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Germplasm Survey of Sri Lankan Rice for *Pup 1* Gene Based Markers: An Approach towards Development of Rice Varieties for Phosphorus Deficient Soil

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ABSTRACT

The major quantitative trait locus (QTL), *Phosphorus uptake 1* (*Pup1*), confers tolerance to phosphorus deficiency in soil and is currently considered as one of the most promising QTLs for the development of phosphorus deficiency tolerant rice (*Oryza sativa*) varieties. Marker-assisted breeding is a useful tool for breeders to track the genetic makeup of plants during the variety development process. It has been revealed that *Pup1* K-46 and *Pup1* K-52 are markers that are associated with *Pup1* gene to confer phosphorus deficiency tolerance. Therefore, this study was conducted to detect the possibility of applying the *Pup1* based markers for the phenotypic assessment of Sri Lankan rice varieties on phosphorus deficiency tolerance. Twenty varieties were genotyped by using *Pup1* K-46 and *Pup1* K-52 markers. Root length, shoot length, root width, root volume, dry weight of root and dry weight of shoot were measured under phosphorus deficient and supplemented soil. The results revealed that except shoot length, all other traits were significantly different between the varieties possessing *Pup 1* gene and lacking *Pup 1* gene. These data suggest that *Pup1* gene based markers are useful in detecting phosphorus deficiency tolerance and therefore, varieties tested in this study would be useful in selecting parental population in rice breeding programs.

KEYWORDS: *Oryza sativa*, Phosphorus deficiency, *Pup1* locus, Sri Lanka

INTRODUCTION

Rice (*Oryza sativa* L.) is the most important cereal crop in human nutrition in South and Southeastern Asia. It has been cultivated for thousands of years in the Tropics and Subtropics and is still one of the crops in which scientists try to improve yield potential and to adapt to various environments.

Phosphorus (P) deficiency is one of the most important abiotic stress factors that limit the rice yield. Therefore P deficiency has been identified as a main factor in preventing the realization of high yield potentials of modern varieties in lowland rice production (Chin *et al.*, 2010; Chin *et al.*, 2011). Phosphorus deficiency can be alleviated by fertilizer application, but farmers are constantly facing financial difficulties when increasing higher input. Development of rice cultivars with an improved tolerance to P-deficiency may therefore be a cost-effective solution to this problem.

Phosphorus uptake 1 (*Pup1*) is a major quantitative trait locus (QTL) located on rice (*Oryza sativa*) chromosome 12, that is associated with tolerance of phosphorus (P) deficiency in soil (Wissuwa *et al.*, 1998 and 2002). Quantitative trait loci (QTLs) for P deficiency tolerance had been identified in a rice population derived from a cross of the intolerant Japonica cultivar, Nipponbare and the tolerant Indica landrace, Kasalath (Wissuwa *et al.*, 1998). It was found that tolerance to P deficiency was largely caused by genotypic differences in P uptake and their internal P use efficiency had a negligible effect. The impact of *Pup1* and other QTLs that enhance yield in P-deficient soil and/or under drought stress is potentially very high (Bernier *et al.*, 2009; Venuprasad *et al.*, 2009), since about 50 % of the rain-fed rice in Asia is grown on problematic soils (Haefele and Hijmans, 2007). Both P deficiency and drought are widespread problems, particularly in soils with acidic pH and high concentrations of cations, for example, Aluminum and Iron, which complexate phosphate (Kochian *et al.*, 2005; Ismail *et al.*, 2007; Xue *et al.*, 2007) and cause the occurrence of companion stresses (e.g. Aluminum toxicity) that restrict root growth. Therefore it is important to develop cultivars tolerance to phosphorus deficiency to enhance the yield potential under problematic soil. This study was conducted to screen rice germplasms that contain *Pup 1* locus and to detect the applicability of them under Sri Lankan soil condition aiming at increasing the varietal choice for rice breeders.

MATERIALS AND METHODS

Experimental Site

This study was conducted at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka during the period of January to April 2013.

Planting Materials

Sri Lankan rice varieties representing modern and traditional were used for this study. Seeds of 20 rice accessions were obtained from the Rice Research and Development Institute, Bathalegoda and Plant Genetic Resourse Center, Gannoruwa, Sri Lanka.

