Original research paper

The *promoted lateral root 1* (*plr1*) mutation is involved in reduced basal shoot starch accumulation and increased root sugars for enhanced lateral root growth in rice

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ABSTRACT

Lateral roots (LRs) are indispensable for plant growth, adaptability and productivity. We previously reported a rice mutant, exhibiting a high density of thick and long LRs (L-type LRs) with long parental roots and herein referred to as *promoted lateral root1* (*plr1*). In this study, we describe that the mutant exhibited decreased basal shoot starch accumulation, suggesting that carbohydrates might regulate the mutant root phenotype. Further analysis revealed that *plr1* mutation gene regulated reduced starch accumulation resulting in increased root sugars for the regulation of promoted LR development. This was supported by the exogenous glucose application that promoted L-type LRs. Moreover, nitrogen (N) application was found to reduce basal shoot starch accumulation in both *plr1* mutant and wild-type seedlings, which was due to the repressed expression of starch biosynthesis genes. However, unlike the wild-type that responded to N treatment only at seedling stage, the *plr1* mutant regulated LR development under low to increasing N levels, both at seedling and higher growth stages. These results suggest that *plr1* mutation gene is involved in reduced basal shoot starch accumulation and increased root sugar level for the promotion of L-type LR development, and thus would be very useful in improving rice root architecture.

Keywords: Rice, lateral root, mutant, carbohydrates, root system architecture, starch accumulation

1. Introduction

26 The root system architecture of rice is determined by several root components,
27 including the embryonic seminal root, post-embryonic crown roots, and lateral roots (LRs)
28 whose overall growth angle, elongation, and branching through LR formation [1–2] reflects
29 its structure and spatial configuration for the absorption of soil resources. Extensive studies
30 on the importance of root system architectural traits in rice have been carried out in rainfed
31 ecosystems (lowland and upland) that comprised 40% of the global rice production area [3],
32 where rice plants were constrained by several abiotic stresses such as drought, soil moisture
33 fluctuations, and low soil fertility. This led to reduced shoot dry matter production, root
34 growth, and yield during drought periods [4], and thus production is not surprisingly low [3].
35 This occurrence can potentially be further aggravated over time, given recent challenges in
36 global climatic changes, which were predicted to cause long-term warming patterns and
37 extreme temperature changes [5]. To address these problems, the development of an enhanced
38 root system, especially promoted LRs, can help maintain root system function [6–8], shoot
39 dry matter production [9] and yield [10] through sustained water uptake and to some extent,
40 nutrient uptake. Therefore, the development of rice varieties with a highly branched root
41 system under drought and adverse soil conditions is a rational approach to improve
42 productivity and enhance food security.

43 Morphologically, rice LRs are classified into two distinct types: the S-type (thin in
44 diameter and short), and the L-type, (thick in diameter and long) [11–12]. L-type LRs are
45 important for the entire root system expansion, owing to its ability to form a higher order of
46 branching, and thus determines the plant's ability to exploit soil resources. Furthermore, LRs,
47 especially the L-type, manifest a promoted response when subjected to environmental
48 conditions, compared to parental roots [13]. To improve root system architecture in rice,
49 L-type LR formation is, therefore, necessary. However, despite the notable importance of
50 promoted L-type LRs in plant productivity and stress adaptability, their mechanisms and

51 genetic determinants remain largely unknown, thus limiting their application as a breeding
52 index. Additionally, because extensive root formation studies in rice mutant lines revealed
53 specific defects in seminal roots, crown roots, or S-type LRs, root studies were focused on
54 these root components [14]. Our previous paper reported a newly identified rice mutant line,
55 11NB10, which exhibits a high number of L-type LRs with enhanced parent roots [15], herein
56 referred to as *promoted lateral root 1* (*plr1*) mutant. Interestingly, although root growth in the
57 wild-type remained unchanged under different soil nitrogen (N) conditions, the *plr1* mutant
58 root system was further enhanced at high N levels, based on its total root length, which was
59 greatly contributed to by L-type LRs. With timely and optimized N application, this gene
60 mutation can be exploited to further improve the development of L-type LRs with a more
61 vigorous root system that, as demonstrated in several studies, can withstand environmental
62 stress conditions such as drought and soil moisture fluctuations [4].
63 Photosynthates are transported to root sinks in the form of sucrose (glucose + fructose),
64 which, together with glucose, promoted LR development in Arabidopsis [16] and maize
65 [17,18]. Furthermore, carbohydrate allocation in Arabidopsis roots is affected by nutrient
66 supply. While starch is stored in the shoot under N-deficiency conditions, this does not occur
67 under N-supplied conditions, corresponding to promoted and reduced root system growth as
68 well as root-to-shoot biomass ratio [19]. In cereals, the basal shoot serves to buffer
69 source-sink interactions during different stages of growth and under varying environmental
70 conditions [20]. In the present study, we investigated the mechanisms of the *plr1* mutant that
71 could induce L-type LR formation under applied-N conditions, through carbohydrate
72 allocation.
73
74 **2. Materials and Methods**

75 Two separate, rice-based experiments were set up according to plant growth stages, in
76 order to analyse several parameters. Experiment 1 was conducted at the seedling stage and
77 Experiment 2 was performed at the higher plant growth stage.

78 2.1. Experiment 1: Seedling stage

79 *2.1.1 Plant materials and growth conditions*

80 Before sowing, seeds of the wild-type, Nipponbare, and *plr1* mutants were
81 pre-germinated in water mixed with fungicide (benomyl benlate, 0.15 % w/v) and incubated
82 in a chamber (LH-241SP, Biotron, Osaka, Japan) for 3 d. After 2 d of sowing, a gentle
83 aeration was supplied using an aerator placed inside the chamber under the following
84 conditions: 28 °C/ 23 °C (maximum/minimum temperature), light (06:00 am – 06:00 pm) and
85 dark (06:00 pm – 06:00 am), at 350 μ mol^{-1} m^{-2} s^{-1} light intensity (PPFD) and 70 % humidity.

86 To test the effects of sugars on mutant root growth specifically on the type of LRs,
87 germinating seeds were treated with various concentrations of glucose (Wako Chemicals,
88 Tokyo Japan) that include 0, 0.05, 0.5 and 1.0 mM concentrations, and were cultured for 10 d
89 under similar chamber conditions as above. Exogenous glucose treatment is generally applied
90 as a carbon source in culture media for in *vitro* growth stimulation and is directly absorbed by
91 plant cells from the extracellular space (plasma membrane), moving from cell to cell through
92 plasmodesmata (symplastic loading) [21, 22]. Exogenous glucose treatment to the roots has
93 been reported to have positive effects on root growth in several studies in rice [23, 24] and in
94 Arabidopsis [25].

95 To investigate whether the mutation gene first affected basal shoot starch
96 accumulation or root growth, seedlings were grown for 6 d in tap water, and the seminal root
97 region of non-emerged LRs and newly emerging crown roots were excised for 8 d of growth.

98 To evaluate the wild-type and *plr1* mutant under N conditions, eight-day-old seedlings
99 were grown using 1/10- strength of the nutrient solution described by Colmer (2003) [26], to

which different N levels were provided in the solution: 0, 20, 40, and 80 % N, which received reduction of N to 0%, 20%, 40% and 80% N, respectively. The nutrient solution contents and the adjusted N supplied for the different N levels are presented in Supplementary Table 1. The solution pH was systematically maintained at 6.5, using 0.5 % HCl and 1M KOH. Seedlings were cultured under similar conditions as described above, in the same chamber.

2.1.2. Morphological characterization of plr1 mutant at the seedling stage

The seminal root and shoot length was measured manually using a ruler. The LRs along the seminal root were classified into different types and counted using a microscope. LRs were classified into two types: S-type, which are short, slender, and non-branching but normally numerous, and L-type, which are generally long and thick, and capable of a higher order of branching [11–12]. LR density was computed as the ratio of LR number and seminal root.

Following manual measurements, root samples were scanned at 720 dpi using the EPSON 10000X A3 size scanner (Seiko Epson Corporation, USA). Scanned images were analyzed for root lengths using WinRHIZO v. 2007d (Régent Instruments, Québec, Canada) at a pixel threshold value set at 175. LR lengths with a thin diameter were described as $0 < D \leq 0.08$ mm, while those with a thick diameter were $0.08 < D \leq 0.30$ mm.

2.1.3. Basal shoot starch staining and internal sugar measurement

Ten-day-old *plr1* mutant and wild-type seedlings, germinated and grown in tap water under chamber conditions, were subjected to starch staining. The basal part of the shoot was gently pressed with a hammer and starch was stained using a solution containing 2 g I and 5% KI_2 (IKI solution) for 20 min [27]. The excess stain was then gently rinsed with water for 2 min, and photographs were captured.

