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Simple sequence repeats (SSR) polymorphism survey between Co51 and Ptb33 for marker assisted backcross breeding to introgress multiple stress resistance in Rice (*Oryza sativa* L.)

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Abstract
Simple sequence repeats (SSR) polymorphism in two rice varieties *viz*., Co51(High yielding, short duration, fine grain variety but susceptible to Brown Plant Hopper) was pyramided with bacterial blight resistance genes *xa5*, *xa13* and *Xa21*, blast resistance gene *Pi54* and sheath blight resistance QTLs *qSBR7-1*, *qSBR11-1* and *qSBR11-2* and Ptb33 (BPH resistant line harboring *Bph2* and *Bph32*) was investigated to identify the ready to use SSR markers which can be employed in background selection of marker assisted back cross breeding programme for the introgression of brown plant hopper resistance. A total of 120 genome wide SSR primers were utilized for this parental polymorphism survey equally distributed on all the 12 chromosomes of rice. The results of this survey revealed distinct polymorphism among Co51 and Ptb33 for 51 genome wide SSR markers with a polymorphism information content (PIC) of about 0.5 and polymorphism percentage of about 42.5%. These identified polymorphic SSR markers can be used in crop improvement programs involving Co51 and Ptb33 aiding in marker assisted back ground selection in recombinants there by developing a desirable cultivar resistant to multiple stress.

Keywords: Marker assisted backcross breeding, background selection, simple sequence repeats polymorphism, multiple stress, rice

Introduction
Rice (*Oryza sativa* L.) is an oldest domesticated crop and has wider adaptability for varied climatic conditions, ranges from tropics to sub-tropics. It is the stable food crop for half billion population in world and providing main source of calories for people in urban and rural areas. In India, rice is mainly cultivated as irrigated and rainfed crop with wide coverage of 16 million ha. World population needs about 135 to 140 million tons of rice in 2030 to maintain its self-sufficiency (IRRI vision 2050) [6]. India is the second largest producer and consumer after China and it accounts for 21 per cent of the world's total rice production and 76% of calorically uptake. Rice production is affected by various biotic stress and abiotic stress. Therefore there is a need to develop improved rice varieties resistant to biotic and abiotic stress.

Rice brown plant hopper (BPH) *Nilaparvata lugens* Stal is a sap sucking pest which is highly destructive in rice causing yellowing, browning and drying of plants ultimately leading to the typical symptom "Hopper burn". Marker assisted pyramiding of R genes against BPH has been proved to be extraordinarily effective against BPH (Jena *et al*., 2017) [7]. Bacterial blight caused by *Xanthomonas oryzae pv. oryzae* is one of the major destructive diseases of rice, causing substantial yield loss in many rice growing areas of the world. Rice blast (*Magnaporthe oryzae*) and rice sheath blight (*Rhizoctonia solani)* were the most devasting diseases of rice growing areas around the world. It causes loss to grain yield up to 90 percent (Thakur *et al*., 2015) [13]. Marker assisted backcross breeding is a very successful strategy to introgress resistance genes from the donor parent to the recurrent parent. Marker assisted gene pyramiding is proved to be very effective against multiple biotic stress in rice (Ramalingam *et al.*, 2020) [9]. Marker assisted background selection is employed to select the recombinant

plants with high recurrent parent genome (RPG) recovery. Simple sequence repeats (SSRs), often called microsatellites, are DNA segments containing a 1–6 nucleotide tandem repeat motif. SSR markers provide a number of appealing characteristics, including genome-wide coverage, good repeatability, co-dominant inheritance, and high polymorphism with several alleles per locus and transferability between species. Fingerprinting, background genome recovery study, genetic diversity research, population structure analysis, association mapping, and linkage mapping have all used SSR markers (Singh *et al.*, 2010) [12].

