpr inhibition by azide calculated as:

$$145 \quad 100 - ((Lpr_{azide_individual_replicate} \times 100) / Lpr_{no_azide_variety_mean}) \quad (1)$$

146

147 **Aquaporins genome-wide identification**

148 A total of 772 AQP protein sequences from 19 plant species (*Arabidopsis thaliana*, *Beta*

149 *vulgaris*, *Brachypodium distachyon*, *Cicer arietinum*, *Gossypium hirsutum*, *Glycine max*,

150 *Hordeum vulgare*, *Linum usitatissimum*, *Musa acuminate*, *Panicum virgatum*, *Pennisetum*

151 *glaucum*, *Populus tremula*, *Oryza sativa*, *Setaria italica*, *Solanum lycopersicum*, *Solanum*

152 *tuberosum*, *Sorghum bicolor*, *Vitis vinifera* and *Zea mays*) were aligned against the pearl millet

153 genome (ASM217483v2) and the non-assembled scaffolds [43] using tblastn with an e-value

154 of 10^{-5} as initial cut-off to identify high scoring pairs. High scoring pairs were further filtered

155 to keep those with a bit score ≥ 100 . Hot-spots of high scoring pairs were identified and

156 redundant high scoring pairs were filtered to keep those with highest bit-score for further

157 analysis (S2 Table). The filtered high scoring pairs locations in the pearl millet genome were

158 used to identify regions with homologies to AQP genes.

159

160 **Structural annotation**

161 Correspondence between selected high scoring pairs and annotated genes in the pearl millet

162 genome was determined. Potential AQP genes were identified and their genomic sequence \pm

163 1000pb upstream and downstream of the start/end position was retrieved as well as the

164 predicted gene structure [43]. When predicted AQP did not correspond to previously

165 annotated genes, the GENSCAN Web Server (<http://hollywood.mit.edu/GENSCAN.html>) was

166 used to predict the exon-intron structure of the genomic region. Putative AQP genomic
167 sequences were aligned against the Plant EST (downloaded in August 2018) and the
168 UniProt/Swiss-Prot plant protein (February 2016) databases and manually annotated using
169 the Artemis software (version 17.0.1, Sanger Institute, UK; S3 Table). Annotation was
170 confirmed by aligning reads from pearl millet transcriptomes [49] on the pearl millet genome
171 using the Tablet software (version 1.19.09.03) [50] and coding and protein sequences were
172 generated. AQP gene structure were further visualized using GSDS2.0 software [51].

173

174 **Sequencing**

175 AQP genes sowing missing sequences in or bordering coding regions were resequenced (S3
176 Table) using genomic DNA or cDNA from Tift 23D2B1 (genotype used to draft the pearl millet
177 whole genome sequence). DNA was prepared using DNeasy Plant mini extraction kit (Qiagen,
178 Germany) while cDNA was prepared by first extracting RNA using the RNeasy Plant mini
179 extraction kit (Qiagen, Germany) followed by DNase treatment (RNase-free DNase set;
180 Qiagen, Germany) and reverse transcription reaction (Omniscript RT kit; Qiagen, Germany)
181 according to the manufacturer's instructions. Corresponding DNA/cDNA fragments were
182 amplified using the Phusion high-fidelity DNA polymerase (Thermo Scientific, USA), purified
183 (Geneclean turbo kit, MP Biomedicals, USA) and sent for sequencing (Eurofins Genomics,
184 Germany). Primers used for amplification are presented in S4 Table. Difficulties in amplifying
185 the missing sequence of PgTIP5-1 were encountered. In that specific case, we used
186 unpublished MINION reads (Mariac, Vigouroux, Berthouly-Salazar; unpublished) to complete
187 its sequence. Missing nucleotides were added to the pearl millet genomic sequence and
188 coding frame of the corresponding new protein was carefully checked.

189

190 **Identification of functional motifs and transmembrane domains**

191 The NCBI conserved domain database (CDD) was used to identify NPA motifs and
192 aromatic/arginine (ar/R) selectivity filters in the putative AQP protein sequences. Froger's
193 residues were identified according to [6]. The number and location of the transmembrane
194 domains were studied using TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>), TMpred
195 (https://embnet.vital-it.ch/software/TMPRED_form.html) and Phyre2 [52]. Protein
196 sequences were aligned using the CLUSTALW alignment function in the Maga7 software
197 (version 7.0.26) [53]. Alignments were colored using the Color Align Properties program
198 (https://www.bioinformatics.org/sms2/color_align_prop.html). Conserved domains as well
199 as transmembrane domains were further manually analyzed to detect sequence alterations.
200 Three-dimensional geometry structure and pore morphology of PIP was obtained using the
201 PoreWalker software (<https://www.ebi.ac.uk/thornton-srv/software/PoreWalker/>).

202

203 **Phylogenetic analysis**

204 Phylogenetic analyses of *P. glaucum* AQP (PgAQP) was analyzed in relation to AQP identified
205 in *A. thaliana* (AtAQP), *O. sativa* (OsAQP) and *P. tremula* (PtAQP) using the Mega7 software
206 (version 7.0.26) [53]. PgAQP, AtAQP, OsAQP and PtAQP protein sequences were aligned using
207 the CLUSTAW function and phylogenetic tree was built using the maximum likelihood method
208 with 1000 reiterations. This allowed determination of the statistical stability of each node.
209 Based on their position in the phylogenetic tree, PgAQP isoforms were classified into PIP, SIP,
210 TIP and NIP families and named according to their close homologs.

211

212 **Expression profiling**

213 *P. glaucum* PIP (PgPIP) genes expression were measured in root of 15 days old plants grown
214 in hydroponic conditions using quantitative polymerase chain reaction (RT-PCR). Roots
215 (seminal + one crown root) were sampled between 10AM to 12PM. Sampled roots were
216 immediately frozen into liquid nitrogen and ground using a TissueLyser II (Qiagen). RNA and
217 cDNA (from 1µg of RNA) were prepared using extraction kits as described above. RT-PCR was
218 performed with 1µL of diluted cDNA (1:9) in a Brilliant III ultra fast SYBRgreen QPCR master
219 mix (Agilent Technologies, USA) using a StepOnePlus Real-Time PCR System (Applied
220 biosystems, USA). Primers used to amplify the different PgPIP genes were checked for
221 specificity and efficiency prior to the experiment (S5 Table). The pearl millet Ubiquitin gene
222 (*Pgl_GLEAN_10001684*) was used as reference and PgPIP relative expression was calculated
223 according to the delta-delta ct method.

224 PgAQP expression in shoots were retrieved from [49]. Data from leaves and
225 inflorescence of ten open pollinated cultivated pearl millet varieties were used [49].

226

227 **Statistics**

228 Statistical analyses were performed using R version 3.5.2 (R Development Core Team, 2018)
229 using ANOVA (aov script) to detect significant differences. Least Significant Difference (LSD)
230 test within the Agricolae package was used to group differences in letter classes.

