RESEARCH ARTICLE

Phosphate acquisition efficiency and phosphate starvation tolerance locus (*PSTOL1*) in rice

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Abstract

Phosphate availability is a major factor limiting tillering, grain filling vis-à-vis productivity of rice. Rice is often cultivated in soil like red and lateritic or acid, with low soluble phosphate content. To identify the best genotype suitable for these types of soils, P acquisition efficiency was estimated from 108 genotypes. Gobindabhog, Tulaipanji, Radhunipagal and Raghusail accumulated almost equal amounts of phosphate even when they were grown on P-sufficient soil. Here, we have reported the presence as well as the expression of a previously characterized rice gene, phosphate starvation tolerance locus (*PSTOL1*) in a set of selected genotypes. Two of four genotypes did not show any detectable expression but carried the gene. One mega cultivar, Swarna did not possess this gene but showed high P-deficiency tolerance ability. Increase of root biomass, not length, in P-limiting situations might be considered as one of the selecting criteria at the seedling stage. Neither the presence of *PSTOL1* gene nor its closely-linked SSR RM1261, showed any association with P-deficiency tolerance among the 108 genotypes. Not only this, but the presence of *PSTOL1* in recombinant inbred line (RIL) developed from a cross between Gobindabhog and Satabdi, also did not show any linkage with P-deficiency tolerance ability. Thus, before considering *PSTOL1* gene in MAB, its expression and role in P-deficiency tolerance in the donor parent must be ascertained.

[Mukherjee A., Sarkar S., Chakraborty A. S., Yelne R., Kavishetty V., Biswas T., Mandal N. and Bhattacharyya S. 2014 Phosphate acquisition efficiency and phosphate starvation tolerance locus (*PSTOL1*) in rice. *J. Genet.* **93**, 683–688]

Introduction

Tropical soil suffers from the deficiency of plant available phosphorus (P), although total P is sufficient in most of the cases. As rice is an important crop in tropics, phosphorus deficiency (PD) has been identified as a major factor limiting rice yields. Stunted growth with dark green leaves, reduced tillering, late maturity and high chaffiness are common in PD. Under P-starvation, rice retranslocates more assimilates to roots, hences favouring root growth over shoot growth. Side by side, rice has also evolved several mechanisms for overcoming PD situations like, arbuscular mycorrhizal association in root (Paszkowski *et al.* 2002), activation of high affinity phosphate transporter (Ai *et al.* 2009; Jia *et al.* 2011), exudation of organic acid and phosphatase from rice roots (Hou *et al.* 2005; Desnos 2008) favouring maximum release of locked phosphate from the soil. It also undergoes modifications in root architecture (Jiang *et al.* 2007) in such a way so as to facilitate extraction of P from larger volumes of soil. Consequently, numerous genes are required to be activated in P-limiting conditions which work in a cascade and initiate several changes in molecular, cellular and physiological processes resulting in plants adapting efficiently to the P-depleted condition (Panigrahy *et al.* 2009; Rouached *et al.* 2010).

Apparently, genes like *LPR1* (which encodes multicopper oxidase) and *LPR2* (which encodes P5-type ATPase) are present in the root tips of *Arabidopsis* for sensing and responding to P-starvation (Sanchez-Calderon *et al.* 2006), but such genes are yet to be identified in rice roots. Several transcription factors like, PTF1 (Yi *et al.* 2005), MYB2P-1 (Dai *et al.* 2012), PHR2 (Zhou *et al.* 2008), PHO2 (Wang *et al.* 2004), SPX1 (Wang *et al.* 2009) and IPS1 (Franco-Zorrilla *et al.* 2007) starts operating during P-depletion, and ultimately activates the high affinity transporter genes in rice

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Keywords. rice; P-deficiency tolerance; *PSTOL1*; Gobindabhog.

like *OsPT2*, *OsPT3*, *OsPT6* and *OsPT8*, or reduce availability of miR399 (Franco-Zorrilla *et al.* 2007), which finally activates the P-mobilization genes like *PHO2*.

