MOLECULAR CHARACTERIZATION OF SELECTED RICE GENOTYPES FOR THE MAJOR QTL *PHOSPHORUS UPTAKE ONE* (*PUP1)**

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ABSTRACT

Marker-assisted breeding is a very useful instrument for breeders but still needs much research work because information on the effect of quantitative trait loci (QTLs) in different genetic backgrounds is uncertain and ideal molecular markers are unavailable. Therefore, some research efforts toward the investigation and validation of the major rice QTL *Phosphate uptake 1* (*Pup1*) that confers tolerance of phosphorus (P) deficiency in rice (Oryza sativa L.) have been made throughout assessing the effect of the presence of the *Pup1* allele on growth parameters of selected genotypes. Six markers were selected that target firstly putative genes that are partially conserved in the Nipponbare reference genome and secondly Kasalath-specific genes that are located in a large insertion-deletion (INDEL) region that is absent in Nipponbare. Testing these markers in 30 diverse rice genotypes showed that those genotypes possess Kasalath alleles at all analysed loci, apart from Kasalath, were Azucena, Black Gora, FR 13A, IAC 165 and IAC 25. Genotypes that partially possessed Kasalath alleles were only three (Dular, Li-Jiang-Xin-Tuan-Hei-Gu and M 202) while the other genotypes lacked Kasalath alleles at all loci analysed. These contrasting Pup1 genotypes were subsequently grown in two different P-deficient soils and environments. Whether P applied in liquid form as Yoshida's nutrient solution (YNS) or solid form as rock phosphate added either homogenously or in 10 cm shallow layer, genotypes with the Kasalath alleles in *Pup1* locus maintained significantly higher shoot dry weight (SDW) plant^{-1} under P deprivation in comparison with intolerant genotypes with the Nipponbare alleles in this locus. Overall, the data provide evidence that *Pup1* has the potential to improve SDW in P-deficient conditions and in diverse genetic backgrounds.

Key words: Oryza sativa, phosphorus deficiency, shoot dry weight.

***Part of Ph.D. dissertation of first author.**

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الوصف الجزيئي لموقع صفة كمية يتعلق بامتصاص الفوسفور *(Pup1)* في تراكيب وراثية منتخبة لمحصول الرز*

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المستخلص

تعد التربية بالاستعانة بالمعلمات الجزيئية اداة مفيدة جدا لمربي النبات ولكن لا تزال بحاجة إلى الكثير من العمل البحثي لأن المعلومات عن تأثير مواقع الصفات الكمية (QTLs) في تراكيب وراثية مختلفة هي غير مؤكده ولا توجد هناك معلمات جزيئيه مثالية، وعليه بعض الجهود البحثية باتجاه التحقيق والتثبت من موقع الصفه الكمية فيما يختص الفوسفور (*Pup1*) الذي يمنح صفة مقاومة شحة الفوسفور في الرز (.Oryza sativa L) كانت قد عُملت من خلال تقييم تائير تباين الاليلات في الموقع *Pup1* على صفات النمو المقاسة في تراكيب منتخبة من الرز. لقد تم اختيار ستة معلمات جزيئية لتستهدف جينات مفترضة ومحفوظة جزئيا في جينوم صنف الرز نيبونباري (Nipponbare) وكذالك تستهدف الجينات الخاصة بصنف الكزلاث (Kasalath) وان هذه الجينات الاخيره هي غير موجودة في صنف ال Nipponbare وتقع في موقع جينومي كبير يحتوي على مسح او وادخال (INDEL). بعد اختبار 30 صنف لمحصول الرز بواسطة المعلمات اظهرت النتائج بأن الأصناف التي تمتلك لاليلات الصنف كزلاث في كل مواقع الصفه الكميه المدروسه من غير صنف الكزلاث كانت Azucena, Black Gora, FR 13A, IAC 165 وكذالك IAC 25 اما الأصناف التي تمتلك لاليلات كزلاث بشكل جزئي فهي ثلاث فقط وهي Dular, Li-Jiang-Xin-Tuan-Hei-Gu و202 M بينما باقي الأصناف لا تمتلك لاليلات كزلاث في كل المواقع المدروسه. ثم بعد ذلك تمت زراعة هذه الأصناف المتباينه باليلاتها في تربتين تفتقر لعنصر الفوسفور وقد أضيف عنصر الفوسفور الى المعاملات بإسلوبين مختلفين وبتجربتين (أما بشكل سائل كمحلول يوشيدا (Yoshida) المغذي او بشكل صلب على شكل الفوسفات الصخري والذي اظيف هو الاخر بطريقتين أما خُلط بشكل متجانس مع كل التربة او اقتصر على 10 سم من الطبقه السطحية فقط). لقد اظهرت النتائج بانه تحت ظروف شحة الفوسفور استطاعت الأصناف التي تمتلك لاليلات صنف الكزلاث في *Pup1* ان تديم وزن جاف للمجموع الخظري عالي بشكل معنوي مقارنتاً بالأصناف الغير مقاومه التي تمتلك اليلات صنف النيبونباري في هذا الموقع للصفة الكمية. بشكل عام ان البيانات تثبت بأن *Pup1* اثر وبشكل معنوي على رفع الوزن الجاف للمجموع الخضري لاصناف من الرز تختلف في تركيبها الوراثي في ظروف شحة الفوسفور.

كلمات مفتاحية: Oryza sativa، نقص الفسفور، الوزن الجاف للمجموع الخضري.

