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Development of blast resistant versions of improved samba Mahsuri variety of rice (*Oryza sativa* L.) through marker assisted breeding

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Abstract
Globally, blast incited by *Magnaporthe oryzae* forms major fungal diseases that can cause severe loss in yield to the extent of 70-80% in various rice ecosystems. Marker assisted backcross breeding approach was employed to incorporate blast resistance genes *viz.*, *Pi1*, *Pi2* and *Pi54*, from the donor lines BPT/lac/tetep and B95-1/A51 into blast susceptible otherwise superior cultivar Improved Samba Mahsuri. Foreground selection coupled with stringent phenotypic selection lead to the identification of 15 superior BC_2F_2 plants homozygous for three genes. Agro-morphological evaluation of the pyramided lines revealed that three of the selections *viz.*, ISM10-15-101, ISM10-15-119 and ISM10-15-154 showed significant increase in no. of filled grains per panicle (30.22%) and yield (24.23%). $BC_2F_{2:3}$ derived lines, varied in their disease reaction to DRR isolates. Three of the *Pi1+Pi2+Pi54* pyramided lines showed complete resistance to mixture of virulent races under artificial inoculation as well as in UBN test locations.

Keywords: Blast resistance, foreground selection, pyramided lines, SSR, uniform blast nursery

1. Introduction
Rice is the staple food crop of India and occupies second prominent position in global agriculture. The projected increase in global population to 9 billion by 2050, predicted water scarcity, decrease in arable land, the constant battle against new emerging pathogens and pests and possible adverse effects from climate change will present great challenges for rice breeders and agricultural scientists (Khush 2005) [14].

Rice productivity is adversely impacted by numerous biotic and abiotic factors. Approximately ~52% of the world's rice production is lost annually owing to the damage caused by biotic factors, of which ~30% is attributable to the attack of diseases (Yarasi *et al*. 2008). Among the several diseases, Blast disease of rice caused by *Magnaporthe grisea* (Hebert) Barr. (Anamorph *Pyricularia grisea* Sacc) is one of the devastating and destructive diseases of rice worldwide (Zeigler *et al*. 1994; Srinivas Prasad *et al*. 2009) [9, 26]. It can cause severe loss in yield to the extent of 70-80% in various rice ecosystems (Rajarajeswari and Muralidharan 2006) and the economic loss due to blast is high as the quality of the harvested grain is also affected in addition to actual yield loss (Variar *et al*. 2009) [30]. This constraint can be effectively addressed by developing cultivars with built in resistance. Breeding for high yielding and improved resistance are the two most economically and environmentally benign methods of maintaining stable rice production.

Till date, 85 blast resistance genes have been identified and the genes *Pi-1*, *Pi-2* and *Pi-54* were reported to provide broad spectrum resistance. The gene *Pi1*, from LAC23 (Mackill and Bonman 1992; Inukai *et al*. 1994) [17] an indica rice line which is highly effective against the pathogen population found in the tropical area of South Asia and Southeast Asia, found to inherit as a major dominant gene (Hittalmani *et al*. 2000; Fuentes *et al*. 2008; Yang *et al*. 2008; Tacconi *et al*. 2010) [11, 9, 33, 28]. The *Pi2 (t)* gene was mapped onto rice chromosome 6, the gene *Pi2* from C101A51 (an indica rice line) which found to inherit as a major dominant gene (Mackill and Bonman 1992) [17]. *Pi54* was identified and mapped to chromosome 11L in a rice variety Tetep and was reported to govern resistance against predominant races of the blast pathogens in India (Sharma *et al*. 2010) [20]. Therefore, we utilized these genes in marker assisted breeding program to develop blast resistant lines. Marker assisted selection (MAS) has been advocated as a highly efficient breeding method as it makes possible rapid and precise selection of the targeted gene (Tanksley *et al*. 1989) [27]. Marker-assisted backcross breeding (MABB), which involves two steps: (1) MAS for the gene of interest, known as foreground selection and (2) MAS for recovery of the recurrent parent genome, known as background

selection (Hospital *et al*. 1992) [12], is the most effective way of transferring specific gene (s) to an otherwise agronomically superior variety or parental lines. In rice, the feasibility of MABB to pyramid bacterial blight resistance genes has been well demonstrated (Joseph *et al*. 2004; Luo *et al*. 2005; Gopalakrishnan *et al*. 2008; Sundaram *et al*. 2008, 2009) [10, 16, 12].