A major QTL, phosphate uptake (*Pup1*) was identified from *aus* type PD-tolerant rice variety of India, Kasalath, and recommended for marker-assisted breeding (MAB) for developing tolerant varieties Wissuwa *et al* 2002; Chin *et al*. Chin-etal:2010. Recently, candidate gene for this QTL from Pup1-NIL line was identified by a map-based cloning approach. This gene was named as phosphate starvation tolerance locus, *PSTOL1* and which was missing in the nontolerant rice genome, Nipponbare. *PSTOL1* is a protein kinase and its expression is upregulated under PD condition (Gamuyao *et al.* 2012). Pup1-NIL lines showed improved root growth under stress but the known P-starvation genes (like *OsPHT* members), were not differentially expressed in *35S:PSTOL1* transgenic roots. Rather, root cell differentiation gene, *HOX1* (Scarpella *et al.* 2005), gene for delayed leaf senescence, *DOS* and *WRKY* gene for transcription factor of chromosome 1 were upregulated due to the presence of *PSTOL1* locus in a diverse collection of tolerant rice accessions (Gamuyao *et al.* 2012) *PSTOL1* was suggested as an easy target for marker-assisted introgression from tolerant to nontolerant high yielding varieties due to its larger effect.

Significant genotypic variation is present in rice for its ability to acquire P under low P-available soil (Sarkar *et al*. 2011). The status of *PSTOL1* alleles among these PD genotypes will help in selecting donor parent in rice breeding programmes. It has been also observed that not all PD tolerant genotypes contain *PSTOL1* gene, contrarily, several genotypes with *PSTOL1* gene do not express during P-deficient situation. Even after transfer of *PSTOL1* (Gamuyao *et al.* 2012) allele into a nontolerant cultivar it did not confer resistance in P-depleted soil.

Materials and methods

For the analysis, 108 rice genotypes collected from several rice growing areas of India and 180 RILs developed by crossing Gobindabhog and Satabdi were used. They were grown at the same time in the Gangetic alluvial soil, where plant-available P was >30 mg/kg as well as in red-lateritic soil, where the availability was <3.5 mg/kg. No external P fertilizer was applied to red and lateritic soils during the entire growth stage of rice and bunding was properly constructed surrounding the field to prevent any invasion of phosphate through other irrigation channels. Normal fertilizer dose of NPK was applied to alluvial soil. The P-estimation of the plant sample (preflowering shoot) with three replications for each line was carried out with Agilent 8453 spectrophotometer (California, USA) after tri-acid digestion as described earlier (Sarkar *et al.* 2011).

Genotyping for PSTOL1

DNA was extracted from ~40 mg of fresh leaf tissue as described in other works (Sarkar *et al*. 2011). Standard PCR was achieved using thermal cycler (Gene Amp PCR System 9700, California, USA). The reaction volume (25 μL) contained diluted DNA sample of 20 ng with 100 ng each of forward and reverse primers, (*PSTOL1*, 5′-ATGCTGCTCTGTCAAAGGGCAT-3′ (forward) and 5′-CAAGCTCAAAGCCCTTTTGGTG-3′ (reverse)) along with 2.5 μL 10× buffer, 1.5 mM $MgCl_2$, 1 μL 2.5 mM dNTPs, 16.5 μL deionized sterile water and 0.5 U *Taq* DNA polymerase enzyme (Invitrogen, California, USA). Amplification was set with the reaction conditions at 94°C for 5 min of initial denaturation followed by 35 cycles each of denaturation at 94°C for 45 s, annealing was done at 58°C and polymerization at 72°C for 1 min. Further extension was followed at 72°C for 7 min. PCR products were size fractionated in agarose gel and photographs were taken with the ethidium bromide staining.