***البحث مستل من اطروحة دكتوراه للباحث الأول.**

INTRODUCTION

P deficiency is a major abiotic stress that limits crop productivity on 30 − 40% of the World's arable land (17). P is an immobile element and is readily bound with soil particles. In most soils, P availability is therefore suboptimal and inadequate for high yield production. P is also expensive and the majority of farmers especially in developing countries cannot afford the rising prices of P fertilizers. So P availability in soil is a matter of concern and invites research attention to find an alternative way for sustainable production and food security for the world's growing population. The importance of P for plant growth is a key factor in crop production worldwide (16). Almost all over the world, optimum crop production relies upon chemical fertilizers especially N and P (3). Unlike nitrogen, P is a problematic nutrient because when applied to soil it is readily bound to soil particles and becomes immobile (14). The ability of an individual or species in acquiring resources determines its adaptation and productivity in a given environment (1). Soil resources are usually unevenly distributed in space and time and often subject to localized depletion that make root architecture of great importance for plant productivity (11). For example greater nutrient acquisition especially in case of immobile resources such as P has been associated with topsoil foraging (2). Plant residues, remaining roots and P applied as fertilisers constitute the main sources of soil P, most of which is bound by soil particles within the shallow surface layer of the soil. This has made P concentration and availability more at the soil surface than at depth. Therefore, genotypes with a deep rooted system may lose the opportunity to access shallow P and are less tolerance of P deficiency. Hence root class may be of great importance in terms of P uptake. Genetic variation is responsible for differences in tolerance of P deficiency between genotypes. The major QTL *Pup1* was identified to confer tolerance of P deficiency and its potential influence to enhance yield in different genetic backgrounds and P-deficient environments had been verified by field-based phenotypic trials in Japan (19, 20). The impact of *Pup1* and other QTLs in enhancing yield under P-deficient conditions has been reported by Chin *et al*. (4). The cloning of the *Pup1*-specific protein kinase gene, which has been named phosphorus starvation tolerance 1 (*PSTOL1*) (**7**), can provide supportive information for the development of tolerant rice varieties. Chin *et al*. (4) reported that an improved and extended set of molecular markers were developed based on the preliminary gene models that have been verified through gene expression and allelic sequencing data that were reported earlier by Heuer *et al*. (9) and the genomic *Pup1* sequence of Kasalath (GenBank accession no. AB458444.1). These gene-based *Pup1* molecular markers differentiate between three main groups of genotypes with different *Pup1* haplotypes which may be used for an assessment of the *Pup1* locus in diverse rice genotypes (4). These three main groups are with different *Pup1* allele constitutions. Group I includes accessions with tolerant *Pup1* alleles and those with intolerant belong to group III. Genotypes containing partial *Pup1* (some of the loci include Kasalath alleles) belong to group II. The development of these molecular markers is of particular importance to complement and/or support or even replace phenotypic evaluations in the field for the development of P-efficient rice varieties. It is of particular importance to characterise selected rice genotypes with some *Pup1* molecular markers. This will allow classifying genotypes according to the three *Pup1* haplotype groups in order to assess statistically the influence of the presence of tolerant *Pup1* alleles (Kasalath alleles) on the growth parameters and P uptake of genotypes used in this study. Exploring the genetic variations of adaptive responses among crop species and genotypes for enhanced P efficiency and soil P acquisition ensures sustainable agricultural production in P-limiting soils. Therefore the objectives of this research were to screen 30 rice genotypes for genetic diversity and allelic variation using six molecular markers of *Pup1* locus and assess the influence of *Pup1* locus on growth performance and P uptake of these rice genotypes in P deficient soil either with YNS-P and YNS+P as a source of P in liquid form or with embedded rock phosphate in

different depths and distributions. Special emphasis is given to check the hypothesis that Kasalath alleles in *Pup1* locus confer tolerance of P deficiency in soil.

MATERIAL AND METHODS

Rice genotype selection

A total of 30 different rice genotypes were mostly obtained from the International Rice Research Institute and used in YNS experiment (experiment one). Twenty of these genotypes belong to the Oryza SNP set (13): Akihikari, Aswina, Azucena, Bala, Black Gora, CT 9993, Cypress, Dom Sufid, Dular, FR 13A, IAC 165, IAC 25, IR 64, Kinandang Patong, Labelle, Lemont, M 202, Minghui 63, Moroberekan, N22, Nipponbare, Rayada, Sadu Cho, Sanhuangzhan No 2, Swarna, Tainung 67 and Zhenshan 97. This Oryza SNP panel was selected because they have received extensive genetic (13) and phenotypic (8) studies. Two genotypes are mutants of the Aux1 gene which is known to affect root growth (Aux1Mutant 1 and Aux1Mutant 2) while the genotype called Aux1 Wild type is genotype Zhonghua 11 in which genotype the mutants were made In the rock phosphate experiment (experiment two), all rice genotypes already used in the YNS experiment were included except for Minghui 63 and M 202, as seed germination was too poor. These were replaced by two new genotypes: Li-Jiang-Xin-Tuan-Hei-Gu and Kasalath. The latter is a traditional *aus*-type rice variety, in which the major QTL for P-deficiency tolerance *Pup1* was identified, which received much research attention (5, 12, 19, 20) and has very recently been cloned (7).