231

232 **Results**

233 **Pearl millet aquaporin contribution to root hydraulic conductivity**

234 In order to evaluate if AQP function in root radial water flow could be associated with water
235 use in pearl millet, we measured root hydraulic conductivity (Lpr) in IP4952 and IP17150,

236 previously described as low and high water use efficiency lines respectively. IP4952 showed
237 1.4 times higher Lpr than IP17150 ($1.30E-07 \pm 2.36E-08$ versus $9.27E-08 \pm 8.33E-09$ $m^3 m^{-2} s^{-1}$
238 MPa^{-1} respectively; n=15) but this difference was not significant ($p = 0.148$; Fig 1 and S1 Table).
239 In both IP4952 and IP17150, treatment with azide, an inhibitor of AQP activity, led to
240 significant Lpr reduction to $2.00E-08 \pm 2.44E-09$ and $2.19E-08 \pm 2.32E-09$ $m^3 m^{-2} s^{-1} MPa^{-1}$
241 respectively ($p < 0.001$; S1 Table). This effect of azide application on Lpr was mostly reversible
242 after treating the same roots with azide-free solution (S1 Fig). AQP contribution to Lpr was
243 significantly higher in IP4952 (low water use efficiency) as compared to IP17150 (high water
244 use efficiency; $84.64 \pm 1.98\%$ versus $76.40 \pm 2.61\%$, $p < 0.05$; S1 Table). These data indicate
245 that AQP could contribute more than 75% to Lpr in pearl millet.

246

247 **Fig 1. Root hydraulic conductivity and aquaporin contribution in roots of IP4952 and**
248 **IP17150.** Root hydraulic conductivity (Lpr) were measured in plants grown in hydroponic
249 conditions between 9AM to 12PM in presence or not of 2mM azide. (A) Lpr values measured
250 in absence of azide. (B) Aquaporin contribution to Lpr was calculated as relative Lpr inhibition
251 by azide. Bars represent mean values \pm se of n=10-15 plants. *: $p < 0.05$.

252

253 **Aquaporin identification and annotation**

254 To have more insight in the AQP isoforms contributing to Lpr in pearl millet, we characterized
255 the AQP genes family in the pearl millet genome. We blasted 772 AQP from the PIP, TIP, SIP,
256 NIP and XIP families identified in 19 different species on the pearl millet reference genome
257 (chromosome assembly and scaffolds) [43]. A total of 7005 sequences with bit score >100 ,
258 representing 50 specific hits spread on the genome were identified (S2 Table). Forty-seven of
259 the hits fall into previously annotated genes, one fall in a non-annotated part of the genome

260 on chromosome 5, and two in non-assembled parts of the genome (scaffold763 and
261 scaffold8428).

262 Manual *de novo* annotation of the 50 putative AQP genes allowed the identification of
263 eight genes with no start or with early stop codon in the first exon that were classified as
264 pseudo-genes (S3 Table). Nine genes did not encode AQP isoforms but zinc-finger protein/LRR
265 receptor-like serine-threonine protein kinase families or DEAD-like helicase-N superfamily,
266 respectively. The absence of AQP signature domains (NPA and Ar/R motifs) in their protein
267 sequence confirmed their non-affiliation to the AQP family. A number of genes showed an
268 excessive number of exons or longer first exon and were re-annotated on the basis of
269 alignment with transcriptome sequences or with close protein homologs (Uniprot/Swiss-Prot
270 blast results) and presence of AQP isoform conserved domains. In addition, ten genes showed
271 missing sequences in coding regions that were completed after sequencing. Overall, 33
272 *Pennisetum glaucum* AQP (PgAQP) genes were identified in the pearl millet genome, among
273 which sixteen were *de novo* annotated (Table 1).

274 **Table 1. Description and distribution of the aquaporins genes identified in pearl millet.**

ID	Gene	Pgl_Glean ID	Gene length (bp)	Transcript length (bp)	Protein length (aa)	Protein MW (kD)	Chr	Start	End
Plasma membrane intrinsic proteins (PIP)									
1	PgPIP1-1	Pgl_GLEAN_10001520	3097	867	288	30.70	3	5721622	5724718
2	PgPIP1-3	Pgl_GLEAN_10010809	1601	867	288	30.76	3	274573998	274575598
3	PgPIP1-4	Pgl_GLEAN_10005724	992	900	298	31.38	2	104247932	104248932
4	PgPIP2-1	Pgl_GLEAN_10028064	2920	873	290	30.35	3	12453415	12456334
5	PgPIP2-2	Pgl_GLEAN_10028876	2830	867	288	30.12	3	45603209	45606038
6	PgPIP2-3	Pgl_GLEAN_10035675	1903	873	290	30.39	3	257669631	257671533
7	PgPIP2-5	Pgl_GLEAN_10028056	1062	834	277	28.93	3	12167791	12168852
8	PgPIP2-6	Pgl_GLEAN_10028055	1053	861	286	29.94	3	12156953	12158005
9	PgPIP2-7	Pgl_GLEAN_10010255	1170	861	286	29.79	Scaffold763	240584	241753
10	PgPIP2-8	Pgl_GLEAN_10009812	837	837	278	29.17	2	64966663	64967453
Tonoplast intrinsic proteins (TIP)									
11	PgTIP1-1	Pgl_GLEAN_10002147	1499	750	249	25.72	5	153303973	153305471
13	PgTIP2-1	Pgl_GLEAN_10000631	844	744	247	24.88	2	30657555	30658398
16	PgTIP2-2	Pgl_GLEAN_10030617	933	747	248	25.03	3	100540239	100541171
12	PgTIP2-3	Pgl_GLEAN_10009584	851	750	249	25.06	3	33622445	33623295
15	PgTIP3-1	Pgl_GLEAN_10028702	911	801	266	27.41	2	44624536	44625481
14	PgTIP4-1	Pgl_GLEAN_10002901	2586	738	245	25.58	1	263875434	263878070
18	PgTIP4-2	Pgl_GLEAN_10003219	1455	744	247	25.15	3	148813464	148814777
17	PgTIP4-3	Pgl_GLEAN_10003218	844	747	248	25.10	3	148807632	148808412
19	PgTIP5-1	Pgl_GLEAN_10033583	1087	813	270	26.69	3	272599938	272601028
Noduline-26 like intrinsic proteins (NIP)									
20	PgNIP1-1	Pgl_GLEAN_10012175	2316	846	281	29.52	2	195368171	195370486
21	PgNIP1-2	Pgl_GLEAN_10028618	2560	846	281	29.53	1	261387113	261389753
22	PgNIP1-4	Pgl_GLEAN_10028339	1137	837	278	29.36	3	145385690	145386826
23	PgNIP2-1	Pgl_GLEAN_10018521	3364	891	296	31.82	3	14105646	14109009
24	PgNIP2-2	Pgl_GLEAN_10019286	3821	894	297	31.57	2	103033018	103036838
25	PgNIP3-1	Pgl_GLEAN_10034621	3855	909	302	31.43	2	40497062	40500916
26	PgNIP3-2	Pgl_GLEAN_10030882	1151	846	281	29.60	4	55609410	55610656
27	PgNIP3-3	Pgl_GLEAN_10030883	1087	780	259	27.05	4	55648949	55650374
28	PgNIP3-4	Pgl_GLEAN_10030881	1258	750	249	25.15	4	55573901	55575272
29	PgNIP3-5	Pgl_GLEAN_10030872	934	837	278	27.79	4	55508562	55509419
30	PgNIP4-1	Pgl_GLEAN_10012100	1319	921	306	31.52	6	110090448	110091766
Small intrinsic proteins (SIP)									
31	PgSIP1-1	Pgl_GLEAN_10003744	2818	726	241	25.32	1	175152169	175154986
32	PgSIP1-2	Pgl_GLEAN_10014008	3375	759	252	25.91	4	93394125	93397493
33	PgSIP2-1	Pgl_GLEAN_10026167	1899	759	252	27.10	5	126489209	126491163

275 PgPIP2-7 is located on scaffold763 that was not assembled to the pearl millet genome. bp:
276 base pairs; aa: amino-acids; MW: molecular weight; kD: kilo Dalton; Chr: chromosome; Start:
277 position of the ATG; End: position of the stop codon.

