บทความวิจัย

Application of the *Pup1-K46* Marker to Evaluate Phosphorus-Deficient Tolerance in Lowland Rice Cultivars from Southern Thailand

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ABSTRACT

Phosphorus (P) deficiency severely limits rice production, which threats to global food security. Selecting rice cultivars tolerant to P deficiency is a sustainable approach for this issue. In rice, a gene-based marker *Phosphate uptake 1* (*Pup1-K46*) has been used to evaluate P-deficient tolerance. Indisputably, the *Pup1-K46* region is highly conserved in an upland rice ecotype. Its existence reflects to the tolerance of P deficiency. Unfortunately, the influence of *Pup1-K46* and its application in lowland rice cultivars remains neglect. In this study, we investigated the *Pup1-K46* locus among 61 lowland rice cultivars. Subsequently, several lowland rice cultivars with and without the *Pup1* locus were grown under the 0.5x low P (0.25 mg/l) and high P (5 mg/l) Yoshida solution for three weeks to examine the P-deficient tolerance of rice seedlings based on the existence of *Pup1-K46.* The results showed that the low P solution reduced rice biomass up to 20 percent reduction with diverse degrees, depending on the rice cultivars. It markedly lowered the total P concentration but raised P use efficiency (PUE) in the shoot and root tissues. The reduction of shoot growth due to the low P availability in the *Pup1* positive group was significantly lower than the *Pup1* negative group. Additionally, the higher shoot PUE in the *Pup1* positive group confirmed their growth performance against P deficiency. These suggest that the *Pup1* locus contributes to P-deficient tolerance in lowland rice cultivars.

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Introduction

P deficiency is one of the major problems in rainfed lowland rice ecosystem since most of the P contents are naturally in unavailable forms [1-2]. Only 0.1 to 15% of the total P is present as soluble phosphate (Pi) [1, 3]. Although rice paddies are well-fertilized, the available Pi portion remains insufficient for rice growth [4-5]. Unless high amounts of fertilizer are frequently added to sustain the Pi level in rice paddies [5-6]. These affect production of rice (Rice, *Oryza sativa* L.) which is an important staple food crops, feeding more than one-third of the human population.

Environmental Pi levels in the range of 0 to 3 ppm have been considered as a P-deficient condition for rice [7-9]. These low P conditions reduce rice cellular Pi levels. Decrease in Pi concentrations in shoots of rice seedlings develops within three days after Pi is removed from the culture condition while that in roots takes place rapidly within a day. However, the decreased Pi level in shoot and root tissues are suddenly recovered by a day after Pi resupply [10-11]. This illustrates the rapid internal Pi change in rice plants due to the external Pi availabilities. Pi starvation holistically reduces rice shoot and root growth by deteriorating their net photosynthetic rates [12-14]. It also lessens chlorophyll contents and causes oxidative stress in rice leaves [15-16]. Additionally, rice grown in the P-deficient condition reduces accumulation of the other essential elements [17]. Thus, to physiologically maintain internal Pi level in the tissues, especially during long-term Pi starvation, rice elevates the efficiency of Pi acquisition and utilization, both of which are counted for P use efficiency (PUE) [18]. Moreover, in plant breeding programs, PUE has commonly been used to evaluate the efficiency of P-deficient tolerance in several plant species [19] including rice [20-22]. The higher PUE rice plants are, the more tolerant to P deficiency they are.

P-deficient tolerance in rice is a useful trait in selection of rice cultivars for improvement of rice production. The QTL *Pup1* awards rice with outstanding P-deficient tolerance [23]. This region empowers rice plants to handle P deficiency by enhancing its phosphorus acquisition [24-25]. The *Pup1-K46* gene-based marker is annotated as a serine/threonine protein kinase and subsequently designated as phosphorus-starvation tolerance 1 *(PSTOL1)* [26]. *PSTOL1* is up-regulated by Pi deficiency. It exclusively expresses in crown root primordia and parenchyma cell located outside the peripheral vascular cylinder where crown roots are generated [26]. The *Pup1-K46* marker is dominantly conserved in the P-deficient tolerant varieties, mainly in upland ecotype or rain-fed varieties [27].

