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Early Vigor of a Pyramiding Line Containing Two Quantitative Trait Loci, *Phosphorus Uptake 1* (*Pup1*) and *Anaerobic Germination 1* (*AG1*) in Rice (*O. Sativa* L.)

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Abstract: Direct-seeded rice is one of the solutions against the issues of limited labor and time in the rice cropping system. Improved useful traits, such as fertilizer uptake and anaerobic germination, are needed to increase yield and efficiency in the direct seeding system in rice. *Pup1* (*Phosphorous uptake1*) containing *PSTOL1* is useful in improving the phosphate uptake under rainfed/upland conditions. *OsTPP7* is the major gene of *AG1* (*Anaerobic Germination*), which shows anaerobic germination. IR64-Pup1-AG1 (I-PA) was developed by pyramiding *Pup1* and *AG1*. Around 20% of the chromosomal segments from the donor remained in I-PA. Phenotypic analysis revealed that I-PA showed better phenotypic performance under low and normal P conditions by enhancing the root system and tiller numbers during the early stage. Significantly better P uptake capacity of I-PA was observed upon a P-supplied soil condition. The coleoptile length and germination rate of I-PA showed tolerance under anaerobic-germinated conditions. *PSTOL1* and *OsTPP7* were independently expressed under different P conditions of soils, as well as anaerobic conditions. The newly developed breeding lines, I-PA, showed early vigor capacity through a high number of tillers, better P uptake, and germination in low-oxygen conditions. It will be a useful and improved breeding line for direct seeding rice breeding programs.

Keywords: phosphorous uptake; anaerobic germination; early vigor; Pup1; OsTPP7; QTL pyramiding; rice

1. Introduction

Rice is a staple food for more than half of the human population. Increasing water scarcity in paddy fields and increasing labor costs in rice cropping systems are challenging the food security systems of Asian countries dependent on rice. Pre-germinated rice seeds or seedlings are broadcasted in the field in the case of direct-seeded rice (DSR) [1]. In the DSR system, abiotic stresses in the early

seedling stage should be considered, such as drought, nutrition uptake, seedling vigor, anaerobic germination, etc. The development of rice for the wet direct seeding system needs those traits, because the manpower to take care of fields is lacking, and the uneven surface of soils is common, especially at the seedling period.

Rice is one of the unique crops that is cultivated in submerged soils, due to its developed aerenchyma that can mitigate the stresses from anoxia [2]. *qAG-9-2*, or *AG1*, derived from Khao Hlan On, is a major quantitative trait locus (QTL) for anaerobic germination tolerance, and confers anaerobic germination tolerance to plants through the submergence escape strategy with rapid coleoptile elongation and delay of radicle developments [3]. *OsTPP7*, the trehalose-6-phosphate phosphatase gene in the *AG1* QTL, was identified as a key gene for anaerobic germination tolerance and is absent in susceptible varieties, such as IR64 [3,4].

In contrast, *Pup1* (*phosphorus uptake 1*) is responsible for improving phosphate uptake in rice, especially under rainfed/upland conditions [5]. *Pup1* was identified in the traditional *aus*-type rice variety Kasalath and mapped on chromosome 12 [6]. *Pup1* consisted of 69 genes including *PSTOL1*, which is absent from phosphorus-starvation-intolerant modern varieties. It has a major function for early root growth and acquisition of more phosphorous by encoding protein kinase [7]. Interestingly, *Pup1* can be commonly identified from upland/drought tolerant rice varieties, and the beneficial effect on the higher P-uptake and biomass of *Pup1* activity was clearly observed in rainfed soil conditions [8,9]. *PSTOL1*, a major gene of *Pup1*, increases the number of the crown root in early stage. Thus, *Pup1*-containing rice gets more benefit in taking up more nutrients from the soil [7,8,10].

Toledo et al. and Mondal et al. showed normal functions of *AG1* in Ciherang, when it was combined with *Sub1* and *AG2*, respectively [11,12]. However, *Pup1* has never been combined with other abiotic related QTLs. IR64-Pup1-AG1 (I-PA) is the first trial to combine *Pup1* with abiotic stress tolerant QTLs. In this study, we expected to see beneficial effects from pyramiding *Pup1* and *AG1*. Thus, *Pup1* and *qAG-9-2* (*AG1*) were combined into IR64, which is a high yield and high quality *indica* rice. The materials were tested under different water and P conditions, and the expression and combination effects were observed.

