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Screening for phosphorus(P) tolerance and validation of *Pup-1* linked markers in *indica* rice

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Abstract

Phosphorous (P) fixation in soils with high free ferric oxides and aluminum in the clay fraction is a widespread problem and limits access of plants to P even if it is present in the soil. Problem is acute in upland situation. Therefore, a set of 31 rice genotypes comprising of adopted upland and lowland lines as well as aromatic and semi-dwarf high yielding cultivars were grown both in P-deficient as well as P-sufficient soil. P-uptake ability of these genotypes was compared when they were grown in P-sufficient as well as in P-deficient soil. Six genotypes (Gitanjali, Gobindabhog, Jaladhi, Pusa Saugandh, Radhunipagol, Tulaipanji) accumulated significantly more P per plant under both P-sufficient ($p < 0.01$) and P-deficient ($p < 0.01$) conditions. Rice genotypes were also characterized by PCR-based markers, *Pup-1* K42 and *Pup-1* K29 which were linked with a major QTL for phosphate uptake-1 (*Pup-1*) locus. Haplotyping of *Pup-1*-K42 markers showed 918 bp amplification in nine genotypes but among them, only three genotypes showed higher P-uptake and dry-matter -weight in P-limiting condition. Unlike *japonica* germplasm, both K42 and K29 were not diagnostics in assessing *Pup-1* locus in *indica* germplasm. Three Bengal landraces, Bhutmuri, Gobindabhog and Radhunipagol can serve as ideal donor parent for introgression of Pup1 locus. The markers validated in this study will help in the marker assisted introgression of P-deficiency tolerance in rice.

Key words: P-deficient, rice *Pup-1*, MAS, Bengal land race

Introduction

Approximately sixty percent rainfed rice in Asia is grown on soils affected by multiple stresses and one of them is Phosphorus (P) deficiency [1]. Phosphorus is one of the least available of all essential nutrients in the soil and its concentration is generally below that of many other micronutrients [2]. A high content of free ferric oxides and high aluminium (Al) limit the P availability to plants even when present in the soil and has become a widespread problem. Although the application of P fertilizer can rectify the problem to some extent, it is expensive for the marginal rice growers and results in eutrophication. Thus selection of rice cultivars which can extract Phosphorus from P-limiting soils, which have a higher P fertilizer use efficiency, is therefore considered an important cost-effective management. Wissuwa *et al*. [3] identified QTLs for P deficiency tolerance in rice which mainly confers increased P uptake (*Pup-1*). A major QTL for P-uptake was mapped on chromosome 12 with additional minor QTLs on chromosome 2, 6 and 10. Though substitution mapping by Wissuwa *et al*. [4], it was found that the *Pup-1* on chromosome 12 alone explains more than 70 % of total variability. *Pup-1*, locus increases P-uptake under adverse conditions rather than increasing internal P-use efficiency [5]. Due to lack of polymorphic SSR markers surrounding the *Pup-1* locus, marker assisted introgression of this locus was not easy [6]. Recently, Chin *et al*. [7] reported that the *Pup-1* locus-specific genomic sequence of the rice genotype Kasalath (tolerant to P-deficiency) is located in large insertion-deletion (INDEL) region, which is absent in P-deficient-susceptible genotype Nipponbare. They have also recommended number of PCR based markers derived from the sequence polymorphic between Kasalath and Nipponbare [7].

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Additionally, Bernier *et al*. [8,9] mapped a major QTL (*Yld qtl12.1*) for yield under drought that overlaps with the Pup1. They also hypothesized that two QTLs *Pup-1* and *Yldqtl* 12.1 might be pleiotropic and introgression of this region might help select simultaneous P-deficiency tolerance as well as for yield under drought. The main objective of this study was therefore to screen the locally available rice genotypes suitable for P-deficient tolerance and validate two *Pup-1* linked INDEL markers for diagnostic value.

