Screening tolerance to phosphorus starvation and haplotype analysis using phosphorus uptake 1 (*Pup1*) QTL linked markers in rice genotypes

V. Nirubana[1] · C. Vanniarajan[1] · N. Aananthi[2] · J. Ramalingam[3]

Abstract Phosphorus (P) deficiency tolerance is a pivotal trait for plant growth and development. Most of the commercial modern cultivars lack this trait and reported it as a very serious problem limiting crop productivity. This trait is advantageous if present in modern high yielding varieties as it increases the yield under the phosphorus-deficient soil conditions. With the importance of phosphorus deficiency tolerance, the present investigation was carried out with an objective to screen for tolerance to phosphorus deficiency using solution culture and phosphorus uptake 1 (*Pup1*) locus linked markers in 30 diverse rice genotypes. A wide range of varied responses to P deficiency in rice genotypes for all the traits were observed. Root length and enzyme activity showed increased mean performance under the − P condition when compared to + P condition. Medium to high heritability estimates were obtained for most of the traits. Correlation analysis showed that the traits: root P content, fresh shoot weight, dry shoot weight, and shoot length showed highly significant correlations with each other under − P conditions. Based on the hydroponics and molecular screening, three genotypes viz., ADT (R) 48, Improved Pusa Basmati 1 and UPLRI 5 were classified as tolerant for its response to P deficiency as they possessed significant increase in desirable root and shoot traits, increased acid phosphatase enzyme and these genotypes also possessed the *Pup1* allele for all the five markers. The selected genotypes may be useful for the exploration of novel genes conferring phosphorus deficiency tolerance and used as donor parents in the breeding programs. Absence of this allele in the rice genotypes viz., drought tolerant (Anna (R) 4) and submergence tolerant (CR 1009 *Sub* 1) may warrant the development of multiple abiotic stress tolerance cultivars for upland and submergence cropping systems in future rice breeding program.

Keywords *Pup1* · Phosphorus deficiency tolerance · Hydroponics · Markers · Rice

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Introduction

Rice (*Oryza sativa* L.), the most vital crop and holds a unique position among cereals, serves as the staple food for the world population. It provides a total caloric intake of the Asian population where it is a prime source of energy. The world population would may reach to 9 billion by the end of 2050 (Ray et al. 2013; FAO 2018). The increasing population demands more production than usual throughout the world. Phosphorus (P) is referred to as "king-pin" in Indian agriculture (Dey et al. 2017) and it is one of the important macronutrients required for normal growth and development of all crop plants. Phosphorus interacts with calcium/magnesium and iron/aluminum in alkaline and acidic soils respectively, to form insoluble compounds and becomes limitedly (20 per cent) available to the plants

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(Kochian et al. 2004). The potential yield of rice cultivars is decreasing due to the phosphorus deficiency in problematic soils, thereby increasing the application of P fertilizer throughout the world. About 50% of the rice fields are experiencing a deficiency of P in Asia (Prasetiyono et al. 2010) and about 60% of rainfed rice is grown on soils affected by multiple stresses including the P deficiency (Haefele and Hijmans 2007). The P reserves are depleting at a faster rate, as the farmers are applying more P fertilizers to tackle the situation and consequently it increased the environmental and health problems. Therefore, an alternative strategy is required to overcome the crisis; and to address this development of phosphorus-deficient tolerant rice varieties holds significance for beneficial rice farming.

In rice, a major quantitative trait locus (QTL) phosphorus uptake 1 (Pup1) detected on chromosome 12 exhibited 78.8% of the phenotypic variance for the phosphorus uptake. It was found related to the phosphorus deficiency or efficient phosphorus uptake in low phosphorus soil (Wissuwa et al. 1998; 2002). Heuer et al. (2009) sequenced the Pup1 genomic region in the tolerant donor parent aus-type rice variety Kasalath and performed the comparative genomic analysis with indica (93–11) and japonica (Nipponbare) reference genomes to discover the genes present in the region. It revealed the complex genomic structure between the Kasalath Pup1 region (280 kb) and the syntenic regions in the indica (750 kb) and japonica (150 kb). Further, fine mapping and candidate gene analysis of Pup1 locus identified PSTOL1 (Phosphorus Starvation Tolerance 1), which was very closely associated with tolerance to phosphorus deficiency and stress-tolerant rice genotypes (Chin et al. 2010; 2011; Gamuyao et al. 2012). Based on detailed sequence information, the allele-specific, insertion-deletion (InDel) and DNA markers for Pup1 locus have been developed for use in gene identification and marker-assisted selection (Chin et al. 2011).

Many researchers detailed the effectiveness of these markers to characterize the rice germplasm lines for tolerance to phosphorus deficiency and detect the Pup1 locus. Pandit et al. (2016) characterized the 96 genotypes including landraces and upland cultivars based on the Pup1 specific markers and identified the new Pup1 containing lines tolerant to phosphorus deficiency. Similarly, Swamy et al. (2019) evaluated the 98 germplasm lines from the North-Eastern part of India and identified the new Pup1 positive lines with low soil P tolerance. Besides, this study also found the novel rice line 'Wazuhophek' tolerant to phosphorus deficiency and devoid of the tolerance allele at Pup1 locus. In another study, one hundred sixty-eight Thai indigenous upland rice germplasm was screened using Pup1 specific markers and identified the novel sources for

tolerance to phosphorus deficiency (Chankaew et al. 2019). Most of the modern varieties in commercial cultivation are lacking PSTOL1 gene and didn't perform well in soils with low P levels (Anila et al. 2018).

Generally, hydroponics culture, sand culture, and soil culture are typically used at the seedling stage for coarse screening and the genotypes preliminarily identified are re-screened and tested at the seedling or maturity in the field. Among these methods, hydroponics is a commonly used convenient method for controlling P concentration (Guo et al. 2002; 2006). Preparation and maintenance of a low P field require more attention than a normal field. Moreover, some researchers consider field study for low P tolerance is less trustworthy because of the conflicting climatic and soil factors (Panigrahy et al. 2014). Deficiency of P can affect crop growth throughout the season, so evaluation at the seedling stage is a viable approach, as it is high throughput, low cost, which saves space and time (Meeks et al. 2013). Therefore, seedling stage screening can serve as an alternative for tedious field screening for low P tolerance (Panigrahy et al. 2014). Several researchers used hydroponic and field experiments for identifying promising traits involved in P deficiency tolerance applied with different doses of P fertilizer (Ali et al. 2018). Generally, an increase in root length, root/shoot fresh weight, dry root and shoot weight had a relation in response to phosphorus deficiency can be used as indicators for tolerance towards low P (Li et al. 2009; Panigrahy et al. 2014; Yugandhar et al. 2018). Keeping this in mind, our study was aimed to screen the rice genotypes for phosphorus deficiency tolerance considering the root and shoot morphological traits at the seedling stage under low P condition. Also, Pup1 locus associated markers were used to identify the genuine phosphorus deficient-tolerant genotypes that can be exploited for future rice breeding programme.

