Parental polymorphic survey between rice varieties (*Oryza sativa* L.) for phosphorus starvation tolerance gene by using SSR markers

Abstract: Insufficient soil phosphorus has been the most important factor affecting rice yield under the rice grown conditions. KMR-3R is a popular restorer line used for commercial rice hybrids production. For majority of the rice ecosystems, most of the rice hybrids released using three line systems were suited for normal phosphorus conditions only which exhibits severe yield reduction under low phosphorus conditions. To facilitate the efficient use of complex polygenic traits in hybrid rice, molecular breeding approaches were developed using KMR-3R BILs with *Pup1* QTL by introgression of major QTL *Pup1* controlling genes yield under low phosphorus stress. Parental polymorphic survey is a pre requisite before starting marker assisted backcrossing, it is used for introgression of a desirable traits from a donor genotype into a recipient genotype. In this context, I have screened all reported SSR primers linked to low phosphorus tolerance and observed K-46-1 and K-46-2 linked to *Pup1* QTL was polymorphic between Kasalath and KMR-3R. Simultaneously I have screened 300 full-genotype SSR markers covering entire genome and observed 86 SSR primers as polymorphic between Kasalath and KMR-3R. After polymorphic survey, I have evaluated Kasalath and KMR-3R.under low phosphorus stress and normal phosphorus conditions and recorded significant differences among the parents. Kasalath performed well when compared to KMR-3R for all the characters studied under low phosphorus stress conditions. With the generated genotypic data, a linkage map can be constructed which will further ensues the identification of a new markers linked to low phosphorus stress.

Keywords: Rice, KMR-3R, low phosphorus stress, Yield, BILs, Marker assisted backcross breeding.

INTRODUCTION

"Rice is one of the most important food crops for more than half of the world's population. Over 90% of global rice production is in Asia, and it contributes 20% of total caloric intake on a daily basis. The green revolution has revolutionized the world's rice production in the last six decades, and the production of rice is equivalent to the population growth in India". [19]

"The demand for rice is expected to reach 140 M T by 2050 with the present average annual population (1.5%) and average daily rice consumption (250 g) per person. India has to rise its rice production by 2.5 M T per year to meet the population demand 2050. Worldwide, the rice cultivation is affected due to the scarcity of natural resources like land, water, and nutrients coupled with climatic change"[15-18]. The development of rice cultivars tolerant to abiotic stress like low phosphorus stress is presently needed. As of now, *Pup1* QTL (Wissuwa*et al.* 1998) has been identified has major QTL controlling grain yield low phosphorus stress and the markers flanking/tightly linked/functional markers for these traits can be used to transfer into elite varieties through MAS. However, identification of background markers for each pair of donor and recipient parental varieties is very laborious and time consuming.

Hence, the current study was carriedout to identify ready to use SSR markers forbackground selection of two widely used donor and recipient varieties. One of the readily available and economicallyviable technological options for meeting theprojected global demand of rice is exploitationof heterosis through large-scale cultivation ofrice hybrids. "To increase the nation's rice yield, hybrid rice technology is essential.According to the draft proceedings of 58th annual rice group meetings of 2022-23, a total of 137 rice hybrids have been certified for commercial cultivation in India.103 million hectares of land is covered by hybrid rice in india in 2022. Majority of the rice hybrids were developed for irrigated conditions. Currently, three-line hybrid seed production using CMS system was widely used for exploitation of hybrid vigour in rice".[19]