125 Sampling for internal sugar measurements was carried out on ice. Basal shoot and
126 seminal roots were excised from seedlings. For basal shoot sampling, tissues were excised
127 from the shoot base up to the junction before leaf blade. Basal shoot tissues were the first
128 ones to be sampled and were the quickest to sample (approximately 1~2 min each genotype
129 tissue set) as compared to the root samples. The seminal roots were further divided into two
130 zones representing stages of LR development: with emerged and non-emerged LRs. The zone
131 with emerged LRs was comprised of emerged and elongated LRs, while the zone without LRs
132 referred to the early stage of LR development up to the apical root zone. 50 mg (fresh weight)
133 of each desired tissue was transferred into a 2-mL tube, immediately frozen in liquid N, and
134 stored at –80 °C until further analysis. For the diurnal experiment, seedlings were harvested
135 successively at 06:00 am, 12:00 noon, 06:00 pm and 12:00 midnight. Sampling during the
136 dark period was conducted using foil as a cover to eliminate light effect.

137 Tissue samples were crushed using a multi-bead shocker (Yasui Kikai Corporation,
138 Osaka, Japan) at 2000 rpm for 3 min. The contents of glucose, sucrose, starch and total
139 non-structural carbohydrate (NSC) of the root and leaf sheath segments were determined
140 following the method described by Sugiura et al. (2015) [28] with modifications. Briefly,
141 soluble sugars were extracted with 750 μL and 500 μL of 80 % ethanol at 78 °C successively,
142 and the precipitate was used for starch determination. The supernatant was concentrated
143 centrifugally for 2 h using a concentrator (Eppendorf AG, Hamburg, Germany). Up to 150 μL
144 each of distilled water and chloroform were added to the concentrated supernatant, in order to
145 separate sugars from soluble proteins. After centrifugation, the upper clear water phase was
146 used to determine glucose and sucrose content. Up to 200 μL of the water was incubated with
147 10 μL of invertase solution (Wako Chemicals, Tokyo, Japan) for 30 min at 25–28 °C (room
148 temperature), in order to break down sucrose into glucose and fructose. On the other hand, the
149 precipitate reserved for starch determination was mixed with 200 μL of distilled water and

150 boiled at 98 °C for 1 h, followed by treatment with 200 μM amyloglucosidase (A-9228;
151 Sigma-Aldrich, St. Louis, MO) in a 50 mM Na-acetate buffer solution (pH 4.5) at 55 °C for 1 h.
152 Finally, the glucose content and glucose equivalents of sucrose and starch were quantified
153 using a Glucose CII test kit (Wako Chemicals, Osaka, Japan). A microplate spectrophotometer
154 (Biotek EPOCH2, USA) was used to measure absorbance at 505 nm.

155

156 *2.1.4 RNA extraction and gene expression*

157 To check the gene expressions of stem starch synthesis-related genes under N
158 conditions, total RNA was extracted from frozen tissues collected from the basal region of the
159 stem using Macherey-Nagel NucleoSpin® RNA Plant kit according to the manufacturer's
160 instructions. RNA quantity was checked by optical density at 260 nm and 25 ng total RNA
161 was used as a template to measure transcript levels. A quantitative real-time PCR (qRT-PCR)
162 was carried out using the One-Step SYBR PrimeScript RT-PCR Kit II (Perfect Real Time)
163 (Takara, Bio) with a StepOnePlus Real-Time PCR (Life Technologies) for 40 cycles of 95°C
164 for 1 min, 60°C for 1 min and 60°C for 1 min. A set of genes for starch synthesis
165 predominantly expressed in the stem as described previously [29] was used and the target
166 gene expression was normalized to the expression level of *Actin1* as internal control. The
167 primers used are presented in Supplementary Table 1.

168

169 2.2. Experiment 2: Higher plant growth stage (65-day-old plants)

170 *2.2.1. Plant handling and growth conditions*

171 To evaluate the *plr1* mutant and wild-type at a higher growth stage under different N
172 conditions, eight-day-old seedlings grown in tap water were transferred to seedling plates
173 containing soil pre-mixed with fertilizer. The seedling plates were placed in the field for 52 d
174 (maximum tillering stage of the plants). Plants with only the main stem (i.e., no tillers) were

175 then selected and the roots were gently washed. Old roots were tied 1 cm from the base to
176 separate new emerging crown roots from the main stem during the treatment. The plants were
177 randomly placed in foam blocks allocated to black 9-L buckets containing nutrient solution at
178 different N levels (0, 25, 50, and 100 % N of full-strength nutrient solution, similar to
179 Experiment 1) under greenhouse conditions for 5 days. Similarly, the solution pH was
180 systematically maintained at 6.5, using 0.5% HCl and 1M KOH. To monitor the effectivity of
181 N treatments, relative chlorophyll content was measured daily using a chlorophyll meter
182 (SPAD-502Plus, Konica Minolta) and expressed as SPAD value.

183 *2.2.2. Plant sampling and processing*

184 Leaf photosynthesis and stomatal conductance of second youngest fully-expanded
185 leaves were measured using a portable photosynthesis system (LI-6400XTP/S, LiCOR Inc.,
186 Lincoln, Nebraska, USA) 5 d after treatment, i.e., on the last day of the experiment. The
187 chamber settings for measurements include the following: flow- 500 μ ms, carbon dioxide
188 concentration- 400 mmol, light intensity-1200 μ m and temperature was set at the temperature
189 at that time (32°C). Next, leaf sheaths numbered third from the newest leaf were harvested for
190 starch staining since the stem starch accumulation at this growth stage of rice plant is high [30]
191 and thus obvious starch stain levels could be obtained. The entire leaf sheaths were gently
192 pressed with a hammer and stained with IKI solution following the method described by
193 Togari and Sato (1954) [27], and images were captured using a digital camera. The percentage
194 stain was calculated as the ratio of the length of the stain to the total leaf sheath length.
195 Thereafter, new crown roots that were generated during the treatment period and separated
196 from the old roots through the tie were collected. Three new crown roots of similar length and
197 relatively the same age (distinguished by the presence of emerged and elongated LRs) per
198 plant were randomly selected for morphological measurements and root length analysis,
199 following the same method used for the seedling stage.

200

201 2.3. Data analysis

202 Shoot and root characteristics were compared using either the Student's *t*-test or
203 analysis of variance (ANOVA), as well as a multiple-comparison LSD's test using the
204 Statistical Tool for Agricultural Research (STAR) software version 2.0.1. developed by IRRI
205 [31].

206

207 **3. Results**

208 *3.1. Morphological characterization of the plr1 mutant*

209 While the majority of studied mutant lines for root analysis were identified based on
210 specific root component defects [1, 14, 32], the isolated 11NB10 mutant line exhibited
211 enhanced development both in LRs and in parental roots (i.e., in seminal and crown roots)
212 [15]. Compared to wild-type seedlings, seminal root length was 20 % longer (Fig. 1A–B and
213 F) and L-type LR density was greater than double, from eight days after germination (DAG)
214 (Fig. 1H) in 11NB10 mutant seedlings, which were consequently named as *promoted lateral*
215 *root 1* or *plr1* mutants. Other root component traits in the *plr1* mutant, such as total LR and
216 S-type LR density, were either slightly lower or comparable to the wild-type (Fig. 1G and I).
217 Additionally, the shoot length of the mutant plant was longer than that of the wild-type plant
218 from six DAG (Fig. 1A–B and E).