Growing genotypes under study in greenhouse

A box experiments were conducted in the glasshouse of the Cruickshank Building, Aberdeen, UK during June and continued to July 2011. A total of 30 rice (*Oryza sativa* L.) genotypes were evaluated for their growth response in a mixture of 25 % P-limited (814 µg g^{-1}dw) Insch subsoil uniformly added to 75 % blast sand (P content = 12.2 µg g^{-1}dw) in two experiments. The first (YNS experiment) was with two treatments, Yoshida's nutrient solution (22) either with P (YNS+P) or without P (YNS-P). The second (rock phosphate experiment) was when two levels of rock phosphate treatment were used. The first of the P treatments was by adding 59 mg of phosphorus pentoxide (P_2O_5) per plant to the soil mixture, achieved by adding 200 mg rock phosphate per plant that was distributed homogenously throughout soil profile (homogenous P). The second treatment was created where the same amount of rock phosphate (200 mg $plant^{-1}$) was given in a band in the 10 cm surface layer (shallow P), while the control treatment had no P added to the soil mixture (zero P). Both experiments were conducted with three replicate blocks (boxes) for each treatment, with two plants of each genotype in each box arranged in two randomised sub-blocks. At the bottom of each box (53 x 33 cm at the top, 49 x 27 cm at the bottom and 39 cm depth), five drainage holes of five mm diameter were introduced then a non-woven fabric (Teram, UK) sheet was placed inside. The Insch subsoil and sand were thoroughly mixed and distributed among clear 60 litre plastic boxes. In YNS experiment, six boxes were prepared while in rock phosphate experiment nine boxes were used. A plastic sheet (52 x 32 cm length x width) was place on the soil surface; the plastic sheets had 60 perforations (2 cm diameter) for sowing plants maintaining a 5 x 5 cm distance. A black/white plastic sheet was wrapped around the box to prevent heat gain and light entry. Before sowing, each box was saturated with eight litres of suitable YNS either -P or +P (pH 5.5) for YNS experiment and with -P for rock phosphate experiment. In both experiments, seeds were surface sterilised in diluted bleach (1% Na hypochlorite) before being germinated at 30 ^{o}C for two days. Two pre-germinated, uniform and healthy seedlings for each genotype were sown in each hole on the 10th June 2011. At the second leaf stage, the seedlings were thinned to one per hole. Each box was watered with four litres of suitable YNS, three times a week for the first two weeks and five litres three times a week for another two weeks. In the final week, four litres of nutrient solution a day were supplied until harvested on day 35 so that each plant was supplied with 1.5 litres of YNS. To minimize the accumulation of nutrients in the growth medum, each box was watered with six

litres of deionised water once a week. In YNS experiment plants were grown in a glasshouse with natural light and dark hours. The average day/night temperature was 28/24 ^{o}C and the relative humidity ranged from 55 to 70 %. Whereas rock phosphate experiment was grown in a controlled condition in a growth room under a 12 hr light regime with a light intensity of approximately 350−400 µmol $m^{-2}s^{-1}$ photosynthetically active radiation with 25 ± 2 ^{o}C at night and 28 ± 2 ^{o}C in the day. Relative humidity was maintained between 55 and 70% throughout. In both experiments, weeds were controlled by hand weeding. Plant height was monitored on weekly basis. After 35 days the plants were harvested and the shoot samples were oven-dried for two days at 70 ^{o}C to constant weight and the SDW was measured. Before analysis, each box was treated as two randomised replicate blocks and the mean for each genotype per box was calculated. The resulting data (one value for each genotype in a box) were treated as a randomised complete block with three replicates. The effect of block on traits was assessed by analysis of variance and data were checked for normality and log transformed when needed by producing residuals of the data then adding these residuals to the mean of these data to produce the corrected data.

Molecular marker analysis

A total of six microsatellite markers, which have been derived from markers sequences and described by Chin *et al*. (4), were tested in the present study as polymerase chain reaction (PCR) fragments produced by amplifying the DNA from genotypes used in this study.

Preparation of plant material

The 30 rice genotypes used in this study were sown in propagation trays with soil, in the greenhouse for germination. All the genotypes germinated well. After 3 weeks of growth, a few leaves were separated from each genotype and subjected to DNA extraction.

DNA extraction

For each individual genotype, 100 mg of fresh leaf was sampled, ground to a fine powder in liquid nitrogen using a pestle and mortar and had genomic DNA extracted using the DNeasy Plant Mini Kit (QIAGEN, UK Ltd.) and was stored at 4ºC.

Amplification of DNA

DNA amplification was carried out by PCR using an automated thermal-cycler (PCR Express, Hybaid, UK, Ltd.). The PCR was initially done separately for each primer and then collectively as multiplex groups. Each PCR vial contained 5 µl of DNA suspended in buffer, 0.1 µl primer (50 mM), 0.3 µl *Taq* DNA polymerase enzyme (50 units μl^{-1}), 0.5 µl nucleotides (DNTPs, 10 mM), 0.75 µl $MgCl_2$ (50 mM), 2.5 µl 10 x enzyme buffer and complementary amount of distilled water so that the total mixture making a volume of 25 µl per run per individual sample. Negative and positive controls were included in each amplification test. Negative control was done with all the reagents except for the target DNA which was replaced by deionised water. This enables the verification of the absence of contamination (10). The already tested DNA of Bala cultivar was used in another vial as a positive control. Six molecular markers, which had initially been used by Chin *et al*. (4), were used in this study. The primers are listed in Table 1 and these were tested for polymorphism on 30 samples from the genotypes used in this study.

PCR

A total of 5 µl of the loading buffer was added to the DNA samples and the DNA amplification was performed in the automated thermal-cycler beginning with initial denaturing stage of the double stranded DNA at 95 °C for five minutes. The second stage of 34 cycles consisted of denaturing step of the double stranded DNA at 95 °C for 30 seconds, primer annealing step at 55 or 58 ^{o}C (see Table 1) for 30 seconds and primer extension step at 72 °C for one minute. A final extension stage of 72 ºC for five minutes followed the completion of all the cycles. This last elongation stage guarantees the complete extension of the DNA (6). The PCR product samples were kept at 4 °C for electrophoresis.

Electrophoresis

PCR products were run on a 3 % agarose gel. Typically 3.25 g agarose powder was dissolved in 75 ml of TAE buffer **(**1 x Tris-Acetate EDTA); the gel melted in a

microwave oven and was allowed to cool. When the molten Agarose was at about 60 °C, 3µl of ethidium bromide (10 mg ml^{-1} stock solution) was added to give a final concentration approximately 400 ng ml^{-1}. About 10 µl from each amplified DNA sample was separately loaded into each well of the gel. Before electrophoresis commenced, the loaded wells were flanked with one well at either sides loaded with standard DNA size marker (Bioline Hyper Ladder I). Electrophoresis was conducted at 100 volts for about 60 minutes. The visualisation of the separated DNA fragments was performed by ultraviolet transilluminator at 302 nm wave length with AlphaImager 2200 software installed on a connected computer and an UV photo was recorded by a camera linked to a computer.