Materials and methods

Plant material grown on P-suffiicient and P-deficient soil and P-estimation

Thirty one rice genotypes comprising of popular cultivars, land races adopted for up land and low land ecology, were grown in P-sufficient soil (available P-60 mg/kg), experimental Farm, Bidhan Chandra Krishi Viswavidyalaya, Balindi, Nadia and P-deficient soil (available P< 3.50 mg/kg) of Regional research substation for Red and Lateritic zone of the same Viswavidyalaya, located at Sekhampur, Birbhum in the *kharif* season of 2009. To estimate soil phosphate, Bray method [10] was followed in case of Sekhampur soil and Olsen method [11] for Balindi soil. External P-fertilizer was not applied in Sekhampur soil during entire growth stage of rice and bunding was properly made surrounding the field to prevent any invasion of phosphate through other irrigation channels. Recommended fertilizer dose of NPK was applied for Balindi soil. The P-estimation of plant sample (mature shoot) with three replications for each line was done with Agilent 8453 spectrophotometer after tri-acid digestion following Barton [12] and Hsu and Jackson [13].

Analysis of Pup1-K42 and Pup1-K29 genes using PCR

DNA was extracted from approximately 40 mg of fresh leaf tissue as described earlier [14]. Standard PCR was carried out using thermal cycler (Gene Amp PCR System 9700). The reaction volume (25 µl) contained diluted DNA sample 20ng with 100 ng each of forward and reverse primer, (*Pup-1*-K42, 5´-CCCGAGAGTTCATCAGAAGGA-3´ and 5´-AGTGAGTGGCGTTTGCGAT-3´ and *Pup-1*-K29, 5´-CCATAGTAGCACAAGAAACC GACA-3´ and 5´-GCTTCAATGAGCCCAGATTACGAA-3´) along with 2.5 ml 10X buffer, 2.0 mM $MgCl_2$ solution, 1 ml 2.5mM dNTPs, 16.5 ml HPLC grade sterile water and 0.5 U *Taq* DNA polymerase enzyme (Chromus Biotech). Amplification was carried out with the reaction condition of 94°C for 5 minutes of initial denaturation followed by 35 cycles each of denaturation at 94°C temperature for 45 seconds, annealing at 58°C and polymerization at 72°C for 60 seconds. Further extension was allowed at 72°C for 7 minutes, followed by holding the samples at 4°C. PCR products were size fractionated in 1 % Agarose gels and stained with ethidium bromide and documented.

Results and discussion

Phenotyping of P-uptake ability in field grown plants

P-uptake in rice plants was measured at maturity and expressed in mg per plant. P-uptake ability in deficient soil was measured when they grown in red and lateritic soil of West Bengal where available P was less than 3.5 mg/kg. P-uptake ability of the same set of plants was measured in P-sufficient soil when they were grown in alluvial soil where available P is 60 mg/kg. P-acquisition efficiency varied significantly among the genotypes both in P-deficient and sufficient soil (Table 1). Thirty one genotypes comprising of upland-adopted lines, aromatic lines/cultivars, low land varieties and popular cultivars were used in this study. Bhutmuri, Gitanjali, Gobindabhog, Jaladhi, Lalat, Neigersail, Pankaj, Pusa Saugandh, Radhunipagol, Ranjit, Swarna and Tulaipanji showed high P-uptake under low-P conditions in field trial. The twelve genotypes showed an average P-uptake of 17.5 mg $plant^{-1}$ which is higher than total average of 9.63 mg $plant^{-1}$ or average of remaining nineteen genotypes that were 5.295 mg $plant^{-1}$. Among these twelve genotypes, six (shown in bold) accumulated significantly more P per plant under both high-P ($p < 0.01$) and low-P ($p < 0.01$) conditions. Semi-dwarf high yielding varieties for irrigated agro-ecosystem, like, IR36, IR64, Palman, Satabdi uptake less P than traditional landraces both in high as well as P-limiting conditions. It has been found that genotypes with high P-uptake ability have significantly ($p < 0.01$) higher dry-mass-weight, 30.72 mg $plant^{-1}$, than that of average 21.66 mg $plant^{-1}$. Like P-sufficient soil, a significant correlation between P-uptake and dry-mass-weight ($r = 0.81$) of the rice genotypes was found in P-deficient soil. Genotypes like, Gitanjali, Gobindabhog, Jaladhi, Pusa Saugandh, Radhunipagol and Tulaipanji may be considered as donor parents where P-acquisition efficiency both in P-limiting and non-limiting condition was higher than the average.