The present study was aimed at incorporation of three major blast resistance genes, *Pi1*, *Pi2* and *Pi54* into the genetic background of an elite Bacterial Blight resistant rice cultivar Improved Samba Mahsuri through MABB, and evaluation of different gene combinations involving the improved versions of ISM lines for agronomic performance, grain and grain type traits and reaction to blast.

Materials and methods

Plant materials and breeding strategy

Improved Samba Mahsuri (ISM) is an improved, bacterial blight resistant, high yielding, fine grain variety known for its premium grain and cooking quality but highly susceptible to blast disease was used as the recurrent parent for incorporation of blast resistance genes from two donors namely, BPT/lac/tetep (*Pi1* & *Pi54*) and ISM/A51 (*Pi2*). The gene linked SSR markers RM224, RM527 and RM206 were used to identify the *Pi1*, *Pi2* and *Pi54* genes respectively. The recurrent parent Improved Samba Mahsuri, was crossed as female parent with the donor, BPT/lac/tetep to generate hybrid and the F_1's positive for the respective genes were crossed with the donor ISM/A51 to obtain three way cross hybrid with all the target genes under study. F_1 positive for all the three genes were backcrossed with ISM to produce the BC_1F_1 seeds. Foreground selection in the BC_1F_1 generation for the genes *Pi1*, *Pi2* and *Pi54* was performed using gene linked markers. The gene positive plants with maximum phenotypic similarity to the recurrent parent were backcrossed to ISM to generate BC_2F_1 seeds. Foreground and stringent phenotypic selections were carried out to select elite plants from backcross derived population to produce BC_2F_3 progenies following pedigree selection (Figure 1).

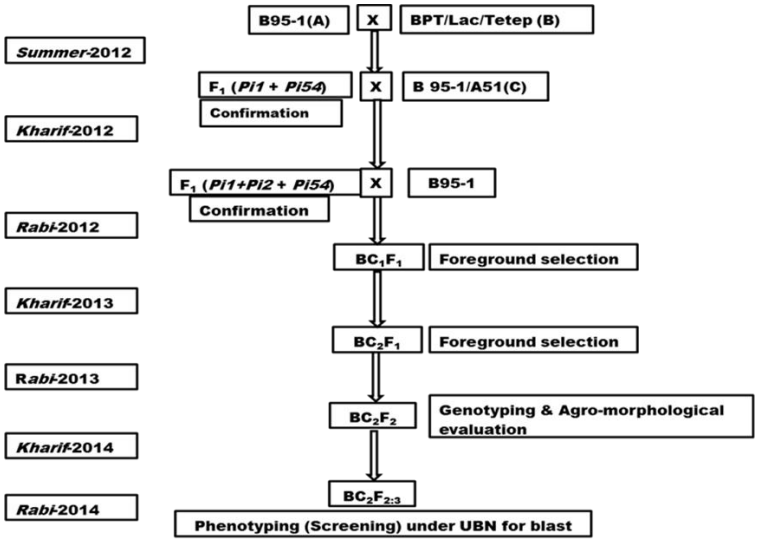

Fig 1: Simultaneous Gene Transfer Backcross Scheme employed

Screening for blast resistance

Artificial inoculation: Twenty-one-day-old seedlings of selected families homozygous for three blast resistance genes were artificially screened against the DRR blast isolates. Stored cultures of each pathogen isolate were revived by inoculating the colonized filter discs on oat meal agar slants. The inocula were prepared following the standard procedures established by Bonman *et al*. 1986. About 30-40 ml of the spore suspension containing gelatin (0.1%) and Tween-20 (0.02%) was sprayed onto seedlings using a glass atomizer. Inoculated seedlings were kept in a humid chamber with the temperature maintained at 25± 1°C. The distilled water was sprinkled three to four times a day to maintain high humidity. The disease reaction of each rice line was recorded 7 days after inoculation using a 0–5 disease scoring scale (Bonman *et al*. 1986) [4]. The plants exhibiting 0-2 reaction were considered as resistant, reactions of 3 were considered moderately resistant while those showing 4-5 reaction were categorized as susceptible. The resistant genotypes were further tested in Uniform Blast Nurseries.