2. Materials and Methods

2.1. Plant Materials

IR64, I-Pup1 (IR64-Pup1), I-AG1 (IR64-AG1) and I-SA (IR64-Sub1-AG1) were introduced to Sejong University (SJU, Seoul, Korea) by a Seconded Special Material Transfer Agreement (seconded SMTA) via Hankyoung National University (HKNU, Anseong, Korea), with help from the International Rice Research Institute (IRRI, Los Baños, Philippines). In the process to develop I-PSA (IR64-Pup1-Sub1-AG1) using I-Pup1 and I-SA (IR64-Sub1-AG1), plants that lost *Sub1* were found and used as I-PA (IR64-Pup1-AG1) in this study. IR64, I-Pup1, I-AG1, and I-PA were used for testing the P uptake ability under different P application conditions and anaerobic germination. I-Pup1 and I-AG1 were known as tolerance plants to low phosphorus and anaerobic germination conditions [7,11].

2.2. Genomic DNA Extraction and Genotyping

Genomic DNA was extracted using the cetyl-trimethyl-ammonium bromide (CTAB) method [13]. Two-week-old leaf samples were obtained from the testing plants. Foreground selections were conducted by PCR using gene-specific markers, which were reported in Chin et al. [9] and Kretzschmar et al. [3]. Primer sequences are listed in Table S1, Supplementary Materials. PCR amplification was conducted in a PCR thermocycler (SimpliAmp, Thermo Scientific, Waltham, MA, USA). Reaction mix was 20 μL (IN5001-0500, Inclone, Yongin, Korea) and constituted with 50 ng of template DNA, and 10 pmol each of forward and reverse primers (Bioneer, Daejeon, Korea). The thermal cycling conditions were as follows: initial denaturation was conducted for 4 min at 94 °C, followed by 30–35 cycles of 94 °C for 1 min, annealing at 55–65 °C for 30 s, a 1 min extension at 72 °C, with a final extension for

5 min at 72 °C. Amplified products were electrophoresed (BioFACT, Daejeon, Korea) in 1–2.5% agarose gel, and visualized using a gel imager (GDS-200D, Korea Lab Tech, Seongnam, Korea). Background genotyping was conducted with 405 KASP (Kompetative Allele Specific PCR) markers [14] using CFX384 (BIO-RAD, Hercules, CA, USA).

2.3. RNA Extraction and Gene Expression Analysis of Pup1 and AG1

The three plants were harvested at two weeks after transplanting (WAT) and RNA was extracted to test *PSTOL1* expression. The three plants of germinating seeds were used for RNA extraction to test the *OsTPP7* gene expression. For each line, the shoot and root parts of the three plants were divided and separately used. RNA was extracted using the RNeasy Plant Mini Kit (50) (QIAGEN, Hilden, Germany). The first-strand cDNA was synthesized to 1000 ng/µL using the Easy cDNA Synthesis Kit (NanoHelix, Daejeon, Korea) and was diluted five times with distilled water. Primers used in gene expression levels were used in reference to Gamuyao et al. [7] and Toledo et al. [11]. The sequences are listed in Table S1. RT-qPCR was performed using a Corbett RG-6000 device (Qiagen, Hilden, Germany) under the following conditions: 95 °C for 2 min followed by 52 cycles at 95 °C for 5 s and 60 °C for 15 s. The reaction mix was 15 µL using the SensiFAST SYBR No-Rox Kit (Bioline, Meridian, London, UK), with 10 ng of cDNA, and 10 pmol of forward and reverse primers (Bioneer, Daejeon, Korea). qPCR results were normalized to *OsUBQ5* using the $2^{-\Delta\Delta Ct}$ method [15].