Relative expression of PSTOL1

After germination, eight genotypes were allowed to grow in a glass plate dipped into Yoshida solution (Yoshida *et al*. 1976) supplemented with 10 mg of inorganic P/L (P-sufficient solution) and 0.05 mg of inorganic P/L (P-depleted solution). After 15 days, the total RNA of eight genotypes were extracted using RNeasy plant mini kit (Qiagen, Germany), and treated with RNase free DNase from the seedlings root following manufacturer's instructions. Transcript level of *PSTOL1* was measured by quantitative RT-PCR as described previously (Bhattacharyya *et al.* 2003). First-strand cDNA was synthesized from 5 μg of total RNA using oligo-dT(18) primer and Super Script First-Strand Synthesis system for RT-PCR (Applied Biosystems, Foster City, USA). Quantitative real-time PCR was performed in 20 μL reaction volume containing 2 μL cDNA, 75 ng each of gene-specific primers, and SYBR Premix using Step One (Applied Biosystems) model. Primer sequences used were: *PSTOL1*-F, 5′-GTTTGTGGTGCATACAACTCGT-3′ and 5′-GGTTCCTCAAAAACAGAAGATG-3′; *β-tubulin*, 5′-GCTGACCACACCTAGCTTTGG-3′(forward) and 5′-AGGGAACCTTAGGCAGCATGT-3′(reverse). The relative quantification of expression for target genes of different genotypes were measured and compared to popular, P-deficiency nontolerant cultivar, Satabdi. Normalization of target gene expression with housekeeping gene (*β-tubulin*) was carried out to compensate sample to sample variations and ensure experimental reliability.

Results

A set of 108 genotypes were evaluated for P accumulation ability when grown in P-sufficient (plant available P, 31 mg/kg of soil) as well as P-depleted (plant available P,

3.2 mg/kg) soil. To this end, vegetative dry mass and P content were measured just before flowering initiation stage. P content varied from 3.683 mg/plant to 27.723 mg/plant as measured in IR20 and Gobindabhog, respectively (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). Forty-nine genotypes showed higher P content than that of the mean value, 11.663 mg/plant and Gobindabhog showed twice the mean value. Genotypes like Tulaipanji, Radhunipagol, Swarna, Raghusail and Laxmisail recorded ~20 mg/plant. They also showed higher dry mass than that of the average and among them, Gobindabhog ranked the highest. So, Gobindabhog, a popular short-grain aromatic land race of a leading rice growing state in India, West Bengal, may be considered as the donor parent for introgression of P-deficiency tolerance character into any semi-dwarf high yielding popular cultivars like Satabdi, IR64, etc.

Amplification of PSTOL1 gene

When gene-specific primer pairs were used, as expected, several genotypes did not amplify any fragment. Fragment of 959 bp was amplified by 62 genotypes (table 1 in electronic supplementary material) but only 28 genotypes recorded P above the mean value. Remaining 46 genotypes did not contain the *PSTOL1* gene but 25 of these showed higher P accumulations than that of the average. Mean P accumulation per plant of 62 genotypes were 11.904 mg, whereas it was 12.709 for the remaining 46. These two mean values were same as per the statistical analysis ($P = 0.498$). RM1261 located near *PSTOL1* of chromosome 12 amplified four alleles of 160, 180, 190 and 290 nucleotides in length. Like *PSTOL1*, the SSR also did not show any association with P deficiency tolerance ability among the 108 genotypes.

Expression of PSTOL1 in rice root of a few selected rice genotypes

The expression patterns under P-deficient and P-sufficient conditions were evaluated by real time RT-PCR using RNA samples extracted from the seedlings root. Nine genotypes were considered for expression analysis but only Gobindabhog and Binni showed detectable expression in the root, although four parents which were amplified the desired *PSTOL1* fragment from the genomic DNA (table 1). Expression of the gene was activated several folds during P-deficient condition (0.05 mg/L) in both the genotypes but Gobindabhog showed approximately five-fold higher expression than that of Binni. Expression of Gobindabhog allele in P-sufficient condition was also higher. Expressions in Radhunipagal and Tulaipanji (two short grain aromatic Bengal land races) were not detectable neither at P-sufficient nor at P-deficient situations, although both of them contain *PSTOL1* gene as observed from the amplification (table 1). As per the gel picture of RT-PCR (figure 1 in electronic supplementary material), expressions of Radhunipagol and Tulaipanji were so mild that they remained undetected. Availability of *tubulin* transcript in all the nine genotypes was almost equal as revealed from similar CT values obtained from the real time expression of data. To determine whether differential P accumulation ability had any effect on plant biomass, both the dry root mass at P deficient and sufficient conditions were compared among the selected nine genotypes. Interestingly, the genotypes that accumulated higher P in deficient soil also manifested higher root biomass at the seedling stage except Radhinipagal (table 2). Contrarily, nontolerant genotypes, IR20, Palman and Satabdi, either reduced or remained same. Root length of Binni, Gobindabhog, Palman and Radhunipagol increased significantly in P-deficient conditions, whereas it was decreased in Satabdi and TN1. Shoot biomass remained same both at P-sufficient and deficient media except in Binni and Palman. Swarna, a popular mega cultivar of India also showed higher root biomass and longer root length although it did not posses the *PSTOL1* gene. On the other hand, Radhunipagol and Tulaipanji consisted the *PSTOL1* gene without detectable expression but showed P-deficiency tolerance ability clearly. DNA sequences of *PSTOL1* alleles from Gobindabhog, when compared, have