Field screening for blast resistance in Uniform Blast Nursery (UBN)

All the promising lines were evaluated for their reaction to blast under Uniform Blast Screening Nursery (UBN) at two locations *viz.*, Agricultural Research Institute (ARI), ANGRAU, Rajendranagar, Hyderabad, Telangana and Directorate of Rice Research, Rajendranagar, Hyderabad (lat 17° 32' N, long 78° 40' and alt 695 m asl). A 50 cm row of each entry was planted in nursery bed with a row spacing of 10 cm. A row of susceptible check was inter-planted after every five entries and also on the borders to ensure uniform spread of the disease. The test materials were surrounded by densely sown spreader rows of susceptible rice cultivar HR 12/TN1. Data on blast reaction of the entries were recorded thrice following 0–9 scale (SES, 1996) at 10 days interval starting after 30 days of sowing. The lines with a score of 0–3 were considered as resistant, 4–5 as moderately resistant, 6 as moderately susceptible and 7–9 as susceptible.

Molecular marker analysis

Marker assisted foreground and Background selection: Sequences of PCR based SSR markers were selected from the Gramene database (www.gramene.org) and selected gene specific markers were employed in the Marker Assisted selection programme. The SSR marker RM224 for Pi1 gene (Fjellstrom *et al*. 2006) [8], RM527, AP56595 for Pi2 gene (Liu *et al*. 2002) [15] and RM206, Pi54-MAS for Pi54 genes

(Sharma *et al*. 2005, Ramkumar *et al*. 2011) [19, 18] were used for the polymorphism study between the parents. The markers which are clearly differentiating between the parents were employed for the confirmation and foreground selection of the target genes (Figure 2a). The marker RM224 linked to Pi1, RM527 linked to Pi2 and RM206 linked to Pi54 were used for foreground selection in backcrossed and selfed generations. The markers used and their primer sequences, chromosomal locations and linkage distance between marker and respective genes are presented in Table 1. A set of 375 SSR markers uniformly spread across the 12-rice chromosomes were screened for polymorphism survey between the recurrent parent, Improved Samba Mahsuri, and the resistant donor, BPT/Lac/Tetep and B95-1/A51, to find out polymorphic background markers. The primer sequences for SSR markers were adapted from the Gramene SSR marker resource (http://www.gramene.org/). At least five polymorphic SSR background markers per chromosome were used for background selection to recover the recipient.

Marker Assisted derived lines were also screened with three STS markers, RG556, RG136 and pTA248, tightly linked to Blight resistance genes *xa5*, *xa13* and *Xa21* respectively to confirm the presence of each gene.

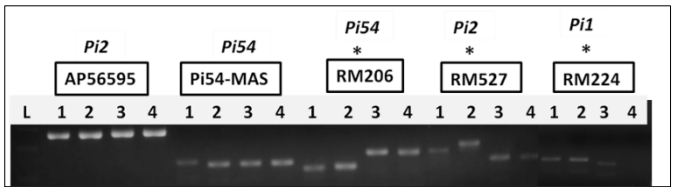

Fig 2: Agarose gel showing polymorphism between the parents by SSR markers (1-ISM/A51; 2-BPT/LAC/TETEP; 3-ISM; 4-BPT)

Table 1: List of primers used for parental polymorphism and foreground selection

Gene	Primer	LG	Amplicon product size (bp)	Forward primer sequence	Reverse primer sequence	Reference
Pi1	RM224	11	157	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	Fuentes *et al*., 2008
Pi2	RM527	6	233	GGCTCGATCTAGAAAATCCG	TTGCACAGGTTGCGATAGAG	Liu *et al.*, 2002
Pi2	AP56595	6	-	CTCCTTCAGCTGCTCCTC	TGATGACTTCCAAACGGTAG	-
Pi54	Pi54-MAS	11	-	CAATCTCCAAAGTTTTCAGG	GCTTCAATCACTGCTAGACC	Ramkumar *et al.*, 2011
Pi54	RM206	11	175	CCCATGCGTTAACTATTCT	CGTTCCATCGATCCGTATGG	Sharma *et al*. (2005)

Table 2: Agronomic performance & Blast disease reaction of three blast gene pyramided (*Pi1+Pi2+Pi54*) plants into the genetic background of Improved Samba Mahsuri