Genomic DNA Extraction and Quantification

Five seeds from each accession were planted on wetted filter paper in a Petry dish. Leaves of three week old rice seedlings were used for the extraction of genomic DNA. Three to five leaves were cut in to small pieces and inserted into 1.5 ml eppendorf tubes. The leaf pieces were homogenized using a micro pipette tip with 300 µl of DNA extraction buffer (1 M KCl, 1 M Tris HCl, 0.5 M EDTA). Capped eppendorf tubes were incubated at 70 ^{0}C for 20 min. Extracts were centrifuged at 13,000 rpm for 15 min under room temperature. 100 µl of ice cold iso-propanol was added into new eppendorf tubes, and the supernatant of the above centrifuged samples were transferred into them. Solutions were mixed gently. 100 µl of ice cold iso-propanol was added to precipitate the DNA. After mixing gently, tubes were kept at 4 ^{0}C for 15-30 min and centrifuged at 13,000 rpm for 15 min under room temperature. After removing the supernatant, DNA pellets were washed with 150 µl of 70 %, ice cold ethanol by centrifuging at 13,000 rpm for 10 min. Supernatants were removed and pellets were air dried in dark for 2 hr. Pellets were dissoloved in 150 µl of 1/10th TE buffer (10 mM Tris, 1 mM EDTA). Samples were kept at 4 ^{0}C overnight after adding TE buffer. Then they were transferred to -20 ^{0}C and used for the experiments.The extracted DNA samples were quantified by 0.8 % agarose gel containing 0.5 µg/ml ethidium bromide.

PCR Amplification

PCR reactions were carried out on BioRad (My Cycler^{TM}) Thermal cycler in a final volume of 12 µl containing, 5 µl of DNA sample, 1.5 µl of 10X PCR buffers with 2.5 mM $MgCl_2$, 1.2 µl of 2.5 mM dNTPs (Promega Madison, WI U.S.A.), 1.0 µl of primer (20 pmol/µl), (2 primers for 2 master mixers), and 0.14 µl of 5 u/µl *Taq* DNA polymerase (Dream Taq, Fermentas) in a thermal profile of initial denaturation (5 min at 95 ^{0}C) followed by 35cycles of denaturation (at 95 ^{0}C for 1 min), annealing (at 58 ^{0}C for 30 sec), extension (at 72 ^{0}C for 1 min) and final extension at 72 ^{0}C for 5 min.

Table 1.DNA sequence of the *Pup1*markers (Heuer *et al.*, 2009)

primer	Fragment Size(bp)	F/R	Sequence
Pup1 K46	523	F	5'TGAGATAGCCCGTCAA GATGCT3'
		R	5'AAGGACCACCATTCCA TAGC3'
Pup1 K 52	505	F	5'ACCGTTCCCAACAGAT TCCAT3'
		R	5'CCCGTAATAGCAACAA CCCAA3'

F-forward, R-reverse

Analysis of PCR Products

Amplified PCR products were electrophoresed (Electrophoresis unit, MUPID-exu, England) on 1 % agarose gel stained with ethidium bromide. The gel was run at 100 V/cm using 0.5X TBE buffer. After electrophoresis the gel was visualized and photographed under ultra violet light. A 100-bp DNA ladder was used as the marker to determine the fragment size.

Pup1 Phenotyping in Phosphorus Deficient Soil

For the soil experiment, the above 20 varieties 5 plants from each variety were grown in a plant house with natural light conditions for 46 days. Plants were grown in a black polyethene pots with the height of 30 cm and diameter of 15 cm, filled with 2.5 kg top soil collected from the university field. The average P content of the soil was determined by Olsen method (Olsen *et al.*, 1954) and it was accounted for 78 ppm. Nitrogen (N; 0.39 g urea; at basal stage) and potassium (K; 0.2 g Muriate of potash) were added to all pots. Phosphorus fertilizer (0.16 g Triple Super Phosphate) was added only to +P control pots, that was considered as P supplemented treatment. No P was added to the other pots that were considered as P deficient treatment. One plant was grown in one pot. Soil was kept aerobic but well watered at all times. Six quantitative morphological characters were measured in each plant. Root length (cm), shoot length (cm), root width (cm), root volume (ml), root dry weight (g) and shoot dry weight (g) were measured. Root volume was measured by measuring the spilled content of water.