Genotype grouping according to Kasalath alleles

The band size in base pair can be assigned by comparing the band of the PCR product with known sizes of a standard DNA size marker. Typical photos of the gels are given in Figure 1 and 2. In some cases, alleles were scored as either presence or absence of a PCR product. To display the results of allele scoring a graphical genotype was produced (Figure 3) where green represented the Kasalath allele and red the Nipponbare allele while the absent score was represented as light-blue. This was used to classify the genotypes into three groups according to method described by Chin *et al*. (4). The grouping was used as a fixed factor for one-way ANOVA analysis to assess the influence of the presence of tolerant *Pup1* alleles.

Statistics

After classifying the genotypes into three groups according to the alleles they had, the grouping was used as a fixed factor for one-way ANOVA analysis to assess the influence of the presence of tolerant *Pup1* alleles (Kasalath alleles). This used the genotype mean values of the growth parameters and P uptake of the genotypes used in both YNS and rock phosphate experiments.

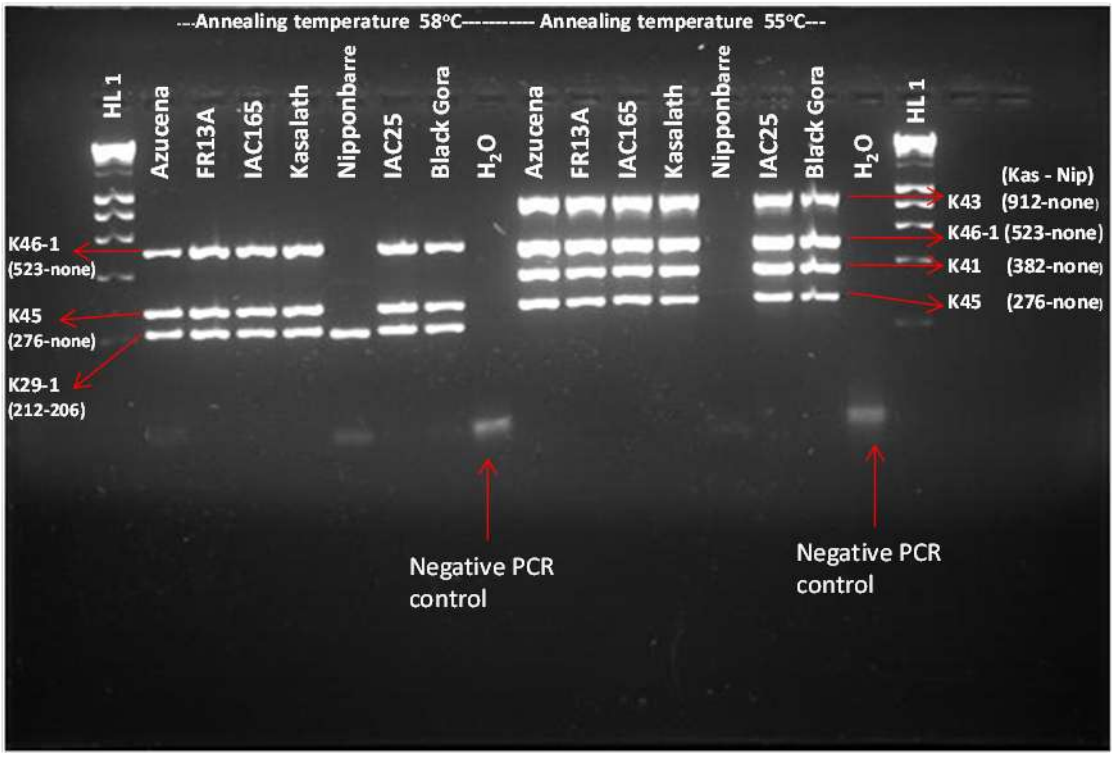

Figure 1. Testing the markers K29-1, K41, K43, K45 and K46-1 on a 1% agarose gel with seven genotypes to find a suitable annealing temperature. HL1= Hyper Ladder 1 DNA size marker (Bioloine, UK). Molecular marker nomenclature used the primer name followed by the band molecular weight of Kasalath and Nipponbarre respectively between brackets