"Phosphorus (P), one of the most important macronutrients necessary for healthy rice plant growth and development, is one of the most deficient elements in rice soils, and its low availability in the soil is one of the major yield-limiting factor in rice production" (Wissuwa*et al.*, 1999). "Global demand for P fertilizer is steadily rising, despite the fact that commercial phosphate supplies are expected to run out in a few decades. In India, soils are either low (49.3% of soils) or medium (48.8% of soils) in terms of available Pnecessitating the country's importation of phosphorus-based fertilizers on a massive scale"(Elser, 2012). "As a result, it is critical to be prepared with alternate solutions to this problem, such as improved crop residue management, adoption of integrated nutrient management, and development of low soil P tolerant rice cultivars to handle low soil P levels.Genetic enhancement of rice tolerance to P-limiting soils should be one of the focus areas of rice research and development in order to

reduce the use of phosphatic fertilizers, which is required not only to increase farmers' income by lowering fertilizer costs, but also to maintain rice production. Therefore, creating hybrids resistant to low phosphorus stress will aid in preventing losses in quantum yield.Though conventional plant breeding made significant impact on increasing the yield coupled with resistance to various biotic and abiotic stresses for target environments, these efforts are slow, need expertise labour and resource intensive".[19]

Before initiating marker assisted selection or marker assisted backcross breeding for transferring a desired trait into a variety from the donor parent, an analysis of parental polymorphism is required. Further selectionof plants possessing the traits of interest isnot achievable in the progenies unless theparents are polymorphic for the traits of interest.Polymorphism is an index of geneticvariation that shifts with different sets of parents.According to the survey of parentalpolymorphism, there is demonstrable variationbetween parents. Due to their many advantageous features, microsatellites (SSRs) have been used more than any other kind of molecular marker. These include having a large genomic distribution, being co-dominant and highly polymorphic, and being amplified by PCR. Through their ability to facilitate precise transfer of specific genomic regions, these markers have also demonstrated significant promise in enhancing breeding efficiency.

SSRs have been widely utilized in most crop species for which sequence information is available, including rice which have wider applications like gene tagging, genetic diversity research, population genotyping mapping, linkage map creation, tracking marker-trait associations, single marker analysis, and quantitative trait loci (QTL) mapping. Finding the relevant polymorphic SSR markers for the donor and recipient parents was the goal of the current investigation. After that, these markers were included to a backcross breeding experiment using polymorphic markers, which was used to introgresslow phosphorus deficiency tolerance QTLs from the donor parent Kasalath into the background of the extensively used restorer line of hybrid rice *i.e.*, KMR-3R.

MATERIALS AND METHODS

The details of the parents used for the study were presented below.

1. **Kasalath**: Kasalath is a low P tolerant *indica* ricegenotype harboring major effect QTL *Pup1* controlling grain yield under phosphorus stress.

2. **KMR-3R**: KMR-3R is a restorer line of the popular hybrid KRH2**.**

Leaf sample collection and DNA isolation

Total genomic DNA was isolated from the parental linesusing the method of Zheng e (1991) with modifications.Healthy leaf pieces (2-3 cm) from the youngest leaves of a 20-25 day old rice seedlings were taken into the well of a spot testplate (M/s Thomas Scientific, USA). 400 ml of extractionbuffer (50 mM Tris HCL, pH 8.0, 25 mM EDTA, 300 mM NaCland 1 % SLS) was added to the well. The leaf tissues weremacerated for about 15-20 seconds till it was completelyhomogenized. Another 400 µl of extraction buffer was addedto the well containing the leaf sample. Using a micropipette of1 ml capacity, the entire content from the well was transferredto a 1.5 ml capacity micro centrifuge tube. About 400-500 µlPhenol (pH 8.0) : Chloroform : Isoamyl alcohol (25:24:1) wasthen added to the micro centrifuge tube. The contents weremixed well by inversion for about 10 minutes and centrifugedat 12000 rpm for about 10 minutes at room temperature. Afterthe centrifugation, the supernatant was aliquoted from themicro-centrifuge tube without disturbing the intermediatelayer into a fresh 1.5 ml micro-centrifuge tube. To the clearsupernatant, 5-10 µl of RNAase (10 mg per ml) was addedand incubated for 45-60 minutes at room temperature (~ 37^0C). After completion of the incubation period, about 500 µl ofChloroform: Isoamyl alcohol (24:1) was added to the micro-centrifuge tube and mixed well by inversion for 10 minutes.The content was centrifuged at 12000 rpm for 10 minutes andthe supernatant was collected into another fresh 1.5 ml micro-centrifuge tube without disturbing the intermediate layer. Tothe clear supernatant equal volume (~500-600 ml) of chilledisopropanol was added. The contents were mixed gently andcentrifuged at 12000 rpm for 10 minutes at room temperature.The supernatant was drained gently without disturbing theDNA pellet. About 150-200 µl of 70 % ethanol was added tothe pellet at the bottom of the micro-centrifuge tube. It wasensured that the pellet was completely immersed in 70%alcohol. Then the content was centrifuged at 5000 rpm for 5minutes at room temperature. After centrifugation, thesupernatant was drained and 70% alcohol wash wasrepeated once again. Finally, after centrifugation and drainingout the supernatant, the pellet was left overnight air drying atroom temperature. After complete drying of the pellet,depending upon the size of the pellet, about 50-100 µlofsterile distilled water was added for dissolving the pellet.About 3 µl of the DNA sample was taken and loaded in 0.8 %ethidium bromide stained agarose-TBE