278

279 Pearl millet aquaporins phylogenetic analysis

280 To classify the PgAQP into families and name them, a phylogenetic tree was built using the
281 PgAQP protein sequences along with protein sequences from *Arabidopsis thaliana*, *Oryza*
282 *sativa* and *Populus tremula* (Fig 2). The PgAQP were named according to their grouping into
283 families (PIP, TIP, SIP or NIP) and close homologs (Table 1). Ten isoforms showed homologies
284 to the PIP family with three isoforms falling in the PIP1 sub-family (PgPIP1-1, PgPIP1-3 and
285 PgPIP1-4) and seven isoforms falling in the PIP2 sub-family (PgPIP2-1, PgPIP2-2, PgPIP2-3,

286 PgPIP2-5, PgPIP2-6, PgPIP2-7 and PIP2-8). Nine isoforms from the *P. glaucum* TIP family
287 (PgTIP), eleven isoforms from the *P. glaucum* NIP family (PgNIP) and three isoforms from the
288 *P. glaucum* SIP family (PgSIP) were further identified. No isoforms from the XIP family were
289 identified in pearl millet. This analysis further confirmed the classification of PgPIP1-1, PgPIP2-
290 1, PgPIP2-3, PgPIP2-6, PgTIP1-1 and PgTIP2-2 cloned by [30]. However, PgPIP1-2 from [30] was
291 renamed as PgPIP1-3 in this study.

292 PgAQP genes distribution in the pearl millet genome denoted that most of them were
293 localized on chromosome 3 while none were localized on chromosome 7 (Fig 3). Two hot-
294 spots of PgAQP genes were observed, one located in a region of 11899bp on chromosome 3
295 containing *PgPIP2-1*, *PgPIP2-5* and *PgPIP2-6* and the other in a region of 141812bp on
296 chromosome 4 containing *PgNIP3-2*, *PgNIP3-3*, *PgNIP3-4* and *PgNIP3-5*.

297
298 **Fig 2. Phylogenetic relationship among aquaporins isoforms from pearl millet, Arabidopsis,**
299 **rice and poplar.** Tree was generated using the Maximum Likelihood method with 1000
300 reiterations in MEGA7. Bootstrap values above 50% are represented. The PIP, TIP, SIP, NIP and
301 XIP family clades are represented by blue, grey, purple, green and red, respectively. Pearl
302 millet sequences are indicated by colored dots.

303
304 **Fig 3. Distribution of aquaporin genes in the pearl millet genome.** The seven chromosome
305 (Chr) from the pearl millet genome are represented according to their size in megabase (Mb).
306 Positions of PIP, TIP, SIP and NIP genes are represented in blue, purple, orange and green,
307 respectively. *PgPIP2-7* which is located on scaffold763 is not represented.

308
309 **Aquaporin gene structure in pearl millet**

310 PgAQP genes showed large variation in gene length (ranging from 837bp for *PgPIP2-8* to
311 3855bp for *PgNIP3;1*; Table 1). Transcript length were less variable and relatively conserved
312 within families with lengths of around 800-900bp for the PgPIP and PgNIP genes, 750-800bp
313 for the PgTIP genes and 700-750bp for the PgSIP genes.

314 To investigate associations between phylogenetic classification and gene structure, the
315 intron-exon organization of the PgAQP genes was analyzed (S2 Fig). PgAQP genes displayed
316 between one (*PgPIP2-8*) to five exons (*PgNIP2-1*, *PgNIP2-2* and *PgNIP4-1*). Except for the NIP
317 family, intron-exon organization was generally conserved within families with 5/9 PgPIP genes
318 displaying 3 exons, 6/9 PgTIP genes displaying 2 exons and all PgSIP genes displaying 3 exons,
319 supporting their phylogenetic distribution. *PgPIP2-5/PgPIP2-6* and *PgNIP3-2/PgNIP3-3* that
320 were found to be close homologs in the phylogenetic analysis (Fig 2) and closely located in the
321 pearl millet genome (Fig 3) showed similar gene and transcript length (Table 1) as well as gene
322 structure (S2 Fig). PgAQP coding regions encoded proteins with length varying between 250
323 to 300 amino acids, with molecular weight of around 30kD for the PgPIP and PgNIP and 25kD
324 for the PgTIP and PgSIP isoforms (Table 1).

325

326 **Pearl millet AQP conserved domains**

327 To investigate polymorphisms in the PgAQP isoforms conserved motifs that could underlay
328 potential changes in structural and substrate selectivity, analyses of conserved and
329 transmembrane domains were performed. The conserved domain analysis showed that all
330 PgAQP isoforms belonged to the MIP super-family and displayed typical double NPA motifs
331 (S6 Table and Table 2). Although some polymorphisms in the NPA motifs were observed in
332 some isoforms (particularly from the NIP family) the subsequent amino-acids were of the
333 same chemical properties (generally neutral and non-polar; Table 2, Fig 4 and S3-5 Fig). Ar/R

334 selectivity filters and Froger's residues known as AQP markers were well conserved in the
335 PgPIP isoforms except for the Froger's residue on position 1 (P1; Table 2, Fig 4). More
336 polymorphisms were observed in these residues for the PgTIP, PgNIP and PgSIP isoforms
337 although the ar/R residue on Loop E (R on LE2) and the Froger's residues at positions 3 (A) and
338 4 (F/Y) were well conserved across all isoforms (Table 2).

339 Transmembrane domains analyses using three different prediction softwares
340 suggested a high probability for all identified PgAQP to display six transmembrane domains
341 with cytoplasmic N-terminal and C-terminal ends as typically observed for AQP (S7 Table).
342 Predictions of the 3-dimensional geometry structure and pore morphology suggested a
343 continuous pore that runs longitudinally across both sides of the membranes for all PgPIP (S6
344 Fig). Two deviations in the pore center were typically observed at both extremities illustrating
345 the two constraints caused by the NPA motifs structured as alfa-helixes and acting as
346 selectivity filters.

347
348 **Fig 4. Conserved domains and membrane topology of the PIP isoforms from pearl millet.**
349 Alignment of the PIP isoforms were obtained using ClustalW in Mega7. Sequence identities
350 and similarities (80%) are highlighted in colors. The transmembrane domains are represented
351 by orange bars with the N-terminal and C-terminal ends of the protein located in the cytosol.
352 NPA: Asparagine-Proline-Alanine motifs; *: Aromatic/Arginine selectivity filters. #: Froger's
353 residues.