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Table 1. Genotyping of 31 rice genotypes by *Pup*1 linked markers and the allele size (bp) estimated from agrose gel

S.No.	Ecology	Variety	A	B	C mg/kg	D mg/kg	E (gm)	F gm
1	UPLAND	Bhajan	918	200	11.50 ± 2.12	7.86 ± .77	23.98 ± 4.22	380 ± 31.6
2		Bhutmuri	918	200	14.45 ± 1.87	12.03 ± .15	26.3 ±1.68	412 ± 28.7
3		Binni	918	220	10.93 ± 2.12	7.77 ± 1.36	17.97 ±1.44	390 ± 31.2
4		BU 1	918	491	32.24 ± 3.42	6.23 ± 1.42	16.59 ± 0.51	378 ± 29.43
5		Choli 60	Null	200	12.68 ± 3.21	4.89 ± 1.57	25.92 ± 0.31	409 ± 17.4
6		Gitanjali	Null	200	**46.28 ± 4.32**	**33.85 ± 3.5**	37.03 ± 1.74	467 ± 23.9
7		Hamsahamas	null	200	8.81 ± 2.13	7.72 ± 2.05	10.31 ± 2.67	355 ± 23.7
8		Kunti	918	200	13.66 ± 2.08	3.65 ± 0.55	12.11 ± 2.98	398 ± 23.8
9		Lalat	Null	200	17.70 ± 1.34	9.69 ± 1.32	22.33 ± 2.87	571 ± 24.9
10		TN 1	Null	200	15.61 ± 2.13	6.63 ± 0.39	13.16 ± 1.22	444 ± 19.3
11		Tulaipanji	Null	200	**25.29 ± 3.81**	**14.96 ± 2.15**	29.82 ± 0.54	428 ± 31.3
12		Rasi	Null	200	14.45 ±0.76	3.22 ± 1.12	8.94 ± 1.78	452 ± 24.6
13		Satika	918	491	17.70 ± 1.05	6.54 ± 0.45	19.775 ± 1.24	376 ± 18.9
14	LOWLAND	Durgasail	918	200	24.14 ± 5.43	3.77 ± 0.11	16.48 ± 0.16	311 ± 21.4
15		Jaladhi	null	200	**28.47 ± 4.12**	**17.66 ± 2.9**	34.115 ± 4.56	532 ± 27.4
16		Kalikhasa	null	200	32.08 ± 5.23	6.05 ± 0.12	15.235 ± 0.47	473 ± 31.4
17		Neigersail	Null	200	11.99 ± 1.87	17.86 ± 5.6	27.11 ± 3.10	643 ± 29.3
18		Pankaj	Null	200	12.82 ± 1.92	14.70 ± 0.51	23.31 ± 1.43	461 ± 27.3
19		Ranjit	Null	491	15.41 ± 2.1	12.43 ± 4.95	31.435 ± 2.78	583 ± 32.3
20		Vijaya	Null	200	11.37 ± 1.76	4.73 ± 1.34	11.38 ± 0.31	356 ±28.2
21	HYV	IR 20	null	200	16.95 ± 0.98	1.96 ± 0.91	13.05± 0.64	517 ± 17.3
22		IR 36	null	491	12.13 ± 1.63	3.72 ± 0.87	16.71 ± 1.54	493 ± 12.5
23		IR 64	null	491	10.12 ± 1.63	4.05 ± 1.07	15.53 ± 2.31	487 ±14.5
24		Khitish	null	200	23.35 ± 2.32	4.67 ± 0.98	16.34 ± 3.18	556 ± 34.2
25		Swarna	Null	200	12.26 ± 1.96	17.22 ± 0.93	25.325 ± 1.44	589 ± 27.2
26		Palman	Null	200	11.26 ± 2.11	3.25 ± 1.28	17 ± 4.44	521 ± 16.3
27		Satabdi	Null	200	10.53 ± 1.13	4.87 ± 1.87	12.185 ± 2.07	392 ± 11.7
28	AROMATIC	Gobindobhog	918	200	**18.47 ± 2.19**	**24.37 ± 4.12**	40.49 ± 7.62	612 ± 18.6
29		Pusa Basmati	Null	200	26.94 ± 3.32	4.65 ± 0.28	19.72 ± 4.4	411 ± 19.7
30		Pusa Saugandh	Null	200	**25.35 ± 1.32**	**14.57 ± 5.32**	31.08 ± 1.99	516 ± 22.3
31		Radhunipagal	918	200	**22.44 ± 1.98**	**12.94 ± 2.69**	40.795 ± 3.68	494 ± 18.1
		Mean ± SD			**18.37 ± 8.54**	**9.63 ± 7.18**	**21.66 ± 8.95**	**461.5 ± 23.8**