Table 1. Relative expression (RQ) of *PSTOL1* gene in root, P accumulation ability (mg/plant) and dry mass (mg/plant) in P sufficient (P+) and -deficient (P−) soil along with the amplification of whole *PSTOL1* gene and yield in deficient soil of nine genotypes.

	RQ of *PSTOL1*		P accumulation (mg/plant)		Dry mass of aerial parts (mg/plant)		Whole *PSTOL1*	Yield/sq m
Genotype	HP	LP	HP	LP	HP	LP	gene	(LP)
Binni	0.05	0.71	10.93 ± 2.12	9.36 ± 1.869	22.39 ± 3.16	17.97* ± 1.44	959 bp	490 ± 31.2
Gobindabhog	1	3.4	28.47 ± 2.19	27.72 ± 3.918	42.81 ± 12.16	40.49 ± 7.62	959 bp	612 ± 28.6
IR20	N	N	16.95 ± 0.98	3.68* ± 0 .45	22.83 ± 1.53	13.05* ± 0.64	Absent	457 ± 17.3
Palman	N	N	11.26 ± 2.11	7.01* ± 0.345	23.17 ± 2.83	17* ± 4.44	Absent	521 ± 16.3
Radhunipagol	N	N	22.44 ± 1.98	19.79 ± 5.273	31.7 ± 4.31	30.795 ± 3.68	959 bp	494 ± 18.1
Satabdi	N	N	13.38 ± 1.13	6.91* ± 1.22	21.22 ± 1.49	12.18* ± 2.07	Absent	392 ± 11.7
Swarna	N	N	21.06 ± 1.96	20.09 ± 4.852	23.06 ± 2.85	25.325 ± 1.44	Absent	589 ± 37.2
TN1	N	N	15.61 ± 2.13	9.58* ± 0.143	25.63 ± 2.53	13.16* ± 1.22	Absent	444 ± 19.3
Tulaipanji	N	N	25.29 ± 3.81	20.25 ± 7.053	32.35 ± 6.14	29.82* ± 2.54	959 bp	373 ± 31.3

*Significant differences at $P < 0.05$ compared with the high P solution by Students's *t* test. HP, high phosphate; LP, low phosphate; N, no expression.

Table 2. Dry shoot, dry root weights and root length of nine genotypes.

	Shoot biomass (mg)		Root biomass (mg)		Root length (cm)	
Genotype	HP	LP	HP	LP	HP	LP
Binni	21.4	18.27*	6.89	9.14*	8.3	11.1*
Gobindabhog	18.93	19.31	8.72	15.44*	7.6	10.4*
IR20	20.14	19.87	5.32	3.42*	8.7	8.2
Palman	21.65	16.65*	8.11	6.32*	9.3	12.1*
Radhunipagol	16.21	15.72	7.45	8.33	7.8	10.2*
Satabdi	22.2	23.6	10.15	6.62*	11.1	7.10*
Swarna	18.1	19.2	6.87	9.12*	9.4	8.5
TN1	17.8	17.2	9.67	9.42	11.2	7.2*
Tulaipanji	15.6	14.1	7.81	11.23*	11.3	12.1

Plants were grown in Yoshida solution for 14 days supplemented with high phosphate (HP) and low phosphate (LP), later, the plants were sampled for measurements. The mean values of three independent experiments using five seedlings were recorded. * Significant differences at $P < 0.05$ compared with the high P solution by Students's *t* test.

shown 100% similarity with the allele of Kasalath but significant nucleotide polymorphism was detected between Gobindabhog with Radhunipagol and Tulaipanji (figure 2 in electronic supplementary material).