Materials and methods

Plant materials

Thirty rice genotypes used in the present study were collected from Tamil Nadu Agricultural University (TNAU), Coimbatore, India, International Rice Research Institute (IRRI), Philippines, and ICAR-Indian Institute of Rice Research (IIRR), Hyderabad, India. The experiments were conducted during 2017–2019 at Agricultural College and Research Institute, Tamil Nadu Agricultural University (TNAU), Madurai, Tamil Nadu, India.

Hydroponic phosphorus efficient assay

Thirty rice genotypes including two phosphorus-deficient tolerant genotypes (IR 64 *Pup1* and Samba Mahsuri *Pup1*) were grown in both phosphorus sufficient (+ P, 100%) and phosphorus-deficient (− P, 0%) hydroponic solutions in the greenhouse (Fig. 1). IR 64 *Pup1* and Samba Mahsuri *Pup1* possessing the *Pup1* locus were used as positive checks. The hydroponic solution was prepared based on the modified Yoshida solution (Yoshida and Parao 1976) (Table S1). The experiment was formulated in Completely Randomized Design (CRD) with two replications. Initially, the seeds were germinated in a rolled towel for ten days, and on the eleventh day, seedlings were transplanted into thermocol floats, and the floats were suspended in 51 × 35 cm size plastic trays containing 12 L of hydroponic solution. The pH of the solution was checked using a handheld pH meter (Hanna instruments) and maintained at a pH of 5.0 using 1 N sodium hydroxide (SDFCL) and 1 N hydrochloric acid (ISOCHEM). Every two days, the 12 L volume of hydroponic solution in the trays was maintained regularly. Once a week, the hydroponic solution was changed and refilled with the fresh solution using the same size of plastic trays. Care was taken to prevent any algal growth in and around the plastic trays and also on the thermocol floats. After 50 days of transplanting, the individual plants were taken out separately, shoots and roots of each plant of each treatment (+ P and − P) were separated without damaging the shoot and root for evaluating the morphological traits.

Evaluation of morphological traits

The morphological traits like shoot length (cm), root length (cm), fresh shoot weight (g), fresh root weight (g), dry shoot weight (g), dry root weight (g), acid phosphatase enzyme activity (molar p-NPP per min per mg), shoot phosphorus content (mg/g) and root phosphorus content (mg/g) were recorded on three randomly selected plants in each genotype per replication (Chithrameenal et al. 2017).

Estimation of phosphorus content

The phosphorus content (mg/g) in root and shoot samples were estimated using the Vanadomolybdate yellow colour method using a spectrophotometer (Varian CARY 50) (Piper 1966). One gram of powdered sample was taken and subjected to triacid acid (nitric acid (ISOCHEM): sulphuric acid (ISOCHEM): perchloric acid (SDFCL) of 3:2:1 ratio) digestion. The solution was filtered and volume made upto 100 mL using distilled water. About 5 mL of the triacid extract was pipetted out into a 25 mL volumetric flask. Five mL of Barton's reagent was added and the volume was made up with the distilled water. The development of the yellow colour was observed after 30 min and the intensity of colour was measured in a photoelectric colorimeter using blue filter (470 nm) after adjusting the
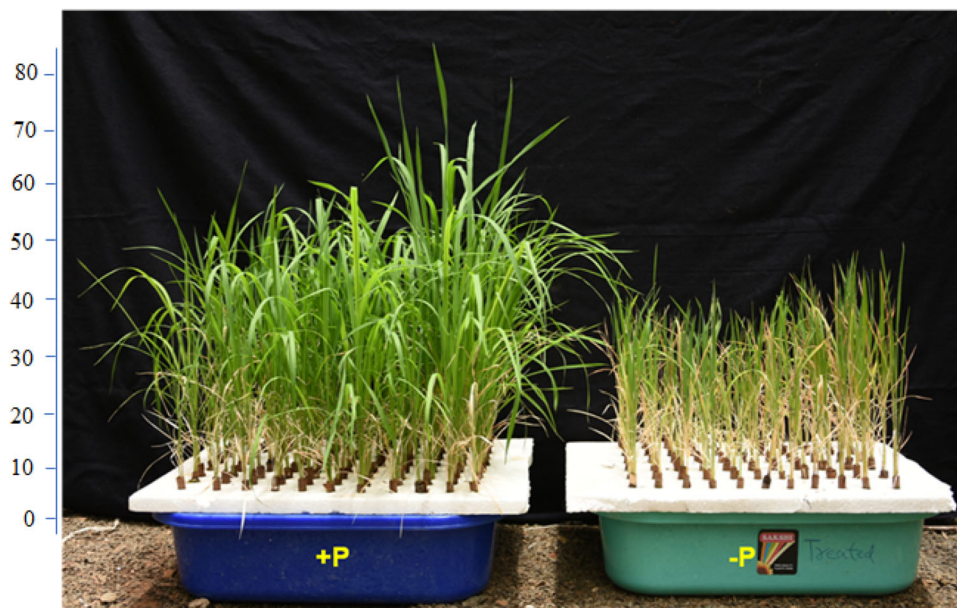

Fig. 1 Screening of genotypes for phosphorus deficiency tolerance under hydroponics (+ P and − P). *Note* Genotypes 11–21 (50 days old seedling)

transmittance of the meter to 100 with a blank. The colour was stable for 24 h. The concentration of phosphorus in the solution was deduced from the standard curve from which the percentage of phosphorus content of the sample was calculated.

P content on moisture free basis

$$= \frac{X}{10^6} \times 25 \times \frac{V}{5} \times \frac{100}{W} \times \frac{100}{100 - M}$$

where X = corresponding ppm from standard graph; V = volume of tri-acid extract; W = weight of the sample taken; M = moisture content of the plant sample.

Acid phosphatase assay

One gram of fresh leaf tissue was taken from 50 days old individual plants. Fresh leaf samples were ground in a cold pestle and mortar using 10 ml of 50 mM citrate buffer at a pH of 5.3. The extract was filtered and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant has been used as an enzyme mean source. Three ml of the acid substrate incubated at 37 °C for 5 min and 0.5 ml of enzyme extract was added and mixed thoroughly. Then, 0.05 ml was taken immediately and mixed with a 9.5 ml of sodium hydroxide (SDFCL) (0.085 N), this served as a blank for the assay. The remaining solution (substrate + enzyme) is incubated for 15 min at 37 °C. For that 0.5 ml of the sample is taken and mixed with 9.5 ml sodium hydroxide solution. Then the absorbance of blank and incubated tubes is measured at 405 nm followed by the standard curve drawn. The enzyme activity is expressed as molar para-nitrophenol released per min per mg of fresh weight (Sadasivam and Manickam 1996).

