RESEARCH NOTE

Looking beyond *PsTOL1*: marker development for two novel rice genes showing differential expression in P deficient conditions

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Introduction

With the availability of the full genome sequence of rice, identification and localization of genes related to stress tolerance has become feasible. Using the rice genome information, better alleles of these genes can be identified in the germplasm, which will be useful for breeding. Insufficient plant-available soil phosphorus (P) is a major constraint for rice production and is apparent under conditions which are commonly characterized by infertile, highly acidic and *P* fixing soils. A few genes such as *PHR1*, *PHR2*, *OsPTF1*, *OsSPX1*, *OsSPX2*, *OsSPX3*, *OsIPS1* and *OsIPS2* (Hou *et al.* 2005; Wang *et al.* 2009) have been reported in P deficiency signalling, but whether they function similarly in different rice genotypes in response to low P is not clear. Only one major quantitative trait loci (QTL) phosphorus uptake1 (Pup1) (Wissuwa *et al.* 1998) has been identified in rice for better uptake of P under deficiency conditions explaining nearly 30% variation for P uptake and has now been narrowed down to gene level (*PsTOL1*) (Gamuyao *et al.* 2012). Molecular genetic understanding of P deficiency tolerance is so far restricted to two genes, i.e. *PsTOL1* and *PTF1*. Phosphorus deficiency tolerance being a complex quantitative trait, where P uptake is only one of the components, it is likely that there would be other molecular mechanisms, loci and genes that contribute to tolerance. Thus, there is a need to generate and evaluate novel molecular breeding resources to capture different molecular mechanisms for P deficiency tolerance. In this study, for the first time, we report the use of rice genotypes adapted to acidic soils of eastern and northeastern India for generating novel molecular tools in terms of characterized germplasm and gene-based markers. These resources will be helpful for understanding molecular mechanism underlying adaptability and performance under acidic soils.

Sahbhagi Dhan (*LR 23*) is a popular variety of eastern India while Chakhao Poreiton (*LR 26*) is a renowned landrace of Manipur in northeastern India. A previous study has identified *LR 23* as a potential donor for tolerance to low phosphorus (Tyagi *et al.* 2012). *LR 23* has Kasalath type allele for *PsTOL1,* while it is absent in *LR 26*. Based on transcriptome data generated on these two tolerant rice genotypes, *LR 23* and *LR 26* (Tyagi *et al.* 2013) with diverse mechanisms for tolerance to low P, functional validation has been attempted in this study with an objective of identifying novel genes from the root transcriptome data for tolerance to low P.

Materials and methods

Selection of candidate genes for study and primer design

Based on available transcriptome data (table 1) for *LR 23* and *LR 26*, six genes were selected to validate their functions under stress and control conditions. DNA was extracted from fresh leaves of rice genotypes by CTAB (cetyl-trimethyl ammonium bromide) method (Murray and Thompson 1980). The coding and the genomic sequences were obtained by searching the loci identity in Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu). The forward and reverse RT-PCR primers were designed from coding sequence to give amplicons of about 100–350 bp using free online software Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Online validation of the designed primers was done by local alignment against Gramene database, (http://www.gramene.org) using BLAST with near exact oligo matches option. Unique primers showing minimum number of possible hits across rice chromosome were selected and synthesized. Based on the differential expression, genes were selected for design of candidate gene-based primers. Candidate gene information was obtained from japonica sequence (NC_008394,

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Table 1. Details of six novel genes selected for validation in present study.

Loci (MSU ver. 6.1)	Chromosome no.	Gene name assigned	Genomic sequence length (bp)	CDS length (bp)	FPKM (gene level relative abundance in fragments per kilobase of exon model per million mapped fragments)
LOC_Os01g68300	1	*MO2*	609	276	12.56
LOC_Os02g29620	2	*MO5*	1672	546	19.31
LOC_Os03g28920	3	*MO7*	378	378	8.26
LOC_Os03g37864	3	*MO8*	895	267	33.44
LOC_Os04g27250	4	*MO9*	2963	429	9.29
LOC_Os06g08990	6	*MO10*	1104	1104	11.08

pseudomolecule 7, (http://rice.plantbiology.msu.edu/). Based on the sequences of candidate genes along with their upstream and downstream regions (1 kb each), two to six primer pairs were designed per gene ensuring the coverage of complete gene along with upstream and downstream regions (1 kb) using Primer 3.0 software (http://frodo.wi.mit.edu) (Sigma Aldrich, Bangalore, India).