354 **Table 2. Amino-acids residues in the conserved domains of the pearl millet aquaporins
355 isoforms.**

Isoform	NPA (LB)	NPA (LE)	Ar/R selectivity filters				Froger's residue				
			H2	H5	LE1	LE2	P1	P2	P3	P4	P5
Plasma membrane intrinsic proteins (PIP)											
PgPIP1-1	NPA	NPA	F	H	T	R	Q	S	A	F	W
PgPIP1-3	NPA	NPA	F	H	T	R	Q	S	A	F	W
PgPIP1-4	NPA	NPA	F	H	T	R	V	S	A	F	W
PgPIP2-1	NPA	NPA	F	H	T	R	Q	S	A	F	W
PgPIP2-2	NPA	NPA	F	H	T	R	Q	S	A	F	W
PgPIP2-3	NPA	NPA	F	H	T	R	Q	S	A	F	W
PgPIP2-5	NPA	NPA	F	H	T	R	-	S	A	F	W
PgPIP2-6	NPA	NPA	F	H	T	R	Q	S	A	F	W
PgPIP2-7	NPA	NPA	F	H	T	R	T	S	A	F	W
PgPIP2-8	NPA	NPA	F	H	T	R	H	S	A	F	W
Tonoplast intrinsic proteins (TIP)											
PgTIP1-1	NPA	NPA	H	I	A	V	T	S	A	Y	W
PgTIP2-1	NPA	TPA	H	I	G	R	T	S	A	Y	W
PgTIP2-2	NPA	NPA	H	I	G	R	T	S	A	Y	W
PgTIP2-3	NPA	NPA	H	I	G	R	T	S	A	Y	W
PgTIP3-1	NPA	NPA	H	V	A	R	T	V	A	Y	W
PgTIP4-1	NPS	NPA	N	S	A	R	T	S	A	Y	W
PgTIP4-2	NPA	NPA	Q	S	A	R	T	S	A	Y	W
PgTIP4-3	NPA	NPA	H	I	A	H	T	S	A	Y	W
PgTIP5-1	NPA	NPA	Q	V	A	R	R	S	A	Y	W
Noduline-26 like intrinsic proteins (NIP)											
PgNIP1-1	NPA	NPA	W	V	A	R	F	T	A	Y	V
PgNIP1-2	NPA	NPA	W	V	A	R	F	T	A	Y	F
PgNIP1-4	NPA	NPV	W	A	A	R	F	S	A	Y	I
PgNIP2-1	NPA	NPA	G	S	G	R	L	T	A	Y	F
PgNIP2-2	NPA	NPA	G	S	G	R	L	T	A	Y	F
PgNIP3-1	NPS	NPV	A	I	G	R	F	T	A	Y	L
PgNIP3-2	NPA	NPA	A	A	A	R	Y	T	A	Y	M
PgNIP3-3	NPA	NPA	A	A	A	R	Y	T	A	Y	M
PgNIP3-4	NPA	NPA	A	A	A	R	Y	T	A	Y	M
PgNIP3-5	NPA	NPA	A	A	A	R	Y	T	A	Y	M
PgNIP4-1	NPA	NPI	M	G	G	R	M	T	A	Y	L
Small intrinsic proteins (SIP)											
PgSIP1-1	NPT	NPA	V	V	P	N	M	A	A	Y	W
PgSIP1-2	NPT	NPA	L	I	P	N	M	A	A	Y	W
PgSIP2-1	NPL	NPA	S	H	G	S	F	A	A	Y	W

356 The two NPA (Asparagine-Proline-Alanine) motifs are located on loop B (LB) and loop E (LE). Aromatic/Arginine
357 motifs (Ar/R) are located on helix 2 (H2), helix 5 (H5), and loop E (LE1 and LE2). P1-5 designed the five Froger's
358 position.

359

360 **Aquaporin expression profiling in pearl millet**

361 Because of their localization at the plasma membrane, PIP isoforms play major roles in root
362 hydraulic conductivity. To infer PgPIP isoforms putative importance to Lpr in pearl millet, we
363 analyzed PgPIP gene expression pattern in roots of IP4952 and IP17150 using quantitative PCR.

364 PgPIP genes were generally more expressed in IP4952 as compared to IP17150 (Fig 5). In both
365 lines, the most expressed genes in roots were *PgPIP1-1*, *PgPIP1-3*, *PgPIP1-4*, and *PgPIP2-3*
366 while *PgPIP2-5*, *PgPIP2-6*, *PgPIP2-7* and *PgPIP2-8* were lowly expressed. *PgPIP1-3* and *PgPIP1-4*
367 were the only genes significantly differentially expressed between both lines.

368 Expression of PgPIP in shoots (leaves and inflorescence) were retrieved from [49].
369 Transcriptomic analyses from ten pearl millet varieties suggest that *PgPIP1-4* and *PgPIP2-3*,
370 two of the most expressed genes in roots, are lowly expressed in shoots (S7 Fig). Conversely,
371 *PgPIP1-1* and *PgPIP1-3* are highly expressed in roots and shoots.

372
373 **Fig 5. Relative expression of PIP genes in roots of IP4952 and IP17150.** Transcript abundance
374 of each PIP genes were measured between 9AM and 12PM on plants grown in hydroponic
375 conditions and normalized to the expression of *PgPIP2-5* in IP4952. Bars show mean values ±
376 se of n=6-8 biological replicates, each with technical triplicates. Letters indicate different
377 significance groups.

378
379 **Discussion**
380 Here, we studied the role of AQP in root water transport in pearl millet, a heat and drought-
381 adapted crop. Root hydraulic conductivity (Lpr) varied around $1E-07 \text{ m}^3 \text{ m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$, in a
382 range that has been previously reported in other plants [54]. Root treatment with a common
383 AQP inhibitor (azide) suggested that AQP contribute up to 84% to the Lpr in pearl millet (S1
384 Table). This figure is higher than what has been observed in Arabidopsis (57 to 64%) [55] and
385 rice (42 to 79%) [56]. However, as complete reversion of Lpr inhibition by azide could be
386 observed (S1 Fig), we do not think AQP contribution might have been over-estimated due to

387 azide secondary effects. Hence, our results suggest that PgAQP are major regulators of root
388 water flow in pearl millet.

389 We used the pearl millet genome sequence to characterize the AQP gene family and
390 identified thirty-three putatively functional AQP isoforms (based on conserved domains and
391 protein topology) that belonged to the PIP (10), TIP (9), SIP (3) and NIP (11) families. No XIP
392 were identified in pearl millet which confirm the absence of isoforms from this family in the
393 monocotyledon clade [8]. The number of identified AQP in pearl millet is similar to what has
394 been observed in *A. thaliana* (35) [57], rice (33) [22] and maize (31) [19]. Interestingly, AQP
395 genes were over-represented on Chromosome 3 with fourteen genes (Fig 3). *PgPIP2-5* and
396 *PgPIP2-6* closely located in Chromosome 3 are phylogenetically related and possess similar
397 gene structure (Fig 3 and S2 Fig) suggesting possible tandem duplication events in this region
398 [58]. Similarly, *PgNIP3-2* and *PgNIP3-3* located on Chromosome 4 may be the result of
399 duplication events. Furthermore, *PgPIP2-7* was identified in scaffold763 suggesting that genes
400 may be missing on parts of the pearl millet genome assembly.

401 The selectivity of plant AQP is defined by amino-acids structuring the pore that
402 constitute their signatures. Among these amino-acids, the NPA motifs on loops B and E
403 contribute with the dipole moment of the α -helices to prevent proton permeation [59,60].
404 These motifs were strictly conserved in the PgPIP but showed some polymorphisms for other
405 isoforms (Table 2). However, these substitutions did not drastically change the positive
406 electrostatic potential at the NPA motifs suggesting that proton exclusion from AQP pore is
407 conserved in pearl millet. Furthermore, the Ile preceding the Froger's residue P4 and P5 at the
408 end of Loop E, shown to be essential for CO₂ transport in PIP [61], is conserved in the PgPIP
409 (Fig 4).