219 To further analyze shoot growth and yield patterns in *plr1* mutants, we transferred the
220 mutant seedlings to pots containing soil and allowed them to grow until maturity, under
221 greenhouse conditions. The aerial parts of mutant plants were able to grow vigorously (Fig. 1J
222 and K), reaching a significantly greater height than the wild-type (Fig. 1K, L and M);
223 however, tiller number was significantly reduced (Fig. 1L). Despite *plr1* mutant plants
224 generating a low number of tillers, almost all of the latter bore panicles, resulting in a

225 comparable number of panicles (Fig. 1N) to wild-type plants. However, *plr1* mutant panicles
226 produced a higher number of spikelets (Fig. 1O).
227
228 *3.2. plr1 mutation affected carbohydrate metabolism and allocation*
229 Starch staining was carried out at the basal shoot of ten-day-old seedlings using IKI
230 solution. The degree of starch staining in the *plr1* mutant was lower than in the wild-type,
231 which had accumulated more starch as indicated by the dark purple stain (Fig. 2A and B).
232 This suggested the possible transport of sugar to the roots for LR development, supported by
233 our internal carbohydrate analysis, which confirmed that starch concentration in the basal
234 shoot was indeed significantly lower in *plr1* mutant seedlings than in wild-type seedlings (Fig.
235 2C). Additionally, the concentration of the other carbohydrate forms such as sucrose and the
236 total non-structural carbohydrate (NSC) in the mutant basal shoot were similar to that in the
237 wild-type basal shoot (Fig. 2E and F). By contrast, glucose concentration was significantly
238 higher in the *plr1* mutant basal shoot (Fig. 2D) than in the wild-type basal shoot, which likely
239 suggests that the *plr1* mutant could not utilize glucose (the building units of starch) to
240 synthesize starch in the basal leaf sheath, and that these sugars were subsequently transported
241 to the roots.
242 As previously mentioned, carbohydrates are translocated as sucrose and their
243 metabolism is important in development processes, depending on cell type and developmental
244 stage. To test this hypothesis in roots, we segmented whole seminal roots into two distinct
245 zones representing stages of LR development: zone of emerged and elongated LRs (Fig. 2G)
246 and zone of non-emerged LRs (Fig. 2H), and examined carbohydrate distribution in these
247 zones. *plr1* mutant roots possessed significantly higher sucrose and glucose concentrations
248 limited to the zone of non-emerged LRs, as compared to wild-type roots (Fig. 2G and H),
249 suggesting that sugars were necessary for the early LR development stage, such as L-type LR

250 primordium formation in *plr1* mutants. Furthermore, the difference between *plr1* mutant and
251 wild-type sugar concentrations in the roots was more pronounced in glucose than sucrose,
252 whereas starch concentration was comparable (Fig. 2H).

253 Carbohydrates in cereals are temporarily stored as starch in sink tissues such as stem
254 and leaf sheaths [20]. Thus, sampling at different time points over a one-day period, under
255 12-h light (06:00 am – 12:00 noon) and 12-h dark (06:00 pm – 12:00 midnight) conditions,
256 was carried out to investigate in detail the diurnal patterns of carbohydrate distribution and
257 accumulation in the basal parts of the shoot and seminal root regions of non-emerged LRs
258 (Supplementary Fig. 1). Significantly lower starch levels (Supplementary Fig. 1C) and higher
259 glucose levels (Supplementary Fig. 1A) were consistently recorded in the basal shoot of *plr1*
260 mutant seedlings, regardless of the time of sampling, compared to wild-type seedlings,
261 confirming the likely inability of *plr1* mutants to convert glucose into starch, resulting in low
262 basal shoot starch accumulation. Other carbohydrate concentrations in *plr1* mutant basal
263 shoots, such as sucrose and total NSC, were comparable to those of wild-type basal shoots
264 (Supplementary Fig. 1B and D). On the other hand, *plr1* mutant roots produced significantly
265 higher levels of glucose and total NSC at different time points (Supplementary Fig. 1E and H),
266 while sucrose levels were significantly higher only at 6 h after exposure to light, in
267 comparison to wild-type roots (Supplementary Fig. 1F), suggesting that glucose likely exerted
268 a greater effect on LR growth. Conversely to these carbohydrate forms, starch levels in *plr1*
269 mutant roots were comparable to those in wild-type roots (Supplementary Fig. 1G). From
270 these observations, the sampling time point (at 6 h after exposure to light) that corresponded
271 to the lowest basal shoot starch and highest root sugar concentrations was consistently used in
272 our prior and all subsequent experiments.

273

274 *3.3. plr1 mutation regulated basal shoot starch accumulation and then LR growth*

275 The *plr1* mutation affected both LR growth (Fig. 1A-D and H) and basal shoot starch
276 accumulation (Fig. 2A–C). To determine which of the two is regulated by this mutation first,
277 excision of the seminal root region with non-emerged LRs and newly emerged crown roots
278 was performed in seedlings, in order to restrict root growth and assess the effects on starch
279 accumulation in basal shoots (Fig. 2I and J). Compared to basal shoot starch level in
280 wild-type seedlings with intact roots (i.e., with no excised roots), the level in wild-type
281 seedlings with excised roots was significantly higher (Fig. 2J). Although the same tendency
282 was observed in *plr1* mutants, starch levels in mutant seedlings with excised roots were
283 clearly lower and did not reach similar levels those of wild-type seedlings with intact roots
284 (Fig. 2J). These results indicate that the gene mutation affected lower starch accumulation in
285 the basal shoot, which subsequently affected promoted root growth, via sugar transport to the
286 roots.
287
288 *3.4. Effect of glucose and nitrogen on wild-type and plr1 mutant seedlings*
289 As reported earlier, *plr1* mutant roots contained significantly higher glucose
290 concentrations compared to wild-type roots (Fig. 2H), suggesting that glucose was important
291 for L-type LR primordium formation. Therefore, *plr1* mutant and wild-type seedlings were
292 exogenously treated with glucose. To avoid the possibility of osmotic stress that could be
293 elicited by glucose, we treated a glucose concentration range of 0-1 mM since it was reported
294 that 88 mM sugar concentration supplied in MS media serves as a main carbon source for
295 growth and development [33, 34] whereas 111-444 mM sugar concentration serves as osmotic
296 regulation [35]. We showed that the number of L-type LRs in *plr1* mutant seedlings increased
297 in a dose-responsive manner (Fig. 3A). Further, L-type LRs in wild-type seedlings were
298 observed at only the highest glucose concentration (1 mM), but were generated at almost the
299 same number as that of the *plr1* mutant (Fig. 3A). On the other hand, S-type LR density was

300 less responsive to the glucose treatments (Fig. 3B). These results indicate that transported
301 sugars, especially the glucose form of carbohydrates, are important and had positive effects
302 on L-type LR promotion and that this regulation is controlled by the *plr1* mutation, resulting
303 in the promoted root phenotype in the mutant.

304 N application was found to reduce starch accumulation in *Arabidopsis* shoots, in
305 contrast to shoots under N-deficiency conditions, where starch accumulated, resulting in
306 altered root growth [19]. Thus, we modified basal shoot starch accumulation in wild-type and
307 *plr1* mutant seedlings by manipulating N conditions and examined the effects on LR
308 formation. Wild-type seedlings reduced basal shoot starch accumulation (Fig. 3C) and
309 increased total LR length (Fig. 3F–H). L-type LR density generated on wild-type seminal root
310 was also significantly promoted by N treatment (Fig. 3I). Most notably, there were no clear
311 differences between the wild-type and *plr1* mutant on their starch concentrations in the basal
312 shoot, glucose concentrations in the seminal root, and L-type LR density under 80% N
313 treatment conditions (Fig. 3C–I). These results indicate that reduced basal shoot starch
314 accumulation was indeed important for increased root sugar levels in the regulation of L-type
315 LR formation.

316

317 *3.5. Correlation between starch biosynthesis transcripts and basal shoot starch levels*

318 Starch biosynthesis genes that were previously comprehensively analyzed in rice leaf
319 sheaths [29, 36, Fig. 4A] were used to check their expression levels in the basal shoot of *plr1*
320 and wild-type seedlings, in order to assess their association with basal shoot starch
321 accumulation. Compared to the wild-type, the expression levels of *BEIIa*, *GBSSI,* and *GBSSII*,
322 which encode starch branching (*BEIIa*) and granule-bound starch synthase (*GBSSI,* and
323 *GBSSII*) enzymes for accumulation in leaf sheaths [37], were lower in *plr1* mutants grown
324 without supplied N (Fig. 4D–F). This suggests that the low accumulation of basal shoot starch