Marker-assisted backcross breeding (MABB) approach recovers 99% of recurrent parent genome within three backcrosses as compared to conventional backcross; it takes six generations to recover. Background selection is usually referred to in the context of trait introgression. The process of integrating a trait from one species into another, is a common strategy used for crop improvement. Selection against background genomic regions can be greatly facilitated by knowledge of molecular markers. When introgressing desired traits from wild species, background selection must often be applied to help recover the desired background genome (Hasan *et al.*, 2015) [5]. Selection based on molecular marker data and graphical genotypes can be useful to accelerate the background genome recovery process. In the present study genome wide marker survey was carried out using a very popular high yielding variety of India, Co51 and donor Ptb33 which might be helpful to select desired background genome in rice

Plant materials

Co51 is a short duration, high yielding and fine grain variety released from Tamil Nadu Agricultural University, Coimbatore. It is very popular in India but susceptible to brown plant hopper, bacterial blight, blast and sheath blight attack. Ptb33, a variety native to Kerala is hardy variety which exhibits a higher level of resistance to BPH were used in this study to identify the polymorphic genome wide SSR markers to be used in Marker Assisted Backcross Breeding (MABB) for the purpose of background selection.

Genome wide markers

One hundred and twenty random genome wide simple sequence repeat (SSR) primers were selected for this study from Gramene marker view (https://archive.gramene.org/markers/). The markers were chosen in such a way that 10 each of the 120 selected primers represent all the 12 chromosomes in rice giving an overall representation of the genome (Table. 1.).

Table 1: List of SSR primers used for polymorphism survey

Linkage group	Simple Sequence Repeat (SSR) primer
1	RM 490, RM 493, RM 495, RM 576, RM 3403, RM 3412, RM 6515, RM 8051, RM 8070, RM 8077
2	RM 6, RM 145, RM 262, RM 290, RM 482, RM 1081, RM 1313, RM 2792, RM 3212, RM 3316
3	RM 16, RM 251, RM 514, RM 520, RM 545, RM 1038, RM 1278, RM 6283, RM 6594RM 7565
4	RM 124, RM 142, RM 252, RM 255, RM 280, RM 307, RM 451, RM 1153, RM 5633, RM16337
5	RM 13, RM 31, RM 146, RM 161, RM 163, RM 164, RM 178, RM 430, RM 440, RM 4837
6	RM 162, RM 276, RM 345, RM 469, RM 585, RM 587, RM 597, RM 3805, RM 7488, RM 19388
7	RM 11, RM 125, RM 214, RM 234, RM 336, RM 436, RM 445, RM 1243, RM 2006, RM 3718
8	RM 44, RM 152, RM 223, RM 310, RM 339, RM 404, RM 408, RM 544, RM 6863, RM 6999
9	RM 242, RM 285, RM 1026, RM 1328, RM 3025, RM 6867, RM 7481, RM 23858, RM 24325, RM 24390
10	RM 222, RM 228, RM 3019, RM 3123, RM 3510, RM 3773, RM 5629, RM 5708, RM 6477, RM 25439
11	RM 181, RM 202, RM 287, RM 1233, RM 1240, RM 2738, RM 3717, RM 4844, RM 6115, RM 7120
12	RM 17, RM 19, RM 20, RM 179, RM 247, RM 309, RM 1302, RM 3813, RM 5196, RM 28048

Methods

Both the varieties Co51 and Ptb33 were raised in pots and genomic DNA is extracted by using the modified CTAB method (Dellaporta *et al.*, 1983) [4]. Young leaves from 20 days old plant were selected for the extraction of genomic DNA. One gram of fresh leaves were homogenized in CTAB buffer (1.5% CTAB, 0.2M Tris HCl, 0.02M EDTA, 1.4M NaCl), consequent steps of modified CTAB method is followed to extract DNA and the quality and quantity of the extracted DNA was judged by agarose gel electrophoresis and the purity and quantity was checked by using Genova Jenway Nanodrop spectrophotometer. The DNA with desirable quantity and purity is selected and diluted to a concentration of 100 ng/µl desirable for polymerase chain reaction. A total of 120 SSR markers were used. The information regarding the chromosomal location, sequence of the primers, repeat motifs and size of the expected product (bp) were obtained from (https://archive.gramene.org/markers/).

