

Morphological and SSR marker-assisted parental polymorphism survey for drought tolerance in maintainer lines of hybrid rice (*Oryza sativa* L.)

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ABSTRACT

Drought stress is now a major abiotic factor influencing rice yield under the changing climatic conditions. IR58025B is one of the maintainer lines of the well-known WA CMS line (IR58025A) used in commercial rice hybrid production in India. The majority of rice hybrids are released for various ecosystem conditions using the three-line system. As a result, these derived rice hybrids suffer from drastic drop in yield under the conditions of moisture stress. To facilitate the efficient use of complex polygenic traits in hybrid rice molecular breeding research, we developed drought tolerant introgression lines in background of IR58025B by introgression of major QTL controlling grain yield under moisture stress into IR58025B by marker assisted backcrossing. To begin marker assisted back cross breeding for introgression of a desirable trait into a variety from the donor genotype, study of parental polymorphism is a pre-requisite. Unless the parents are polymorphic for the traits of interest, the further selection of plants carrying the traits of interest is not possible in the progenies. We screened all reported SSR primers linked to drought tolerance and observed RM 324 linked to *qDTY_{2.1}* and RM 520 linked to *qDTY_{3.1}* was polymorphic between Apo and IR58025B. Simultaneously we screened 300 full-genotype SSR markers covering entire genome and observed 88 SSR primers as polymorphic between IR58025B and Apo. After polymorphic survey, we evaluated Apo and IR58025B under moisture stress and control conditions and recorded significant differences among the parents. Apo performed well when compared to IR58025B for all the characters studied under stress conditions. Construction of a Linkage Map could be ensued procuring the generated genotyping data which could further avail QTL analysis and identification of markers linked to drought tolerance.

Key words: Rice, IR58025B, Drought, Yield, BILs and 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 produc-

tion of rice is equivalent to the population growth in India. With an average annual population growth rate (1.5%) and an average daily rice consumption of 250g per person, the demand for rice is expected to reach at least 140 million tonnes (Mt) by 2050. By 2050, India's population is going to need production of rice to rise by 2.5 M T annually (<http://www.fao.org/rice2004/en/pdf/khush.pdf>). The global problem of cli-

mate change is currently affecting rice cultivation worldwide by influencing resources like land, water, and nutrients to become scarcer. The development of rice cultivars resistant to climate change is urgently needed (6). As of now, as many as 8000 QTLs governing various traits have been identified and 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 also.

Hence, the current study was carried out to identify ready to use SSR markers for background selection of two widely used donor and recipient varieties. One of the readily available and economically viable technological options for meeting the projected global demand of rice is exploitation of heterosis through large-scale cultivation of rice hybrids. To increase the nation's rice yield, hybrid rice technology is essential. According to the AICRIP progress report 2021, 127 rice hybrids have been certified for commercial cultivation in India as of now. In *Kharif* 2017, 3.5 million tonnes of hybrid rice were produced on 3 million hectares of land (Hariprasad *et al.*, 2018). Majority of the rice hybrids were developed for irrigated conditions. Currently, three line CMS system was widely used for exploitation of hybrid vigour in rice. Therefore, creating hybrids resistant to moisture 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.

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 selection of plants possessing the traits of interest is not achievable in the progenies unless the parents are polymorphic for the traits of interest. Polymorphism is an index of genetic variation that shifts with different sets of parents. According to the survey of parental polymorphism, there is demonstrable variation between 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 polymor-

phic, 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 by most crop species for which sequence information is available, including rice. Many genetics-related domains, such as gene tagging, genetic diversity research, population genotyping mapping, linkage map creation, tracking marker-trait associations, single marker analysis, and quantitative trait loci (QTL) mapping, can benefit from these. 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 marker support, which was used to insert drought-resistant QTLs from the donor parent Apo into the background of the extensively planted rice maintenance line, IR58025B.

Materials and Methods

The parents used for the study were IR58025B and Apo and their characteristics were mentioned below.

1. **APO:** Apo is a drought tolerant upland indica genotype developed at IRRI, Philippines harboring major effect QTL $qDTY_{3.1}$ controlling grain yield under moisture stress.
2. **IR58025B:** IR58025B is a maintainer line of widely used CMS line IR58025A in commercial rice hybrids production.