2.4. Phenotyping under Different P Concentrations of Soil and Hydroponic Conditions

To assess *Pup1* function, soil and hydroponic experiments were conducted. Seeds of IR64, IR64-Pup1, IR64-AG1, and I-PA were sterilized with disinfectants in 50 mL conical tubes for 24 h. Washed seeds were transferred to Petri dishes and incubated in darkness for 3 days at 30 °C. Only sieved, cleaned, and dry soils, whose particle size was below 1 mm in diameter, were used in the soil conditions of this study. Cylinder shape pots (r × h: 3.75 × 25 cm) having two holes of 1 cm in diameter for drainage were used. Thin, round-shaped aluminum plates (H × W: 5 × 23 cm) were placed inside the pots near the holes to prevent the loss of soil. Pots were filled with sieved soils with an 18 cm depth, and fertilizers were applied on the surface of the soils as follows: 0.518 g of N and 0.352 g of K_2O were applied to each pot as basal fertilization, and 0.42 g of P_2O_5 was applied to P-supplied condition pots. After finishing the application, additional soil (5 cm depth) was added to every pot. So that all pots had the same level of mild-drought (or rainfed) condition, every pot containing soil was weighed and checked to be considered completely dry. The pots were placed in a plastic box with water to soak the soil completely for 24 h, by watering through the bottom holes (100% field capacity). After watering, two pre-germinated seeds were sowed into the pots and covered with approximately 1 cm depth of soil. After 1 week, 1 plant was thinned. All pots were weighed every 3 days and water contents were maintained at an 80% level of field capacity to replicate mild-drought conditions by adding the calculated amount of water only. Phenotyping was conducted at two time points, i.e., at two WAT using eleven plants and at seven WAT using five plants. Plant height and tiller number per plant were measured. The SPAD (single-photon avalanche diode) value for chlorophyll content was measured using a SPAD-502 device (Konica Minolta, Tokyo, Japan). Root indexes including crown root number and root length were measured using photo images taken right after sampling. For hydroponic culture, the germinated seeds were transferred to two float trays and were grown under a 10 L Yoshida solution [16] with 10 mg/L (normal concentration of phosphorus) and 0.01 mg/L (low concentration of phosphorous) of $NaH_2PO_4H_2O$, respectively. The pH of the solution was measured every day and the solution was changed every seven days. After two weeks, longest root length, total root length, crown root number, and root width were measured using a digital microscope (SMZ-745T, Nikon, Tokyo, Japan). Plant height and tiller numbers were measured at the same time. Phosphorus content in 7-week-old plants were measured in soil P experiments. Only three plants, those closest to the average value of phenotype data, were selected and analyzed after phenotyping. The plant samples from the same treatment were bulked for each variety. The selected plants were labeled and placed in a 65 °C

dry oven for two weeks. Phosphorus contents of the completely dried root and shoot samples were measured at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University, Korea) using an inductively coupled plasma (ICP) atomic emission spectrometer (ICP-730ES, Varian, Australia).

2.5. Phenotyping Germinability under Anaerobic Conditions

To test the *AG1* function in I-PA, hypoxia and anoxia experiments were conducted in growth chambers (Hanbaek Scientific Technology, Bucheon, Korea). The temperature condition was 30 °C (day)/25 °C (night). A common paddy field soil was prepared in a plastic box with an 8 cm depth and was flattened by 5 cm depth of water for five days without touching the water surface. Dry seeds of IR64, I-Pup1, I-AG1 and I-PA were placed directly at about 1 cm beneath the soil surface, using forceps at the 1 cm depth mark. Tap water was carefully filled into the plastic box using a thin hose, up to 10 cm in depth. In the first experiment (hypoxia), water was not added. In the second experiment, water was fully filled in the box, until the end of the experiment (anoxia). Due to the difference of sprouting and germination days under the two conditions, seed germinability and survival rate were measured on different days: 21 days after sowing (DAS) for hypoxia and 28 DAS for anoxia. The germination date of each condition was 10 DAS for hypoxia and 21 DAS for anoxia. Coleoptile length (CL) is the distance from seed embryo to coleoptile tip. Survival rate (SR) was measured by counting the number of seedlings that grew over the water surface.

2.6. Plant Growth Conditions in Paddy Field

The seeds of IR64, I-Pup1, I-AG1, and I-PA were soaked in 65 °C sterilized water for 10 min, with disinfectant for 24 h in paper bags. After washing, the seeds were incubated for four days at 30 °C in a dark chamber. For the seed nursery, commercial topsoil was filled in plates with 6 x 12 holes, which were afterward soaked with water. Then, the ten pre-germinated seeds were sown in each hole and were covered with 1 cm depth of soil. The plate pot was covered with breathable fabric and incubated for four days in the greenhouse. Plants were transplanted to two different paddy fields after 37 DAS. The location of field A was in Suwon, Korea (37°16′08.7″ N 126°59′24.0″ E) and B was in Hwaseong, Korea (37°09′49.3″ N 126°49′03.2″ E). Nitrogen, phosphate, and potassium were treated as 21-11-21 kg/10a. Plants were grown using conventional practices. Ripened seeds were harvested 38 days after pollination.

3. Results

3.1. Genomic Structure of I-PA

The plant that lost the *Sub1* QTL was found in the process of crossing I-Pup1 and I-SA, and was developed to I-PA in this study. The developed I-PA was validated using QTL-specific markers (Figure 1a). The tested I-PA lines showed to contain *Pup1* and *AG1* QTLs through PCR analyses using three gene-specific markers of *Pup1* [9] and the gene-specific markers for *AG1* [3]. The selected I-PA plants were identified by background genotyping using KASP markers with other lines (Figure 1b). Non-IR64 chromosomal segments remained in each line. Thus, non-IR64-alleles were kept in I-PA (20%), I-Pup1 (13%), and I-AG1 (8%), by counting the number of corresponding alleles of the used polymorphic markers. Chromosome segments originating from I-Pup1 and I-AG1 were detected in I-PA. Chromosomal differences were detected in overall chromosomes except chromosomes 2 and 3 (Figure 1b).