(concentration ofTBE) gel, electrophoresed for about an hour and the bands ofgenomic DNA were visualized and documented in a UV geldocumentation system (Syngene).

Polymerase Chain Reaction (PCR) using SSR markers

The genomic DNA of parental lines subjected to PCRamplification as per the procedure described by Chen (1997). .PCR amplification was carried out in a thermocycler usingthe temperature profile, followed by rapid cooling to 4°C prior to analysis. For electrophoretic studies, The SSR-PCR products were resolved in a 3%Agarose gel prepared in 0.5 X TBE buffer stained with Ethidium Bromide. The gel was run ata constant voltage of 90 V for 1 h and visualized under UV light and documented using gel documentation system. The temperature specifications for the denaturation of DNA strands, Annealing of primers and extension steps are as following .

List 1. Temperature specifications for the denaturation of DNA strands

S.No.	Steps	Temperature	Time
1	Initial denaturation	94^0C	5 min
2	Denaturation	94^0C	30 sec
3	Annealing	55^0C	1 min
4	Extension	72^0C	1 min
5	Final extension	72^0C	7 min
6	Cooling	4^0C	A

Agarose gel electrophoresis and image visualization

PCR products were analyzed by electrophoresis using a 3.0% agarose gel. About 9.0 g of agarose was weighed and applied to a conical flask and 400 ml of 1X TAE buffer was added and mixed well. The ingredients were boiled slowly in the microwave by stirring occasionally. Complete melting of the agarose and a clear solution were maintained during the process. To clean the gel cast plate, it was soaked in water and then wiped with ethanol. After cooling the agarose to room temperature, two milliliters of ethidium bromide (10 mg/ml) was added to the melt and the mixture was then poured onto a gel casting tray with the necessary gel combs and allowed to settle at 20°Cfor 30 minutes.The gel was transferred

to an electrophoresis apparatus containing 1X TAE buffer. Before loading, PCR-amplified products were mixed with 1/6 volumes of gel loading dye (ethidium bromide) and loaded into wells. A 100 bp DNA ladder was added to one well to determine the size of the amplified fragments. Ethidium bromide-stained gels were documented in a UV gel documentation system (Syngene) and the subsequent banding pattern was evaluated.

Statistical Analysis

The percentage of polymorphism was calculated by using the formula:

$$\text{Polymorphism (\%)} = \frac{\text{Number of molecular markers showing polymorphism}}{\text{Total number of molecular markers run}} \text{ X } 100$$

Graphical representation of the genome

Graphical representation of molecular marker data was performed using Graphical Genotype (GGT2.0) software.

Phenotypic evaluation of KMR-3R and Kasalathin field under normal phosphorus and low phosphorus stress conditions

In normal phosphorus experiment, regular irrigation and flooded conditions were maintained until maturity of the crop and soil was maintained with $P > 18.3$ ppm. In low phosphorus experiment,regular irrigation and flooded conditions were maintained until maturity of the crop and soil was maintained with $P < 2$ ppm. The crop was harvested when the grains reached physiological maturity.Low 'P' level in the field was maintained without external application of P fertilizers. The levels of phosphorus in the field were estimated from the collected soil sample in each season through Olsen P method before taking up of planting of experimental breeding material in the field plot.The differences between the parents on yield and low phosphorus stress tolerant traits like leaf P content and seed P content were tested using paired t-test.