Functional validation of PSTOL1 alleles

A recombinant inbred population comprising 180 lines, developed from Gobindabhog and Satabdi were grown both in P deficient and sufficient soil for assessing their P accumulation ability as well as to estimate filled grain per spikelet. Available P in depleted soil was only 3.36 mg/kg of soil and no external P fertilizer was applied during cultivation. Based on the filled-spikelet per panicle in P-depleted soil, 10 lines from two extreme groups were selected. Average filled spikelet per panicle in two extreme groups were 38.3 and 131.8, respectively; named as groups 1 and 2 (table 2 in electronic supplementary material). Mean chaffy grains of two groups were 71.2 and 23.8, respectively, whereas test weight of the seed was almost equal, 1.979 and 1.920 gm, respectively. As P concentration in shoot was not same for all the lines in P sufficient condition, absolute value of P concentration was not considered for comparison, instead, ratio of P accumulation in sufficient by deficient solution was considered for analysis. Independent lines of each group along with two parents were subjected to PCR amplification of *PSTOL1* gene by specific primer pairs. Seven lines carried the *PSTOL1* gene, six from group 1 and one from group 2. Rest of the lines, did not amplify anything i.e., they were of null alleles. P concentrations of two groups at P sufficient condition were almost same ($P = 0.99$) but when the same lines were grown on P-depleted soil, accumulation varied significantly ($P = 0.04$). On the other hand, genotyping of *PSTOL1* did not show the expected banding pattern with the members of two extreme groups which were categorized based on filled grains per panicle on P-depleted soil. Rather, six of 10 lines from group 1 (mean grain per panicle was 38.3) amplified the gene and only one line of group 2 amplified the same (mean grain per panicle was 131.8).

Discussion

The P level in 53 genotypes was higher in P-depleted soil and exhibited higher dry mass per plant and correlation between P accumulation and dry mass was almost 0.7 in this study, when calculated considering all the genotypes. It was not only due to their differential accumulation, but also difference in remobilization or internal use efficiency. A small aromatic Bengal landrace, Gobindabhog, was finalized as the best donor parent as it manifested higher P accumulation and yield both at P deficient and sufficient conditions. Genotypes like Tulaipanji, Radhunipagal and Raghusail are popular landraces adopted by the farmers of the red and lateritic zones before the popularization of semi-dwarf type cultivars, perhaps due to their P deficiency ability. P levels of HYV like IR36 and IR64 even at sufficient soil were medium, probably because of high internal P use efficiency or remobilization ability. Presence of *PSTOL1* gene or closely linked SSR and RM1261 was not associated with the P status of rice, when grown on PD soil. P level was poor (below the mean value) in more than 50% genotypes where *PSTOL1* gene was present. When single point ANOVA analysis was carried out among the *PSTOL1* containing null genotypes, mean P accumulation between two groups did not differ significantly. So, among the studied genotypes, *PSTOL1* amplification was not associated with P deficient tolerance. Another close marker, K46 as suggested by Chin *et al.* (2011) was actually designed from the region material which was denoted as *PSTOL1* later on. Thus, K46 was not considered for analysis again. Sequence-based markers surrounding the Pup1 locus, like, PupK52 and PupK42 were validated among the genotypes as described in table 1 in electronic supplementary material and showed no correlation with P deficiency tolerance. Additionally, one codominant