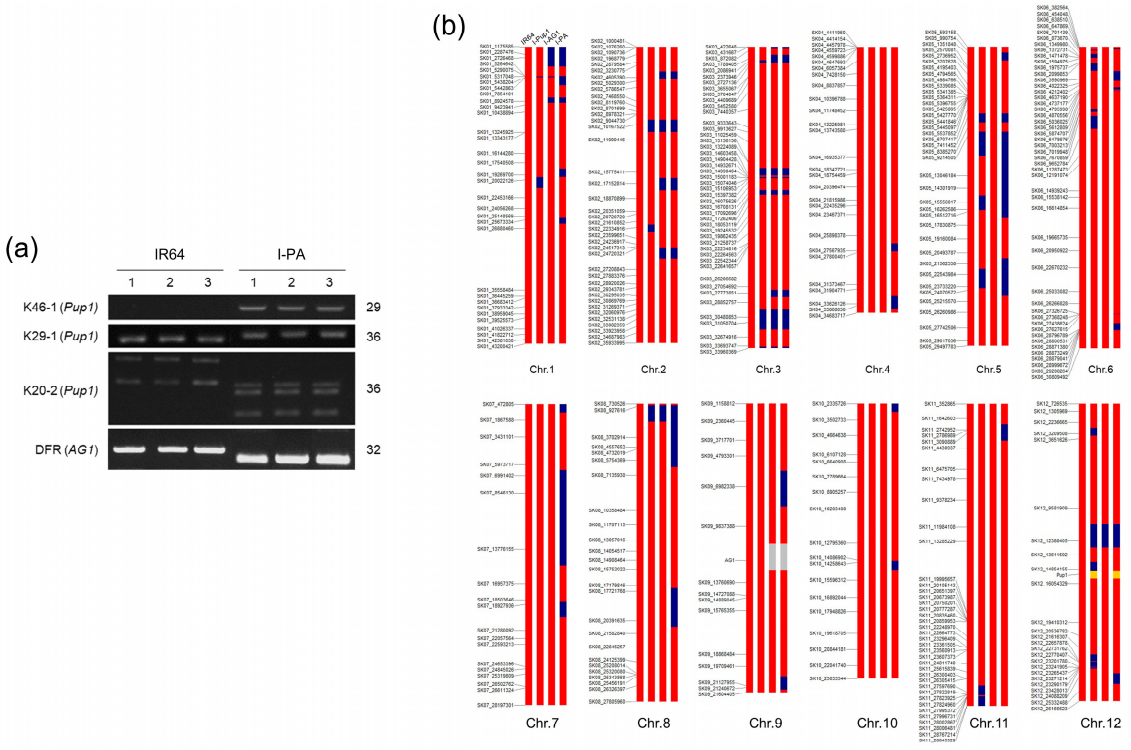

Figure 1. Development and selection of IR64-Pup1-AG1 (I-PA). (**a**) Foreground genotyping using *Pup1* and *AG1* QTL gene-specific markers. (**b**) Background genotype of I-PA and near isogenic lines (NILs). Blue bar: unique regions of NILs against IR64; grey bar: *AG1* QTL; yellow bar: *Pup1* QTL.

3.2. Independent Pup1 Function of I-PA under Different P Supply Conditions

In the soil experiment, to assess the *Pup1* function under *AG1* co-existence, root phenotypic analysis was conducted with P non-supplied soils (non; -g P_2O_5/pot) and P-supplied soils (sup; 0.42 g P_2O_5/pot). The root and shoot phenotypes of IR64, I-Pup1, I-AG1, and I-PA were measured at two WAT and seven WAT. I-PA started to show differences in phenotypes from other lines after seven WAT (Figure 2). In terms of plant height, I-PA was significantly shorter than other lines under both conditions (Figure 2c). The SPAD value of I-PA was lower than the others under the sup condition (Figure 2d). However, the tiller number of I-PA was larger than others under the sup condition (Figure 2e). Root length under the sup of I-PA was larger than the others (Figure 2g). In the earlier growth stage, the phenotypic performance of I-PA was not better than the others. At two WAT, no plants started tillering and new crown roots were not identified (Figure S1a,b, Supplementary Materials). In the shoot division, I-Pup1 was taller than the others, but I-PA was shorter than other plants under non and sup conditions (Figure S1c). The SPAD value of I-PA was small under both conditions (Figure S1d). On the contrary, I-PA did not show statistical differences regarding root phenotypes, including crown root number and length from those of IR64 under non and sup conditions (Figure S1f). I-PA showed better P-uptake capacity in the soil experiment (Figure 3). The P content of I-PA from whole plant samples, root, and shoot at seven WAT was significantly higher than that in IR64 and I-AG1, whereas it was indifferent in I-Pup1 (Figure 3a,b). The increased ratios of P content in I-PA (359%) and I-Pup1 (370%) were higher than those in I-AG1 and IR64 (Figure 3c). With these results, we can assume that *Pup1* was independently functioning from *AG1* to increase P uptake in the soil system, during the seven WAT stage.