325 in *plr1* mutants was caused by the lower levels of these starch synthesis enzymes. Notably,
326 N-supplied wild-type plants that presented with reduced basal shoot starch accumulation
327 compared to N-deficient plants exhibited a downregulation of the same genes (*BEIIa*, *GBSSI*,
328 and *GBSSII)*, as well as the genes encoding the small subunit ADP-glucose
329 pyrophosphorylase *(AGPSI)* and starch synthase *I* (*SSI*) (Fig. 4C–G). Conversely, transcript
330 levels of the genes encoding the large subunit ADP-glucose pyrophosphorylase *(AGPLI)* and
331 starch synthase (*SSIIb,* and *SSIIIb)* remained unchanged in N-supplied wild-type plants,
332 compared to levels in N-deficient wild-type plants (Fig. 4B, H–I). Furthermore, transcript
333 levels of almost all the examined genes, including *AGPLI*, *SSI,* and *SSIIIb*, were
334 downregulated in *plr1* mutants under applied-N conditions (Fig. 4). These results suggest that
335 starch accumulation in the basal shoot of *plr1* mutant and wild-type seedlings grown under N
336 conditions was low due to the repressed expression of genes essential in starch biosynthesis.
337
338 *3.6. Effect of starch reduction on older wild-type and plr1 mutant plants*
339 At the seedling stage, rice plants depend on a dual carbon source that includes the
340 maternal seed reserves as a renewable carbon source, which later switches on to
341 photosynthesis-derived carbon. To further examine and confirm the mechanism involved in
342 photosynthesis-derived carbon usage, *plr1* mutant and wild-type seedlings were grown until
343 the higher growth stage. Sixty-day-old plants initially grown under soil conditions and which
344 possessed only the main stem (i.e., no tillers) were selected. Their roots were gently washed
345 to remove the soil and older roots were tied (Fig. 6A) before subjecting them to different N
346 treatments under greenhouse conditions for 5 d.
347 Generally, the SPAD value, photosynthesis, and stomatal conductance of *plr1* mutant
348 and wild-type plants at different N levels were comparable (Fig. 5C–E). These physiological
349 traits, however, were lowered under N-deficient conditions and increased under N-supplied

350 conditions. On the other hand, a substantial difference in the level of basal shoot starch
351 (represented by the percentage stain in the 3rd leaf sheath numbered from the newest leaf, Fig.
352 5F) and LR morphology (Fig. 6A–E) was observed between *plr1* mutant and wild-type plants
353 at each N level. These results show that, under N-deficient conditions, *plr1* mutant plants
354 presented a significantly lower basal shoot starch level (30.8 %) compared to wild-type plants
355 (57.6 %), that was sharply and significantly reduced under treatments with N up to 1.6 % (Fig.
356 5F). Wild-type plants, on the other hand, displayed a relatively slight reduction in basal shoot
357 starch level under N conditions, with the minimum value being 33.6 % when treated with
358 50 % N (Fig. 5F).

359 Consistent with the reduced starch level in the shoot, promotion of the LR trait was
360 observed in the root. *plr1* mutant plants with lower basal shoot starch produced a significantly
361 higher number and longer L-type LRs than wild-type plants under N-deficient conditions (Fig.
362 6A–B and D–E). Under treatment with increasing N levels, where the starch level in *plr1*
363 mutant basal shoots was sharply reduced (Fig. 5F), mutant L-type LR traits were further
364 significantly enhanced (Fig. 6A–B and D–E). Although wild-type plants tended to promote
365 L-type LR traits under treatment with 25 % N, no further promotion was observed at higher N
366 levels (Fig. 6A–B and D–E). Thus, differences in L-type LR development between *plr1* and
367 wild-type plants became more pronounced with increasing N levels. On the other hand, the
368 number of S-type LRs increased under treatment with N, but no apparent differences were
369 observed between *plr1* mutant and wild-type plants at each N level (Fig. 6C). We therefore
370 confirm and strongly support that, at the higher growth stage, the lower basal shoot starch
371 accumulation regulated L-type LR formation in *plr1* mutants at different N conditions, but
372 that this regulation was not clearly observed in the wild-type.
373
374 **4. Discussions**

375 *4.1. plr1 is an identified mutation gene promoting lateral root growth*

376 Several mutants with impaired root development have been identified to uncover the
377 genes and mechanisms regulating root formation. However, most of them exhibit specific
378 defects on root components due to repressed cellular processes, e.g., cell division and
379 elongation in *short lateral root length 1* [38] and *short-root 5* [39], impaired cell wall
380 construction in *root growth inhibiting 1* [40], as well as abnormalities in other physiological
381 processes including auxin signalling, e.g., *crown rootless 1* [41] and *Osiaa13* [42]. Owing to
382 their root defects, these mutants cannot be utilized for improving water and nutrient uptake
383 efficiency. We therefore focused on mutants with a promoted root system, in order to
384 determine the mechanisms regulating L-type LR development. In the present study, we
385 characterized the *plr1* mutant, which exhibited a higher number of L-type LRs, and possessed
386 normal and longer seminal roots as compared to the wild-type (Fig. 1A–D, F and H). These
387 traits were consistently observed at later stages of growth, as described previously [15].
388 Furthermore, the *plr1* mutant plant presented normal and vigorous aerial parts (Fig. 1E and J–
389 O), with a notable increase in height, both at the seedling and mature stages. Despite a
390 reduced tiller number in the *plr1* mutant, panicle number was comparable, whereas spikelet
391 number was significantly higher, compared to those of the wild-type (Fig. 1L and N–O).
392 These results indicate that *plr1* mutation positively affected root growth and had no
393 detrimental effects to shoot growth and yield, highlighting the mutant's suitability for studies
394 on root development and as an appropriate material for breeding purposes.

395

396 *4.2. plr1 gene mutation promotes lateral root development through carbohydrate regulation*

397 Studies limited to Arabidopsis [16] and maize [17, 18] have determined the positive
398 effects of sugars on LR development. In addition to the *plr1* mutant's apparent root
399 characteristics, basal shoot starch accumulation was notably lower than in the wild-type

400 anytime of the day (Fig. 2C, Supplementary Fig. 1C), under tap water conditions. We
401 revealed that the gene mutation firstly reduced starch accumulation in the basal shoot,
402 consequently enhancing LR development in the *plr1* mutant (Fig. 2I and J). A higher amount
403 of glucose was detected in the *plr1* mutant basal shoot compared to the wild-type (Fig. 2D,
404 Supplementary Fig. 1A), further suggesting that the *plr1* mutant was likely unable to convert
405 glucose units into starch, transporting these sugars to the roots instead, where they were
406 utilized for LR development. Additionally, these traits were observed in *plr1* mutants
407 subjected to various N levels, as well as in wild-type seedlings grown under applied-N
408 conditions (Fig. 3C–I). Expression analysis of starch biosynthesis genes in the basal shoot
409 revealed reduced transcript levels, in agreement with the low basal shoot starch concentration
410 levels (Fig. 4), meaning that the repressed expression of genes essential for starch
411 biosynthesis in the mutant resulted in low starch accumulation in the *plr1* mutant and
412 wild-type basal shoot under N conditions.

413 Our internal sugar analysis in the roots confirmed that *plr1* mutant roots contained
414 higher sugar levels, especially at the seminal root region of non-emerged LRs (Fig. 2H,
415 Supplementary Fig. 1E-F), suggesting that sugars most notably affected early stage of LR
416 development. This was supported by exogenous glucose application (Fig. 3A and B), which
417 significantly increased L-type LR density in the *plr1* mutant, highlighting the promotive
418 effects of sugars on LR development, which, when transported, in turn promoted the mutant
419 root phenotype. Interestingly, glucose application also increased L-type LR formation in
420 wild-type seedlings (Fig. 3A). This was further illustrated under applied-N conditions,
421 wherein wild-type seedlings with significantly reduced basal shoot starch relative to those
422 under N-deficient conditions, showed increased root glucose levels resulting to enhanced LR
423 development (Fig. 3).

424 In cereals, the basal shoot serves to buffer source-sink interactions during different
425 stages of growth and/or under varying environmental conditions [20]. The low starch level in
426 the mutant (as compared to the wild-type), and the lower basal shoot starch level in the
427 N-supplied wild-type (as compared to the N-deficient wild-type) may suggest the modulation
428 of basal shoot starch accumulation to channel sugars for LR development, as manifested by
429 increased sugar levels in mutant and wild-type roots under N conditions. The promotion of
430 root growth via sugars is in agreement with reports on Arabidopsis, wherein LR proliferation
431 in terms of number and length occurred in response to increased sugar concentrations [43–44].
432 Additionally, Ogawa et al. (2005, 2009) [17, 18] showed that the content of sucrose, glucose,
433 and fructose was higher in the apical root section and in the surrounding tissue of maize root
434 LR primordia. Furthermore, a study by Albrecht and Mustroph (2003) [45] on wheat roots
435 revealed that the highest concentration of sucrose and hexose occurred in the root apex.
436 Although the studied plants produce only L-type LR unlike rice, these reports support our
437 findings, which indicate that sugars are important in L-type LR regulation in *plr1* mutant and
438 we showed here the relationship between sugars and the type of LRs.
439
440 *4.3. plr1 gene mutation is consistently functional under low to high N conditions at seedling*
441 *and higher plant growth stages*
442 The ability of rice plants to promote root system development, especially LR
443 formation, is a desirable trait for efficiently acquiring water and nutrients from larger soil
444 areas, in order to boost productivity [4]. These root characteristics were associated with *plr1*
445 mutants that displayed a highly branched root system under various N conditions (from
446 N-deficient to N-supplied) at different growth stages (seedling and maximum tillering) (Fig. 1,
447 3, and 6). The promoted root system was regulated by lower basal shoot starch accumulation,
448 which consequently increased root sugar levels, with promotive effects on LR development.