The Polymerase Chain Reaction (PCR) was carried out in Eppendorf thermo cycler using 120 SSR markers. The PCR reaction of 10µl was set up using 1µl template DNA (100 ng/µl concentration), 0.5µl of forward primer, 0.5µl of reverse primer, 4µl of 2 x Emerald Takara master mix and 4µl of nuclease free water. The PCR cocktail is made and the cycler is run in a programme included i) Initial denaturaion at 94 ^{0}C for 5 min, ii) denaturation 94 ^{0}C for 1 min, iii) primer annealing at 55 ^{0}C for 1 min, iv) extension 72 ^{0}C for 1 min, v) final extension 72 ^{0}C for 7 min and vi) hold at 4 ^{0}C. Steps from ii) to iv) were repeated for 35 cycles for amplification if the template DNA. After the completion of amplification the PCR products were visualized by agarose gel electrophoresis. The amplified PCR products were separated by using 2.5% agarose gel. 5µl of amplified PCR product was loaded in the solidified agarose gel well along with 2µl of 100 bp ladder and ran for 1.30 hrs at 100v and then visualized under the UV light in gel documentation unit (Bio Rad Gel Doc EZ Imager) for visual scoring of amplified bands. The banding pattern was visualized on the gel documentation unit under the UV light. The identified banding pattern was scored according to the banding pattern of 100 bp ladder.

Quantitatively, the polymorphism degree was measured by Polymorphism Information Content (PIC) for co dominant markers (Botstein *et al.*, 1980) [2] and was measured using the following equation

$$PIC_j = 1-\sum_{i=1}^{n} P_i^2$$

Where i is i-th allele of the j-th marker, n is the number of the j-th marker's alleles, P is allele frequency. Parental polymorphism percentage is calculated by

$$\text{Parental polymorphism percentage} = \frac{\text{Number of Polymorphic SSR markers used}}{\text{Total number of SSR markers used}} /$$

Results and Discussion

The purity and concentration of DNA of both the varieties was measured by using Geneva Jenway Nanodrop spectrophotometer, the absorbance ratio at OD260/OD280 was 1.80 for Co51 and 1.82 for Ptb33 respectively and the quantity of DNA in these samples was 2350 ng/µl for Co51 and 1860 ng/µl for Ptb33. Lucena-Aguilar *et al.*, (2016) [8] stated that the absorbance ratio (OD260/OD280) for pure DNA is 1.8 and the extracted genomic DNA from the two varieties were on par with their results.

PCR was performed with SSR markers using the template DNA from Co51 and Ptb33, the parental lines to be used in MABB. Parental polymorphism at molecular level was identified by genotyping them with SSR markers. The selected 120 rice microsatellites markers distributed throughout the genome over twelve chromosomes of rice. In the parental polymorphism study, the number of bands in the different RM markers of the two parents ranged from one (RM 514) to three (RM 262). The sizes of the amplicons resolved among the RM markers were found to be in the range of 93bp (RM 28048) to 259bp (RM 514). Out of 120 RM markers used, 51 markers were observed to be polymorphic (Table.2.).