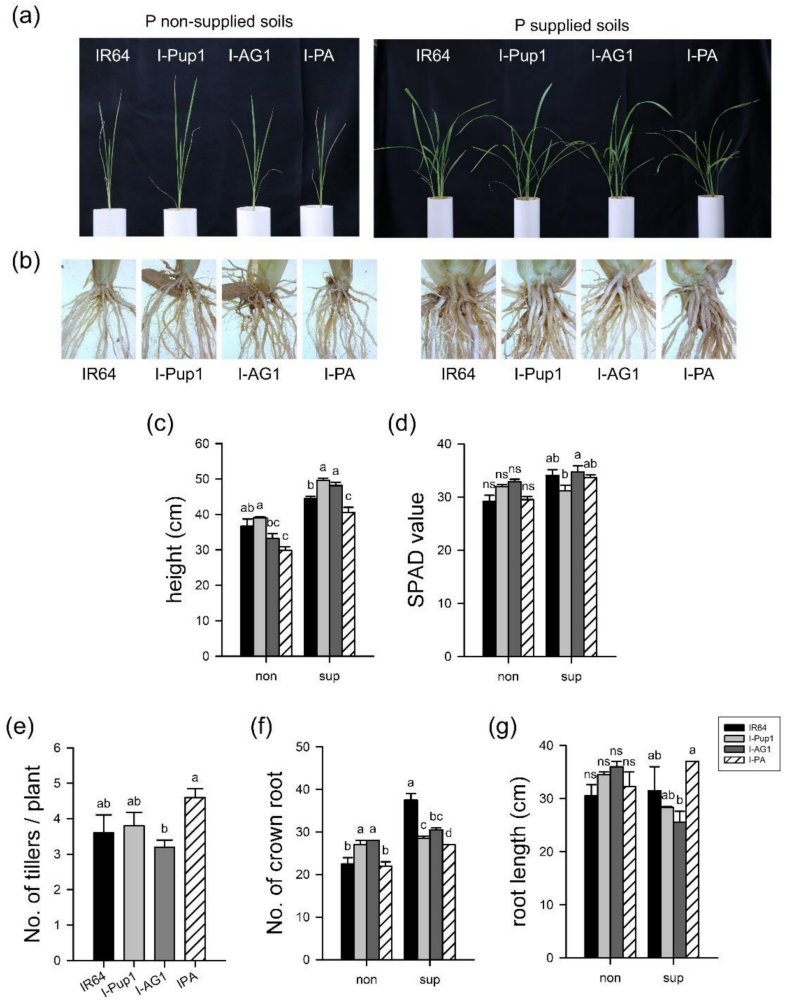

Figure 2. Phenotypic analysis of IR64, I-Pup1, I-AG1, and I-PA under different phosphorous conditions. (**a**,**b**) Phenotype of plants that were grown under P non-supplied and P-supplied conditions, respectively. (**c**,**d**) Plant height and SPAD value. (**e**) Tiller numbers per plant under P-supplied condition. (**f**,**g**) Crown root number per plant, root length. I-Pup1: IR64-Pup1; I-AG1: IR64-AG1; IPA: IR64-Pup1-AG1. The letters above bars represent statistical significance ($p < 0.01$) as measured by Duncan's multiple range test. ns: no significant difference.