S. No	Plant identity	Days to maturity	Plant height (cm)	No. of productive tillers /plant	Panicle weight (g)	Panicle length (cm)	No. of filled grains/ panicle	Spikelet fertility (%)	Plant yield (g)	1000 seeds weight (g)	Blast disease score	Grain type
	ISM	**140.00**	**85.00**	**12.50**	**2.80**	**22.00**	**94.52**	**77.45**	**20.00**	**16.5**	**9**	**MS**
1	ISM10-15-12	130.00	89.00	16.00	3.50	23.50	145.00	81.20	38.2	17.50	0	MS
2	ISM10-15-16	140.00	92.00	18.00	3.20	24.25	176.00	85.00	42.3	16.80	2	MS
3	ISM10-15-17	136.50	89.50	15.00	3.00	21.00	169.00	84.00	40.5	17.20	1	MS
4	ISM10-15-51	138.20	90.00	19.25	3.42	23.60	192.00	89.00	41.25	18.00	1	MS
5	ISM10-15-77	130.20	98.00	16.00	2.80	22.46	145.00	90.00	45.2	17.30	1	MS
6	ISM10-15-82	130.50	108.00	12.50	3.60	24.00	190.00	75.00	33.21	17.25	1	MS
7	ISM10-15-101	130.50	92.00	16.60	3.80	24.50	185.00	93.50	46	17.50	0	MS
8	ISM10-15-105	134.00	88.50	14.20	3.10	20.00	190.00	79.50	39.95	17.80	2	MS
9	ISM10-15-109	131.00	95.20	16.50	3.75	25.00	188.00	86.50	43.6	17.50	0	MS
10	ISM10-15-119	125.40	90.00	21.00	3.75	26.00	201.00	94.20	52.8	16.50	0	MS
11	ISM10-15-154	128.00	90.50	18.36	3.65	23.50	195.00	92.00	49.2	17.20	0	MS
12	ISM10-15-167	129.00	91.00	18.54	3.80	26.50	186.00	90.50	48.5	17.80	2	MS
13	ISM10-15-177	136.00	88.00	16.47	3.60	24.00	190.00	87.80	45.8	17.50	1	MS
14	ISM10-15-180	131.00	105.00	14.50	3.75	26.40	182.00	85.50	42.8	18.00	1	MS
15	ISM10-15-195	133.20	94.00	16.00	3.50	26.00	180.00	84.00	40.8	17.20	1	MS
Mean		132.230	93.363	16.595	3.4813	24.047	180.93	86.51	43.34	17.40	0.9	
Range		14.60	20.00	8.50	1.00	6.50	56.00	19.20	19.59	1.50	2	
CD (0.01)		2.21	0.91	1.5	0.88	1.02	0.94	1.15	3.6	1.23		

DNA isolation, PCR conditions and electrophoresis: DNA was extracted from the frozen leaf sample (at –70° C) using CTAB protocol of Wen - Yue, *et al*. (2006). The PCR mixture contained, 25–30 ng template DNA, 5 pmol of each primer, 0.05 mM dNTPs (MBI, Fermentas, Lithuania, USA), 10x PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM MgCl2) and 0.5 U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India) in a reaction volume of 15 µl. Template DNA was initially denatured at 94 ◦C for 5 min followed by 35 cycles of PCR amplification with the following parameters: 1 min denaturation at 94 ◦C, 1 min annealing at 55 ◦C and 2 min of primer extension at 72 ◦C followed by final extension of 72 ◦C for 7 min. The amplified products were resolved in 3.5% agarose gel. The resolved amplified products were visualized under UV transuliminator and documented in gel documentation system (Bio-Rad Laboratories Inc., USA).

Evaluation of agronomic performance of improved lines: Twenty-five-day-old seedlings of 15 improved BC_2F_2 lines along with the recurrent parent were transplanted with a spacing of 20 cm × 15 cm in a randomized complete block design (RCBD) with two replications, in plots of 5 m^2 during *Kharif* 2014 at the research farm of the Biotechnology, Directorate of Rice Research, Rajendranagar, Hyderabad. Data was recorded on five plants taken from central rows of each family for agronomic traits *viz.*, days to 50% flowering, plant height (cm), effective tillers/plant, panicle length (cm), number of filled grains/panicle, spikelet fertility (%), 1000-grain weight (g) and grain yield (q/ha).