Results and Discussion

Genotypic polymorphism by molecular markers

The presence of two or more discontinuousgenotypes or alleles on a specific locus in agiven population is referred to as geneticpolymorphism. In the present study, clear polymorphism was observed between the parents both for full genome coverage markers and drought tolerant markers. A total of 300full genome coverage SSRmarkers (forward and reverse) and 10 phosphorus uptake yield QTL markers reported by Chin *et al.* (2010) were analyzed for molecular polymorphism.Polymerase Chain Reaction was done to check eachsample for parental polymorphism using specific Rice Microsatellite (RM) primers. Out of 300full genome coverage primers used, the parents showed polymorphic only to 86 RM primers listed in Table 1 whereas K-46-1 and K-46-2 markers linked to *Pup1* QTL listed in Table 2 were polymorphic between the parents for low phosphorus deficiency tolerant markers. These are used in foreground selection. K-46-1 and K-46-2 markers are dominant markers. They will show only the presence or absence of *Pup1* QTL. This type of parental polymorphic survey was done by previous workers *i.e.,*Collard *et al.* (2006), Chin*et al.* (2010), Sarkar*et al.* (2011), Tyagi*et al.* (2012), Aluwihare*et al.* (2014), Goncharova and Kharitonov(2016), Neelam *et al.* (2017), Zhang *et al.* (2018) and Lapitan and Mercado (2023), before starting marker assisted backcross breeding for introgression of desired yield controlling *qDTYs* under moisture stresses in rice. Chromosome wise distribution of polymorphic markers used in this study was presented in the Table 3. Highest polymorphic percent was displayed on 1 chromosomeand least was displayed on 8 chromosomes respectively. The average polymorphism percent on all the chromosomes was observed as 28.74.The lack ofdetectable polymorphism between two parents could be due to the fact that both parents, Kasalath and KMR-3Rare*indica* lines. Lack of molecular marker polymorphismbetween indica genotypes has been earlier noticed in studiesby Xu *et al.*(2002) and Biradar *et al.*(2004). In the present study dinucleotide repeats in the microsatellite region showed more polymorphism than tri and tetra nucleotide repeats. It may be due to the abudance of dinucleotide repeats distributed throughout the genome of rice. The prevalence of dinucleotide repeats in parental polymorphism was reported by previous workers. The usage of molecular marker technology in breedingprograms has greatly increased its efficiency and fastens the transfer of desirable genes among varieties and to introgressnovel genes from related wild species. Polygenic markerswhich were previously difficult to analyze using traditional breeding methods, would now be easily tagged using molecular markers. The screening of markers for parental polymorphism among the rice cultivars forms the basis for tagging of the desired gene,

fine mapping of the gene in the rice chromosome and in the subsequent Marker Assisted Selection (MAS) programmes.