Genomic DNA isolation and PCR analysis

Genomic DNA was isolated from the young leaves of each genotype using CTAB (Cetyl Trimethyl Ammonium Bromide) method (Murray and Thompson 1980). The DNA was quantified using Nanodrop Spectrophotometer ND-1000 and the final concentration of DNA was adjusted to 50 ng/μl and stored at − 20 °C. Five SSR markers comprised of three dominant (K46-1, K52 and K59) markers in INDEL region and two co-dominant markers (K29-2 and K29-3) in *Pup1* genomic region (Chin et al. 2010, 2011) were used for genotyping to screen for the presence or absence of *Pup1* locus in the genotypes (Table S2). PCR reaction was carried out using Eppendorf master cycler with the following thermal cycler conditions: 94 °C for 5 min of initial denaturation followed by 35 cycles of each denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 45 s followed by final extension at 72 °C for 7 min. The amplified PCR products were

separated on 3.5 percent agarose gel stained with ethidium bromide and the bands were visualized in BioRAD-Gel Doc™ XR gel documentation system (Bio-Rad laboratories, USA) under UV light.

Statistical analysis

The data from the experiments were analysed by performing analysis of variance (ANOVA) using AGRES statistical software. Heritability in broad sense was calculated according to the method formulated by Lush (1940) and heritability values were categorized as suggested by Robinson et al. (1949) as follows: low (0–30%), moderate (30–60%) and high (> 60%). The correlation coefficient between the parameters was calculated using the formulae suggested by Johnson et al. (1955) in the Microsoft Excel program. Significance was tested by comparing correlation coefficients with the correlation table values at (n − 2) degrees of freedom. Where 'n' denotes the number of genotypes used in the study. The values of the correlation coefficient were classified based on the scale given by Searle (1965) as follows: very strong (> 0.65), moderately strong (0.50–0.64), moderately weak (0.30–0.49) and very weak (< 0.30). The cluster analysis using molecular data was performed using the STAR (Statistical tool for Agricultural Research) 2.0.1 software.

Results

Phenotypic variation of traits under + P condition

Analysis of variance revealed significant variation among genotypes for all the traits except shoot length, fresh root weight, dry root weight, and root P content (Table S3). The estimated heritability values were high for enzyme activity (98.35%), total P content (98.11%), shoot P content (81.30%) and fresh shoot weight (62.03%), whereas those for the other traits were medium and ranged from 32.33% (root P content) to 59.09% (dry shoot weight) (Table S3). The significance of the genotypes was tested against grand mean ± CD (critical difference) for identifying the genotypes with superior performance.

The shoot length varied from 36.53 to 80.50 cm, with ADT 47 recorded the highest (80.50 cm) and Improved Samba Mahsuri recorded the lowest (36.53 cm) (Table 1; Fig. 2). The highest root length was recorded in IR 64 (26.00 cm) and the lowest in ADT 39 (12.20 cm). CO 43 genotype has performed better under + P condition than other genotypes in the study with significantly deviating mean for fresh root weight (g), fresh shoot weight (g), dry shoot weight (g), and total phosphorus content (Table 1).

Table 1 Root and shoot characters and enzyme activity of genotypes grown under hydroponic conditions at two levels of phosphorus treatments

S. No.	Genotypes	Shoot length (cm) +P	Shoot length (cm) −P	Root length (cm) +P	Root length (cm) −P	Fresh shoot weight (g) +P	Fresh shoot weight (g) −P	Fresh root weight (g) +P	Fresh root weight (g) −P	Dry shoot weight (g) +P	Dry shoot weight (g) −P	Dry root weight (g) +P	Dry root weight (g) −P
1	ADT 37	70.20	33.38	14.17	23.48	3.77	0.64	0.62	0.06	1.02	0.23	0.12	0.02
2	ADT 39	53.65	32.85	12.20	29.30	1.83	0.67	0.37	0.36	0.59	0.20	0.04	0.09*
3	ADT 43	60.55	30.50	17.60	21.58	5.61*	0.49	1.36	0.20	1.56**	0.17	0.17	0.05
4	ADT 47	80.50	37.30	15.35	19.00	3.86	0.49	0.76	0.19	1.08	0.24	0.11	0.04
5	ADT (R) 48	51.40	41.08	13.73	26.53	2.26	1.20*	0.47	0.53**	0.64	0.37**	0.06	0.08
6	ADT 49	60.10	34.35	17.20	32.05	2.40	0.70	0.58	0.34	0.43	0.22	0.27*	0.08
7	Anna (R) 4	75.00	45.90	17.65	33.88*	5.48*	1.12	0.98	0.27	1.46*	0.36**	0.20	0.10**
8	APO	64.35	43.05	14.30	24.55	3.11	0.76	1.00	0.31	0.81	0.25	0.12	0.03
9	ASD 16	62.33	36.63	13.63	22.60	2.96	0.88	0.54	0.34	0.76	0.22	0.08	0.08
10	CO 43	57.20	28.95	13.10	23.70	6.60**	0.42	1.65*	1.16**	1.60**	0.17	0.18	0.08
11	CO 51	55.15	39.45	18.80	26.85	1.41	0.75	0.46	0.50*	0.39	0.20	0.05	0.08
12	CO 52	55.10	38.95	20.08	33.05	1.82	0.52	0.33	0.15	0.42	0.14	0.08	0.06
13	CR 1009 Sub 1	49.00	30.28	15.85	20.80	1.83	0.76	0.44	0.30	0.42	0.20	0.07	0.07
14	IPB 1	61.03	38.03	22.80*	27.48	2.88	0.85	1.22	0.53**	0.59	0.25	0.14	0.11**
15	ISM	36.53	34.83	18.00	36.40**	0.66	0.55	0.21	0.26	0.16	0.18	0.04	0.07
16	IR 20	55.88	27.48	21.63	16.00	1.71	0.30	0.68	0.13	0.40	0.10	0.11	0.04
17	IR 36	52.68	35.05	16.98	22.23	2.13	0.54	0.49	0.26	0.53	0.16	0.09	0.06
18	IR 50	52.25	39.15	19.35	20.20	2.15	0.61	0.45	0.23	0.59	0.19	0.10	0.06
19	PMK (R) 3	70.00	60.70**	16.80	23.40	1.16	1.05	0.62	0.26	0.55	0.22	0.08	0.07
20	IR 64	65.80	37.68	26.00**	28.65	3.88	0.69	1.02	0.32	0.96	0.24	0.17	0.08
21	MDU 5	67.10	37.50	19.13	21.30	3.20	0.71	1.08	0.35	0.86	0.24	0.13	0.07
22	MDU 6	53.78	37.90	17.80	26.43	1.13	0.77	0.20	0.36	0.42	0.19	0.05	0.04
23	PMK 1	72.95	61.75**	18.10	25.65	1.60	1.01	0.40	0.35	0.50	0.28	0.05	0.05
24	PMK 2	77.25	61.83**	18.95	22.48	2.49	1.11	0.59	0.25	0.65	0.31	0.10	0.06
25	RMD (R) 1	62.80	47.75	18.30	26.30	2.42	1.57**	0.61	0.41	0.59	0.40**	0.08	0.07
26	TKM (R) 12	72.30	47.80	17.00	21.48	3.65	0.82	1.08	0.17	0.78	0.23	0.17	0.03
27	TKM 13	45.25	31.93	16.00	23.23	1.10	0.34	0.27	0.13	0.29	0.14	0.03	0.03
28	UPLRI 5	70.85	52.35**	18.08	21.60	3.14	1.56**	1.17	0.24	0.86	0.49**	0.16	0.06
29	IR 64 Pup1	64.10	46.38	18.28	23.63	3.05	0.88	0.89	0.17	0.83	0.30	0.14	0.05
30	SM Pup1	54.85	31.20	15.85	34.05*	1.28	0.43	0.50	0.13	0.48	0.15	0.05	0.03
	Mean ± SE	61.00 ± 1.84	40.06 ± 1.72	17.42 ± 0.53	25.26 ± 0.89	2.69 ± 0.26	0.77 ± 0.06	0.70 ± 0.07	0.31 ± 0.04	0.71 ± 0.07	0.23 ± 0.02	0.11 ± 0.01	0.06 ± 0.004