Hydroponics experiment

Seeds were pregerminated on wet filter paper at 30°C for 3 days. For hydroponics experiment, 15 day old seedlings were divided into two groups. One group was grown in the normal culture solution with 10 mg inorganic phosphate per litre (P/L) (Yoshida *et al.* 1976) and another group was grown in P-deficient culture solution with 0.5 mg P/L. After 15 days of growth, plants were harvested as per Dohling (2011). During the entire experiment, the pH was maintained at 5.

RNA extraction; cDNA synthesis and RT–PCR

Root tissue from 30-day-old rice genotypes grown under control and treatment conditions were harvested and frozen in liquid nitrogen till further use. Total RNA was extracted from roots using Trizol reagent (Sigma, St Louis, USA) according to the manufacturer's protocol. The first strand cDNA synthesis was carried out using 5 μg RNA in a tube containing 1× RT buffer, 100 mM DTT, 0.8 mM dNTPs, 200 μM of oligo dT (D1806; Sigma Aldrich, USA) and incubated at 42°C for 10–15 min. Then, the enzyme reverse transcriptase (1 U) was added, and incubated at 42°C for 1 h. RNase H (1 unit) was added and incubated for 20–30 min at 37°C. The cDNA was subsequently used as a template for RT-PCR after 10× dilution. PCR reactions consisted of around 100 ng of cDNA, 0.2 U of *Taq* DNA polymerase (D1806; Sigma Aldrich, St Loius, USA), 1× PCR assay buffer with 1.5 mM $MgCl_2$, 12 ng each of forward and reverse primer and 200 μM of dNTP mix with a thermal cycling profile of initial denaturation at 94°C for 5 min followed by 35 cycles consisting of denaturation at 94°C for 40 s, annealing at 55–60°C for 45 s, extension at 72°C for 1 min and a final extension of 7 min at 72°C. Ubiquitin housekeeping gene was used as internal control. Scoring was carried out using 3–3.5% agarose gel (UltraPure Agarose 1000; Sigma,). The gel was stained with ethidium bromide (0.5 μg/mL) and visualized under UV light and the image was captured using Biorad gel documentation unit.

Table 2. List of primers with names, sequence and amplicon size used in the present study.

	Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
RT-PCR	*MO2*	GGCTCACTACCAGGAGGT	GGAACGTCTCCTTGACG	116
	MO5	GAGGACGGATTTTAGGGA	AGGGTTGTGCATTAGCTCT	73
	MO7	CGGATCCATCAGTCCTC	GAGAAGCTTGGGACGAA	176
	MO8	TAGCAACTGGAGGGGTG	ATCGCCGGATGAAGAG	98
	MO9	TCACATGCAACAAGGGA	CAACTTTGCGTGTTGGA	182
	MO10	ACGGGGTTGGTACTTATTG	ACACCAGTCAGGACAGAAAC	361
Candidate gene amplification	CAUCG-PR5-1	CCATTGACATGAGCATATGTAACTATT	TATACCTGAACGGCGGAATC	1045
	CAUCG-PR5-2	TCATTGGTACGTGCTTCGAG	AACGTGGACAAATGGCAAAT	984
	CAUCG-PR5-3	ATTTGCCATTTGTCCACGTT	GGAGCCTCTCATGCTGTTTC	1011
	CAUCG-PR5-4	CCACCACGTGTGAGAAAATG	GGAAAGACCCATGGACAGAA	982
	CAUCG-PR6-1	ATACGGAATCCACGCACACT	CCACCACAAGAGAAGCTTGG	992
	CAUCG-PR6-2	CCAAGCTTCTCCCACATTTC	GGAATTTGCCAAGAAATCACA	999
	CAUCG-PR6-3	GATTGATTTTGAGGGCCAAG	CTTACCATGTCGTCCCATGA	981

Results and discussion

Semi-qPCR validation

As roots are the main organs involved in sensing and responding to low P levels (Richardson 2009), hydroponics-based experiment was designed to find genes upregulated in low P in two different tolerant rice genotypes. The main aim of the experiment was to identify novel low P responsive genes which could be targeted for understanding tolerance mechanism to low P in rice. Based on the transcriptome data, six genes highly expressed in roots of *LR 26* (table 1) were selected for functional validation by RT-PCR (table 2).

From the RT-PCR data generated (figure 1A), it was observed that two genes (*MO2* and *MO10*) showed similar expression in both control and treatment conditions suggesting that these genes may not play a role in low P tolerance mechanism in the conditions tested in the present study.