A & B = Allele sizes (bp) of K42 and K29 respectively; C = P-uptake value per plant is estimated from field grown plants grown in Balindi farm (P-availability is 60mg/kg of soil); D= P-uptake value per plant at Sekhampur farm (P-availability is 3.5mg/kg of soil); E = Dry mass weight and F = corresponding yield at P-deficient soil for the same season.

Genotyping by Pup-1 linked INDEL markers

First step towards marker assisted backcrossing is to identify suitable donor parent for the trait of interest and identification of polymorphic markers between donor and recipient parents which can be used either for foreground and background selection in future. *Pup-1* was a desirable QTL of rice for improvement of P-uptake under P-limiting condition as it explains approximately 70% of total variance [4]. From the sequence of a P-tolerant rice genotype, Kasalath [7], two primer pairs, *Pup-1*-K42 and *Pup-1*-K29 which were developed from inside and outside of INDEL region respectively, were used in this study for genotyping in 1% agarose gel. Nine genotypes amplified a 918 bp fragment as expected by using a primer pair *Pup-1*-K42 (Fig. 1). The Pup K 29 primer pairs did not amplify the expected 480/491 bp (tolerant/susceptible) for the selected 31 genotypes showed in Fig. 1. It amplified two alleles of 480 bp and 1050 bp i.e. instead of 491 bp it amplified a fragment of 1050bp. Surprisingly, two genotypes, Khitish and Ranjit did not amplify any fragment (Fig. 1) even of repeated attempt although they responded well in other SSR amplification. Bhutmuri, Gobindabhog and Radhunipagol with 918 bp amplification have high P-uptake as well as better dry-weight when grown in P-deficient soil. Durgasail and Kunti neither uptake high-P in P-deficient or sufficient soil and it also showed low dry weight but remaining six genotypes showed medium uptake. Twenty seven rice genotypes amplified a fragment of 480bp and only four rice genotypes amplified a fragment of 1050bp. Genotypes which were showing high P-uptake in P-sufficient soil but low uptake in P-deficient soil can be used as recipient parent for introgression of *Pup-1* locus. Haplotyping of *Pup-1*-K42 markers showed 918bp amplification in nine genotypes, among them, three genotypes showed higher P-uptake and dry-matter -weight in P-limiting condition. Three Bengal landraces, Bhutmuri, Gobindabhog and Radhunipagol can serve as ideal donor parent for introgression of *Pup-1* locus following *Pup-1*-K42 markers. It was to note that two genotypes, Durgasail and Kunti amplified 918 bp fragments like *Pup-1* linked markers but uptake less P and dry-weight than those of average in P-deficient soil. Similarly, Gitanjali, Jaladhi, Lalat, Neigersail, Tulaipanji did not possess *Pup-1*-K42 allele although they uptake significantly higher amount of P in P-limiting condition with higher dry-weight. So *Pup-1*-K42 was less diagnostic in *indica* germplasm. It may be due to location of this marker in hypervariable INDEL region and it has been developed mainly following *japonica* rice sequence. Amplification of dissimilar banding pattern than that of *japonica* rice by *Pup-1*-K29 also strengthens the same hypothesis. *Pup-1*-K29 amplified a 480bp fragment in twenty seven genotyopes; only of which nine were P-deficient tolerant. Only four rice genotypes which amplified a fragment of 1050bp were P-deficient susceptible. So, *Pup-1*-K29 was also not diagnostic for monitoring *Pup-1* locus in *indica* background. But *Pup-1*-K29 marker was suitable for marker assisted introgression of Pup1 locus from Bhutmuri, Gobindabhog and Radhunipagol into IR36 or IR64 background, especially for reducing the linkage drag from the donor parent. Therefore in our study K42 was showing dominant behavior but K29 showed co-dominant behavior. These two markers can be used in marker assisted introgression of *Pup-1* locus from Gobindabhog, Bhutmuri and Radhunipagol into IR36 and IR64 background. It will be better if a co-dominant SSR marker near K42 can be included for distinguishing heterozygote from homozygote of Pup-1 locus in BC_1 or BC_2 population.