S. No.	Genotypes	Enzyme activity conc. (molar p-NPP per min per mg) +P	Enzyme activity conc. (molar p-NPP per min per mg) −P	Shoot P content (mg/g shoot weight) +P	Shoot P content (mg/g shoot weight) −P	Root P content (mg/g root weight) +P	Root P content (mg/g root weight) −P	Total P content (mg/g plant dry weight) +P	Total P content (mg/g plant dry weight) −P
1	ADT 37	1.070	1.389**	0.496**	0.036	0.289	0.082	0.785**	0.118
2	ADT 39	1.106	1.265	0.402*	0.053	0.208	0.100	0.610**	0.153
3	ADT 43	1.136	1.270	0.381	0.050	0.154	0.094	0.535	0.144
4	ADT 47	1.166	1.332**	0.274	0.047	0.191	0.169	0.465	0.216**
5	ADT (R) 48	1.158	1.444**	0.428**	0.048	0.179	0.063	0.607**	0.111

Table 1 continued

S. No.	Genotypes	Enzyme activity conc. (molar p-NPP per min per mg)		Shoot P content (mg/g shoot weight)		Root P content (mg/g root weight)		Total P content (mg/g plant dry weight)	
		+ P	− P	+ P	− P	+ P	− P	+ P	− P
6	ADT 49	1.061	1.034	0.387*	0.058	0.214	0.065	0.601**	0.123
7	Anna (R) 4	1.083	1.316**	0.363	0.036	0.203	0.041	0.566*	0.077
8	APO	1.102	1.104	0.336	0.052	0.117	0.099	0.453	0.151
9	ASD 16	1.113	1.406**	0.324	0.050	0.223	0.057	0.547	0.107
10	CO 43	1.092	1.073	0.371	0.055	0.222	0.073	0.593**	0.128
11	CO 51	1.278	1.236	0.332	0.043	0.233	0.076	0.565*	0.119
12	CO 52	1.158	1.320**	0.381	0.064	0.224	0.325**	0.605**	0.389**
13	CR 1009 Sub 1	1.301	1.173	0.344	0.045	0.228	0.079	0.572**	0.124
14	IPB 1	1.226	1.148	0.296	0.047	0.185	0.058	0.481	0.105
15	ISM	1.270	1.215	0.253	0.171**	0.238	0.153	0.491	0.324**
16	IR 20	1.473**	1.208	0.359	0.049	0.148	0.218**	0.507	0.267**
17	IR 36	1.303	1.388**	0.326	0.050	0.154	0.086	0.480	0.136
18	IR 50	1.324**	1.181	0.280	0.129**	0.258	0.192*	0.538	0.321**
19	PMK (R) 3	1.232	1.246	0.264	0.043	0.122	0.049	0.386	0.092
20	IR 64	1.301	1.190	0.337	0.156**	0.250	0.067	0.587**	0.223**
21	MDU 5	1.311	1.360**	0.235	0.036	0.148	0.031	0.383	0.067
22	MDU 6	1.294	1.383**	0.346	0.057	0.282	0.022	0.628**	0.079
23	PMK 1	1.252	1.278	0.315	0.042	0.142	0.048	0.457	0.090
24	PMK 2	1.216	1.242	0.259	0.036	0.176	0.046	0.435	0.082
25	RMD (R) 1	1.290	1.467**	0.304	0.056	0.224	0.052	0.528	0.108
26	TKM (R) 12	1.289	1.324**	0.333	0.046	0.163	0.060	0.496	0.106
27	TKM 13	1.349**	1.387**	0.260	0.047	0.186	0.047	0.446	0.094
28	UPLRI 5	1.384**	1.426**	0.231	0.057	0.221	0.053	0.452	0.110
29	IR 64 Pup1	1.297	1.398**	0.356	0.044	0.207	0.062	0.563*	0.106
30	SM Pup1	1.190	1.250	0.321	0.052	0.343	0.125	0.664**	0.177**
	Mean ± SE	1.228 ± 0.02	1.282 ± 0.02	0.330 ± 0.01	0.058 ± 0.01	0.204 ± 0.01	0.090 ± 0.01	0.534 ± 0.02	0.148 ± 0.01

IPB 1 Improved Pusa Basmati 1, ISM Improved Samba Mahsuri, SM Pup1 Samba Mahsuri Pup1, SE standard error

*Significant at 5% level, **Significant at 1% level

Fig. 2 Comparisons of genotypes for shoot and root length in hydroponics. **a** ADT 47 shows higher shoot length (cm) in + P condition; **b** PMK 2 shows larger shoot length (cm) in − P condition; **c** IR 64 shows higher root length (cm) in + P condition; **d** ISM (Improved Samba Mahsuri) shows larger root length (cm) in − P condition; **e** and **f** (*Pup1* positive lines) (scale bar in cm)

The highest shoot P content was recorded in ADT 37 (0.496 mg/g) and the lowest in UPLRI 5 (0.231 mg/g). In case of root P content, the genotype Samba Mahsuri *Pup1* (0.343 mg/g) recorded the highest and APO (0.117 mg/g) recorded the lowest. ADT 37 (0.785 mg/g) recorded the highest total P content while MDU 5 (0.383 mg/g) was recorded as lowest. The activity of acid phosphatase enzyme ranged from 1.061 to 1.473 molar of para-nitrophenol (p-NPP) released per minute per mg of fresh weight, ADT 49 was found to have the lowest enzyme activity and IR 20 has recorded the highest activity (Table 1).