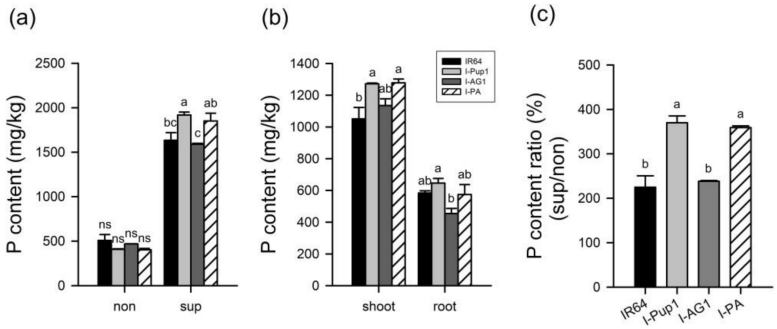

Figure 3. Total P content of I-PA and NILs under P-non and P-sup conditions at 48 days after transplanting (DAT). (**a**) Total P content under P non-supplied and P-supplied conditions at 48 DAT. non: P non-supplied condition; sup: P-supplied condition. (**b**) Total P content of shoots and roots under P-supplied condition. (**c**) Increase in total P content under P-supplied condition versus P non-supplied condition. The letters above bars represent statistical significance ($p < 0.01$) as measured by Duncan's multiple range test. ns: no significant difference.

We checked the gene expression levels of *OsPSTOL1* and *OsTPP7*, which are major genes of introgressed QTLs, to determine the function of *Pup1* in I-PA (Figure 4). The samples of two WAT were used and phenotypes appeared later than the gene expression. *OsPSTOL1* was specifically expressed in shoots under the "non" condition (Figure 4a). In contrast, *OsPSTOL1* expression was observed in shoots and roots under the "sup" condition, where expression levels in I-PA were significantly higher than those in I-Pup1 in both parts (Figure 4b). *OsTPP7* was expressed in I-PA, and the expression level was the same or slightly decreased than that of I-AG1 under both conditions (Figure 4c,d). However, the crown root number did not show phenotypic difference of the *Pup1* line in this study (Figure 2f). I-Pup1 did not have the largest tiller numbers under non and sup conditions. Instead, we observed the root length and root width of I-PA and I-Pup1 in hydroponic experiments (Figure S2, Supplementary Materials). Interestingly, the increase in total root length and root width of I-PA and I-Pup1 in the hydroponic system at different P conditions was observed, implying that the additive effect of *Pup1* in the hydroponic system was shown regardless of *AG1* presence.

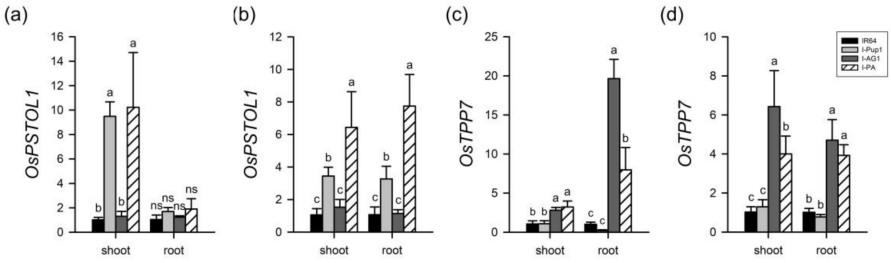

Figure 4. Transcriptional levels of *PSTOL1* and *OsTPP7*, the major genes of *Pup1* and *AG1* QTL, under P non-supplied condition (**a**,**c**) and P-supplied condition (**b**,**d**) using two WAT plants. Expression levels of major genes were normalized using the $2^{-\Delta\Delta Ct}$ method. The letters above bars represent statistical significance ($p < 0.01$) as measured by Duncan's multiple range test. ns: no significant difference.

3.3. Independent AG1 Function of I-PA under Anaerobic Conditions at the Germination Stage

To assess the function of the *AG1* QTL in I-PA, the germinability of IR64, I-Pup1, I-AG1, and I-PA was tested under hypoxia and anoxia experiments (Figure 5). All the lines, including IR64 and I-Pup1, were germinated in hypoxia (Figure 5a). However, only I-PA and I-AG1 were germinated in the anoxia condition (Figure 5d). I-PA showed a 55% survival rate, like that of I-AG1, in the hypoxia condition, whereas the survival rates of IR64 (5%) and I-Pup1 (8.8%) were lower (Figure 5a,b). IR64 and I-Pup1 showed a decreased level of coleoptile length; however, I-PA and I-AG1 showed normal coleoptile lengths (~50 mm) in hypoxia (Figure 5c). Under the anoxia condition, the survival rates of I-AG1 and I-PA were 18% and 12%, respectively. Germinated I-PA showed the same level of coleoptile and shoot elongation as that of I-AG1 (Figure 5e–g). The expression level of *OsTPP7*, a major gene of *AG1*, was significantly increased in the shoot of I-PA. However, the difference was not observed in the root (Figure 5h).