Leaf sample collection and DNA isolation

Total genomic DNA was isolated from the parental lines using the method of Zheng method (1991) with modifications. Healthy leaf pieces (2-3 cm) from the youngest leaves of a 20-25day old rice seedlings were taken into the well of a spot test plate (M/s Thomas Scientific, USA). 400 μ l of extraction buffer (50 mM Tris HCL, pH 8.0, 25 mM EDTA, 300 mM NaCl and 1 % SLS) was added to the well. The leaf tissues were macerated for about 15-20 seconds till it was completely homogenized. Another 400 μ l of extraction buffer was added to the well containing the leaf sample. Using a micropipette of 1 ml capacity, the entire content from the well was transferred to a 1.5 ml capacity micro centrifuge tube. About 400-500 μ l Phenol (pH 8.0): Chloroform: Isoamyl al-

cohol (25:24:1) was then added to the micro centrifuge tube. The contents were mixed well by inversion for about 10 minutes and centrifuged at 12000 rpm for about 10 minutes at room temperature. After the centrifugation, the supernatant was aliquoted from the micro-centrifuge tube without disturbing the intermediate layer into a fresh 1.5 ml micro-centrifuge tube. To the clear supernatant, 5-10 μ l of RNAase (10 mg per ml) was added and incubated for 45-60 minutes at room temperature (~ 37°C). After completion of the incubation period, about 500 μ l of Chloroform: 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 and the supernatant was collected into another fresh 1.5 ml micro-centrifuge tube without disturbing the intermediate layer. To the clear supernatant equal volume (~500-600 μ l) of chilled isopropanol was added. The contents were mixed gently and centrifuged at 12000 rpm for 10 minutes at room temperature. The supernatant was drained gently without disturbing the DNA pellet. About 150-200 μ l of 70 % ethanol was added to the pellet at the bottom of the micro-centrifuge tube. It was ensured that the pellet was completely immersed in 70% alcohol. Then the content was centrifuged at 5000 rpm for 5 minutes at room temperature. After centrifugation, the supernatant was drained and 70% alcohol wash was repeated once again. Finally, after centrifugation and draining out the supernatant, the pellet was left overnight air drying at room temperature. After complete drying of the pellet, depending upon the size of the pellet, about 50-100 μ l of sterile distilled water was added for dissolving the pellet. About 3 μ l of the DNA sample was taken and loaded in 0.8 % ethidium bromide stained agarose-TBE (concentration of TBE) gel, electrophoresed for about an hour and the bands of genomic DNA were visualized and documented in a UV gel documentation system (M/s Alpha Innotech Corporation, USA).

Polymerase Chain Reaction (PCR) using SSR markers

The genomic DNA of parental lines subjected to PCR amplification as per the procedure described by Chen (1997). PCR amplification was carried out in a thermo cycler using the 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 at a 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.

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

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

Graphical representation of the genome

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

Phenotypic evaluation of IR58025B and Apo in field under control and moisture stress conditions

In control experiment, regular irrigation and flooded conditions were maintained until maturity of the crop. In stress experiment irrigation was withheld for 3 weeks at panicle initiation stage. The tolerant genotypes that recovered from stress were tagged and harvested at physiological maturity stage. The differences between the parents on yield and drought tolerated traits were tested using paired t-test.

Results and Discussion

Genotypic polymorphism by molecular markers

The presence of two or more discontinuous genotypes or alleles on a specific locus in a given population is referred to as genetic polymorphism. In the present study, clear polymorphism was observed between the parents both for full genome coverage markers and drought tolerant markers. A total of 300 full genome coverage SSR markers (forward and reverse) and 10 drought tolerant yield QTL markers reported by Venuprasad *et al.* (2009) were analyzed for molecular polymorphism. Polymerase Chain Reaction was done to check each sample for parental polymorphism using specific Rice Microsatellite (RM) primers. Out of 300 full genome coverage primers used the parents showed polymorphic only

to 88 RM primers listed in Table 1 were polymorphic whereas RM 324 linked to *qDTY2.1* and RM 520 linked to *qDTY3.1* listed in Table 2 were polymorphic between the parents for drought tolerant markers. These are used in foreground selection. This type of parental polymorphic survey was done by previous workers *i.e.*, Kanagaraj *et al.* (2010), Singh *et al.* (2012), Suh *et al.* (2014), Henry *et al.* (2015), Sonalikhari *et al.* (2017), Shamsudin *et al.* (2016) and Sandhu *et al.* (2018) 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. Percentage of polymorphism was highest on 1 chromosome and least on 6 chromosome. The average per cent polymorphism on all the chromosomes was 28.72. The lack of detectable polymorphism between two parents could be due to the fact that both parents, Apo and IR58025B are indica lines. Lack of molecular marker polymorphism between indica genotypes has been earlier noticed in studies by Xu *et al.* (2002) and Biradar *et al.* (2004). In