This study aimed to investigate the *Pup1* locus in 61 lowland rice cultivars, mainly originated from southern Thailand by using the *Pup1*-K46 marker. After the rice cultivars were genetically categorized into *Pup1* positive and negative groups, their growth efficiency under

P deficiency was clarified in a hydroponic system. We hypothesized that the *Pup1* locus assists lowland rice to grow under the limitation of P. This research will preliminarily determine application of the *Pup1* QTL marker in the selection of lowland rice cultivars for low P environments, minimizing fertilizer inputs in rice paddies and improving the sustainable rice production.

Materials and Methods

1. Seed collection

Rice seeds were kindly provided by Phatthalung Rice Research Center, Phatthalung, Thailand and additionally collected from local rice farmers from the major rice producing locations of Southern Thailand including Songkhla, Nakhon Sri Thummarat and Phatthalung provinces under the Plant Genetic Conservation Project under the Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhorn. For IR64 and Dular, the seeds were obtained from Department of Agriculture, Bangkok, Thailand.

2. Screening of the *Pup1-K46* locus variation

Genomic DNA was extracted from 7-day old rice seedlings using the protocol modified from Dellaporta et. al. [28]. The 25-μl PCR reaction mixture composed of approximately 100 ng of genomic DNA, 1X ViBuffer S, 0.1 mM dNTPs mix, 0.4 μM each forward and reverse primers and 1 unit Taq DNA polymerase (Vivantis). The 523-bp *Pup1-K46* fragment [25] was determined by the *Pup1-K46* marker primers (Pup1-K46-F: 5$'$-TGAGATAGCCGTCAAGATGCT-3$'$ and Pup1-K46-R: 5$'$-AAGGACCACCATTCCATAGC-3$'$). Moreover, the PCR was verified by the *EF1α* primers as positive control (EF1α-F: 5$'$-TTTCACTCTTGGTGTGAAGCAGAT-3$'$ and EF1α-R: 5$'$-GACTTCCTTCACGATTTCATCGTAA-3$'$) which yield an 805-bp DNA fragment (the data not shown) [29]. The PCR conditions were programmed as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 3 steps PCR as denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min as well as additional final extension at 72°C for 5 min.

3. Plant growth conditions

Seeds of 20 and 21 rice cultivars with and without the *Pup1* locus were sowed on damp tissue papers in petri dishes for a week. Seven-day old seedlings were gently transferred into 1-ml pipette tips with 2 to 3 mm from the end was cut to increase the internal diameter of standard pipette tips allowing for the growth of roots into a pipette tip box containing the 500-ml half-strength Yoshida solution. The 24 seedlings were grown under high P (5 mg/l) and low P (0.25 mg/l) conditions in an open green house for three weeks (Fig 2A). The hydroponic solution was exchanged twice a week while the amount of solution was daily maintained by distilled water. After three weeks, only healthy plants (19-22 plants) were harvested for further analyses.

4. Plant biomass and P concentration analyses

Rice seedlings were dried in an oven at 60°C for three days. Shoot, root and total biomass were individually measured. The data was presented in the relative efficiency of P use (REP, %) [30]. To analyze P concentration in rice tissues, dry tissues from three plants were pooled into a sample. Three samples (three biological replicates) were prepared for the analyses. Briefly, each sample was weighed with a four-digit scale and then recorded. The sample was incubated in 2 ml of 65% HNO_3 at 95°C for an hour before adding 1 ml of 30% H_2O_2 to complete the digestion. The solution was filtered with a whatman paper No.1. Later, the solution volume was adjusted by distilled water up to 10 ml. Total P concentration was measured by ICP-OES (AVO 500, Perkin Elmer). Subsequently, the ratio of dry weight and P content in shoot and root parts was interpreted as PUE, indicating the biomass produced per unit P accumulated [30-31]

5. Statistical analyses

Student's t-test was performed in Excel to compare means of REP and overall PUE between the *Pup1* positive and negative groups. Means of dry weight, P concentration and PUE from each individual cultivar were analyzed by one-way ANOVA following LSD test in R.