marker, PupK29 also did not exhibit any correlation with P deficient tolerance as described earlier (Sarkar *et al.* 2011). PupK29 showed expected co-dominant behaviour but fragment sizes were not same as mentioned by Chin *et al.* (2011). PD tolerance is a complex trait controlled by P-starvation-induced transcription factors followed by activation of high affinity transporter genes and/or modification of root architecture. Lack of association might be due to involvement of independent network other than *PSTOL1*. Thus, the presence of *PSTOL1* gene in rice genotypes does not assure PD tolerance. To check the expression pattern of *PSTOL1*, a set of nine genotypes were grown in hydroponics both at P deficient and sufficient conditions. Five genotypes manifested equivalent or higher root biomass in two-week-old seedlings in PD situation than those at control conditions. This was an indicator of PD tolerance since in depleted situations, plants translocate major amount of assimilates for maintaining higher root biomass (Jain *et al.* 2007) so that it can extract maximum amount of P from the media. During PD, plants sense changes in P availability and transmit the signals by short and long range pathways by controlling sugar, cytokinin, auxin, gibberellins and Fe (Wang *et al.* 2006; Jain *et al.* 2007; Jiang *et al.* 2007; Hammond and White 2011). Thus, augmentation of root biomass in P-limiting solution may be considered for screening criteria at the seedling stage. Root biomass of tolerant genotypes generally, increased due to retranslocation of phloem sucrose flux from shoots to roots as reported in *Arabidopsis* (Al-Ghazi *et al*. 2003) and beans (Ciereszko *et al.* 1996). Sucrose appears to have an effect on the expression level of several genes like high affinity P transporter (PHT1), PHO1 families, acid phosphatase, IPS1, etc. by P starvation (Ciereszko *et al.* 2005; Müller *et al.* 2007; Ribot *et al.* 2008; Fang *et al.* 2009). As a result, rice root undergoes several architectural changes. In this study, root length was elongated in deficient situation in three PD tolerant genotypes along with a nontolerant genotype, Palman. On the other hand, root length remained indifferent in Swarna and Tulaipanji P-limiting condition. So, in rice, screening PD lines based on root elongation or retardation was not recommended. In rice, retardation of root length or shoot biomass in P limiting situation was not a universal phenomenon as reported in *Arabidopsis* (Fang *et al.* 2009).

Of the four genotypes containing *PSTOL1* gene only two, Binni and Gobindabhog, manifested expressions in root. Genotypes with null *PSTOL1* allele, as expected, did not show any expression but surprisingly, Radhunipagol and Tulaipanji too did not exhibit expression. Allele of *PSTOL1* gene with different degrees of expression including lack of expression was possible. Sequence comparision of *PSTOL1* gene is shown in figure 2 in electronic supplementary material. *PSTOL1* gene of Gobindabhog showed 100% similarity with the published sequence of Kasalath (BAK26566.1). Two genotypes, especially, Radhunipagol and Tulaipanji, which did not express sufficiently, showed some dissimilarity in comparison to Gobindabhog or Kasalath. On the other hand, both genotypes, Radhunipagol and Tulaipanji, did not amplify the upstream regulatory regions by the same primers which had given positive results in Gobindabhog. Thus, the failure of gene expression in Radhunipagol and Tulaipanji might be due to modification in the upstream regulatory region. Comparison of sequences of the upstream regulatory elements between Gobindabhog and Kasalath gave more than 98% similarity (figure 3 in electronic supplementary material).

As the number of P deficient tolerance genes of rice become active during P starvation, the influence of the *PSTOL1* on a nontolerant cultivar was examined by analysing 10 lines from two extreme groups of a RIL population developed between Gobindabhog and Satabdi. Ratio of P accumulation in sufficient by deficient condition of seven lines carrying *PSTOL1* gene was almost 1.88 whereas the ratio was 1.16 for the rest of the lines with null alleles. The expectation was reverse. Thus, the presence of *PSTOL1* gene was not sufficient for higher P accumulation and vis-a-vis its manifestation towards higher grains per panicle in P-deficient soil. So, when targeting *PSTOL1* allele during marker-assisted selection, one must be assured of its role in P-deficiency tolerance ability in the recipient parents.

Acknowledgements

The authors sincerely acknowledge the Department of Biotechnology, Government of India for funding vide BT/20/NE/TBP/2010 dated 20.10.11. The authors also thank Soumya Bhattacharyya for his critical review of English.

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Received 7 November 2013; in final revised form 7 May 2014; accepted 14 May 2014
Unedited version published online: 24 June 2014
Final version published online: 15 October 2014