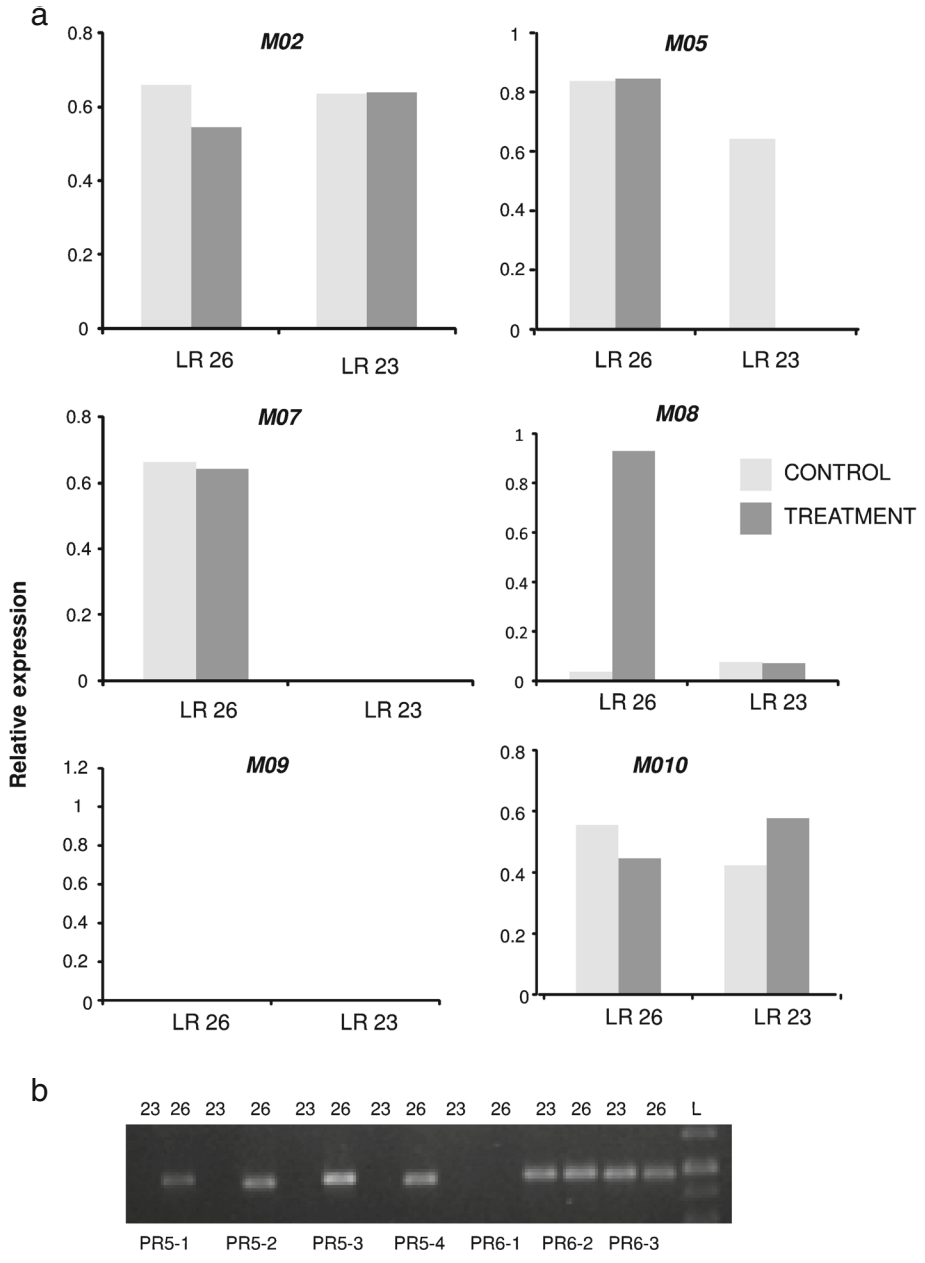

Figure 1. (a) Expression of *MO2*, *MO5*, *MO7*, *MO8*, *MO9* and *MO10* in root tissues of two rice genotypes namely *LR 26* and *LR 23* under P deficiency treatment. 30-day-old seedlings were grown in Yoshida solution with normal (10 mg P/L) or low (0.5 mg P/L) phosphorus. Total RNA from root tissues of hydroponically grown plants were used for semi-qRT-PCR. Values were standardized using ubiquitin gene in rice. Values show a representative result of three independent experiments. *MO10*, *MO9*, *MO8*, *MO7*, *MO5* and *MO2* are the novel genes corresponding to rice loci ID mentioned in table 1. (b) A representative gel picture showing validation of seven gene-based markers genomic DNA of genotypes *LR 23* (23) and *LR 26* (26). The name of the primer is indicated at the bottom of the gel.