Results

1. The *Pup1-K46* locus is investigated in several lowland rice cultivars

To examine the *Pup1-K46* locus in diverse lowland rice cultivars, we exploited 61 lowland rice cultivars, mainly originated from Southern Thailand and one upland rice cultivars namely Dokpayom. Additionally, Dular and IR64 were recruited in the genotyping analysis as positive and negative controls, respectively. After PCR amplification by the Pup1-K46 primers, as expected, *Pup1-K46* was detected in Dular but non-detected in IR64 (Fig. 1). Among the tested rice cultivars, half of them possessed the *Pup1-K46* region (Fig. 1, Table S1).
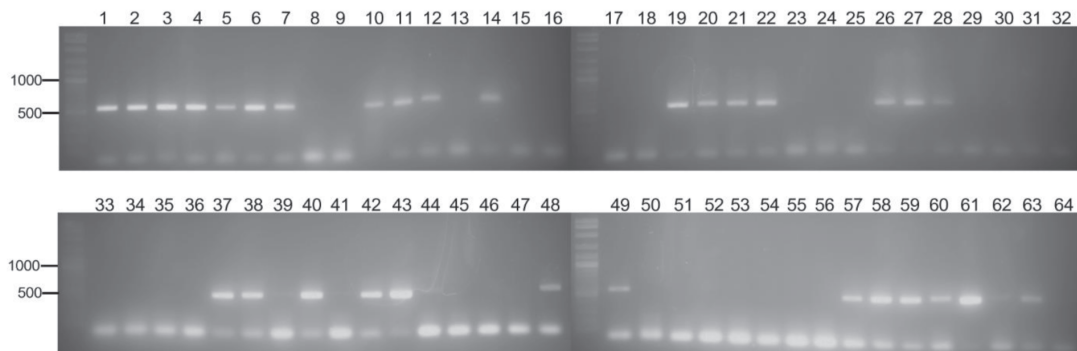
Figure 1 Genotyping analysis of the *Pup1-K46* region in various rice cultivars. The *Pup1-K46* locus was detected in Khawleong (1), Keanchan (2), Chorpleekhaw (3), Maemai (4), Hulhumdoulila (5), Kilo (6), Chorlamai (7), Hua-nadum (10), Buangkhaw (11), Eawmoddang (12), Majanoo (14), Nanglong (19), Lumhak (20), Ra-den (21), Lookdum (22), Koo-ning (26), Kaopan (27), Lookpung (28), Muangsai (37), Moh-aroon (38), Saansuay (40), Riceberry (42), Lepnok (43), Khawdum (48), Sangyod (49), Hombi-toey (57), Homtummasart (58), RD15 (59), Pathumthani 1 (60), KDML105 (61), Homnil (62) but undetected in Looknon (8), Chuktiumdang (9), Lookkheoy (13), Looktor (15), Chana (16), Yakobao (17), Muntom (18), Dokpayom (23), Koomuangluang (24), Kom (25), Raisai (29), Pooyoo (30), Khawrai (31), Nangloy (32), Puangwai (33), Kantang (34), Kleabmek (35), Klanak (36), Saemar (39), Tiamalaydang (41), Chaing (44), Khemthong (45), Lookpla (46), Khawmae (47), Malay (50), I-chiang (51), RD41 (52), Homrachinee (53), Mlea-hom (54), Khawkemthong (55), RD61(56). Dular (63) and IR64 (64) were used as positive and negative controls. Noted that the major bands of DNA ladder at 500 and 1,000 bp are labeled on the left side of the gels.

2. The *Pup1* positive cultivars are more tolerant to the low P condition than the *Pup1* negative cultivars

20 and 21 cultivars with/without *Pup1-K46* were selected to confirm the role of *Pup1* locus on rice growth performance related to P availability under hydroponic condition. Seven-day old seedlings were hydroponically treated with the high and low P conditions in the opened greenhouse for three weeks (Fig 2A). The result showed that the low P condition apparently reduced shoot and root biomass in all rice cultivars (Fig. 2B-C).

Subsequently, shoot, root and total dry mass between the two different P regimes was considered. According to the *Pup1* genotyping analysis, we categorized the rice cultivars into *Pup1* positive and negative groups (Table S2) and then verified effect of the low P condition on their relative biomass reduction. The hydroponic system revealed that the shoot REP in the *Pup1* positive group was significantly higher than the *Pup1* negative group (Fig. 3A and Table S3) whereas the root and total REPs were not different among the two distinct groups (Fig. 3B-C and Table S3).