Table 2. Details of polymorphic primer used for foreground selection of *qDTY_{2.1}* and *qDTY_{3.1}*

QTL	SSR Marker	Chromosome	Size
<i>qDTY_{2.1}</i>	RM 324	2	175bp
<i>qDTY_{3.1}</i>	RM 520	3	247 bp

Table 1. Microsatellite markers exhibiting polymorphism between IR58025B and Apo

1	RM 10694	21	RM 250	41	RM 3558	61	RM 408	81	RM 7391
2	RM 8094	22	RM 29	42	RM17486	62	RM 331	82	RM 209
3	RM 3412	23	RM 3316	43	RM6909	63	RM 256	83	RM 457
4	RM 140	24	RM 262	44	RM2275	64	RM 447	84	RM 2136
5	RM 10794	25	RM 530	45	RM127	65	RM 126	85	RM 28048
6	RM 10825	26	RM 424	46	RM5270	66	RM 342	86	RM 571
7	RM 562	27	RM 569	47	RM 413	67	RM 23679	87	RM 28199
8	RM 10927	28	RM 3766	48	RM 169	68	RM 23911	88	RM 27100
9	RM 543	29	RM 15087	49	RM 5140	69	RM 24173		
10	RM 486	30	RM 231	50	RM 3638	70	RM 24179		
11	RM 315	31	RM 468	51	RM 3321	71	RM 24390		
12	RM 472	32	RM 16232	52	RM 204	72	RM 201		
13	RM 12023	33	RM 16236	53	RM 6836	73	RM 105		
14	RM 165	34	RM 523	54	RM 3827	74	RM 26076		
15	RM 8051	35	RM 15914	55	RM20546	75	RM 286		
16	RM 312	36	RM 15087	56	RM 20801	76	RM596		
17	RM 12611	37	RM 545	57	RM 21330	77	RM 6132		
18	RM 145	38	RM 551	58	RM 21427	78	RM 206		
19	RM 6374	39	RM 16616	59	RM 5481	79	RM 590		
20	RM 3275	40	RM 5757	60	RM 248	80	RM 286		

the present study dinucleotide repeats in the microsatellite region showed more polymorphism than tri and tetra nucleotide repeats. It may be due to the abundance 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 breeding programs has greatly increased its efficiency and fastens the transfer of desirable genes among varieties and to introgress novel genes from related wild species. Polygenic markers which were previously difficult

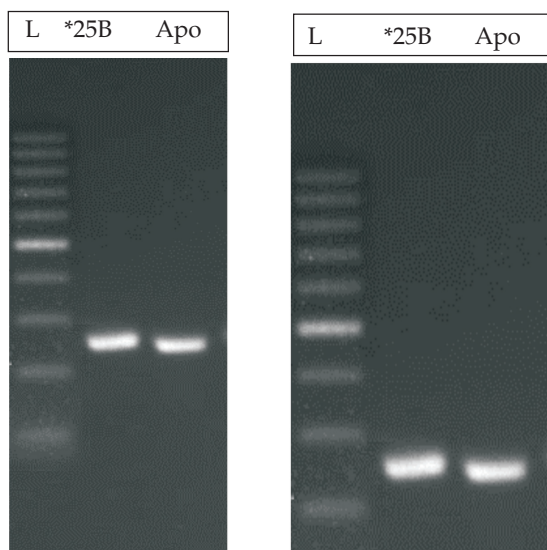
to analyze using traditional breeding methods, would now be easily tagged using molecular markers. The screening of markers for parental polymorphism among the rice cultivars forms the basis for tagging of the desired gene, fine mapping of the gene in the rice chromosome and in the subsequent Marker Assisted Selection (MAS) programmes.

Phenotypic polymorphism by morphological traits under control and moisture stress conditions

IR58025B and Apo were assessed for grain yield and its attributing traits under reproductive stage moisture stress and control conditions (Table 4). IR58025B recorded significant differences for all the traits studied except for unfilled grains per panicle between control and reproductive moisture stress conditions. Similarly, Apo recorded significant differences for all the traits studied except for 1000 grain weight, harvest index and SPAD chlorophyll meter reading between control and reproductive stage moisture stress conditions. This significant difference indicated the effectiveness of introgression the drought tolerance QTLs $qDTY_{2.1}$ and $qDTY_{3.1}$ into IR58025B from Apo by marker assisted backcross breeding.