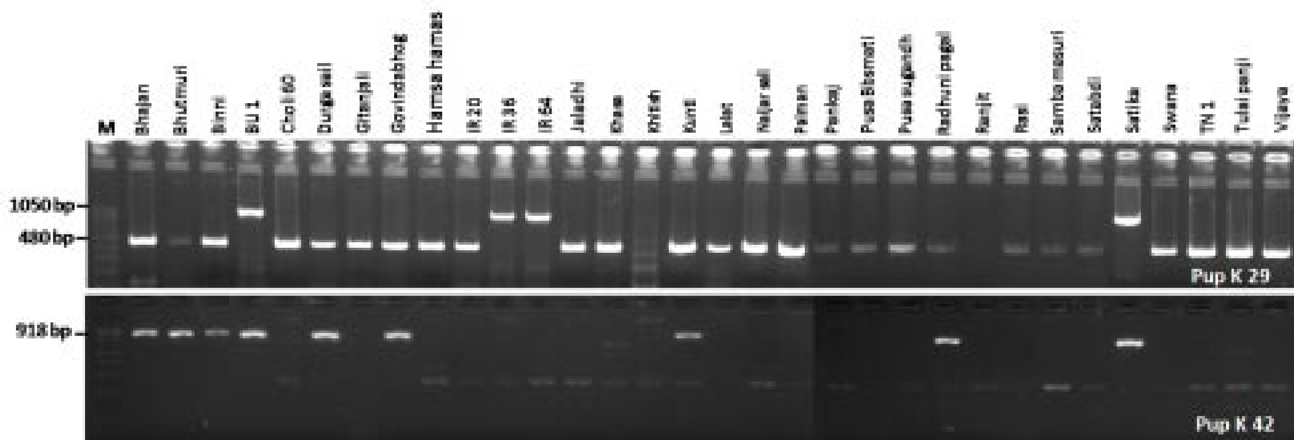

Fig. 1. PCR amplification of 31 rice genotypes by Pup1-K42 and Pup1-K29 primers

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In conclusion, although *Pup-1*-K42 and K-29 markers showed very strong diagnostic value in *japonica* rice lines but it was less diagnostic for assessing *Pup-1* locus in *indica* germplasm. Positive allele in any rice genotypes by these markers should combined with P-acquisition efficiency and dry-weight in P-deficient soil for consideration of donor parent in MAS for introgression of *Pup-1* locus in high yielding semi-dwarf cultivars. Three Bengal Landraces, Bhutmuri, Gobindabhog and Radhunipagol were recommended as donor parents for introgression of high P-uptake locus into IR36, IR64, by following the banding pattern of *Pup-1*-K42 as dominant and *Pup-1*-K29 as a co-dominant marker for foreground selection.

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