3. The tolerance to P deficiency in *Pup1* positive cultivars may be involved in their PUE

To guarantee whether our P conditions modify internal P concentration in rice seedlings, we selected three rice cultivars with and without the *Pup1* locus as representatives for the measurement of total P concentration in shoot and root parts. Apart from dry weight (Fig. 4A), the low P condition significantly reduced total P concentration in shoot and root tissues (Fig. 4B). Under the high P condition, the P concentration in the shoots was markedly greater than the roots whereas under the low P condition their P concentrations became indifferent (Fig. 4B).

Finally, we hypothesized that growth response of rice to the different P availabilities is dependent of their internal PUE. To preliminarily test our hypothesis, PUE was interpreted from the ratio of biomass to its P content. Our result showed that the low P condition significantly elevated the shoot and root PUEs in all of the rice cultivars (Fig. 4C). Nevertheless, the overall shoot PUE in the *Pup1* positive group was relatively higher than the *Pup1* negative group under the low P condition (Fig. 4C).
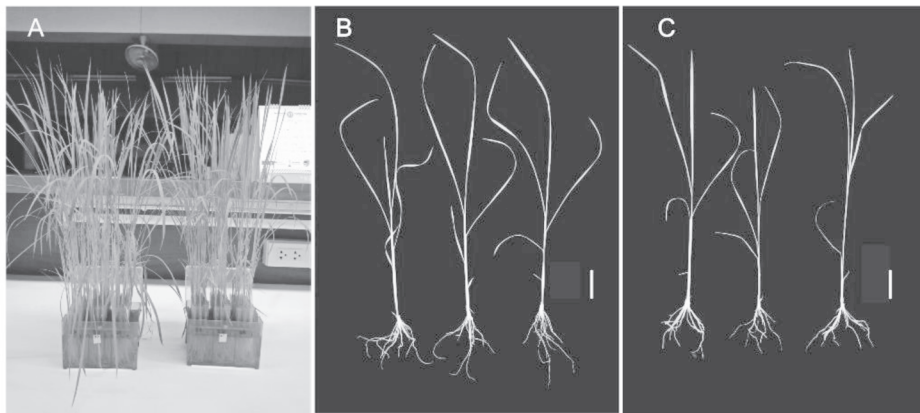