410 The ar/R motifs, composed of four amino-acids forming a constriction at the
411 extracytosolic entry of the pore, represent the main selectivity filter. Modelling approaches
412 based on ar/R signatures were used to predict permeability of plant AQP [12,13]. For instance,
413 the F-H-T-R signature observed in the PgPIP (Table 2) which seems strictly conserved across
414 PIP from different species has been associated with water and H₂O₂ permeability
415 [14,25,26,62]. The H-I-G-R signature observed in PgTIP2-1, PgTIP2-2 and PgTIP2-3 that is
416 conserved in TIP2 from Arabidopsis, maize and rice supposedly allow permeability to water,
417 NH₃, urea and H₂O₂ while the H-I-A-V signature observed in the unique PgTIP1 (PgTIP1-1) may
418 allow NH₃, urea and H₂O₂ but restrict water permeation [63–65]. In PgNIP, the W-V-A-R
419 signature have been associated with water, NH₃ and H₂O₂ transports while the A-I/A-G/A-R
420 signatures were restricted to water and NH₃. Interestingly, PgNIP2-1 and PgNIP2-2 showed
421 similar ar/R signature (G-S-G-R) than OsNIP2-1 that is permeable to silicon [18] and possess
422 precise spacing of 108 amino-acids between the two NPA motifs supposed as essential for
423 silicon permeability [66].

424 AQP in plants are expressed from root to leaf tissues, including inflorescence and
425 pollen. Some PgPIP genes show tissue-specific expression with *PgPIP1-4* and *PgPIP2-3* being
426 more specifically expressed in roots while *PgPIP2-1* is more specifically expressed in shoots
427 and *PgPIP1-1* and *PgPIP1-3* are expressed in both tissues (Fig 5 and S7 Fig). It has been shown
428 that PIP isoforms agglomerate as tetramers in the plasma membrane, each monomer forming
429 functional units. Functional studies as well as protein-protein interactions studies suggest that
430 PIP tetramers can be formed of heteromers of PIP1 and PIP2 with distinct functional
431 properties depending on the isoform combination [67–70]. Based on PgPIP gene expression
432 in pearl millet, PgPIP1-1, PgPIP1-3 and PgPIP1-4 might interact with PgPIP2-3 to form

433 heteromers in roots while PgPIP1-1, PgPIP1-3 and PgPIP2-1 might form different combinations
434 of heteromers in shoots.

435 Intraspecific diversity in AQP isoform expression have been observed in rice and
436 Arabidopsis [55,56,71,72]. In our study, diversity in expression pattern of PgAQP genes were
437 observed between two pearl millet inbred lines with different water use strategies (Fig 5).
438 IP4952 that showed significantly higher AQP contribution to Lpr as compared to IP17150 also
439 showed significantly higher *PgPIP1-3* and *PgPIP1-4* gene expression. These results suggest that
440 differences in expression of these AQP genes can reflect differences in AQP contribution to
441 Lpr in pearl millet. These observations are in line with results from [34] showing that the
442 expression of *VvPIP1-1* is associated to root hydraulics and response to water stress in two
443 isohydric and anisohydric grapevine (*Vitis vinifera*) cultivars. Transpiration response to high
444 VPD in four pearl millet recombinant inbred lines from a high resolution cross have been linked
445 to PgPIP gene expression in roots [30]. These authors suggested that a down-regulation of
446 PgPIP genes under high VPD induced reduction in transpiration and water savings. Our results
447 showing reduced expression of *PgPIP1-3* and *PgPIP1-4* and AQP contribution to Lpr in IP17150,
448 the line with higher water use efficiency, support the observations of [30]. Overall, expression
449 profiling suggests that APQ may have different physiological functions across the pearl millet
450 plant and contribute to its response to the environment. However, expression alone is
451 certainly not fully representative of AQP function due to the many post-translational
452 regulations affecting their activity [8]. Further investigations are needed to better understand
453 the links between reduction in transpiration under high VPD, improved water use efficiency
454 and AQP function in roots.

455 Pearl millet is a drought-adapted crop that will play a major role in the adaptation of
456 agriculture to future climate in arid and semi-arid regions of Africa and India. Here, we provide

457 a comprehensive view of the AQP genes and isoforms present in pearl millet as well as their
458 contribution in root radial water transport. We confirmed the presence of selectivity filters
459 suggesting permeability to water in the PgAQP and point isoforms PgPIP1-3 and PgPIP1-4 as
460 potential main contributors of root water transport in pearl millet. The function of these
461 isoforms may be subjected to natural diversity and associated with plant water use strategies
462 as suggested by their differential expression in pearl millet lines contrasting for Lpr and water
463 use efficiency. Therefore, our study supports a potential role for AQP in regulating pearl millet
464 hydraulics and potentially adaptation to challenging environmental conditions.

465

466 **Declaration of competing interest**

467 No conflicts of interest declared

468

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482

483 **Author contributions**

484 AG, CTD and LL designed the study. AG, PA, CTD, CFC and CM performed the experiments. AG,
485 CTD, PA, CFC, CM, PG, VV, YV and LL analyzed the data and discussed the results. AG and LL
486 wrote the paper. All authors read and approved the manuscript.

487

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712 **Supporting information**

713 **S1 Table. Root hydraulic conductivity (Lpr) and aquaporin (AQP) contribution in IP4952 and**

714 **IP17150.**

715 **S2 Table. High scoring pairs with highest bit score at the fifty hot-spots.**

716 **S3 Table. Functional annotation of the pearl millet genomic regions corresponding to**
717 **hotspots of High Scoring Pairs.**

718 **S4 Table. Primers used for genomic DNA (gDNA) or complementary DNA (cDNA)**
719 **amplification of aquaporins showing missing sequence.**

720 **S5 Table. Primers used for quantitative RT-PCR.**

721 **S6 Table. Analysis of aquaporin conserved domains in pearl millet.**

722 **S7 Table. Transmembrane domain analysis of aquaporins in pearl millet.**

723 **S1 Fig. Reversion of azide-induced root hydraulic conductivity inhibition.**