Results and discussion

Marker assisted foreground & Background selection: For the development of blast resistant versions of Improved Samba Mahsuri with three genes, the F_1s from the cross ISM × BPT/lac/tetep were tested for hybridity using the *Pi1* and *Pi54* linked molecular markers, RM224 and RM206 respectively. Single, two gene positive F_1 was inter-crossed with ISM/A51 to generate 13 three way cross F_1 plants and

were subjected to screening with *Pi1*, *Pi2* and *Pi54* gene linked molecular markers RM224, RM527 and RM206, respectively (Figure 3). Three confirmed triple gene positive plants were backcrossed with recurrent parent ISM to generate 75 BC_1F_1 plants and were screened with RM224, RM527 and RM206 markers for *Pi1*, *Pi2* and *Pi54* genes, respectively. Based on the agro-morphological similarity to recurrent parent, one out of 15 plants heterozygous for all the three genes (*Pi1*, *Pi2* and *Pi54*) was selected and used to generate BC_2F_1 seeds. In BC_2F_1 generation, eight plants heterozygous for three genes were identified and finally five best plants were forwarded to generate BC_2F_2 families. 15 best BC_2F_2s homozygous for genes *Pi1*, *Pi2* and *Pi54* (Figure 4 - 4a, 4b & 4c) were also screened for confirmation of bacterial blight resistance, three STS markers, RG556, RG136 and pTA248, tightly linked to Blight resistance genes xa5, *xa13* and *Xa21* respectively were used to confirm the presence of each gene and results showed that these gene are intact in Improved Samba Mahsuri background. Such lines were advanced to generate BC_2F_3 generation through pedigree selection, exercising selection for agro-morphological, grain and grain type traits in every generation.

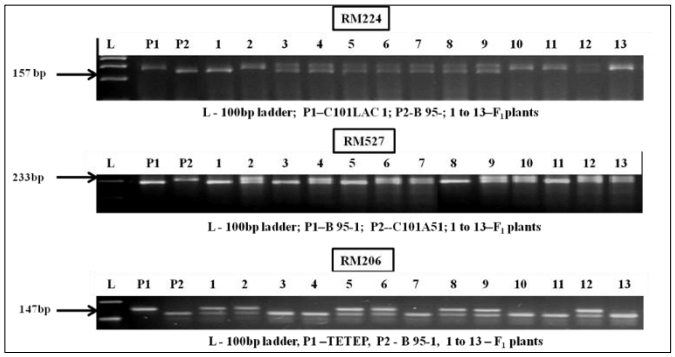

Fig 3: Confirmation of Inter-cross F1s (ISM/Lac /Tetep x B 95-1/A51) using gene linked markers

Agronomic performance of improved lines: The data on agronomic performance of the selected families of ISM are presented in Table 2. ISM-10-15 families showed a significant increase in panicle length, filled grains per panicle, thousand grain weight in comparison to the recurrent parent ISM. One of the selections, ISM-10-15-119 showed significant difference in number of filled grains per panicle (12.22%) and yield (20.23%) in comparison to ISM (Table 2). In all, families of ISM-10-15 showed 12.11% (ISM-10-15-12) to 20.74% (ISM-10-15-119) superiority in yield over ISM.

Disease reaction of improved lines: The blast reaction of the improved lines of ISM (ISM-10-15 + *Pi1+Pi2+Pi54*) is presented in Table 2. The recurrent parent ISM was found susceptible and donor parents ISM/lac/tetep and ISM/A51 were found resistant to DRR isolates both under artificial inoculation as well as in the Uniform Blast Nursery (UBN). The improved lines, varied in their disease reaction to DRR isolates. Three of the *Pi1+Pi2+Pi54* introgressed lines ISM-10-15-119, ISM10-15-101 and ISM10-15-154 showed complete resistance to mixture of virulent races under artificial inoculation as well as in UBN test locations (Fig 5).

Grain and grain type analysis of improved lines: The grain weight and grain type of most of the improved lines of ISM (ISM-10-15) were either on par or superior to the recurrent parent ISM. The parent ISM was showing medium slender grain type. Three gene pyramided lines (ISM-10-15-119, ISM-10-15-101 and ISM-10-15-154) were showing superior grain type character than its parental line ISM with respect to grain size and shape (Fig 6).