Table 2: List of Polymorphic genome wide SSR markers between Co51 and Ptb33

S. No	SSR Marker	Repeat Motif	Primer Sequence (5'-3')	Product Size(bp)	Chromosome Number
1	RM 490	$(CT)^{13}$	F - ATCTGCACACTGCAAACACC	101	Ch.1
			R - AGCAAGCAGTGCTTTCAGAG		
2	RM 493	$(CTT)^{9}$	F - TAGCTCCAACAGGATCGACC	211	Ch.1
			R - GTACGTAAACGCGGAAGGTG		
3	RM 495	$(CTG)^{7}$	F - AATCCAAGGTGCAGAGATGG	159	Ch.1
			R - CAACGATGACGAACACAACC		
4	RM 3412	$(CT)^{17}$	F - AAAGCAGGTTTTCCTCCTCC	211	Ch.1
			R - CCCATGTGCAATGTGTCTTC		
5	RM 8051	$(TC)^{19}$	F - CGCGGTTAATGTCATCTGA	153	Ch.1
			R - CAAGACTGACCCTAAAACCATAC		
6	RM 8070	$(CTT)^{19}$	F - AAATGGACTCGCTCCTAAAC	217	Ch.1
			R - AGGAGCGAATTTTATTGCTACT		
7	RM 6	$(AG)^{16}$	F - GTCCCCTCCACCCAATTC	163	Ch.2
			R - TCGTCTACTGTTGGCTGCAC		
8	RM 262	$(CT)^{16}$	F - CATTCCGTCTCGGCTCAACT	154	Ch.2
			R - CAGAGCAAGGTGGCTTGC		
9	RM 2792	$(AT)^{35}$	F - GAAAATAAGTTCAGGGAGAA	163	Ch.2
			R - AGAATTGTCCCACGTATACT		
10	RM 3212	$(CT)^{12}$	F - AGACGACAAACACCTGCCTC	181	Ch.2
			R - CAAACACAAACGCAGCCTC		
11	RM 3316	$(CT)^{14}$	F - TTCGACGATTCTGTACACGC	207	Ch.2
			R - CATGATCCCAAATGCATGGG		
12	RM 251	$(CT)^{29}$	F - GAATGGCAATGGCGCTAG	147	Ch.3
			R - ATGCGGTTCAAGATTCGAT		
13	RM 514	$(AC)^{12}$	F - AGATTGATCTCCCATTCCCC	259	Ch.3
			R - CACGAGCATATTACTAGTGG		
14	RM 520	$(AG)^{10}$	F - AGGAGCAAGAAAAGTTCCCC	247	Ch.3
			R - GCCAATGTGTGACGCAATAG		
15	RM 545	$(GA)^{30}$	F - CAATGGCAGAGACCCAAAAG	226	Ch.3
			R - CTGGCATGTAACGACAGTGG		
16	RM 6283	$(CTG)^{8}$	F - TGGAGACTGAGCTGATGCC	93	Ch.3
			R - TCAGGTGGTCGGTTCCTTAC		
17	RM 127	$(AGG)^{8}$	F - GTGGGATAGCTGCGTCGCGTCG	223	Ch.4
			R - AGGCCAGGGTGTTGGCATGCTG		
18	RM 142	$(CGG)^{7}$	F - CTCGCTATCGCCATCGCCATCG	240	Ch.4
			R - TCGAGCCATCGCTGGATGGAGG		
19	RM 252	$(CT)^{19}$	F - TTCGCTGACGTGATAGGTTG	216	Ch.4
			R - ATGACTTGATCCCGAGAACG		
20	RM 307	$(AT)^{14}$ $(GT)^{21}$	F - GTACTACCGACCTACCGTTCAC	174	Ch.4
			R - CTGCTATGCATGAACTGCTC		
21	RM 13	$(GA)^{6}$-$(GA)^{16}$	F - TCCAACATGGCAAGAGAGAG	141	Ch.5
			R - GGTGGCATTCGATTCCAG		
22	RM 163	$(GGAGA)^{14}$ $(GA)^{11}$ C $(GA)^{20}$	F - ATCCATGTGCGCCTTTATGAGGA	124	Ch.5
			R - CGCTACCTCCTTCACTTACTAGT		