724 **S2 Fig. Structure of pearl millet aquaporins genes.**

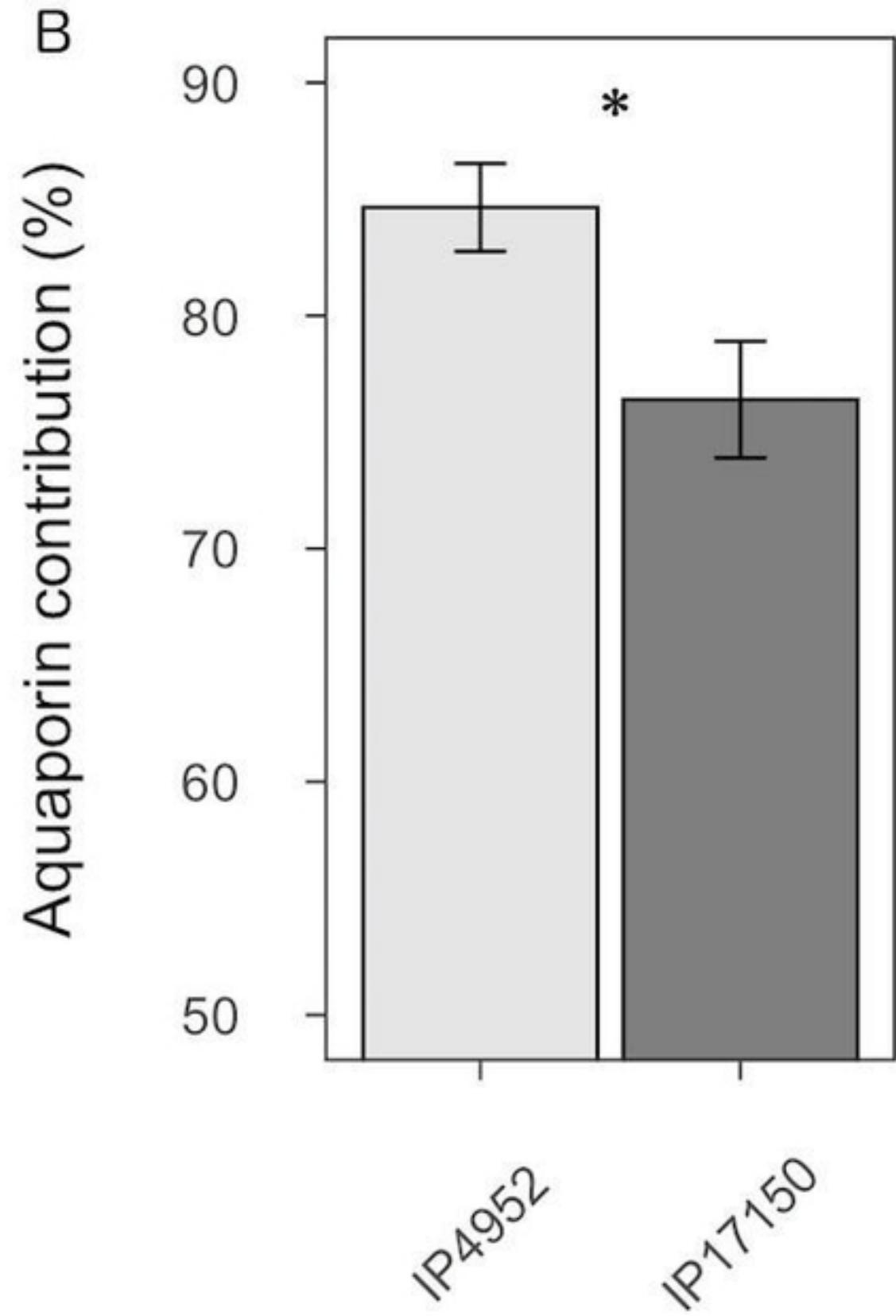
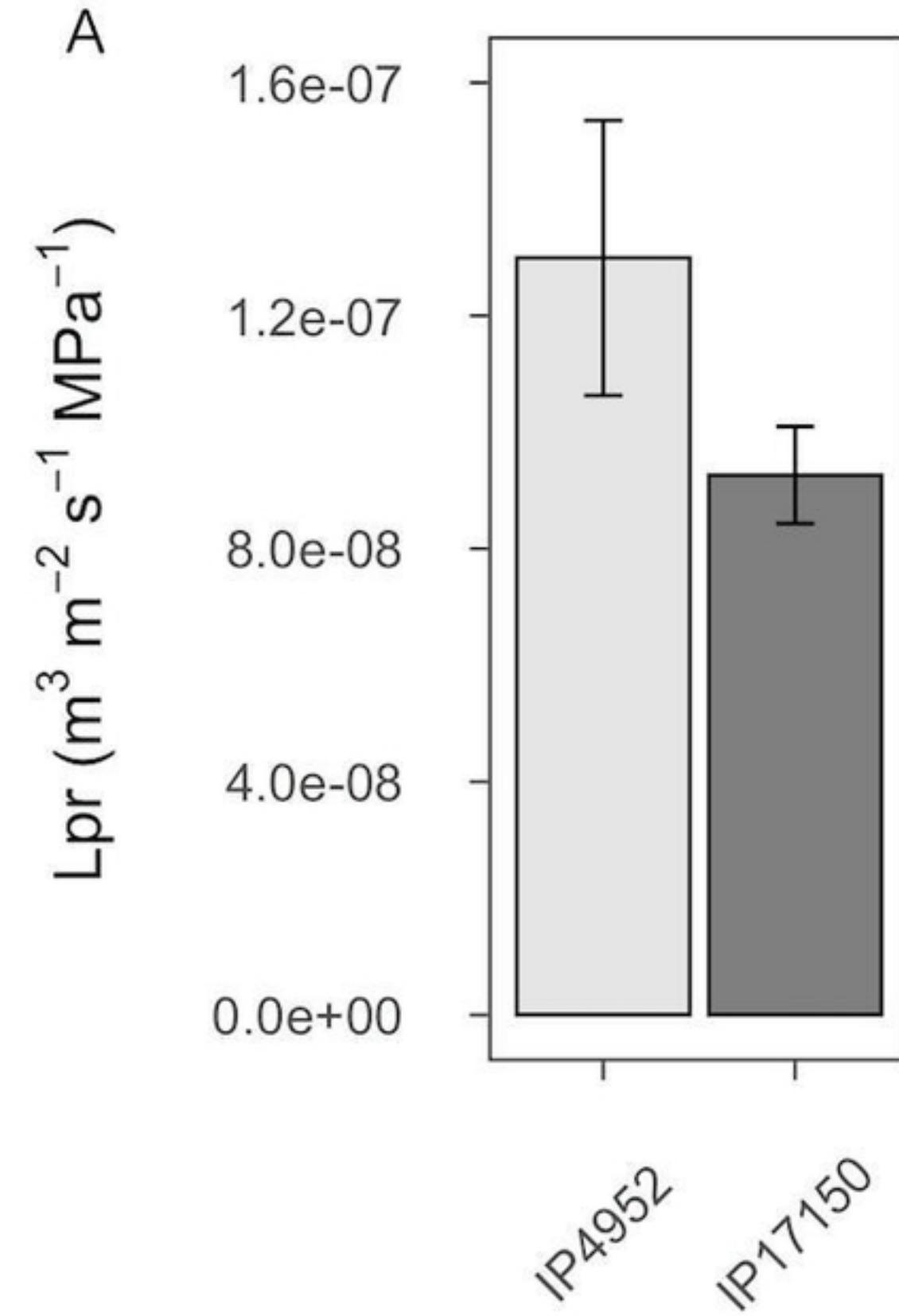
725 **S3 Fig. Conserved domains and membrane topology of the TIP isoforms from pearl millet.**

726 **S4 Fig. Conserved domains and membrane topology of the NIP isoforms from pearl millet.**