Data Analysis

Data were analyesed by Pooled-t test using SAS statistical package, 2002 by SAS institute Inc., Cary, NC, USA, Version 9.2.

RESULTS AND DISCUSSION

Genotyping by Pup1 Markers

Table 2. List of accessions used for the study and the varieties that *Pup1* present and absent

No	Variety	Acc.no*	*Pup1* K 46	*Pup1* K 52
1	Suwanda samba	003507	+	+
2	Hondarawala	003867	+	+
3	Devereddari	006171	+	+
4	AT 405		+	+
5	Hondarawala	003678	+	+
6	Bg 11-139		+	+
7	Basmathi 370	006820	+	+
8	Bw 400	005311	+	+
9	Azuzena		+	+
10	Bg 352		+	+
11	Bg 357		+	+
12	Moroberakan	006897	+	+
13	Bg 5-110		+	+
14	Bg 250		+	+
15	Ld 356		+	+
16	Kalinga		-	-
17	AT 354		-	-
18	11041		-	-
19	BG 359		-	-
20	Hondarawala		-	-

** Varieties with accession numbers were obtained from Plant Genetic Resourse Center, Gannoruwa, Varieties without accession numbers were obtained from Rice Research and Development Institute Bathalegoda. +presence of Pup1*

The used markers *Pup1* K-*46* and *Pup1* K-52 appeared to be closely related markers, because both markers showed same genotyping pattern. *Pup1* K-*46* and *Pup1* K-52 amplified 505 bp and 523 bp fragments respectively in 15 rice varieties out of 20 rice accessions tested. Five varieties did not show any amplification, either *Pup1* K-*46* or *Pup1* K-52 fragments. PCR procedure was repeated two times for all varieties due to the dominant nature of the marker. *Pup 1* null varieties were further tested with the SSR markers in chromosome 12 in order to verify the quality of DNA as *Pup1* K-*46* and *Pup1* K-52 are not supposed to amplify fragments under null allelic condition. SSR markers amplified fragments in *Pup 1* null varieties suggesting that the absence of *Pup 1* allele is accurate.

Phenotyping under P Deficient and P Supplemented Field Condition

As the main organ of plants that take up nutrients, roots play an important role in phosphorous acquisition from soils. It is well documented that the ability of a plant to access phosphorous from soil depends on root physiological and morphological properties such as root length, root exudates etc. Root architecture, the spatial configuration of a root system in the soil, determines the soil exploration by roots. In this study several traditional and modern developed rice varieties were assessed by four root related traits and two shoot related traits under P supplemented soil and P deficient soil (Table 2). Soil P level was determined and it was found as 78 ppm at the 10.58 pH level.