Phenotypic variation of traits under − P condition

Analysis of variance showed significant differences for all the traits among the 30 genotypes studied (Table S3). The

high heritability was observed for enzyme activity (99.39%), total P content (99.32%), shoot P content (93.36%), fresh root weight (87.54%), shoot length (81.14%), fresh shoot weight (77.32%), dry shoot weight (76.49%) and root P content (61.72%), whereas root length (54.77%) and dry root weight (44.12%) exhibited medium heritability (Table S3).

The shoot length under − P condition varied from 27.48 cm (IR 20) to 61.83 cm (PMK 2) and root length ranged from 16.00 cm (IR 20) to 36.40 cm (Improved Samba Mahsuri) (Table 1; Fig. 2). Anna (R) 4 has performed better than other genotypes with significant deviation from mean for root length (cm), shoot length (cm), dry shoot weight (g), dry root weight (g) and enzyme activity (p-NPP). Like wise, ADT (R) 48 for fresh shoot weight (g), fresh root weight (g), dry shoot weight (g) and enzyme activity (p-NPP); UPLRI 5 for shoot length (cm), fresh
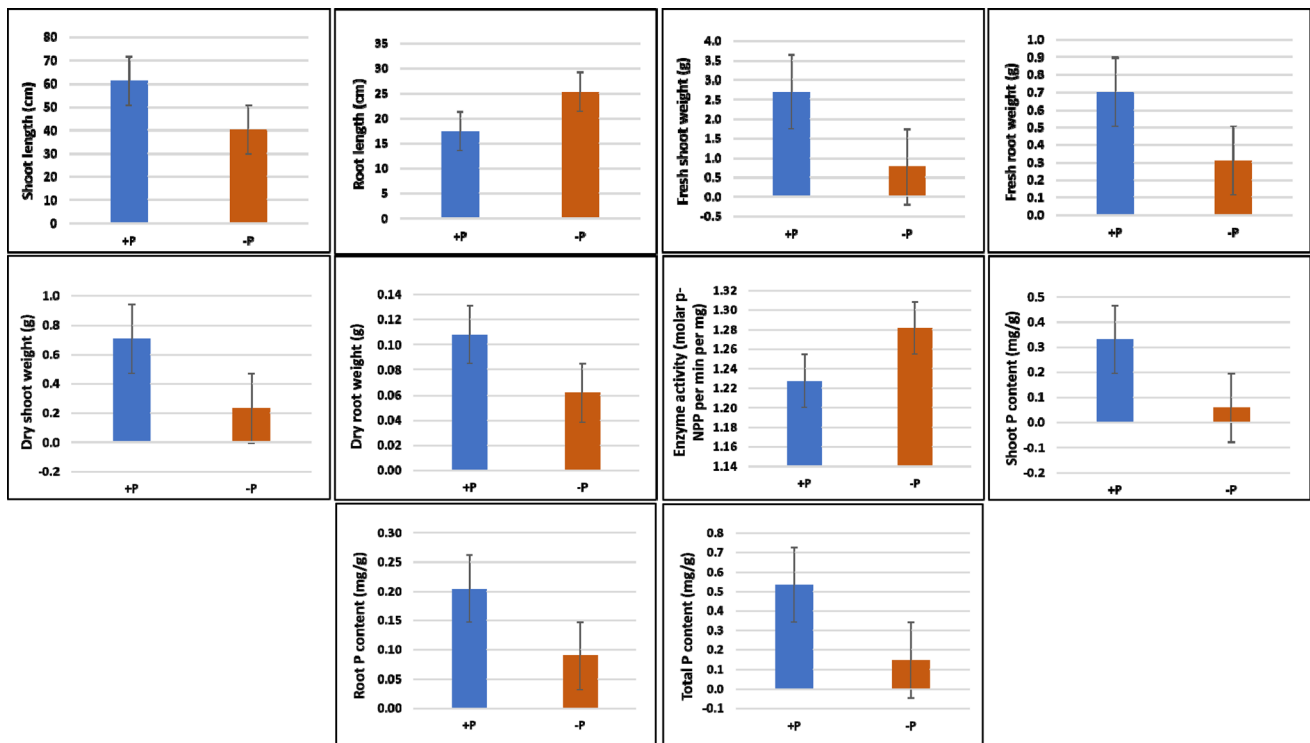
Fig. 3 Changes in ten parameters of rice genotypes grown under + P and − P conditions. Bars are the standard error of means of 1–30 genotypes

shoot weight (g), dry shoot weight (g) and enzyme activity (p-NPP) (Table 1).

Improved Samba Mahsuri (0.171 mg/g) has recorded the highest shoot P content and ADT 37, Anna (R) 4, MDU 5 and PMK 2 have recorded the lowest shoot P (0.036 mg/g). For root P, the highest was observed by CO 52 (0.325 mg/g) and the lowest by MDU 6 (0.022 mg/g). The genotype CO 52 (0.389 mg/g) has recorded the highest total P content while MDU 5 (0.067 mg/g) recorded the lowest. The enzyme activity varied from 1.034 to 1.467 molar of para-nitrophenol (p-NPP) released per minute per mg of fresh weight, the lowest activity was observed by ADT 49 and the highest activity by RMD (R) 1 (Table 1).

Traits' performance and their genetic correlation

Comparing the trait performance under + P and − P conditions, all the traits under + P condition were increased except root length and enzyme activity. Under − P condition, root length and enzyme activity were increased and the remaining traits were decreased. The phenotypic responses of the genotypes under the hydroponic condition at two levels of phosphorus treatments were presented in Fig. 3. Correlation among the different traits has been carried out in both + P and − P conditions to understand the relationship of phosphorus deficiency

tolerance to other morphological traits in the study. In + P condition, correlation coefficients ranged from 0.009 to 0.97. Among the traits studied, a very strong significant and positive correlation was observed between fresh shoot weight and dry shoot weight ($r = 0.966$), fresh root weight ($r = 0.845$), dry root weight ($r = 0.709$); total P content and shoot P content ($r = 0.817$), root P content ($r = 0.740$); fresh root weight and dry shoot weight ($r = 0.795$), dry root weight ($r = 0.712$). Moderately strong positive correlation was observed between dry shoot weight and dry root weight ($r = 0.597$); root length and enzyme activity ($r = 0.542$); shoot length and dry shoot weight ($r = 0.513$). Moderately weak correlation was recorded between shoot length and fresh shoot weight ($r = 0.472$), dry root weight ($r = 0.448$), fresh root weight ($r = 0.447$) (Table 2). While in − P condition, correlation coefficients varied from 0.018 to 0.922. A very strong positive and significant correlation was recorded between total P and root P content ($r = 0.922$), shoot P ($r = 0.650$) content; fresh shoot weight and dry shoot weight ($r = 0.920$); shoot length and fresh shoot weight ($r = 0.723$). Shoot length with dry shoot weight ($r = 0.615$); fresh root weight with dry root weight ($r = 0.551$) exhibited moderately strong significant association. Enzyme activity had moderately weak positive association with fresh ($r = 0.395$) and dry ($r = 0.398$) shoot weight (Table 2).