Figure 2 Application of hydroponic cultures to screen the efficiency of P-deficient tolerance in rice seedlings. Rice seedlings were grown in pipette boxes containing the 0.5X Yoshida solution with 5 mg/l and 0.25 mg/l P for the high (left box) and low P (right box) conditions, respectively (A). Compared with the seedlings grown in the high P medium (B), the low P medium reduced rice vegetative growth (C). Scales represent 5 cm.
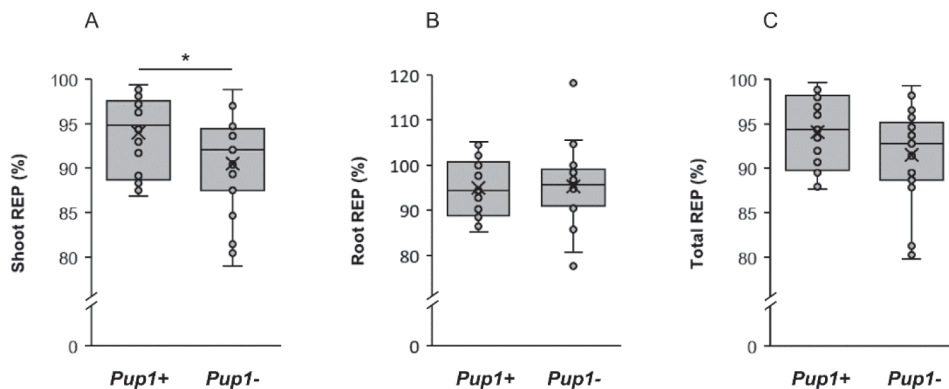
Figure 3 Relative efficiency of P use (REP) in the *Pup1* positive (+) and negative (-) groups. REP (%) was calculated as the ratio between the mean of plant dry weight under low and high P treatment. The box plots give the distribution in percentage of shoot REP (A), root REP (B) and total REP (C). Horizontal lines and crosses in the boxes are the median and mean values of 20 *Pup1* positive cultivars (n=20) and 21 *Pup1* negative cultivars (n=21). Statistical analysis was performed with Student's t-test. Asterisks indicate significant differences ($P \leq 0.05$).
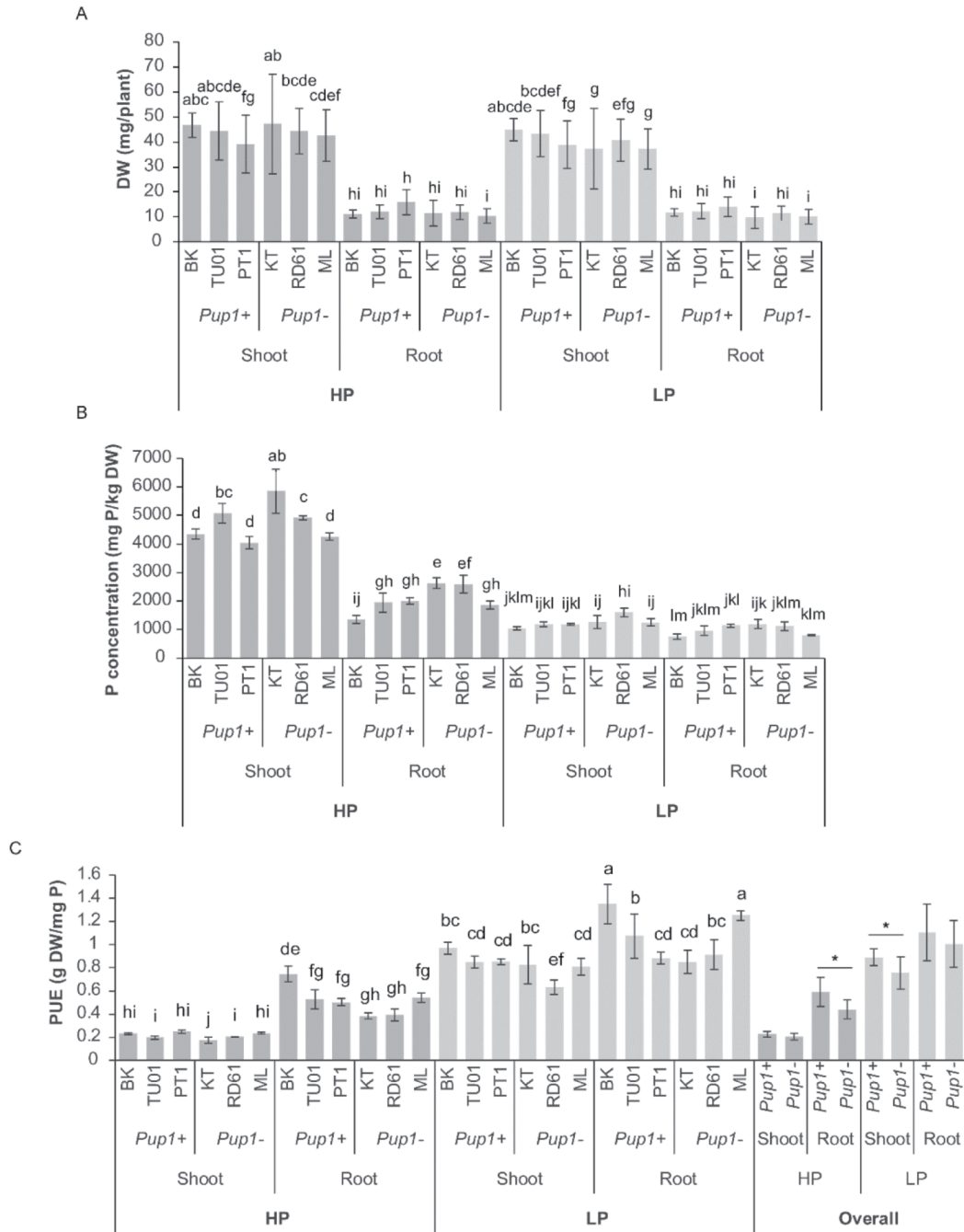
Figure 4   Dry weight (A), Total P concentration (B) and PUE (C) in shoot and root tissues of the *Pup1* positive (+) and negative (-) cultivars under the high and low P conditions. The three representatives of *Pup1+* and *Pup1-* cultivars are Buangkhaw (BK), Homtummasart (TU01), Pathumthani 1 (PT1), Kantang (KT), RD61 and Malay (ML), respectively.