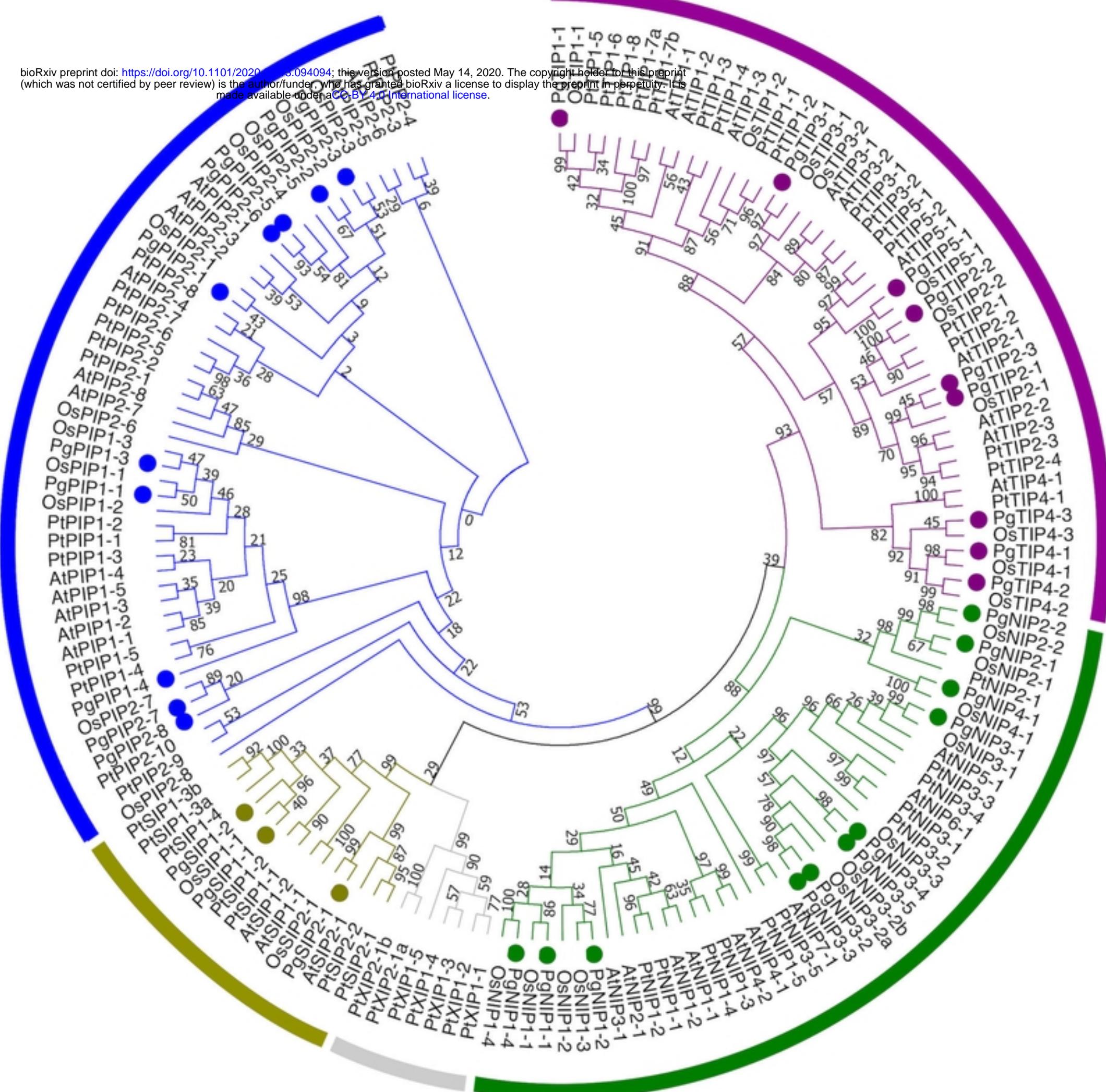
727 **S5 Fig. Conserved domains and membrane topology of the SIP isoforms from pearl millet.**

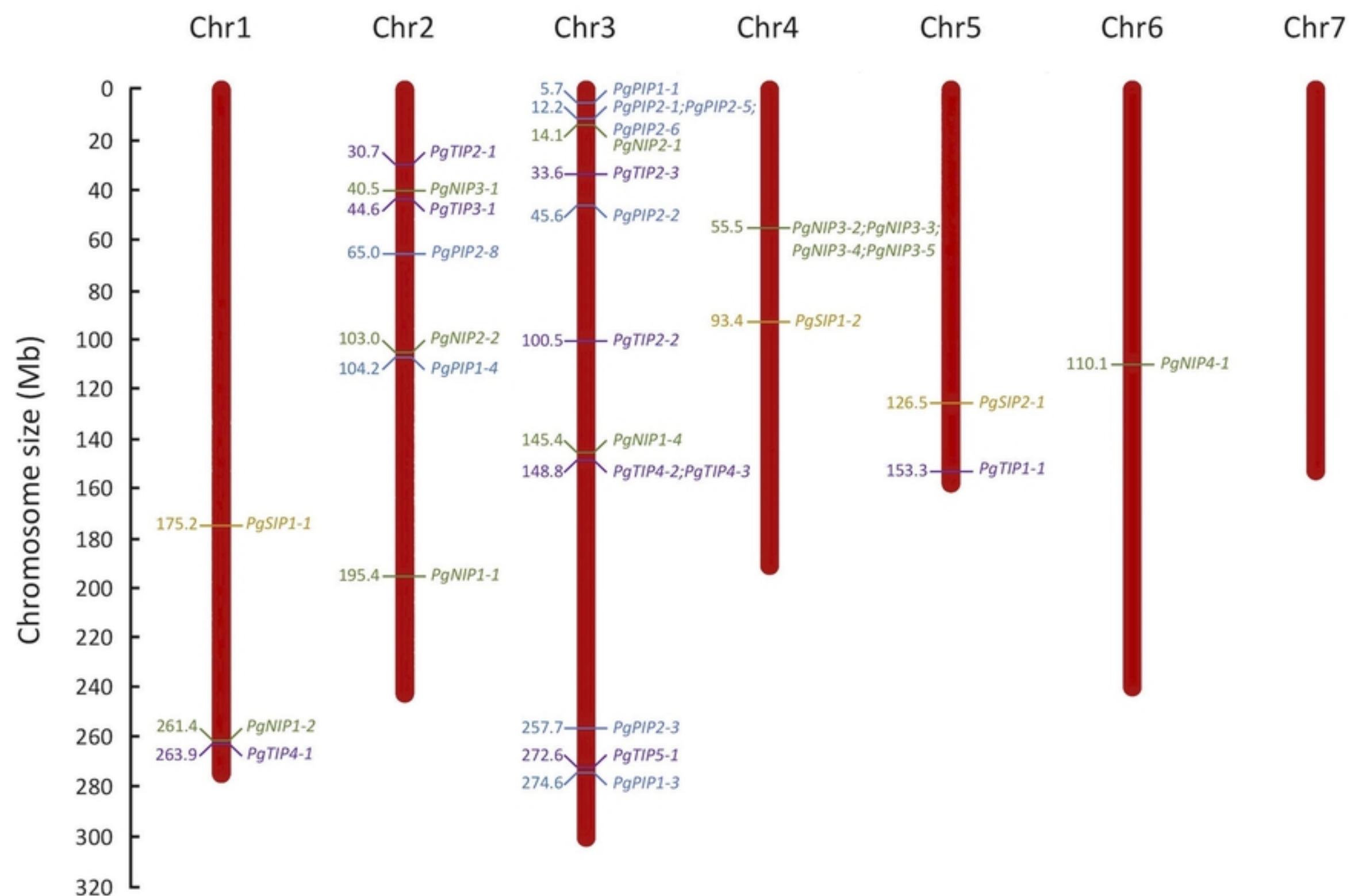
728 **S6 Fig. Cavity features of PgPIP isoforms.**