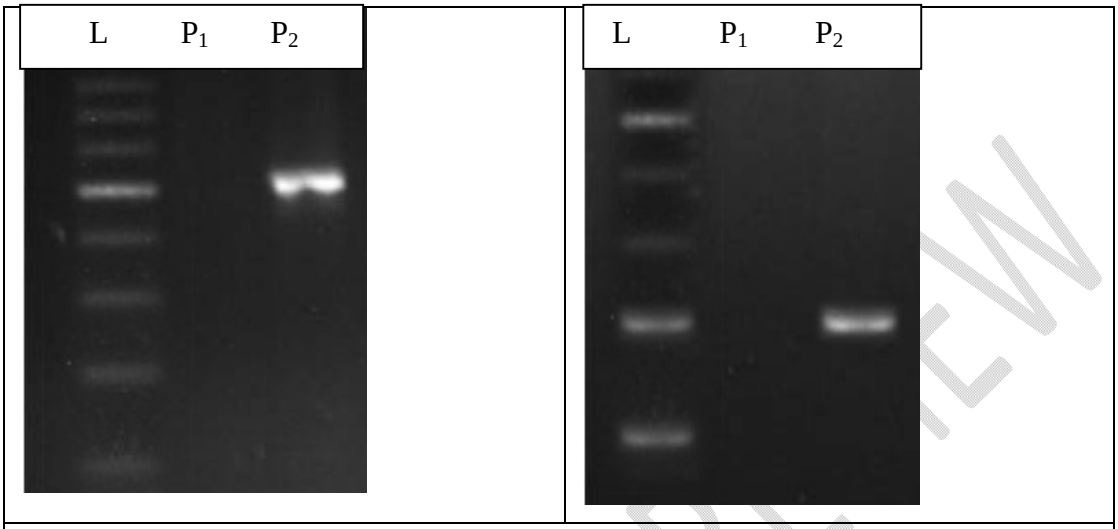

Fig 1 : Parental polymorphism shown by K-46-1 and K-46-2 for *Pup1 QTL*

L : Ladder (100bp) , P_1: KMR-3R, P_2: Kasalath

Phenotypic polymorphism by morphological traits under normal phosphorus and low phosphorus stress conditions

KMR-3R and Kasalath were assessed for grain yield and its attributing traits under low phosphorus stress and normal phosphorus conditions (Table 4). KMR-3R recorded significant differences for all traits studied between normal phosphorus and low phosphorus stress conditions. Similarly, Kasalath recorded significant difference for all the traits studied except for grain yield per plant between normal phosphorus and low phosphorus stress conditions. These findings are used for development of low P tolerant backcross inbred lines of KMR-3R by marker assisted backcross breeding.

CONCLUSION

One of the primary nutrients for plants, phosphorus is essential for raising rice yields. Among all the necessary nutrients in soil, it is one among the least prevalent. P is a limited resource, hence the current state of both high- and low-input farming systems in India is unsustainable. It is challenging to identify mild to moderate P deficiencies in the field. Thus, rice

with low phosphorus content is referred to as having concealed hunger. This issue can be resolved by using fertilizer containing phosphorus. However, it is challenging for India's resource-poor farmers to apply phosphorus fertilizer annually. Therefore selection of rice cultivars capable of extracting phosphorus from phosphorus limiting soils with higher phosphorus use efficiency is considered as a significant cost-effective solution.

Significant genetic variation has been reported in rice for tolerance to soil phosphorus deficiency like leaf phosphorus content and seed phosphorus content (Fageria and Baligar, 1997). A large effect QTL known as *Pup1* controlling grain yield under phosphorusdeficiency has been mapped on chromosome12 of Kasalath rice cultivar (Wissuwa*et al.*, 1998). Marker Assisted Selection (MAS) programmes rely on the identification of parental polymorphism in rice cultivars through the screening of markers. This allows for the subsequent tagging and fine mapping of the target gene within the rice chromosome. Polymorphic markers discovered in this work were used to genotype the entire mapping population and facilitated the introgression of *Pup1* QTL involved in grain production under low phosphorus stress into KMR-3R. This study helped in the development of backcross inbred lines of KMR-3R by introgression of *Pup1* from Kasalath into KMR-3R by marker assisted backcross breeding. These BILs were utilized in development of hybrids tolerant to low phosphorus stress.