449 Although wild-type seedlings showed enhanced LR development under N treatments as
450 compared to the N-deficient condition (Fig. 3C–I), this promotion was not observed at the
451 higher growth stage (Fig. 6). By contrast, *plr1* mutant roots were responsive to N, leading to a
452 more highly branched root system (Fig. 6). This was facilitated by a sharp reduction in mutant
453 basal shoot starch levels, unlike the wild-type basal shoot, which presented minimal starch
454 reduction (Fig. 5). This gene mutation performed similarly under soil conditions with varying
455 N regimes, and its contribution to the shoot was examined [15], revealing that, under low N
456 conditions, the *plr1* mutant generated a larger root system based on its total root length. This
457 was further increased with increasing N levels, possibly owing to the development of highly
458 branched L-type LRs, that might in turn, have contributed to higher leaf area and shoot dry
459 matter production, as compared to the wild-type [15]. Collectively, the gene mutation showed
460 no detrimental effects on shoot growth, was responsive to increasing nutrient levels, and
461 promoted root system development.
462
463 **5. Conclusions**
464 Overall, our study on *plr1* mutation demonstrated the involvement of reduced basal
465 shoot starch accumulation and increased root glucose levels to unravel the associations
466 between shoot and root in the regulation of L-type LR formation. The *plr1* mutant
467 consistently showed enhanced highly developed L-type LRs with promoted parental roots,
468 which was regulated by sharp reduction in basal shoot accumulation, under different N
469 conditions (from low to increasing N levels), both at the seedling and higher growth stages.
470 Additionally, this mutation gene had no detrimental effects on shoot and yield, responded to
471 nutrient levels, promoted a highly branched root system, and thus can considerably improve
472 the rice root system architecture, to overcome soil environment stresses. The findings of this
473 study have potential implications in rice genetic improvement and breeding programs, as well

474 as in crop management practices (i.e., timely N application after sufficient rainfall) that can be
475 applied under different rainfed lowland and upland fields.
476
477 **Acknowledgements**

478 We thank Ms. Kimiyo Inukai, Ms. Eiko Murakami and Ms. Saki Nishiuchi for their
479 valuable technical supports. This work was supported by PRESTO, JST, the Grant-in-Aid
480 from the Japan Society for the Promotion of Science (No. 15H04435), the Japan Science and
481 Technology Agency (JST)/Japan International Cooperation Agency (JICA) and the Science
482 and Technology Research Partnership for Sustainable Development (SATREPS, No.
483 JPMJSA1706), and JSPS KAKENHI Grant Number 18H02174.
484

485 **Fig. 1. Phenotypic characterization of *plr1* mutant.**

486 Root phenotypes of the wild-type (A) and *plr1* mutant (B) seedlings at 8 days after
487 germination, scale bar= 4cm. A closer look of the lateral roots along the seminal root of
488 wild-type (C) and *plr1* mutant (D), scale bar= 1cm. (E-I) Shoot and root traits measured every
489 2 days for 2-week old seedlings of wild-type (white circles) and *plr1* mutant (black circles).
490 Values are means ± SE (n=5). (J-K) Mature shoot phenotypes of wild-type and *plr1* mutant,
491 respectively, scale bar= 20cm. (L-O) Number of tillers, height, number of panicles and
492 number of spikelets per panicle measured at maturity. Values represent means ± SE (n=4). *,
493 ** and *** indicate significant differences between genotypes at $P<0.05$, $P<0.01$ and
494 $P<0.001$, respectively, by two-tailed Student's T-test.
495

496 **Fig. 2. Carbohydrate concentrations in the stem and seminal root.**

497 (A-B) A closer view of the basal shoot starch stain of 8-day-old wild-type and *plr1* mutant
498 seedlings under tap water condition, respectively. Arrows point to the starch stain represented
499 by the dark purple color. (C-F) Starch, glucose, sucrose and the total non-structural carbon

500 (NSC) concentrations in the basal shoot. (G-H) Glucose, sucrose, and starch concentrations at
501 the SR regions of emerged and non-emerged LRs, respectively. SR-seminal root; LRs-lateral
502 roots. Values are means ± SE (*n*= 6 biological repeats). *, and *** indicate significant
503 differences between genotypes at $P<0.05$, and $P<0.001$ by two-tailed Student's T-test,
504 respectively. (I) Non-excised roots (control, left) and the excision of the SR region of
505 non-emerged LRs and newly emerging crown roots (right) of 14-day-old wild-type and *plr1*
506 mutant and their corresponding starch concentrations at the basal shoot (J). Values are means
507 ± SE (*n*= 3). Bars followed by different letters denote significant differences at $P<0.05$ by
508 LSD test.

509 **Fig. 3. Effects of exogenous treatment of D-glucose and nitrogen to the wild-type and**
510 ***plr1* mutant seedlings.**

511 L-type (A) and S-type (B) lateral root density of 8-day-old wild-type and *plr1* mutant
512 seedlings at different exogenous D-glucose concentrations under tap water condition. Values
513 are means ± SE (*n*=5). Starch concentrations at the basal shoot part (C) and glucose
514 concentrations at the SR regions with emerged (D) and non-emerged LRs (E) of 8-day-old
515 wild-type and *plr1* seedlings grown under different nitrogen (N) conditions, respectively.
516 SR: seminal root, LRs: lateral roots. Values are means ± SE (*n*=3 biological repeats). Lateral
517 root formation (F-G), lengths (H) and L-type LR density (I) along the 7-cm SR length from
518 the base. White bars= 2cm. Values are means ± SE (*n*=6). Bars followed by different letters
519 denote significant differences at $P<0.05$ by LSD test.

520 **Fig. 4. Expression analysis of stem-starch biosynthesis genes under different nitrogen**
521 **conditions.**

522 Genes predominantly involved in starch synthesis of leaf sheaths including *AGPL1*, *AGPS1*,
523 *BEIIa*, *GBSSI*, *GBSSII*, *SSI*, *SSIIb*, and *SSIIIb* [29] and with the addition of *GBSSI* (A) and
524 their relative expression levels at the basal shoot of 13-day-old wild-type and *plr1* mutant

525 seedlings grown in tap water for 8 days and subjected to nitrogen (-N, without N and +N, with
526 N) treatments for 5 days. Values are means ± SE (*n*= 3). Bars followed by different letters
527 denote significant differences at $P<0.05$ by LSD test.

528 **Fig. 5. Root system handling, physiological parameter measurements and leaf sheath**
529 **starch staining of wild-type and *plr1* mutant plants under different nitrogen conditions**
530 **at higher plant growth stage.**

531 Old roots of 60-day-old plant tied at 1-cm from the base before exposure to various nitrogen
532 (N) treatments (A), the root system and the new crown roots produced (B), SPAD value (C),
533 leaf photosynthesis (D), stomatal conductance (E) and the staining procedure of the 3rd leaf
534 sheath by IKI solution (adapted from Togari and Sato, 1954) [27] (F) and the starch stain
535 percentage after the 5-day N treatments. Values are means ± SE (*n*= 5). Bars followed by
536 different letters denote significant differences at $P<0.05$ by LSD test.

537 **Fig. 6. Lateral root formation of wild-type and *plr1* mutant plants under different**
538 **nitrogen conditions at higher plant growth stage.**

539 Lateral root formation along the new crown roots of wild-type (left) and *plr1* mutant (right)
540 plants exposed to nitrogen (N)-deficient (0 % N) and N-applied (25 % N) treatments (A-B),
541 respectively. S-type lateral root number, L-type lateral root number and average length at
542 different N treatments, respectively (C-E). Values are means ± SE (*n*= 5, 3 randomly selected
543 new crown roots from each rep were measured). Bars followed by different letters denote
544 significant differences at $P<0.05$ by LSD test.

545 **Supplementary Table 1.** Nutrient solution content and the adjusted N supplied for the
546 different N level treatments for higher growth stage (full-strength solution- 100, 50, 25 and
547 0% N) and seedling stage (1/10 strength solution- 80, 40, 20, 0% N)

548 **Supplementary Table 2.** Primers used for the quantitative RT-PCR

549 **Supplementary Fig. 1. Carbohydrate concentration dynamics in the basal shoot and**

550 **seminal root region with non-emerged lateral roots in a 24 h cycle.**

551 Glucose, sucrose, starch and total NSC (non-structural carbohydrates) concentrations in the
552 basal shoot (A-D) and in the SR zone for non-emerged lateral roots (E-H) during a 24-h cycle
553 (6h- 6h after light, 12h- 12h after light, 18h- 6h after dark and 24h- 12h after dark) in
554 8-day-old wild-type (white bars) and *plr1* mutant (black bars) seedlings grown under tap
555 water conditions. Values are means ± SE (n= 3 biological repeats). Bars followed by different
556 letters denote significant differences at P<0.05 by LSD test.