729 **S7 Fig. Expression pattern of PgPIP isoforms in shoots of pearl millet.**

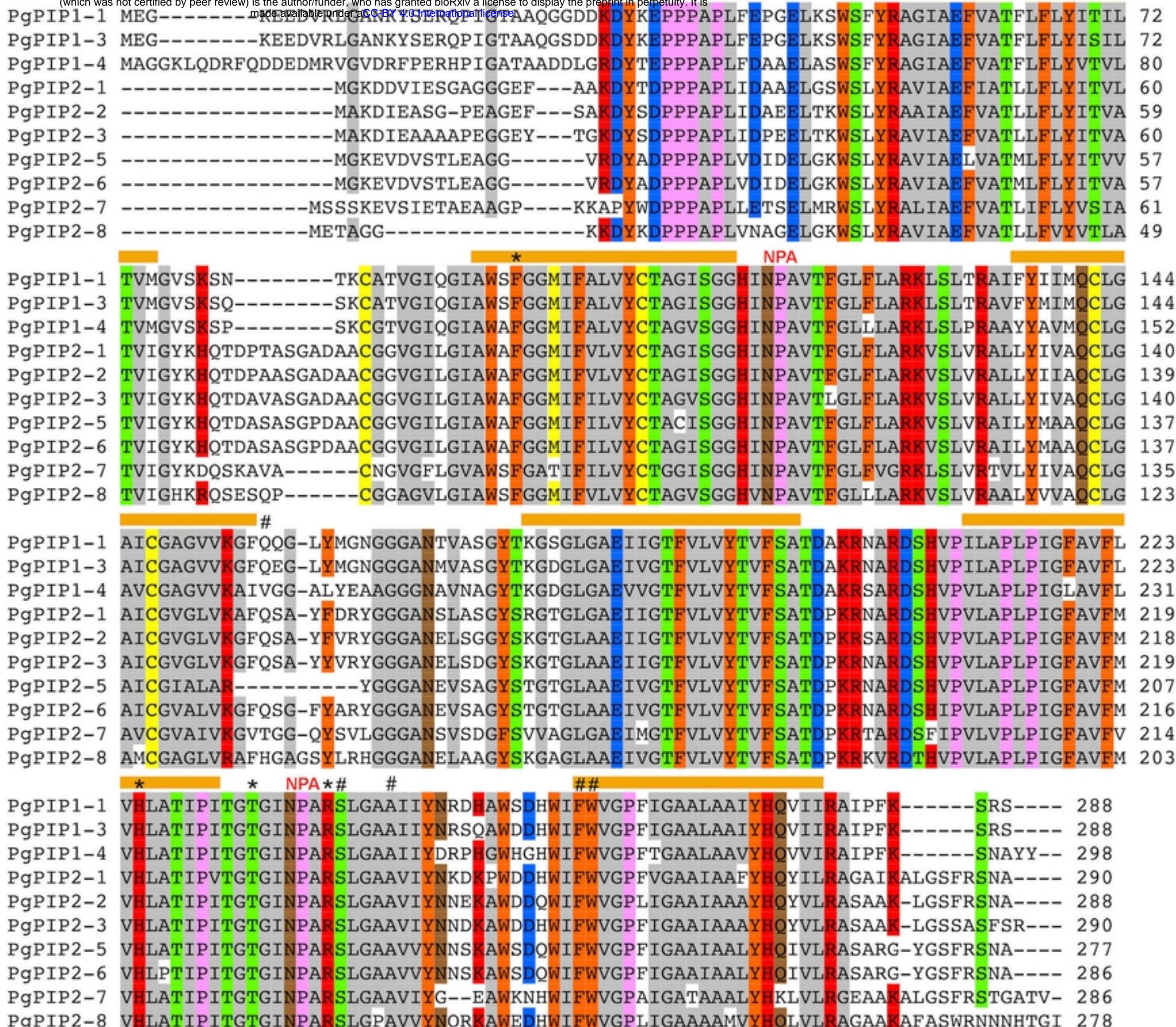


Figure

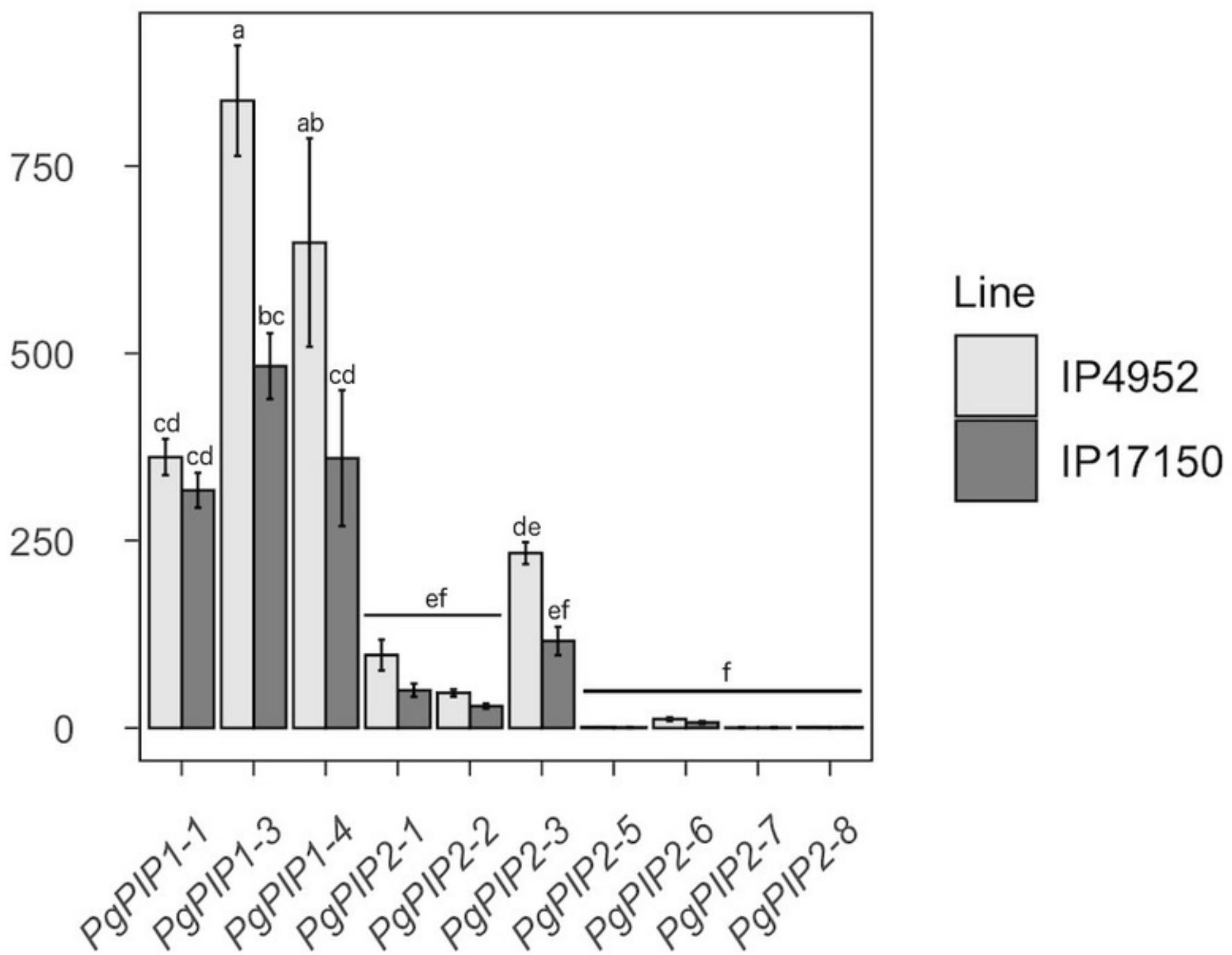




Figure



Relative expression



Figure