Conventional breeding for disease resistance is laborious, time consuming and highly dependent on environmental conditions in comparison to marker assisted selection (MAS), which is simpler, highly efficient and precise. MAS has been successfully utilized for developing improved parental lines of hybrid rice with inbuilt resistance to bacterial blight and blast diseases (Basavaraj *et al*. 2009, 2010; Zhou *et al*. 2011) [1, 34]. The susceptibility of the ISM to blast disease is a major factor offsetting its overwhelming performance. Incorporation of blast resistance into ISM would improve its adaptability to disease endemic regions and also sustain the productivity of the variety. Earlier, Directorate of Rice Research successfully incorporated three genes for BB resistance namely, *Xa5*, *Xa13* and *Xa21* in the Improved Samba Mahsuri using MABB (Sundaram *et al*. 2010). In the present study, we developed Improved Samba Mahsuri lines through incorporation of blast resistance genes *viz.*, *Pi1*, *Pi2* and *Pi54* through MABB breeding strategy.

The application of marker assisted background selection and its potentiality has been discussed at length, especially on the specific issues *viz.*, background selection on carrier chromosome or recombinant selection, background selection on whole genome (Basavaraj *et al*. 2010; Chen *et al*. 2000, 2001; Sundaram *et al*. 2008, 2009) [6, 25, 26] and background selection with trait of interest (Bhatia *et al*. 2011) [3]. Though background selection is very useful for rapid recovery of the recurrent parent genome, its use in breeding programmes is constrained by the limitation to detect polymorphisms with markers such as SSRs, its cost and need for timely execution. A modification in the MAS scheme was advocated by Singh *et al*. 2011, wherein, foreground selection for trait of interest followed by stringent phenotypic selection yields superior results. We adopted this modified approach of MABB in developing improved versions of Improved Samba Mahsuri. The extent of RPG recovery was up to 90.0%, such a high recovery can be attributed to the stringent phenotypic selections made in every generation of backcrossing and selfing. This approach has not only reduced the time but also economized the resources involved. It is evident from the results that phenotypic selection for grain and grain type traits of improved lines coupled with marker assisted selection led to hastening the recovery of recurrent parent genome and phenome. The increase in the yield performance of some of the improved lines were primarily brought about by the increase in panicle length, filled grains/panicle and 1000-grain weight.

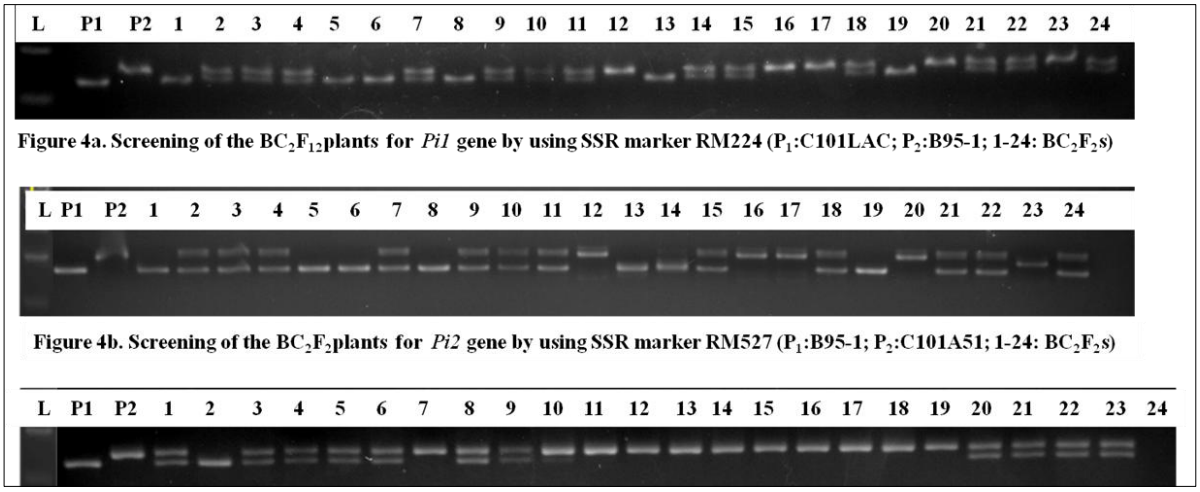

Fig 4: Identification of BC_2F_2 plants homozygous for *Pi1*, *Pi2* and *Pi54* genes using the gene linked marker