Conclusion

Many morphological and environmental factors influence drought resistance, making it a highly nuanced characteristic in rice breeding. The ability of rice cultivars grown in a rainfed ecosystem to endure drought would be useful in maintaining a



Parental polymorphism shown by RM520 for $qDTY_{3.1}$

Parental polymorphism shown by RM 324 for $qDTY_{2.1}$

Table 3. Chromosome wise polymorphic primers between IR58025B and Apo

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	16	19	45.71
2	30	10	20	33.33
3	28	11	17	39.29
4	25	9	16	36.00
5	25	5	20	20.00
6	28	4	24	14.29
7	25	5	20	20.00
8	27	6	21	22.22
9	20	7	13	35.00
10	19	6	13	31.58
11	20	5	15	25.00
12	18	4	14	22.22

Table 4. Comparison of IR58025B and Apo for grain yield and its attributes under control and reproductive stage moisture stress (RSS) conditions

S. No.	Trait	IR58025B			Apo			S.E. of difference
		Control (n=5)	RSS (n=5)	Mean difference (C-RSS)	Control (n=5)	RSS (n=5)	Mean difference (C-RSS)	
1	Plant height (cm)	100.75	87.12	13.63**	113.75	78.63	35.12**	1.53
2	Days to 50% flowering	97.00	94.00	3.00**	90.00	84.00	6.00**	0.82
3	Days to maturity	126.00	121.00	5.00**	118.00	112.00	6.00**	0.82
4	No. of tillers per plant	18.00	12.00	6.00**	20.00	10.00	10.00**	0.41
5	No. of panicles per plant	16.00	10.00	6.00**	18.00	8.00	10.00**	0.44
6	Panicle length (cm)	24.32	19.50	4.82**	26.40	18.12	8.28**	0.45
7	1000 grain weight (g)	18.50	12.80	5.70**	23.04	22.23	0.81	0.61
8	No. of filled grains per panicle	160.00	110.00	50.00**	240.00	162.00	78.00**	5.29
9	No. of unfilled grains per panicle	54.00	60.00	-6.00	41.00	50.00	-9.00*	4.03
10	Spikelet fertility (%)	74.76	64.70	10.06**	85.40	76.41	8.99**	1.87
11	Grain yield per plant (g)	20.25	9.81	10.44**	22.94	18.28	4.66**	1.15
12	Biological yield per plant (g)	38.24	20.14	18.10**	64.12	49.84	14.28**	1.9
13	Harvest index (%)	52.95	48.71	4.24**	35.77	36.68	0.91	1.19
14	Shoot length (cm)	76.43	67.72	8.71**	87.35	60.51	26.84**	1.86
15	Relative water content (%)	79.12	55.38	23.74**	88.50	79.67	8.83**	1.89
16	SPAD chlorophyll meter reading	43.74	34.50	9.24**	42.80	41.61	1.19	0.83
17	Shoot dry weight (g)	8.00	6.12	1.88*	18.00	10.05	7.95**	0.66
18	Specific leaf area (cm ² g ⁻¹)	90.30	64.90	25.40**	78.90	73.20	5.70*	2.62

RSS: Reproductive Stage Moisture Stress

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

steady supply of rice. Genetic development of rice for water-limiting situations has slowed due to a lack of contemporary molecular techniques for screening of breeding lines for drought tolerance and a lack of understanding of the specific mechanisms of drought tolerance and the inheritance of drought tolerance traits. Breeding programmes that make use of molecular marker technologies are more productive, can move desired genes more quickly between varieties, and can introduce novel genes from related or cultivated species. Marker Assisted Selection (MAS) programmes rely on the identification of parental polymorphism in rice cultivars through the screening of markers. This allows for these subsequent tagging and fine mapping of the target gene within the rice chromosome. Polymorphic markers discovered in this work will be used to genotype the entire mapping population and facilitate the introgression of drought-responsive QTLs/genes involved in grain production throughout the reproductive stage. This study helped in the development of backcross inbred lines of IR58025B by introgression of *qDTY_{2.1}* and *qDTY_{3.1}* from Apo into IR58025B by marker assisted backcross breeding. These developed BILs could be further utilized in development of hybrids tolerant to moisture stress.

Conflict of Interest - None

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