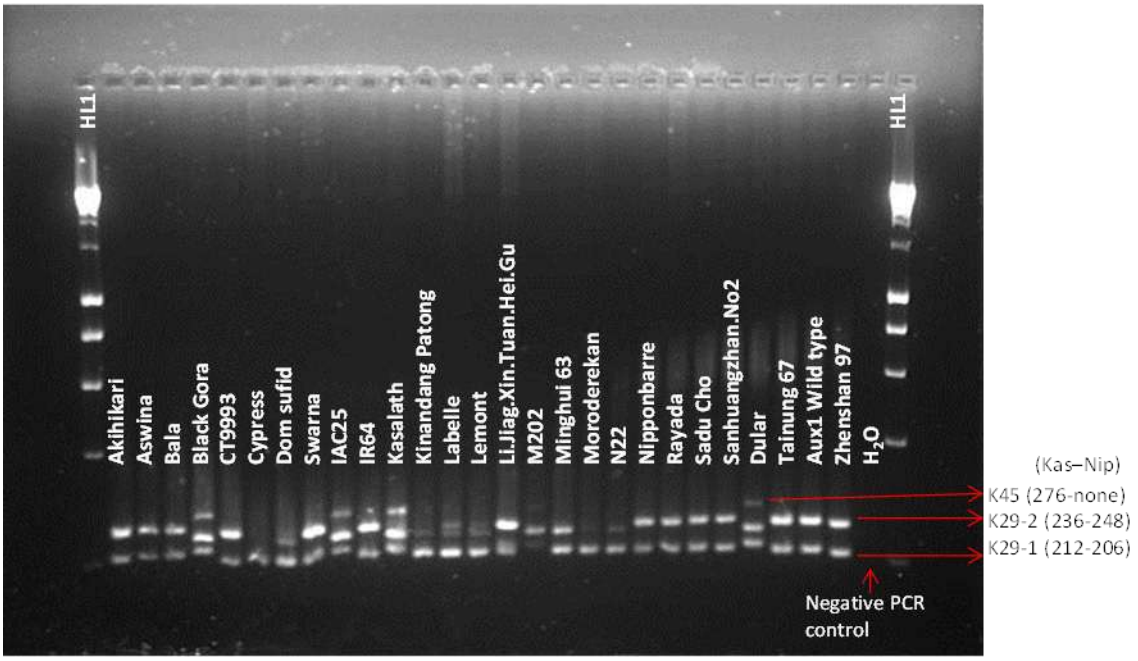

Figure 2. Testing 27 genotypes on a 3 % agarose gel with three markers K45, K29-1 and K29-2 (see Table 1 for primer sequence). HL1= Hyper Ladder 1 DNA size marker (Bioloine, UK)

RESULTS AND DISCUSSION

Two experiments on the response of 30 rice genotypes were conducted in large storage boxes. The plants were sown in the box maintaining five centimetres between each other in order to minimize the box size that can accommodate a large number of genotypes. There is one limitation with this is that the more the plants grow the more the competition will be. To minimize both the competition among plants and the need for a large box and in the meantime to allow the genetic variations to be expressed, the duration of the experiment conducted here was only five weeks. Nonetheless, it is highly likely that above and below ground competition will be operating in these experiments. Below ground may not be unwelcome since it may emphasise the relative ability of genotypes to access the growth limiting P. Above ground competition is not welcome and it would be useful to verify some of these genotype differences detected here in larger pots where above ground competition could be minimised. The two figures 4 and 5 show representative boxes of two experiments [two treatments (YNS-P and YNS+P) for YNS experiment and three treatments (zero P, shallow P and homogenous P for rock phosphate experiment] as they display different shoot growth. Plants in YNS+P treatment, shallow P and homogenous P have long, wide and healthy leaves with high number of tillers while the growth of those in YNS-P and zero P treatments is somewhat stunted with thin stems and reduced number of tillers. In addition the leaves are shorter and narrower than those of plants in the YNS+P, shallow P and homogenous P treatments. In YNS experiment, on average the SDW for genotypes grown in the YNS-P treatment was reduced by 60 % compared to that of plants grown in YNS+P treatment (Table 2). This is clearly indicative of low P availability in this treatment. However, the significant differences in SDW between rice genotypes in the low P treatment are indicative of genotypic variations for P acquisition from conditions lacking of P.

Table 1. List of primer sequences of gene-specific *Pup1* markers used for PCR amplification.

			Physical Location			Amplicon	
Marker Name	*Pup1* Gene Model	Marker Type	Kasalath AB458444.1	Nipponbare Chromosome 12	annealing temperature	Size K/N	Primer Sequence (5' – 3')[a]
K29-1	OsPupK29-1	Codominant	205,067–205,287	15,431,572–15,431,786	58	212/206	F: ATGGCCAACGGGGTAGAG R: GTCCAGGTAACCACGAGGAA
K29-3	OsPupK29-1	Codominant	202,698–202,933	15,419,578–15,419,825	58	236/248	F: TTCGTCCAGATGCTGCTATG R: TCTTCGGTGTAATTGGCACA
K41	OsPupK41-1	Dominant	262,050–262,431	Absent	55	382/–	F: TGATGAATCCATAGGACAGCGT R: TCAGGTGGTGCTTCGTTGGTA
K43	OsPupK43-1	Dominant	268,590–269,501	Absent	55	912/–	F: AGGAGGATGAGCCTGAAGAGA R: TCGCACTAACAGCAGCAGATT
K45	OsPupK45-1	Dominant	274,072–274,344	Absent	55	276/–	F: GCGGAAGAAGAGGATAACGA R: TCTAGGCTTCGTTTGGCAAG
K46-1	OsPupK46-2	Dominant	275,710–276,232	Absent	55	523/–	F: TGAGATAGCCGTCAAGATGCT R: AAGGACCACCATTCCATAGC