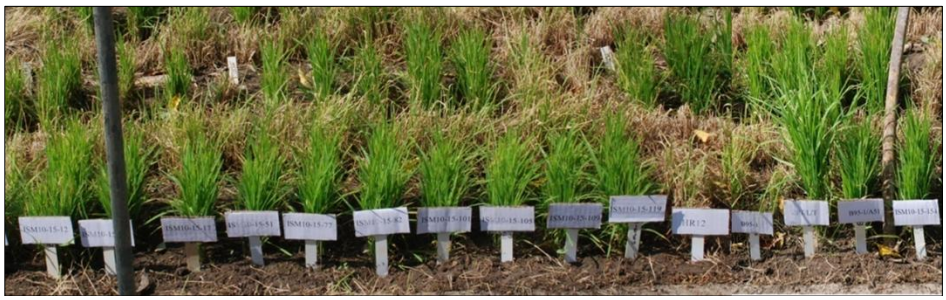

Fig 5: Disease Reaction of the lines with three genes (*Pi 1 + Pi 2 + Pi 54*) in Improved Samba Mahsuri Background

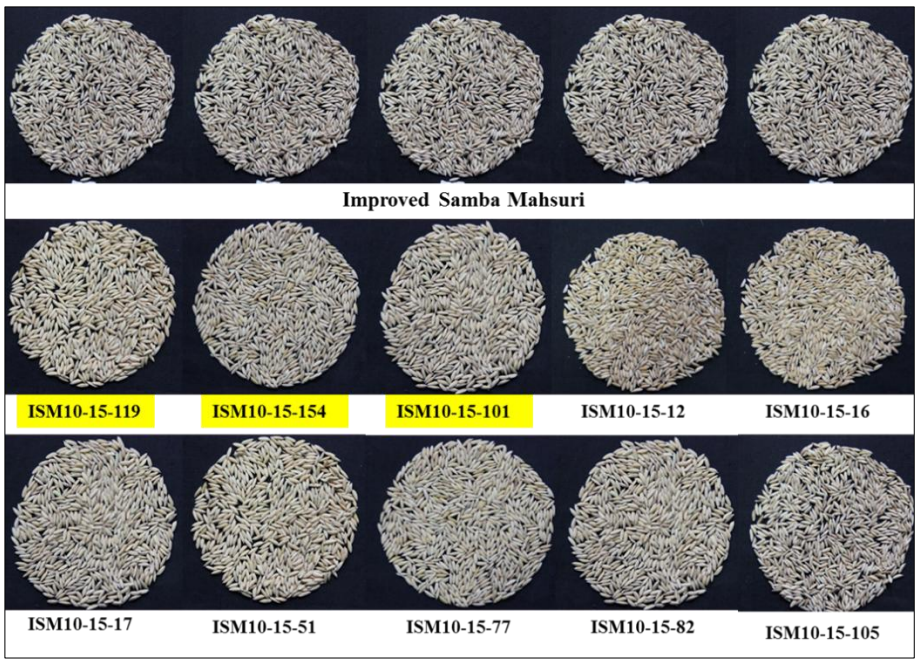

Fig 6: Grain type of improved lines (three gene pyramided lines) of Improved Samba Mahsuri

Conclusion

Marker assisted foreground selection was successfully combined with phenotypic selection to recover yield and its components, grain and grain type traits to develop improved version of ISM with inbuilt resistance to blast. The improved lines of Improved Samba Mahsuri carrying *Pi1+Pi2+Pi54* and *Pi2+Pi54* were either on par or superior in agronomic performance, grain and grain type compared to recurrent parent with an added advantage of blast resistance. Besides developing 15 lines with three gene combination (*Pi1+Pi2+P54*) *viz.*, ISM-10-15-12, ISM-10-15-16, ISM-10-15-17, ISM-10-15-51, ISM-10-15-77, ISM-10-15-82, ISM-10-15-101, ISM-10-15-105, ISM-10-15-109, ISM-10-15-119, ISM-10-15-154, ISM-10-15-167, ISM-10-15-177, ISM-10-15-180 and ISM-10-15-195, 11 lines with *Pi1+Pi54* and one line with *Pi2+Pi54* combination were also identified. Improved versions of 28 lines with *Pi1*, 25 lines with *Pi2* and 30 lines with *Pi54* were developed and suggested to be evaluated in hot spots with differential isolates. The improved lines serve as valuable genetic stocks and valuable as donors for blast resistance and also useful for pyramiding blast resistance genes in rice breeding programmes.

Acknowledgements
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