Table 2 Correlation coefficient among 10 morphological traits related to phosphorus (P) deficiency under + P and − P condition

Conditions	Traits	SL	RL	FSW	FRW	DSW	DRW	EA	SP	RP	TP
+ P	SL	1.000	0.080	0.472**	0.447*	0.513**	0.448*	− 0.201	− 0.081	− 0.283	− 0.223
	RL		1.000	− 0.091	0.119	− 0.161	0.210	0.542**	− 0.306	− 0.012	− 0.218
	FSW			1.000	0.845**	0.966**	0.709**	− 0.433*	0.268	− 0.107	0.121
	FRW				1.000	0.795**	0.712**	− 0.147	− 0.009	− 0.269	− 0.166
	DSW					1.000	0.597**	− 0.447*	0.249	− 0.099	0.113
	DRW						1.000	− 0.273	0.172	− 0.134	0.039
	EA							1.000	− 0.536**	− 0.094	− 0.424*
	SP								1.000	0.217	0.817**
	RP									1.000	0.740**
	TP										1.000
− P	SL	1.000	− 0.037	0.723**	− 0.095	0.615**	0.029	0.211	− 0.171	− 0.324	− 0.328
	RL		1.000	0.037	0.067	0.018	0.359	− 0.160	0.333	0.081	0.200
	FSW			1.000	0.089	0.920**	0.319	0.395*	− 0.183	− 0.486**	− 0.463**
	FRW				1.000	0.082	0.551**	− 0.336	− 0.021	− 0.278	− 0.230
	DSW					1.000	0.248	0.398*	− 0.149	− 0.461*	− 0.429*
	DRW						1.000	− 0.213	0.140	− 0.205	− 0.107
	EA							1.000	− 0.264	− 0.175	− 0.247
	SP								1.000	0.304	0.650**
	RP									1.000	0.922**
	TP										1.000

SL shoot length (cm), *RL* root length (cm), *FSW* fresh shoot weight (g), *FRW* fresh root weight (g), *DSW* dry shoot weight (g), *DRW* dry root weight (g), *EA* acid phosphatase enzyme activity (molar p-NPP per min per mg), *SP* shoot P content (mg/g), *RP* root P content (mg/g), *TP* total P content (mg/g)

*Significant at 5% level; **Significant at 1% level

Based on the phenotypic performance, some of the genotypes showed significantly increased responses for the different traits (Table 1; Fig. 3). Among all the traits, root length and enzyme activity had increased under the − P condition when compared to + P condition. The genotype ADT (R) 48 performed better for fresh shoot weight, fresh root weight, dry shoot weight, and acid phosphatase enzyme activity under − P condition. Likewise, the genotype UPLRI 5 for shoot length, fresh shoot weight, dry root weight, and acid phosphatase enzyme activity; Anna (R) 4 for root length, dry shoot weight, dry root weight, and acid phosphatase enzyme activity; RMD (R) 1 for fresh shoot weight, dry shoot weight and enzyme activity; Improved Samba Mahsuri for root length, shoot P and total P content; CO 52 for enzyme activity, root P and total P; IR 50 for shoot P, root P and total P; Samba Mahsuri *Pup1* for root length and total P; Improved Pusa Basmati for fresh and dry root weight; IR 20 for root P and total P; IR 64 for shoot P and total P; ADT 47 for enzyme activity and total P content under − P condition. Remaining genotypes does not perform well under − P condition (Table 1; Fig. 3). Hence, these genotypes may be selected for the breeding program after screening with *Pup1* linked molecular markers. The traits viz., root P content, fresh and dry shoot weight and shoot length were strongly correlated for P deficiency tolerance under − P condition and these traits may be used as an indicator for screening tolerance to P deficiency.

Genotyping of rice genotypes for *Pup1* locus

Five *Pup1* QTL linked markers viz., K29-2, K29-3, K46-1, K52 and K59 were genotyped for all the 30 rice genotypes and the results were presented in Table 3 and Fig. 4. Amplified bands were scored from 0 (absence of allele in all the markers) to 5 (presence of the allele in all the markers). Among the 30 genotypes, 80 percent of the population have showed the probable presence of the *Pup1* QTL. The marker K46-1 was observed to be the highest (70%) among the genotypes followed by K52 (66.66%), K59 (60%), K29-2 (36.66%), and K29-3 (30%). Five genotypes i.e., ADT (R) 48, APO, Improved Pusa Basmati 1, PMK (R) 3 and UPLRI 5 have shown the *Pup1* allele for all the five markers used in the study. Six genotypes viz., ADT 39, ADT 47, Anna (R) 4, CO 52, CR 1009 *Sub* 1 and TKM 13 did not show any *Pup1* allele amplification. Three

Table 3 Allelic profile of *Pup1* specific markers among the 30 rice genotypes evaluated in the study

S.No.	Genotypes	K29-2	K29-3	K46-1	K52	K59	Total no. of *Pup1* linked marker alleles
1	ADT 37	0	0	0	1	0	1
2	ADT 39	0	0	0	0	0	0
3	ADT 43	1	0	1	0	0	2
4	ADT 47	0	0	0	0	0	0
5	ADT (R) 48	1	1	1	1	1	5
6	ADT 49	0	0	1	0	0	1
7	Anna (R) 4	0	0	0	0	0	0
8	APO	1	1	1	1	1	5
9	ASD 16	0	0	1	1	1	3
10	CO 43	0	0	1	1	1	3
11	CO 51	0	0	1	1	1	3
12	CO 52	0	0	0	0	0	0
13	CR 1009 *Sub* 1	0	0	0	0	0	0
14	Improved Pusa Basmati 1	1	1	1	1	1	5
15	Improved Samba Mahsuri	0	0	1	1	1	3
16	IR 20	0	0	0	1	0	1
17	IR 36	0	0	1	1	1	3
18	IR 50	1	0	1	0	0	2
19	PMK (R) 3	1	1	1	1	1	5
20	IR 64	0	0	1	1	1	3
21	MDU 5	0	0	1	1	1	3
22	MDU 6	0	0	1	1	1	3
23	PMK 1	0	1	1	1	1	4
24	PMK 2	1	1	1	0	1	4
25	RMD (R)1	0	0	0	1	0	1
26	TKM (R)12	1	0	1	1	1	4
27	TKM 13	0	0	0	0	0	0
28	UPLRI 5	1	1	1	1	1	5
29	IR 64 *Pup1*	1	1	1	1	1	5
30	Samba Mahsuri *Pup1*	1	1	1	1	1	5