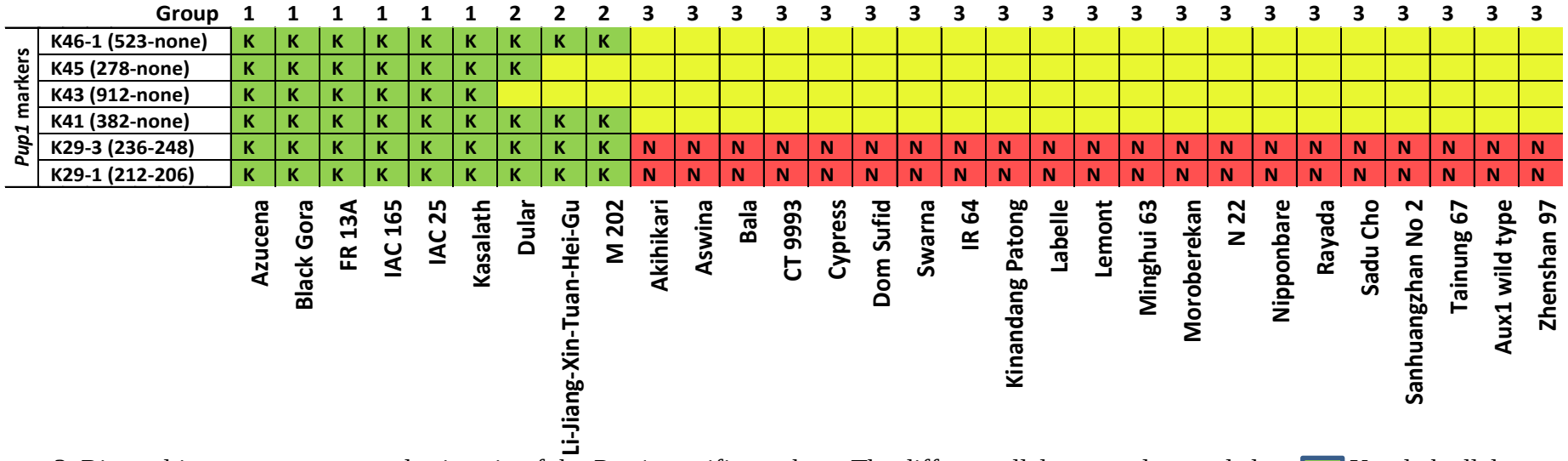

Figure 3. Rice cultivars were genotyped using six of the *Pup1*-specific markers. The different alleles are colour coded as: k Kasalath allele, N Nipponbare allele and for none or deletion. Three main groups (1, 2 and 3) were sorted according to different allelic composition at the *Pup1* QTL (respectively Kasalath, heterozygous and non-Kasalath).

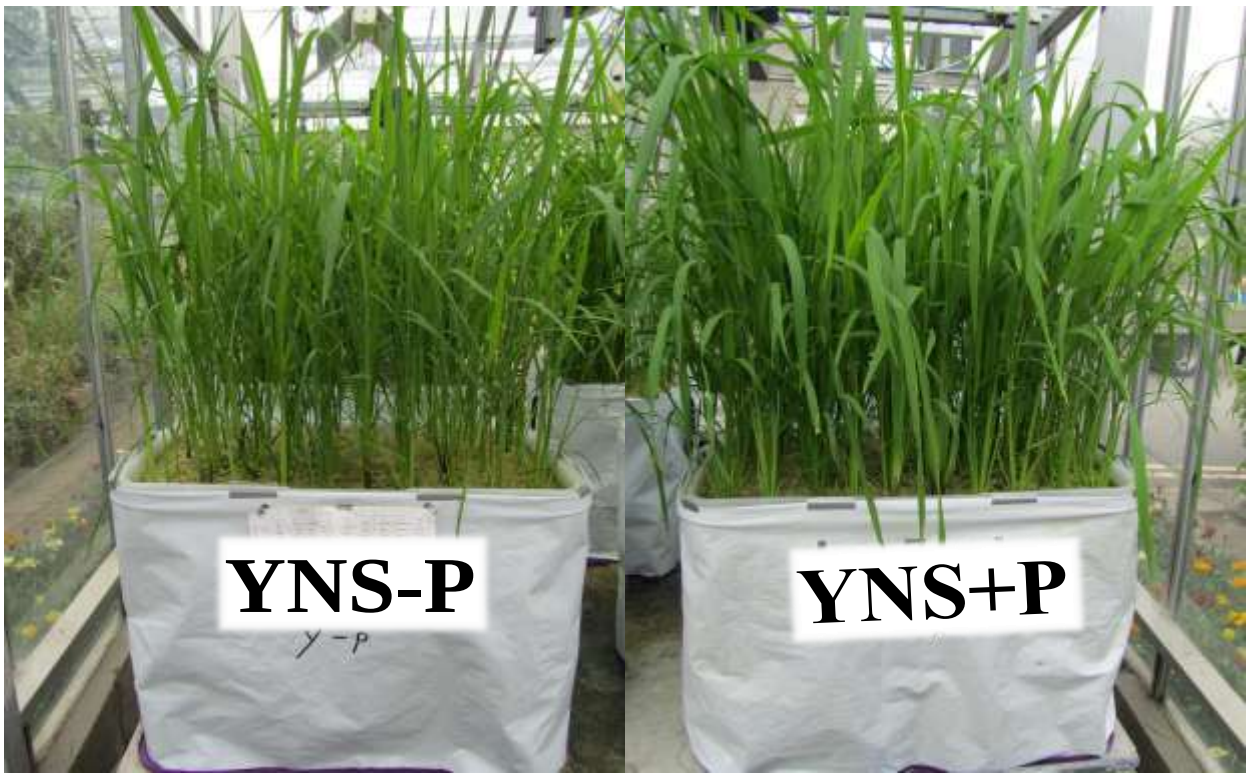

Figure 4. Rice grown in subsoil/sand mix with YNS-P (left) and +P (right)