23	RM 164	$(GT)^{16}$ TT $(GT)^{4}$	F - TCTTGCCCGTCACTGCAGATATCC R - GCAGCCCTAATGCTACAATTCTTC	246	Ch.5
24	RM 440	$(CTT)^{22}$	F - CATGCAACAACGTCACCTC R - ATGGTTGGTAGGCACCAAAG	169	Ch.5
25	RM 4837	$(TA)^{28}$	F - TACAACTGTAACGTTGGAGA R - GTTTAACGAGAGGCATTTAA	107	Ch.5
26	RM 276	$(AG)^{8}$ A^{3} $(GA)^{33}$	F - CTCAACGTTGACACCTCGTG R - TCCTCCATCGAGCAGTATCA	149	Ch.6
27	RM 469	$(AG)^{15}$	F - AGCTGAACAAGCCCTGAAAG R - GACTTGGGCAGTGTGACATG	105	Ch.6
28	RM 585	$(TC)^{45}$	F - CAGTCTTGCTCCGTTTGTTG R - CTGTGACTGACTTGGTCATAGG	233	Ch.6
29	RM 587	$(CTT)^{18}$	F - ACGCGAACAAATTAACAGCC R - CTTTGCTACCAGTAGATCCAGC	217	Ch.6
30	RM 3805	$(GA)^{19}$	F - AGAGGAAGAAGCCAAGGAGG R - CATCAACGTACCAACCATGG	110	Ch.6
31	RM 11	$(GA)^{17}$	F - TCTCCTCTTCCCCCGATC R - ATAGCGGGCGAGGCTTAG	140	Ch.7
32	RM 234	$(CT)^{25}$	F - ACAGTATCCAAGGCCCTGG R - CACGTGAGACAAAGACGGAG	156	Ch.7
33	RM 336	(CTT)18	F - CTTACAGAGAAACGGCATCG R - GCTGGTTTGTTTCAGGTTCG	154	Ch.7
34	RM 44	(GA)16	F - ACGGGCAATCCGAACAACC R - TCGGGAAAACCTACCCTACC	99	Ch.8
35	RM 152	(GGC)10	F - GAAACCACCACACCTCACCG R - CCGTAGACCTTCTTGAAGTAG	151	Ch.8
36	RM 310	(GT)19	F - CCAAAACATTTAAAATATCATG R - GCTTGTTGGTCATTACCATTC	105	Ch.8
37	RM 339	(CTT)8 CTT (CTT)5	F - GTAATCGATGCTGTGGGAAG R - GAGTCATGTGATAGCCGATATG	148	Ch.8
38	RM 404	$(GA)^{33}$	F - CCAATCATTAACCCCTGAGC R - GCCTTCATGCTTCAGAAGAC	236	Ch.8
39	RM 6999	$(TGG)^{15}$	F - TTATCTGGGATCCATCGAGC R - GTGAATTTCCTTGGAGGGAC	157	Ch.8
40	RM 242	$(CT)^{26}$	F - GGCCAACGTGTGTATGTCTC R - TATATGCCAAGACGGATGGG	225	Ch.9
41	RM 3025	$(AT)^{42}$	F - GGTGGCAAGAAGTTCCTAAT R - GATTTCCATACAACCTGTGC	203	Ch.9
42	RM 222	$(CT)^{18}$	F - CTTAAATGGGCCACATGCG R - CAAAGCTTCCGGCCAAAAG	213	Ch.10
43	RM 228	$(CA)^{6}$ $(GA)^{36}$	F - CTGGCCATTAGTCCTTGG R - GCTTGCGGCTCTGCTTAC	154	Ch.10
44	RM 3773	$(GA)^{18}$	F - CTGGATGAAAGGATACAACA R - CACATTATCTGTCAAGGTCC	150	Ch.10
45	RM 21	$(GA)^{18}$	F - ACAGTATTCCGTAGGCACGG R - GCTCCATGAGGGTGGTAGAG	157	Ch.11
46	RM 202	$(CT)^{30}$	F - CAGATTGGAGATGAAGTCCTCC R - CCAGCAAGCATGTCAATGTA	189	Ch.11
47	RM 287	$(GA)^{21}$	F - TTCCCTGTTAAGAGAGAAATC R - GTGTATTTGGTGAAAGCAAC	118	Ch.11
48	RM 1233	$(AG)^{15}$	F - GTGTAAATCATGGGCACGTG R - AGATTGGCTCCTGAAGAAGG	175	Ch.11
49	RM 17	$(GA)^{21}$	F - TGCCCTGTTATTTTCTTCTCTC R - GGTGATCCTTTCCCATTTCA	184	Ch.12
50	RM 3813	$(GA)^{20}$	F - CCTTCTTCTTCACGGCAGAG R - TTAGCAAGACTGGAGGAGACG	206	Ch.12
51	RM 28048	$(CGC)^{8}$	F - TTCAGCCGATCCATTCAATTCC GCTATTGGCCGGAAAGTAGTTAGC	93	Ch.12

In Linkage group 1(LG-1) 6 markers were found to be polymorphic (RM 490, RM 493, RM 495, RM 3412, RM 8051 and RM 8070), 5 in LG-2 (RM 6, RM 262, RM 2792, RM 3212 and RM 3316), 5 in LG-3 (RM 251, RM 514, RM 520, RM 545 and RM 6283), 4 in LG-4 (RM 127, RM 142, RM 252 and 307), 5 in LG-5 (RM 13, RM 163, RM 164, RM 440 and RM 4837), 5 in LG-6 (RM 276, RM 469, RM 585, RM 587 and RM 3085), 3 in LG-7 (RM 11, RM 234 and RM 336), 6 in LG-8 (RM 44, RM 152, RM 310, RM 339, RM 404 and RM 6999), 2 in LG-9 (RM 242 and RM 3025), 3 in LG-10 (RM 222, RM 228 and RM 3773), 4 in LG-11 (RM 21, RM 201, RM 287 and RM 1233) and 3 in LG-12 (RM 17, RM 3813 and RM 28048) (Figure.1 and Figure.2). Similar to the findings of this study 43 polymorphic SSR markers have