In different intensities of anaerobic conditions, I-PA, which harbors *Pup1* and *AG1*, showed significantly increased germinative power and coleoptile elongation than I-Pup1. Based on the result that existing *Pup1* does not affect *AG1* functions, it seems like *AG1* does not interplay with *Pup1* under anaerobic conditions including hypoxia and anoxia.

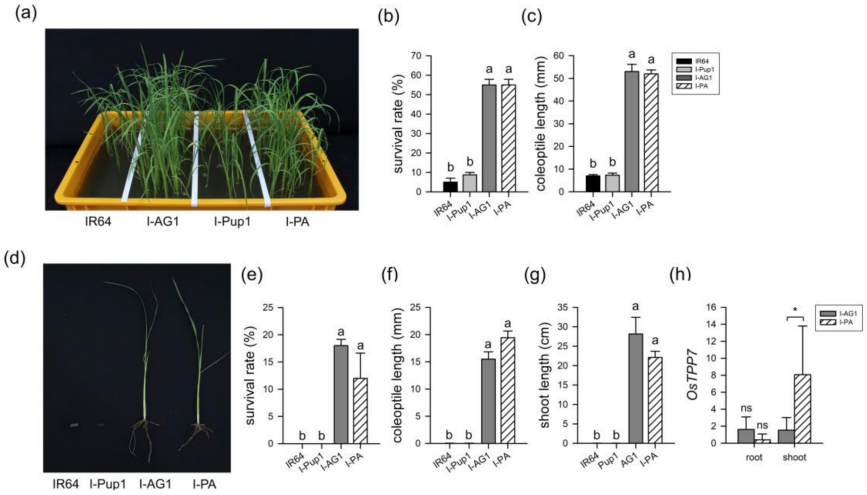

Figure 5. Germination rate, coleoptile elongation, and *OsTPP7* expression levels of I-PA under anaerobic conditions. The tolerance phenotype of I-PA and I-AG1 to anaerobic germination conditions at 21 DAS. (**a**–**c**) and (**d**–**g**) are phenotypic results of hypoxia and anoxia conditions, respectively. (**h**) the transcriptional level of *OsTPP7* in I-PA and I-AG1 under anoxia conditions (IR64 and I-Pup1 did not geminate). The letters above bars represent statistical significance ($p < 0.01$) as measured by Duncan's multiple range test. ns: no significant difference. The asterisk represents statistically significant difference ($p < 0.05$) between I-AG1 and I-PA in (h).

3.4. Improved Tillering Ability of I-PA during the Early Growth Stage in the Paddy Field

I-PA showed an enhanced function of P uptake and anaerobic germination during the early stage. Thus, I-PA and their parents were grown in the paddy field to investigate whether early vigor with enhanced tillering can be made to last to the booting stage in the two different locations—Suwon (field A) and Hwaseong (field B). Plant height and tiller numbers were measured at 40 days after transplanting (DAT) and 60 DAT in the two fields (Figure 6). In terms of plant height, I-PA was shorter than the other plants, including IR64 (Figure 6a). However, I-PA showed a significantly large number of tillers (Figure 6b). The number of tillers of I-PA was 22.1 and 21.1 at fields A and B, respectively, which signifies a 31.5% and 41.6% increase in tiller number in the field than that of IR64. These results were aligned with the results at 40 DAT (not shown) and shown in the hydroponic and different P application experiments (Figure 2; Figures S1 and S2).

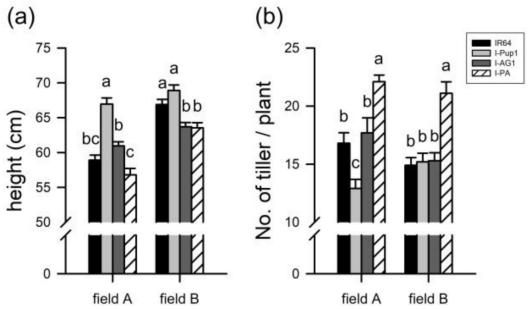

Figure 6. Height and tiller number of I-PA (IR64-Pup1-AG1) at 60 DAT (days after transplanting) in paddy fields. The height (**a**) and tiller number per plant (**b**) of I-PA and NILs at 60 DAT. Locations and conditions of the field are mentioned in Materials and Methods. The letters above bars represent statistical significance ($p < 0.01$) as measured by Duncan's multiple range test.