Figure 5. Growing rice genotypes in plastic boxes in the growth room

In rock phosphate experiment, the P was applied as rock phosphate either homogenously or as a shallow layer of 10 cm depth. It was expected that whether homogenously or in shallow layer the addition of rock phosphate to the soil profile would increase total plant mass. It was also expected that the relative performance of genotypes might differ depending on the distribution of P throughout soil profile. The results reported here are consistent with these predictions. Plant growth was greatly stimulated by the addition of rock phosphate where SDW in both homogenous P and shallow P increased by 1.9 and 2.9 times compared to that in zero P treatment (Table 2). The allele for the *Pup1* locus was determined for the cultivars used in the YNS and rock phosphate experiments and the results used to test if there is evidence that the allele is associated with plant performance. The genotypes that possess Kasalath alleles at all analysed loci, apart from Kasalath, were Azucena, Black Gora, FR 13A, IAC 165 and IAC 25 (Figure 3). Genotypes that partially possessed Kasalath alleles were only three (Dular, Li-Jiang-Xin-Tuan-Hei-Gu and M 202) while the other genotypes lacked Kasalath alleles at all loci analysed. The distribution of these three groups of genotypes in two scatter plots (SDW in -P versus +P YNS treatment, Figure 6 and SDW in -P versus +P shallow treatment, Figure 7), demonstrate that SDW of group one (those possess Kasalath alleles) is higher than group three (those possess Nipponbare alleles). All genotypes with Kasalath alleles (group I) and those with partial Kasalath alleles (group II) appeared to be associated with high SDW except for two genotypes (Sadu Cho and Dom Sufid) with high SDW while do not possess Kasalath alleles. The good performance of these two cultivars in particular needs further investigation. In both experiments, one-way ANOVA revealed significant (P<0.05) differences for SDW due to the presence of Kasalath alleles in the *Pup1* QTL (Table 3). That is to mean the presence of Kasalath alleles in the *Pup1* QTL was found to have a positive effect on SDW in both experiments. This result supports the hypothesis that *Pup1* QTL has a positive effect in enhancing P uptake. For example, SDW of genotypes belong to group one increased by 1.4, 1.5, 1.3, 1.3 and 1.3 times than that in genotypes fit into group three in YNS-P, YNS+P, homogenous P, shallow P and zero P treatment respectively. These results obtained here were consistent with our prediction and confirm the positive effect of *Pup1* QTL being involved in enhancing P uptake. These phenotypic differences between contrasting rice genotypes that possess different alleles of *Pup1* QTL reported here were in agreement with data obtained by the research group of Matthias Wissuwa (5, 18, 21) in terms of showing the beneficial effect of *Pup1* in different treatments and soil types. It is notable that the *Pup1* major gene, the phosphorus starvation tolerance 1 (*PSTOL1*), which has been recently cloned, is absent from the rice reference genome and other modern varieties that are intolerant to P deficiency and its overexpression significantly enhances grain yield in P-starvation conditions (7). The researchers explained that *PSTOL1* enables the plant to take more P and other nutrients by acting as an enhancer of early root growth. Since aerobic conditions were common in all treatments used in YNS experiment and rock phosphate experiment reported within this research, it is not surprising to expect the beneficial effect of *Pup1* on the genotypes used. Yet the positive effect of *Pup1* observed on plant growth under YNS+P treatment poses a challenge since this treatment should not be P deficient so the gene should not confer the advantage it appears to confer in the other treatments. A possible explanation for this *Pup1* beneficial effect is that since the plant were grown in aerobic condition, the availability of P added with YNS may become intermittently low. In aerobic conditions, low moisture content in soil between irrigation intervals severely impaired P diffusion and led to a reduction in P availability in soil (15). Evidence indicated that Kasalath alleles within *Pup1* QTL had the potential to significantly enhance plant growth and P up take in different genetic background and treatments in the greenhouse. However, some cultivars, especially Dom Sufid and Sadu Cho performed well in these experiments despite

lacking the *Pup1* allele so particular attention of research work to investigate other loci in these two cultivars that may reveal their mechanism of P deficiency tolerance is imperative. In addition, identification of candidate genes within *Pup1* QTL will certainly further our understanding of the molecular mechanisms of P acquisition in rice.

Table 2. Genotype means and statistics for two experiments from one-way ANOVA with factor genotype for SDW in each two treatments of UNS experiment and each three treatments of rock phosphorus experiment.

	YNS-P	YNS+P	Homogenous P	Shallow P	Zero P
Genotypes	**SDW (mg)**	**SDW (g)**	**SDW (mg)**	**SDW (mg)**	**SDW (mg)**
Akihikari	**359.2**	**0.745**	**152**	**380**	**138**
Aswina	**528.4**	**1.253**	**495**	**688**	**287**
Azucena	**741.6**	**2.194**	**733**	**1034**	**328**
Bala	**490.7**	**1.279**	**469**	**791**	**246**
Black Gora	**751.0**	**1.792**	**476**	**758**	**273**
CT 9993	**402.2**	**0.956**			
Cypress	**419.8**	**0.911**	**334**	**538**	**198**
Dom Sufid	**793.5**	**2.160**	**655**	**925**	**312**
Dular	**684.9**	**2.161**	**466**	**735**	**241**
FR 13A	**662.9**	**2.184**	**724**	**995**	**380**
IAC 165	**535.1**	**1.236**	**442**	**742**	**238**
IAC 25	**775.1**	**1.849**	**657**	**1127**	**327**
IR64	**621.8**	**1.236**	**505**	**760**	**212**
Kinandang Patong	**474.9**	**1.150**	**517**	**651**	**233**
Labelle	**355.7**	**0.772**	**297**	**492**	**151**
Lemont	**369.9**	**1.032**	**360**	**498**	**170**
Li-Jiang-Xin-Tuan-Hei-Gu			**523**	**784**	**263**
M 202	**757.6**	**1.756**			
Minghui 63	**662.1**	**1.724**			
Moroberekan	**602.7**	**1.187**	**442**	**693**	**251**
N22	**446.5**	**1.118**	**386**	**508**	**229**
Nipponbare	**395.8**	**0.702**	**446**	**523**	**183**
Rayada	**584.3**	**1.806**	**529**	**791**	**304**
Sadu Cho	**732.1**	**2.306**	**954**	**1569**	**414**
Sanhuangzhan No 2	**321.1**	**0.892**	**311**	**565**	**193**
Swarna	**321.8**	**0.769**	**386**	**443**	**193**
Tainung 67	**345.7**	**0.748**	**278**	**411**	**172**
Zhenshan 97	**471.6**	**1.453**	**267**	**595**	**211**
Mean	**541.0**	**1.384**	**472**	**720**	**248**
ANOVA					
F	**16.35**	**21.15**	**7.01**	**7.47**	**14.93**
P	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
R^2	**71.33**	**76.65**	**50.33**	**51.61**	**70.28**

Table 3. One-way ANOVA output and average for SDW versus three levels of *Pup1* alleles for 30 rice genotypes grown in YNS and rock phosphate experiments. Mean of six replicates and appropriate standard deviation.