been identified by using 100 SSR markers in IR68897A (Maternal CMS donor) and Yosen B line (Maintainer line) (Ahmadikhah *et al.*, 2015) [1]. Yadav *et al.*, (2015) [15] reported 70 polymorphic markers among 500 SSR markers for a cross between BPT-5204 AND ARC-15031.

The parental polymorphism survey between Co51 and Ptb33 using 120 SSR markers revealed 51 polymorphic markers with a polymorphism percentage of 42.5% and LG-1 and LG-8 has highest polymorphism (60%) with LG-9 being least (20%) (Table. 3.). Out of 880 SSR markers, 71 were reported polymorphic between two rice varieties Basmathi334 and Swarna with 8.07 percent polymorphism (Vikram *et al.*, 2021) [14]. Salunkhe *et al.*, (2011) [10] have studied 343 SSR markers in two parents Nootripathu and IR 20 and found 96 SSR markers to be polymorphic with about 27.99 percentage of parental polymorphism. In gene pyramiding of bacterial blight (bb), sheath blight (ShB) and blast resistant line using the parents ADT 43, IRBB60 (BB) and Tetep (ShB and blast) and ASD 16, (BB) and Tetep (ShB and blast) Background selection was carried out with a set of 463 SSR markers; 69 and 68 markers were found to be polymorphic for ADT 43 and ASD 16 cross combinations, respectively covering all the twelve chromosomes in rice (Ramalingam *et al.*, 2020) [9]. The Polymorphism information content (PIC) between Co51 and Ptb33 using these 120 random SSR primers is about 0.5 (Serrote *et al.*, 2020) [11]. The polymorphism information content (PIC) of SSR markers is reported to be in the range of 0-1 (Chesnokov *et al.*, 2015) [3].

Table 3: Polymorphism percentage between Co51 and Ptb33

Chromosome Number	Total Number of primers screened	Number of polymorphic markers	Number of monomorphic markers	Polymorphism percentage (%)
1	10	6	4	60
2	10	5	5	50
3	10	5	5	50
4	10	4	6	40
5	10	5	5	50
6	10	5	5	50
7	10	3	7	30
8	10	6	4	60
9	10	2	8	20
10	10	3	7	30
11	10	4	6	40
12	10	3	7	30
Total	120	51	69	42.5

In future Marker Assisted backcross breeding programs can utilize these 51 polymorphic markers for the incorporation of brown plant hopper resistance in Co51 from Ptb33. More over the identified polymorphic SSR markers can be used for diversity analysis and linkage studies for various traits in rice.

Fig 1: Parental polymorphism between Co51 (P1) and Ptb33 (P2) in chromosome 1 to 12 using random genome wide SSR markers

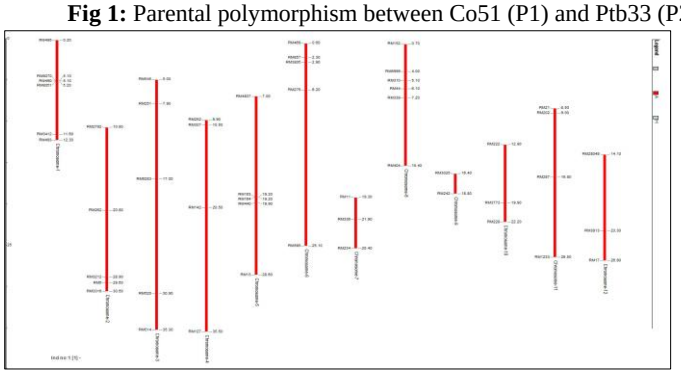

Fig 2: Physical location and distribution of 51 SSR markers showing polymorphism between Co51 and Ptb33

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