(1) present and (0) absent

genotypes had four amplified bands each, eight genotypes with three bands each, two genotypes with two bands and four genotypes with single amplified band were also observed with respect to all the five *Pup1* specific markers. Clustering analysis classified the genotypes into five different clusters based on the scoring of alleles produced by different genotypes (Table 4). The third cluster had eight genotypes including two low soil P tolerant genotypes (ADT (R) 48, APO, Improved Pusa Basmati 1, PMK (R) 3, PMK 1, UPLRI 5, IR 64 *Pup1* and Samba Mahsuri *Pup1*) and all these genotypes except PMK 1 harboring *Pup1* allele for all the markers tested. Whereas the first cluster formed with nine genotypes, among them six genotypes (ADT 39, ADT 47, Anna (R) 4, CO 52, CR 1009 *Sub* 1 and TKM 13) were completely devoid of *Pup1* allele. The

remaining genotypes in different clusters were composed of various combinations with respect to presence and absence for the *Pup1* linked markers.

Discussion

Phosphorus is an essential element for plant growth and becoming a major restraint factor for crop production. To reduce the effects of low soil phosphorus stresses, plants have developed various adaptive mechanisms that involved different root and shoot characters (Raghothama and Karthikeyan 2005). In this study, 30 genotypes were screened phenotypically for P deficiency tolerance under hydroponic conditions. In − P condition, P was obtained only from the
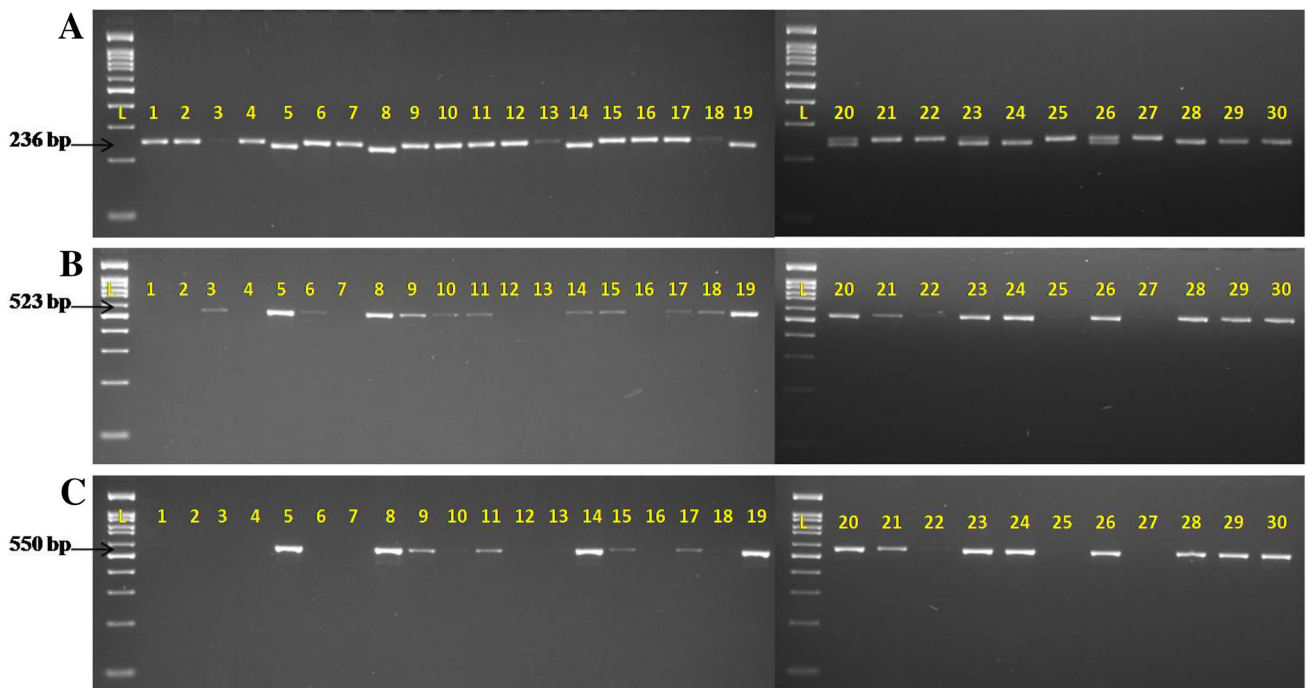
Fig. 4 Genotypic profile using *Pup1* QTL linked markers in 30 rice genotypes. *Note* L—100 bp ladder, **a** K29-3 marker, **b** K46-1 marker and **c** K59 marker

Table 4 Distribution of 30 genotypes into five clusters based on genotypic data

Clusters	Number of genotypes	Name of the genotypes
I	9	ADT 37, ADT 39, ADT 47, Anna (R) 4, CO 52, CR 1009 *Sub* 1, IR 20, RMD (R)1, TKM 13
II	3	ADT 43, ADT 49, IR 50
III	8	ADT (R) 48, APO, Improved Pusa Basmati 1, PMK (R) 3, PMK 1, UPLRI 5, IR64 *Pup1*, Samba Mahsuri *Pup1*
IV	9	ASD 16, CO 43, CO 51, Improved Samba Mahsuri, IR 36, IR 64, MDU 5, MDU 6, TKM (R)12
V	1	PMK 2

reserved form in the endosperm. While in + P condition, P was obtained from endosperm as well as an external nutrient source. In this study, significant variation has been found for morphological traits related to low P response and the mean values for all the traits except root length and enzyme activity substantially decreased under − P condition, indicating that this condition affected the physiology and morphology of the plant (Santos et al. 2016). The high heritability values were observed for enzyme activity, total P content, shoot P content, fresh root weight, shoot length, fresh shoot weight, dry shoot weight and, root P content under − P condition allow the use of these traits as indicators for genotype selection for tolerance to low P conditions. Likewise, Chankaew et al. (2019) noticed the significant variation for root/shoot ratio only under low P

condition, whereas shoot dry weight (Aluwihare et al. 2016; Fageria et al. 1988; Wissuwa and Ae 2001), root dry weight (Wissuwa and Ae 2001), root length (Shimizu et al. 2004; Li et al. 2009; Panigrahy et al. 2014), root/shoot fresh weight, total dry weight (Panigrahy et al. 2014) and P content in different plant tissues (Fageria et al. 1988; Wissuwa and Ae 2001) were used as indicators for P deficiency tolerance screening.