557

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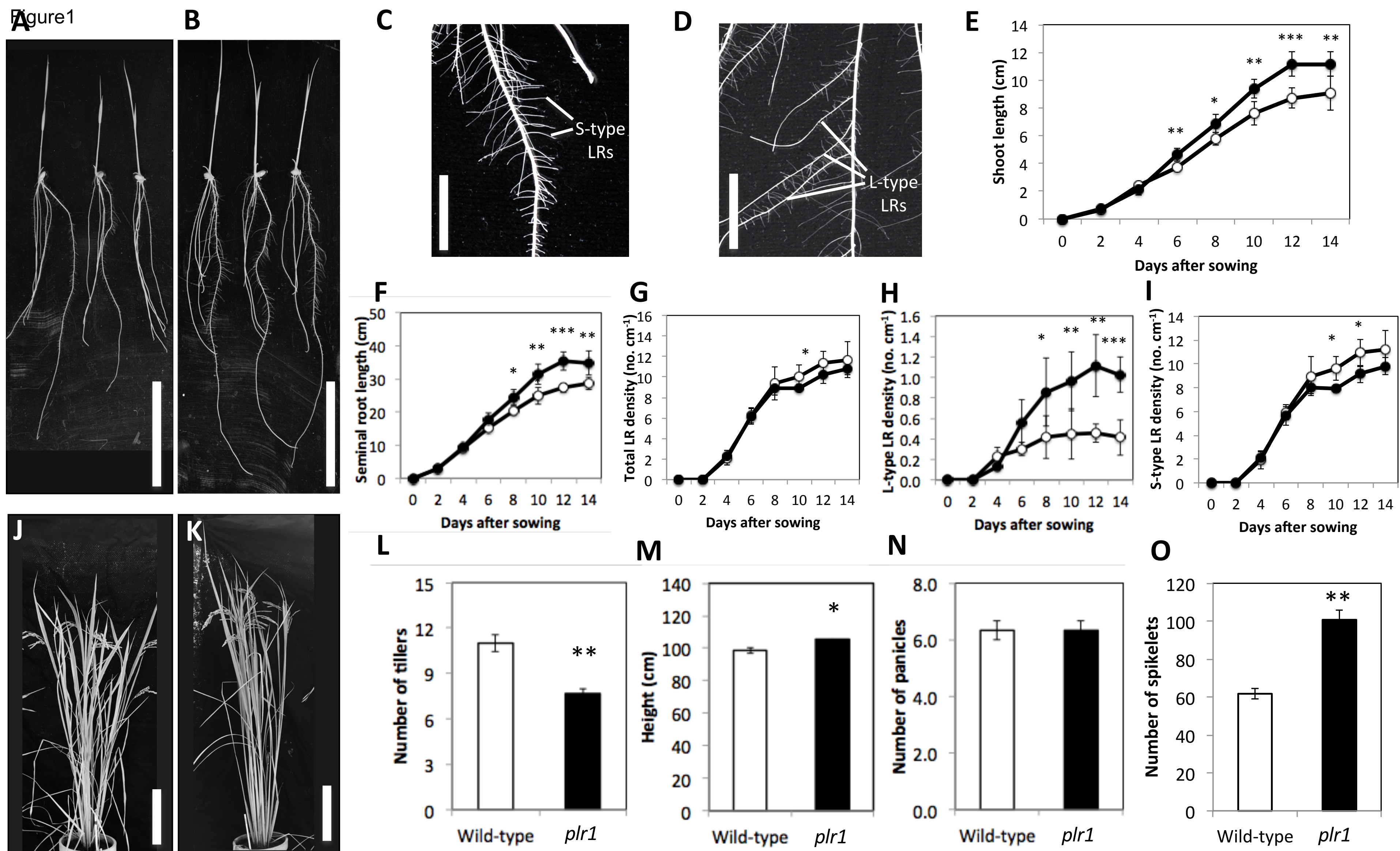