Gene *MO8* was expressed to 30-fold higher level in *LR 26* roots under treatment conditions. Gene *MO9* did not show any expression in either LR 23 or LR 26. Gene *MO7* was expressed in both control and treatment only in *LR 26,* whereas gene *MO5* was expressed only in control conditions in *LR 23* and in both control and treatment in *LR 26*. These two genes namely *MO5* and *MO7* which show differential expression in low P conditions in genotype *LR 26* (lacking *Pup1* locus) indicate different ways of response to low P conditions. They were selected for further marker development. As the molecular genetics understanding of P deficiency tolerance is so far restricted to two genes, i.e. *PsTOL1* and *PTF1*, the selection of novel rice genes induced under low P levels will enable us to generate and evaluate novel molecular breeding resources. The two rice genotypes selected for RT-PCR experiment, namely *LR 23* and *LR 26* will help us in understanding the mechanism of tolerance to low P. As *LR 23* carries *Pup1* locus and *LR 26* does not (Tyagi *et al.* 2012), the RT-PCR data will enable us to identify the different sets of genes working in *Pup1* plus and minus background. Also, the expression data will help us to understand low P tolerant mechanisms across different tolerant genotypes. *MO5* and *MO7* appear to be involved in maintaining cellular homeostasis in LR 26, whereas these genes seem to be either down-regulated in low P conditions or not expressed at all in LR 23. With the availability of the rice genome sequence, candidate gene-based primers for genes *MO5* and *MO7* were developed and validated on the two genotypes. The primer design was done using bioinformatics software Primer3 with an expected amplicon size of 800 bp to 1.2 kb. For *MO5*, four markers (PR5-1, PR5-2, PR5-3 and PR5-4) were designed with the flanking sequences overlapping each other and for *MO7*, three markers (PR6-1, PR6-2, PR6-3) were designed (table 2).

Validation of gene-based markers

The seven gene-based markers were validated on both the rice genotypes *LR 23* and *LR 26*. The markers PR5-1, PR5-2, PR5-3 and PR5-4 were found to be dominant markers which amplified only in LR 26 (figure 1b). Primers designed targeting candidate gene *MO7* were either monomorphic (PR 6-2; PR 6-3) or did not work (PR6-1). The PR6-1 primer can be tested on Nipponbare as the gene sequences are derived from Nipponbare. The use of sequence of candidate genes (CG) from LOC regions and from Nipponbare sequence has resulted in four dominant polymorphic CG based primers for *MO5* (CAUCG-PR5-1, CAUCG-PR5-2, CAUCG-PR5-3 and CAUCG-PR5-4). This gene has been previously reported to be upregulated in roots of arsenic tolerant rice genotype Bala under hydroponics conditions (Norton *et al.* 2010), suggesting role of this gene in roots under adverse conditions. The functional role of this polymorphic candidate gene in P deficiency tolerance needs further validation. These markers when tested on *LR 11* (growing well in acidic soils of northeastern India but lacking the tightly-linked markers for *PUP1* locus) (Tyagi *et al.* 2012) also showed polymorphism with *LR 26*. These markers now need validation in a set of *PUP1* minus P deficiency tolerant lines. *In silico* analysis using coordinated expression search in TIGR database (http://rice.plantbiology.msu.edu/analyses_search_correlations_all_mod.shtml) revealed that *MO5*, *MO8* and *MO10* show coordinated gene expression (cut-off values between 0.5 and 1) in arsenic treated roots (Norton *et al.* 2010). Gramene tool for nucleotide variation (http://plants.ensembl.org/Oryza_sativa/Info/Index?db=otherfeatures) across ~400 *Oryza sativa* has revealed that though no sequence polymorphism is reported for *MO5* and *MO10* in database; two missense SNPs are reported for *MO8*.

In conclusion, two genes namely, *MO5* and *MO7* have been identified as showing differential response to low P condition across two distinct rice genotypes *LR 23* and *LR 26*. The markers for gene *MO5* were shown to be dominant; hence, they can be used for tagging the gene. For *MO7*, sequencing of the monomorphic bands needs to be done to rule out polymorphism due to SNPs or indels. The role of the two genes namely *MO5* and *MO7* in differentiating response of the two selected rice genotypes to low P needs to be targeted for future molecular biology studies.

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