4. Discussion

Rice is relished by more than 3.5 billion people, whereas skilled manpower and labor for rice cultivation is decreasing [17,18]. Direct seeding on the paddy field is a useful way to fulfill the amount of rice consumption with reduced manpower. Since poor early vigor is a major constraint in the direct seeding system, vigorous early establishment is a major key to overcoming the problem and minimizing loss of yield [11]. *Pup1* has never been combined with other abiotic stress tolerance QTLs in previous studies, while *AG1* has been combined with other QTLs like *Sub1* or biotic stress tolerance QTLs [11,19]. In this view, I-PA was the first trial to combine *Pup1* with other stress tolerance QTLs using near isogenic lines (NILs). *Pup1* is known as having a tolerance to the phosphorous deficiency condition. Furthermore, it can also be applied to early root vigor because it was derived from Kasalath, which originates from the uplands, and showed beneficial effects in previous studies [7,8]. However, *Pup1* was poor in anaerobic conditions, such as germination under anoxia/hypoxia (Table 1). In contrast, *AG1* has a tolerance to germination under anaerobic conditions with an escape strategy through the rapid elongation of the coleoptile [3], but showed poor early vigor. In this study, the effects of *AG1* and *Pup1* were shown additively in the pyramiding line (I-PA) under low P and anaerobic conditions (Table 1).

Table 1. Improved early rooting of I-PA under aerobic and anaerobic conditions.

Conditions / NILs	Germination		Low P and Aerobic
	Anoxia	Hypoxia	
I-Pup1	poor	poor	good
I-AG1	good	good	poor
I-PA	good	good	good
IR64	poor	poor	poor

NILs: near isogenic lines; poor/good: phenotypically intolerant/tolerant growth in each condition.

After implementing marker-assisted backcrossing and marker-assisted selection using NILs, 80% of the background with IR64 was recovered in I-PA (Figure 1b). Some chromosomal regions were not inherited from I-Pup1 or I-AG1, because I-SA (IR64-Sub1-AG1) was used as parents during the plant development. However, since the functions of *Pup1* and *AG1* have been checked in I-PA under different P concentrations and anaerobic germination conditions (Figure 3, Figure 4, Figure 5), the effect of the additional fragments on *Pup1* and *AG1* seem to be small, because the additive effects of *Pup1* in I-Pup1 and I-PA were almost the same in different P experiments (Figures 3 and 4). Likewise, the additive effects of *AG1* in I-AG1 and I-PA were almost similar. However, the genetic effect and co-segregation of the genes originated from the donors should be studied further.

Interestingly, the tiller number of I-Pup1 was the same or even smaller than the others at paddy fields (Figure 6b), which means that I-PA's tiller number increase was not solely caused by the effect of *Pup1*. Thus, I-PA showed the additive effects of *Pup1* and *AG1* by pyramiding and the enhanced growth capability only in the early stage, by increasing the number of tillers as compared to vertical growth. Therefore, we examined the presence of the additional background during the material development, where some additional QTLs for tillering capacity were reported in those regions, which showed very small effects in this study (Figure 1).

In rice production systems, I-PA can be used for developing varieties with early vigor under anaerobic germinative conditions. As a result of investigating the agronomic traits in field A, the yield of I-PA did not decrease because of the increased spikelet number per panicle; however, fertility and 100-grain weight were smaller than the parental variety, IR64 (Table S2, Supplementary Materials). In reference to the idea of Wissuwa et al. [20], the decrease in fertility and 100-grain weight in I-PA seems to be due to the increased yield potential, requiring more nutrients, caused by the large biomass at the seedling stage. Strategies such as the treatment with enough fertilizer in the panicle initiating or

booting stage or during late harvesting are needed to fill grains and increase yield. The relationship between the yield potential and maturity will be studied further.

5. Conclusions

A pyramiding line of *Pup1* and *AG1* with IR64 background was developed. In this study, we investigated the function of the QTLs in different P application and anaerobic germination conditions.

The pyramiding line, I-PA, showed shorter height and increased tiller number per plant under P non-supplied and -supplied conditions. Additionally, it showed better P uptake capacity under P-supplied condition. The survival rate and coleoptile length of I-PA increase under anaerobic germination conditions. Shoot elongation of I-PA showed a result similar to that of I-AG1. *OsPSTOL1* and *OsTPP7*, the major genes of *Pup1* and *AG1*, were expressed independently under each tested condition. I-PA showed increased tiller number per plant at 60 DAT under P-supplied conditions. Conclusively, the *Pup1–AG1* pyramiding approach can be considered for direct-seeded rice variety development programs.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/10/10/453/s1, Figure S1: Phenotype of I-PA and near isogenic lines (NILs) under P non-supplied and P-supplied conditions at 14 DAT. Figure S2: Phenotype of I-PA and NILs in hydroponic system under different phosphoric acid concentrations. Table S1: List of primers used in this study. Table S2: Yield of I-PA under fertilizer-sufficient conditions.

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