Figure 4A   Dry weight. Data corresponds to the mean of 19-22 biological replicates and SD (n = 19-22). Different letters indicated the significant difference within the assay (LSD test, ANOVA; P ≤ 0.05).

Figure 4B   Total P concentration. Data corresponds to the mean of three biological replicates and SD (n = 3). Different letters indicate the significant difference within the assay (LSD test, ANOVA; P ≤ 0.05).

Figure 4C   P use efficiency (PUE). PUE was calculated from dry weight divided by P content. Data corresponds to the mean of three biological replicates and SD (n = 3). *(Legend continued on the next page)*

Different letters indicate the significant difference within the assay (LSD test, ANOVA; P ≤ 0.05). In overall, data from the three rice cultivars within the same *Pup1* genotype was assembled (n=9). Asterisks indicate the significant difference of PUE between the *Pup1+ and Pup1-* groups (Student's t-test; P ≤ 0.05).

Discussion and conclusion

The *Pup1* locus is not only restricted in *Oryza sativa* but also widely distributed across the *Oryza* genus [32-33]. However, it is mostly absent in lowland rice varieties [27,32]. To explore the *Pup1-K46* fragment in the lowland rice cultivars in this study, Dular and IR64 were exploited as the positive and negative control [26-27]. Here, we found that half of the tested lowland rice cultivars harbored *Pup1-K46,* discovering that the *Pup1* locus moderately disperses in lowland rice cultivars of Thailand, consistent with the previous report in Thailand [34].

The shoot REP in the *Pup1* positive group was greater than that in the *Pup1* negative group, suggesting that the growth of *Pup1* positive lowland rice cultivars seems less affected by the P deficiency than the *Pup1* negative cultivars. In other plants, REP indicates plant growth efficiency under low P conditions. This parameter has been basically used to describe plant P-deficient tolerance [30, 35, 36]. Furthermore, the biological function of *Pup1-K46 (PsTOL1)* in rice was characterized in IR64, a representative lowland *indica* variety without the *Pup1* locus. The *Pup1* breeding lines in an IR64 background lead to the enlargement of root system and the improvement of aboveground biomass under low P conditions [26, 37]. Together with our study, the presence or absence of *Pup1* in lowland *indica* cultivars have an impact on their P-deficient tolerance, detected at the seedling stage.

After treated with the low P hydroponic solution for three weeks, the rice seedlings at a four-leaf stage demonstrated biomass reduction and decline in total P concentration, particularly in the shoots. Moreover, under the low P condition, P is more partitioned into roots since the different P concentrations between the shoots and roots was no longer observed. This indicates

that P deficiency more dominantly suppresses the shoot growth than the root growth through P partitioning. Like other plants, P deficiency retards rice growth [13] and also reduces their internal P concentration [10]. Roots of rice seedlings actively take up P from environments despite of their endogenous P reserve at the fourth day after germination. Thus, environmental P is essential for rice seedling growth [38]. Under P sufficiency, the total P content is highly accumulated in shoots [10,38] but when Pi deficiency is developed, the proportion of P content is preferentially partitioned into roots, displaying the dynamic of P allocation and partitioning driven by the P demand between the P sink and source organs [13].

Taken consideration into PUE, the superior PUE value was observed in the shoots of *Pup1* positive cultivars under the low P condition. The PUE value refers to the biomass per P content [30,39] and it is lifted up due to P deficiency [31]. This is highly possible that lowland rice seedlings with the *Pup1* locus require less P to maintain shoot growth under the limitation of P. Therefore, the presence of *Pup1* in the lowland ecotype is involved in P-deficient tolerance like the upland ecotype.

According to our results, the above-ground P contents in rice seedlings were markedly reduced when the seedlings grew under a low P condition for a few weeks. Therefore, we further hypothesized that *Pup1* positive cultivars might recover their P level faster than *Pup1* negative cultivars after P is supplied into the growing condition. Moreover, some photosynthetic parameters in seedling leaves would be recruited to evaluate P-deficient symptom in shoots between the two distinct groups of Pup1 genotypes.

Acknowledgements

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