	One-way ANOVA: SDW versus *Pup1* group				
	YNS experiment		Rock phosphate experiment		
Level	**YNS-P SDW (g)**	**YNS+P SDW (g)**	**Homogenous P SDW (g)**	**Shallow P SDW (g)**	**Zero P SDW (g)**
Group 1	0.693a ±0.136	1.851a ±0.441	0.579a ±0.209	0.902a ±0.279	0.3077a ±0.0715
Group 2	0.541b ±0.241	1.263b ±0.610	0.455ab ±0.135	0.598b ±0.369	0.2235b ±0.0459
Group 3	0.508b ±0.163	1.273b ±0.548	0.448b ±0.223	0.681b ±0.321	0.2365b ±0.0751
Mean	**0.543**	**1.376**	**0.479**	**0.726**	**0.2521**
ANOVA					
F	15.27	14.41	3.54	5.24	10.13
P	**0.000**	**0.000**	**0.034**	**0.007**	**0.000**
R^2	14.59	13.91	6.20%	9.92%	19.17%

Using Tukey method, Means that do not share a letter are significantly different.
***P* value in bold is significant.**

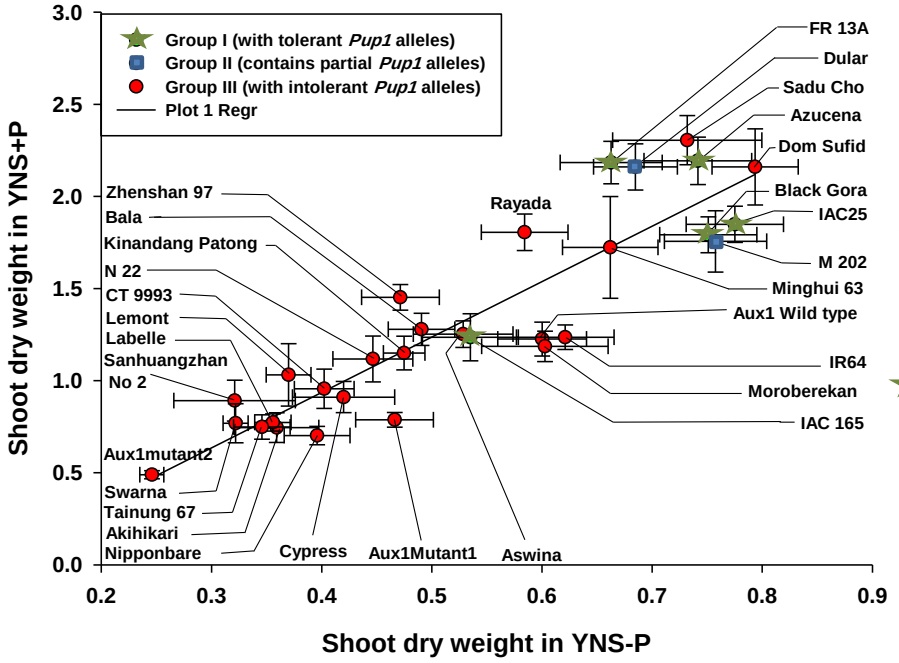

Figure 6. Scatter plot for SDW in –P versus +P treatment in YNS experiment. The three main groups (I, II and III) were sorted according to different allelic composition at the *Pup1* QTL and colour coded as: ★Kasalath allele (group I), ■ heterozygous (group II) and ● for non-Kasalath (Nipponbare) allele (group III). Bars are standard errors.

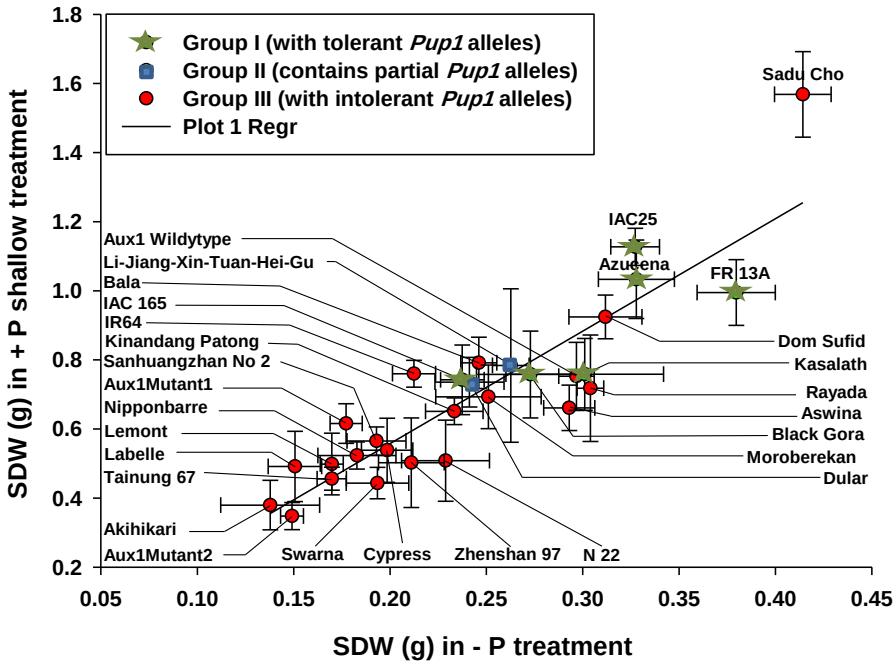

Figure 7. Scatter plot for SDW in -P versus +P shallow treatment in rock phosphate experiment. The three main groups (I, II and III) were sorted according to different allelic composition at the *Pup1* QTL and colour coded as: ● Kasalath allele (group I), ● heterozygous (group II) and ● for non-Kasalath (Nipponbare) allele (group III). Bars are standard errors.

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