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Table 1 Microsatellite markers exhibiting polymorphismbetween KMR-3R and Kasalath

1	RM 8145	21	RM545	41	RM274	61	RM72	81	RM27153
2	RM 10694	22	RM570	42	RM485	62	RM23869	82	RM27953
3	RM140	23	RM14659	43	RM586	63	RM3769	83	RM512
4	RM562	24	RM15081	44	RM3414	64	RM2479	84	RM28166
5	RM11074	25	RM15087	45	RM549	65	RM24832	85	RM28199
6	RM543	26	RM168	46	RM20224	66	RM24330	86	RM484
7	RM486	27	RM16153	47	RM537	67	RM24350		
8	RM472	28	RM16217	48	RM5509	68	RM201		
9	RM12023	29	RM551	49	RM340	69	RM105		
10	RM12146	30	RM16368	50	RM21330	70	RM25282		
11	RM12233	31	RM16655	51	RM21427	71	RM1083		
12	RM8051	32	RM5757	52	RM21500	72	RM1375		
13	RM312	33	RM255	53	RM21700	73	RM6132		
14	RM10794	34	RM609	54	RM21900	74	RM568		
15	RM12702	35	RM507	55	RM234	75	RM26022		
16	RM6374	36	RM413	56	RM1306	76	RM26112		
17	RM6318	37	RM405	57	RM2420	77	RM3863		
18	RM3212	38	RM334	58	RM331	78	RM209		
19	RM205	39	RM5401	59	RM256	79	RM27100		
20	RM262	40	RM3321	60	RM126	80	RM2136		

Table 2 Details of polymorphic primer used for foreground selection of *Pup1* QTL

QTL	Chromosome	Position	Primer
PUP1	12	523 bp	K-46-1
PUP1	12	227 bp	K-46-2

Table 3 Chromosome wise polymorphic primers between KMR-3R and Kasalath

Chromosome	No. of SSR markers used	No. of polymorphic markers on each chromosome	No. of monomorphic markers on each chromosome	Percentage of primers showing polymorphism on each chromosome
1	35	14	21	40.00
2	30	6	24	20.00
3	28	9	19	32.14
4	25	5	20	20.00
5	25	7	18	28.00
6	28	8	20	28.57
7	25	7	18	28.00
8	27	5	22	18.52
9	20	8	12	40.00
10	19	5	14	26.32
11	20	6	14	30.00
12	18	6	12	33.33

Table 4 Comparison of KMR-3R and Kasalath for grain yield and its attributes under normal P and low P stress conditions

S.No.	Trait	KMR-3R			Kasalath			S.E. of difference
		Normal P (N.P.) (n=5)	Low P stress (L.P.) (n=5)	Mean difference (N.P-L.P)	Normal P (N.P.) (n=5)	Low P stress (L.P.) (n=5)	Mean difference (N.P-L.P)	
1	Plant height (cm)	115.12	105.12	10.00**	94.24	83.19	11.05**	1.16
2	Days to 50% flowering	103.00	110.00	-7.00**	85.00	90.00	-5.00**	0.64
3	Days to maturity	128.00	130.00	-2.00**	115.00	120.00	-5.00**	0.58
4	No. of tillers per plant	15.00	8.00	7.00**	16.00	10.00	6.00**	0.37
5	No. of panicles per plant	12.00	7.00	5.00**	14.00	8.00	6.00**	0.35
6	Panicle length (cm)	25.10	19.92	5.18**	23.62	21.24	2.38**	0.64
7	1000 grain weight (g)	24.12	20.21	3.91**	30.56	21.90	8.66**	0.55
8	No. of filled grains per panicle	150.00	74.00	76.00**	135.00	98.00	37.00**	1.24
9	No. of unfilled grains per panicle	8.00	58.00	-50.00**	35.00	48.00	-13.00**	0.96
10	Spikelet fertility (%)	94.93	56.06	38.87**	79.41	65.77	13.64**	0.60
11	Grain yield per plant (g)	24.24	12.41	11.83**	19.12	18.04	1.08	0.71
12	Biological yield per plant (g)	60.14	50.14	10.00**	70.21	65.21	5.00**	0.98
13	Harvest index (%)	40.30	24.75	15.55**	27.23	27.66	-0.43**	1.25
14	Leaf phosphorus content (%)	0.50	0.30	0.20**	0.90	0.80	0.10**	0.03
15	Seed phosphorus content (%)	1.20	1.00	0.20**	1.60	1.50	0.10**	0.03

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