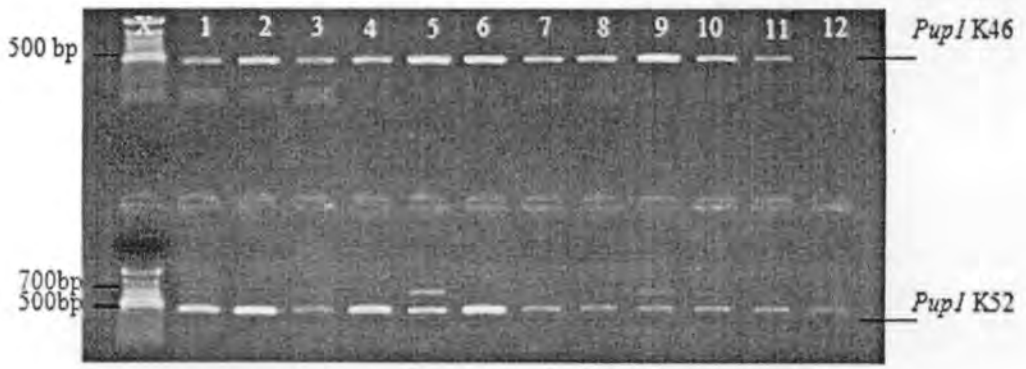

Figure 1. PCR profile generated by *Pup1* markers

Lane X: 100-bp ladder DNA marker, Lane 1: Acc. No. 003507, Lane 2: Acc. No:003867, Lane 3: Acc no.003678, Lane 4: Azuzena, Lane5: Devereddери, Lane 6: Basmathi 370, Lane 7 :AT 405, Lane 8: Bg 250, Lane 9: Bw 400, Lane10: Basmathi 11-139, Lane 11: LD356, Lane 12: Bg 352

Table 3. Mean values and standard error of root and shoot traits of rice in phosphorous supplemented condition

Group	Root** Length (cm)	Shoot^{ns} Length (cm)	Root*** Width (cm)	Root*** Volume (ml)	Root*** Dry weight (g)	Shoot*** Dry Weight(g)
Pup1+	30.95 ± 0.66	82.73 ± 1.64	5.06 ± 0.10	20.44 ± 0.75	3.05 ± 0.23	6.27 ± 0.15
Pup1_	26.69 ± 1.27	86.63 ± 4.14	3.89 ± 0.14	11.62 ± 1.45	1.39 ± 0.29	4.32 ± 0.39
Difference	4.26 ± 0.43	-2.89 ± 3.70	1.17 ± 0.20	8.82 ± 1.55	1.66 ± 0.43	1.94 ± 0.34

**Significant at 0.05 level, **significant at 0.01 level, ***significant at 0.001 level and ^{ns} not significant*

Table 4. Mean values and standard error of root and shoot traits of rice in phosphorous deficient condition

Group	Root*** Length (cm)	Shoot^{ns} Length (cm)	Root*** Width (cm)	Root*** Volume (ml)	Root** Dry weight (g)	Shoot*** Dry Weight(g)
Pup1+	28.88 ± 0.47	85.26 ± 1.46	5.04 ± 0.09	21.56 ± 0.75	1.86 ± 0.11	5.90 ± 0.14
Pup1_	25.18 ± 1.14	87.42 ± 3.48	4.30 ± 0.15	13.88 ± 1.51	1.15 ± 0.18	4.63 ± 0.27
Difference	3.70 ± 1.05	-2.15 ± 3.22	0.73 ± 0.18	7.68 ± 1.57	0.70 ± 0.21	1.26 ± 0.30

**Significant at 0.05 level, **significant at 0.01 level, ***significant at 0.001 level and ^{ns} not significant*

Association between Pup1 Genotype and Rice Phenotypes

Significant differences were observed in the root length between the variety group with *Pup1* gene and variety group without *pup 1* gene in phosphorous supplemented (P<0.01) and deficient soil (P<0.001) conditions. Significant differences were not observed in the shoot length between the *Pup1* present variety group and *Pup1* absent variety group under both phosphorous supplemented and deficient soil conditions.

Significant differences were observed in the root width (P<0.001), root volume (P<0.001) between the *Pup1* present variety group and *Pup1* absent rice variety group under phosphorous supplemented and deficient soil conditions. The *Pup1* gene has increased the dry weight of root by 1.7 g under P supplemented condition (P<0.001) and by 0.7 g (P<0.01) under P deficient soil condition. Under both condition although the shoot length was not significant, shoot dry weight was highly significant at P<0.001 level. *Pup 1* gene has contributed to increase the root volume by more than 50% when compared with null *Pup 1*. (Table 3 and Table 4).

One of the most promising finding of this study is that *Pup1* presenting genotypes integrate different root traits that contribute to the adaptation to low phosphorous availability and therefore more tolerance to phosphorous deficiency is appeared as compared to *Pup1* absent genotypes.

CONCLUSIONS

The results revealed that there is a significant difference between rice variety group with *Pup1* positive genotype and variety group with *Pup1* negative genotype, in root length, root width, root dry weight, root volume and shoot dry weight. Therefore in order to developing varieties that utilize P efficiently, these data would be useful to select parental lines on the basis of presence of *Pup1* markers.

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