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Figure2

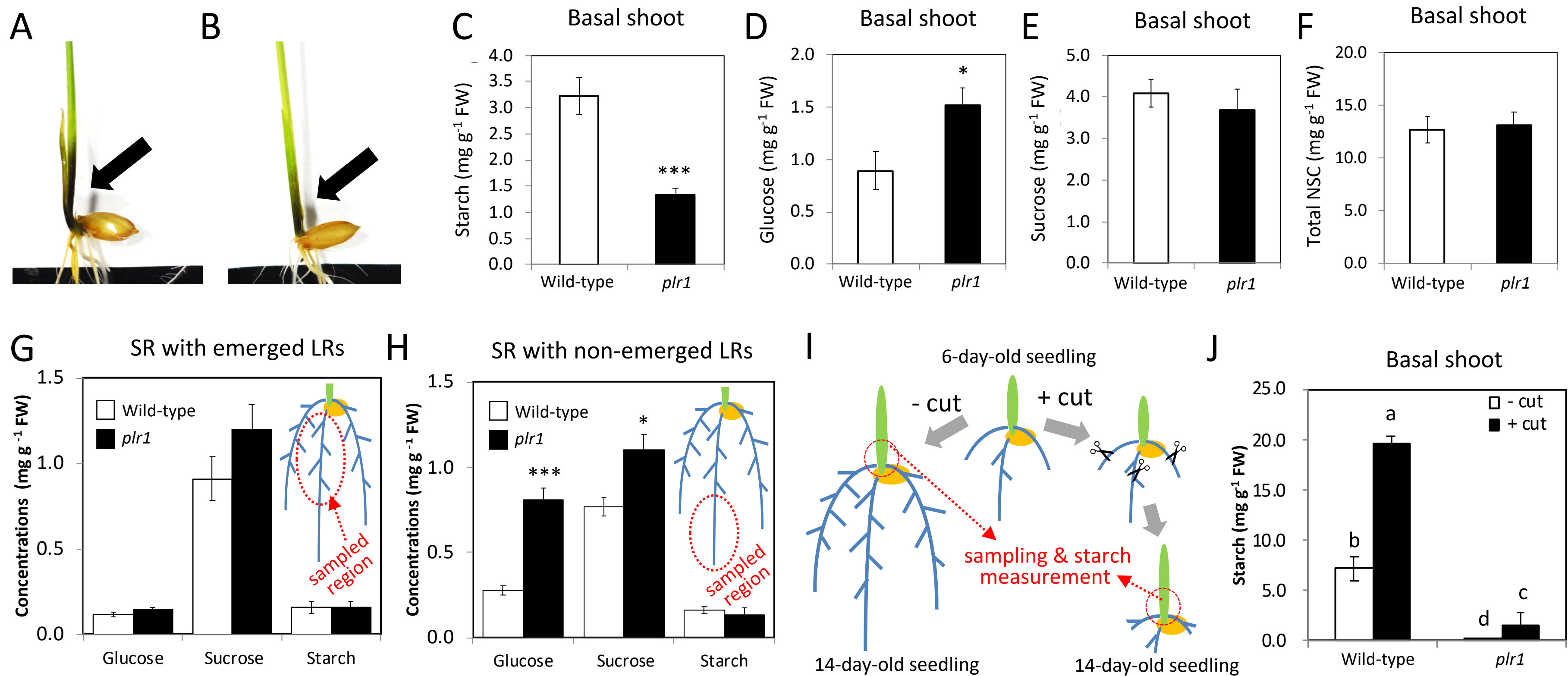

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Figure3

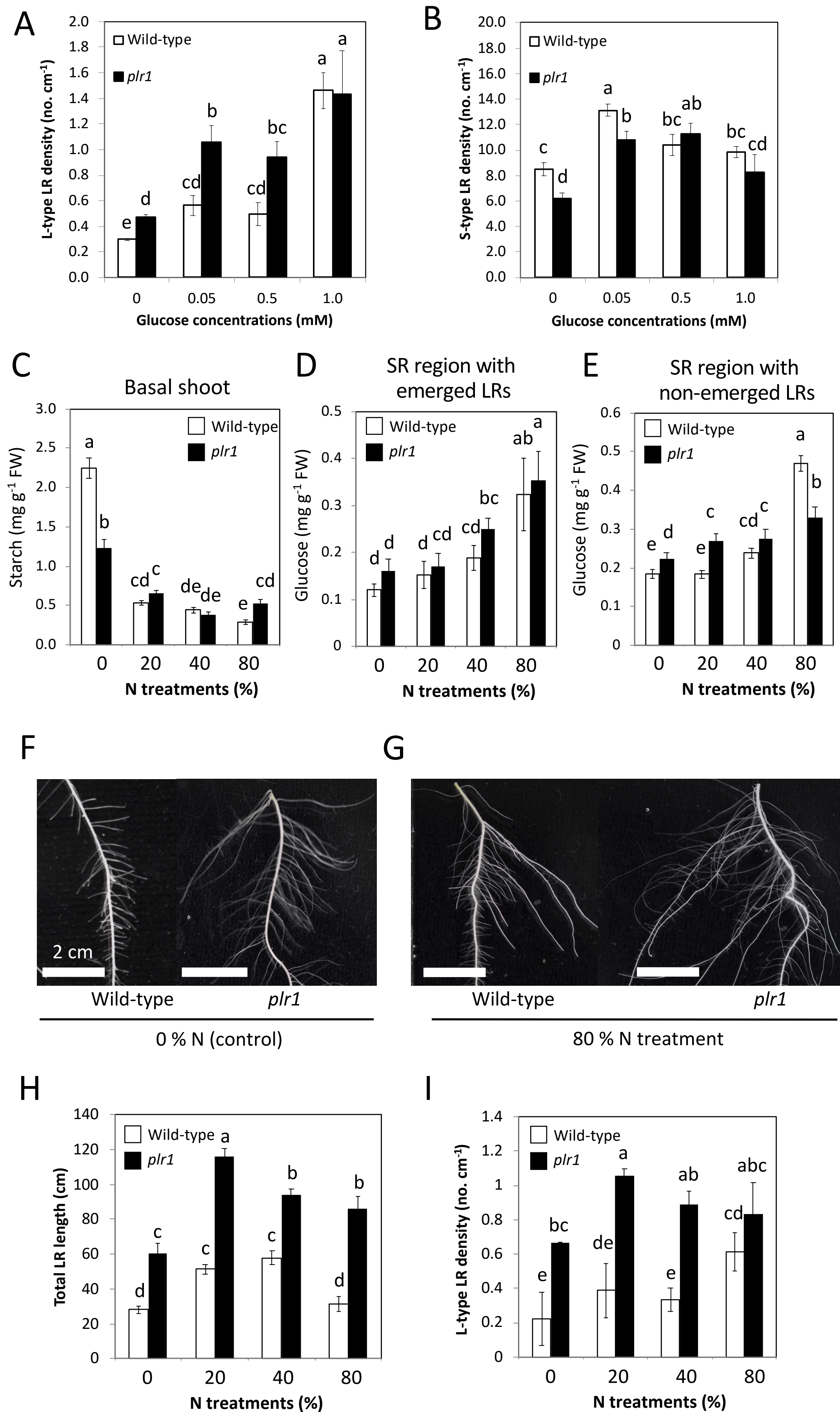

Fig. 3. Effects of exogenous treatment of D-glucose and nitrogen to the wild-type and *plr1* mutant seedlings.

L-type (A) and S-type (B) lateral root density of 8-day-old wild-type and *plr1* mutant seedlings at different exogenous D-glucose concentrations under tap water condition. Values are means ± SE (n=5). Starch concentrations at the basal shoot part (C) and glucose concentrations at the SR regions with emerged (D) and non-emerged LRs (E) of 8-day-old wild-type and *plr1* seedlings grown under different nitrogen (N) conditions, respectively. SR: seminal root, LRs: lateral roots. Values are means ± SE (n=3 biological repeats). Lateral root formation (F-G), lengths (H) and L-type LR density (I) along the 7-cm SR length from the base. White bars= 2cm. Values are means ± SE (n=6). Bars followed by different letters denote significant differences at P<0.05 by LSD test.

Figure4

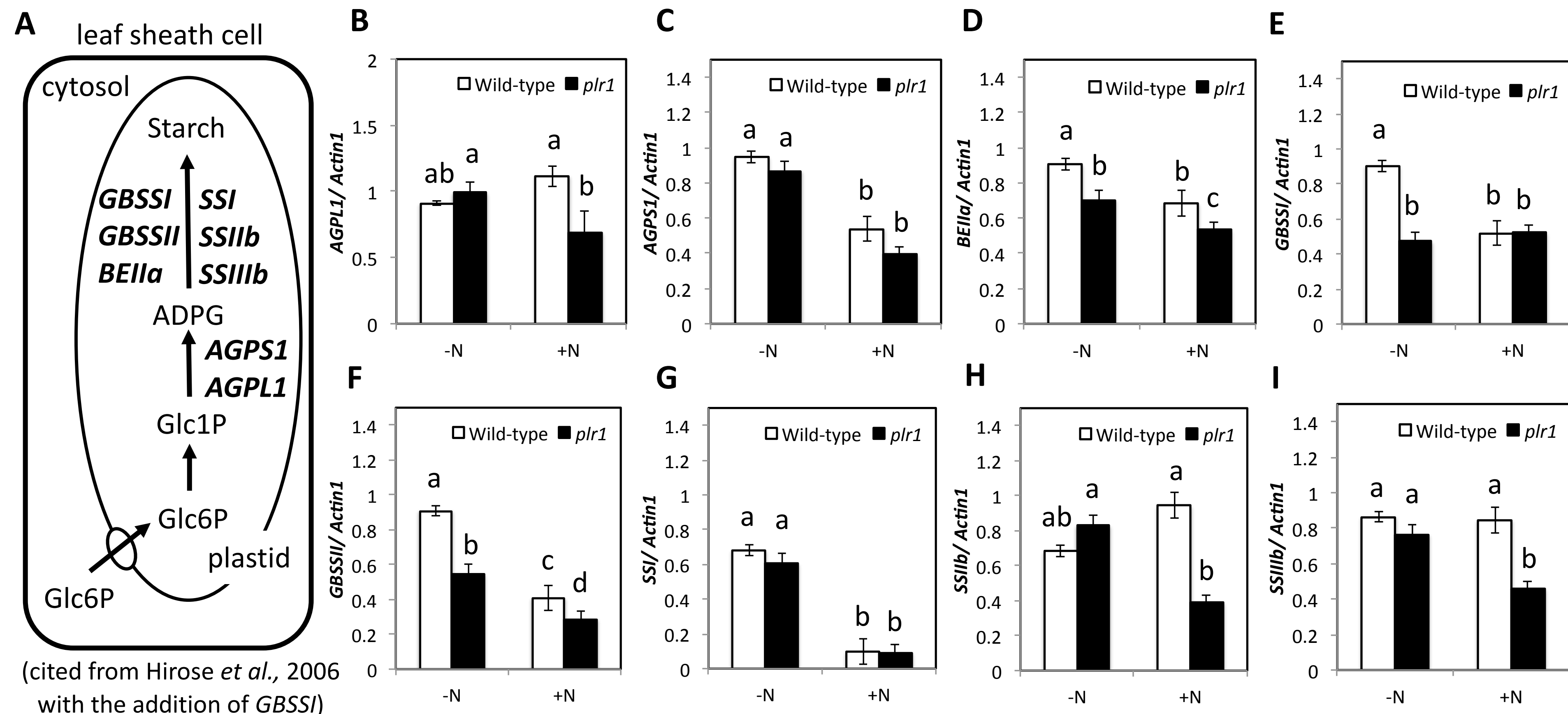

Fig. 4. Expression analysis of stem-starch biosynthesis genes under different nitrogen conditions.

Genes predominantly involved in starch synthesis of leaf sheaths including *AGPL1*, *AGPS1*, *BEIIa*, *GBSSI*, *GBSSII*, *SSI*, *SSIIb*, and *SSIIIb* [29] and with the addition of *GBSSI* (A) and their relative expression levels at the basal shoot of 13-day-old wild-type and *plr1* mutant seedlings grown in tap water for 8 days and subjected to nitrogen (-N, without N and +N, with N) treatments for 5 days. Values are means ± SE (n= 3). Bars followed by different letters denote significant differences at P<0.05 by LSD test.