Under − P condition, a drastic reduction in shoot growth was noticed, this may be due to the reason that rate of tissue expansion is directly related to P status of the tissue growth zone (Kavanova et al. 2006). The genotypes viz., PMK 1, PMK 2, PMK (R) 3 and UPLRI 5 had higher shoot length under − P condition and performed better when compared to other genotypes. Shoot length was

inhibited under low P conditions and therefore this trait is used to measure tolerance to low P stress (Li et al. 2009). For root length, Anna (R) 4, Improved Samba Mahsuri and Samba Mahsuri *Pup1* have exhibited superior performance compared to other genotypes under − P condition. The superior performance of Samba Mahsuri *Pup1* for root length was obviously due to presence of *Pup1* QTL. The Improved Samba Mahsuri has shown probable presence of *Pup1* QTL with linked markers, but Anna (R) 4 genotype did not possess *Pup1* QTL while checking with all linked markers in the study. Still, we observed the increased root length of Anna (R) 4 genotype, this may be due to the presence of novel genes for phosphorus deficiency tolerance beyond *Pup1* locus. Root growth/elongation is the important feature of phosphorus uptake under scarce condition (Fitter et al. 1994) and the cultivars which are tolerant to low P condition were known to have improved root system (Wissuwa 2003) and lateral root growth (López-Bucio et al. 2003). Dissanayaka et al. (2018) also reported that the larger root system potentially promotes P acquisition under low phosphorus availability and acts as a pool of P for remobilization to the shoot. Significant changes were also observed for dry shoot weight, fresh and dry root weight. As mentioned by Fageria et al. (1988), Fageria and Baligar (1997) and Gunes et al. (2006), that selection of genotypes under low P availability in soils could use dry weight of shoot and root as indicators. Therefore, an increase in root weight, root/shoot fresh weight (Panigrahy et al. 2014) and shoot dry weight (Fageria et al. 1988) is considered as a parameter for identifying the genotypes tolerant to P deficiency.

Regarding shoot P content, Improved Samba Mahsuri, IR 50 and IR 64 performed better compared to other genotypes and for root P content, the genotypes CO 52, IR 20 and IR 50 have exhibited superior performance under − P condition. In case of total P content, the genotypes ADT 47, CO 52, Improved Samba Mahsuri, IR 20, IR 50, IR 64 and Samba Mahsuri *Pup1* have superior performance from others in − P condition. Chithrameenal et al. (2017) also studied same traits in NILs and their parents under phosphorus stress condition. For the enzyme acid phosphatase, the highest activity was observed in almost fourteen genotypes under − P condition. Therefore, an increase in P absorption capacity resulting from increased enzyme activity under low P condition. Tadano et al. (1993), Lim et al. (2003), Panigrahy et al. (2014) and Mehra et al. (2017) also revealed that enzyme activity in tolerant genotypes was increased in phosphorus-deficient condition. Among the different morphological traits, enzyme activity and dry shoot weight contributed more in identification of P tolerant genotypes followed by fresh shoot weight, fresh root weight, root length and shoot length. Genotypes with high shoot and root length, shoot and root weight, P content

and enzyme activity at low P conditions can be selected for breeding phosphorus-deficient tolerance after screening with *Pup1* locus linked markers.

Correlation analysis among the morphological traits in both + P and − P conditions revealed that, there is a strong correlation between P content in root to the total P content in the plant. Under − P condition, increase in association between root P and total P content of the plant was observed. This may be due to the increase in the root length which will indirectly contribute to the total P content by absorbing more P in deeper layer of soil. This cannot be observed in + P condition due to availability of phosphorus in the rhizosphere of plant. This was clearly demonstrated that, under stress condition the plant with *Pup1* QTL will express itself through extension of root for nutrition. While the same plant with *Pup1* QTL under normal conditions, continue with their normal metabolic processes without expression for nutrition.

In addition to the phenotypic screening of the rice genotypes, they were also screened genotypically with already reported five *Pup1* locus based markers namely K46-1, K52 and K59 (dominant), K29-2 and K29-3 (codominant) (Chin et al. 2011) to determine the presence or absence of tolerant allele in the 30 genotypes along with two tolerant lines (IR 64 *Pup1* and Samba Mahsuri *Pup1*). Our study revealed that eighty percent of the genotypes possessed probable presence of *Pup1* QTL, but only five genotypes (ADT (R) 48, APO, Improved Pusa Basmati 1, PMK (R) 3 and UPLRI 5) had positive allele for all the markers. Six genotypes viz., ADT 39, ADT 47, Anna (R) 4, CO 52, CR 1009 *Sub* 1 and TKM 13 did not show any amplification from the above five markers. When compared to phenotypic performance of these genotypes, ADT 39 for dry root weight; ADT 47 for enzyme activity and total P content; Anna (R) 4 for root length, dry shoot weight, dry root weight and enzyme activity; CO 52 for enzyme activity, root P and total P content and TKM 13 for enzyme activity had significant responses under − P condition. The genotype CR 1009 *Sub* 1 did not perform well for any of the traits in response to P starvation. Similar kind of genotypic survey was conducted for *Pup1* QTL by Heuer et al. (2009); Tyagi et al. (2012); Pandit et al. (2018); Chankaew et al. (2019).

Cluster analysis based on the molecular data classified the genotypes into different clusters. Among the five clusters formed, seven genotypes in third cluster possessed *Pup1* and in the first cluster composed of six genotypes completely devoid of *Pup1* QTL and remaining groups were composed of partial *Pup1* locus. Similarly, cluster analysis based on genotypic variation with respect to *Pup1* specific markers was carried out by Aluwihare et al. (2015); Pandit et al. 2016; 2018; Swamy et al. (2019). Selection of genotypes in third cluster may be utilized as

potential donors and genotypes in the remaining clusters could be used as recurrent parents in the future breeding program. Cluster analysis gives a sense of the relationships among genotypes and can help breeders for the selection of diverse parents in crop improvement programmes. Identification and introgression of phosphorus tolerance alleles is an important and upcoming breeding strategy to develop the cultivars tolerant to low phosphorus, which improves the yield and productivity for attaining food and nutritional security. The present study has made an attempt to identify the novel alleles of phosphorus deficiency tolerance and identified some of the genotypes with *Pup1* allele using linked molecular markers. The genotypes viz., ADT (R) 48, Improved Pusa Basmati 1 and UPLRI 5 possessing *Pup1* QTL, obviously performed better under − P condition. These genotypes may further explored to identify the new genes for phosphorus deficiency tolerance.

Conclusion

In this study, three genotypes viz., ADT (R) 48, Improved Pusa Basmati 1 and UPLRI 5 were identified as phosphorus-deficient tolerant genotypes which could provide and display the tolerance sources for effective breeding of rice cultivars tolerant to phosphorus deficiency as they possessed *Pup1* QTL. Absence of this allele in the rice genotypes viz., drought tolerant [Anna (R) 4] and submergence tolerant (CR 1009 *Sub* 1) may warrant development of high yielding abiotic stress resistant rice cultivars to increase the rice production. In addition, genome sequencing of Anna (R) 4 need to be carried out to identify the candidate genes for low phosphorus tolerance beyond *Pup1* locus.

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