Figure5

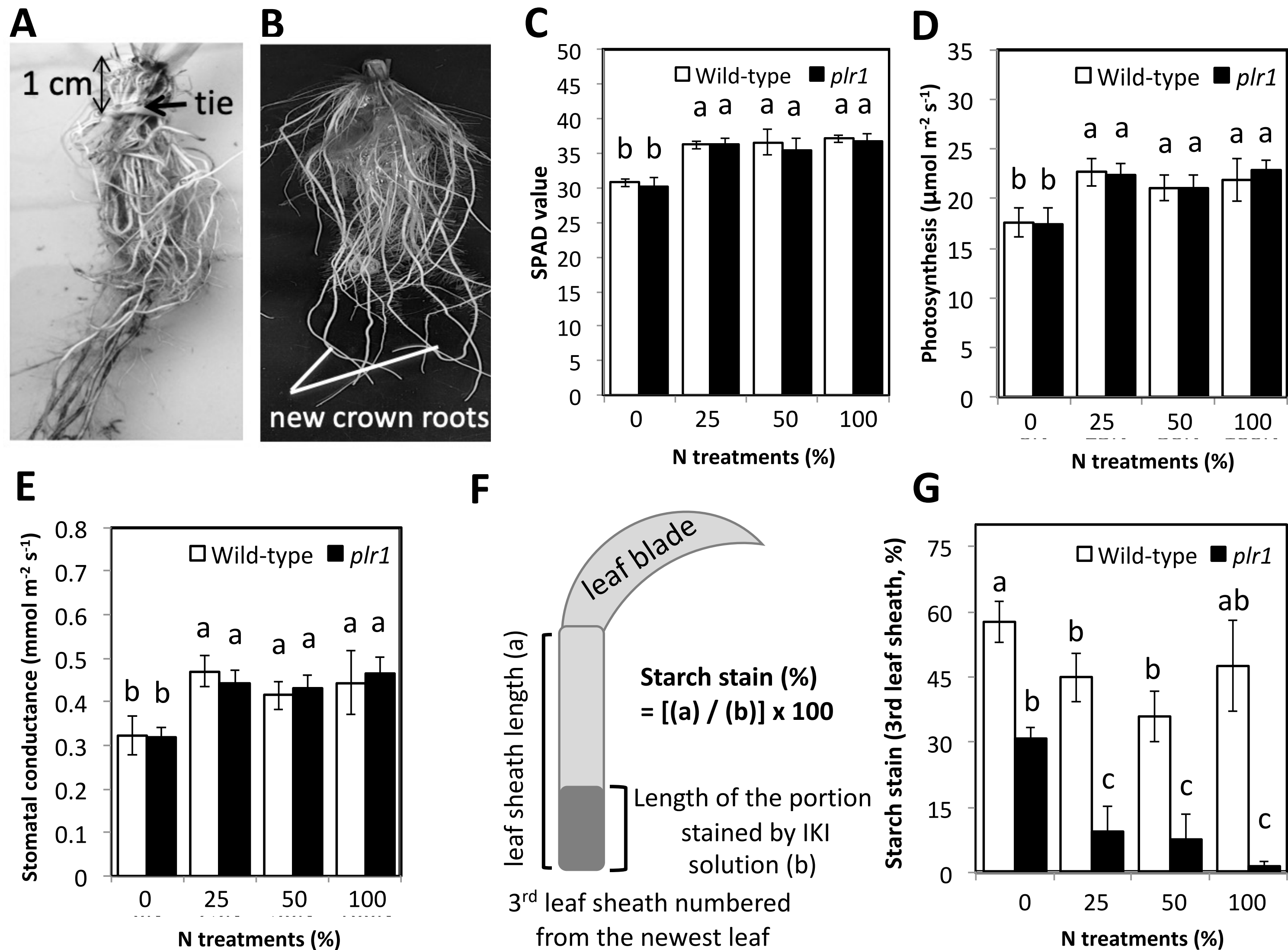

Fig. 5. Root system handling, physiological parameter measurements and leaf sheath starch staining of wild-type and *plr1* mutant plants under different nitrogen conditions at higher plant growth stage.

Old roots of 60-day-old plant tied at 1-cm from the base before exposure to various nitrogen (N) treatments (A), the root system and the new crown roots produced (B), SPAD value (C), leaf photosynthesis (D), stomatal conductance (E) and the staining procedure of the 3rd leaf sheath by IKI solution (adapted from Togari and Sato, 1954) [27] (F) and the starch stain percentage after the 5-day N treatments. Values are means ± SE (*n*= 5). Bars followed by different letters denote significant differences at $P<0.05$ by LSD test.

Figure6

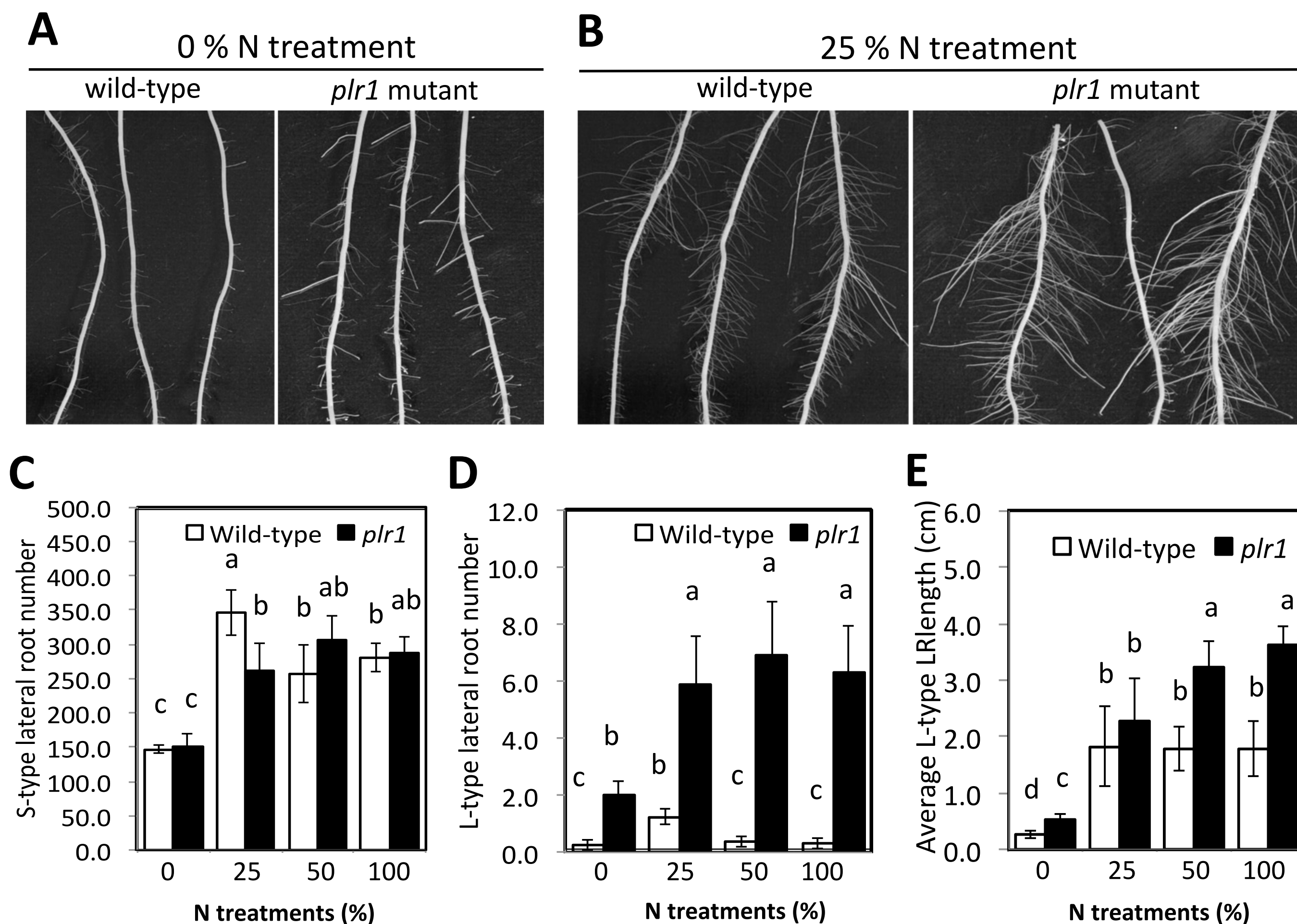

Fig. 6. Lateral root formation of wild-type and *plr1* mutant plants under different nitrogen conditions at higher plant growth stage.

Lateral root formation along the new crown roots of wild-type (left) and *plr1* mutant (right) plants exposed to nitrogen (N)-deficient (0 % N) and N-applied (25 % N) treatments (A-B), respectively. S-type lateral root number, L-type lateral root number and average length at different N treatments, respectively (C-E). Values are means ± SE (n= 5, 3 randomly selected new crown roots from each rep were measured). Bars followed by different letters denote significant differences at P<0.05 by LSD test.