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RESEARCH ARTICLE

Improving bacterial blight and blast resistance in aromatic medicinal rice through marker-assisted selection

S Thirumalraj[1], J B Antony[2], R P Gnanamalar[3], S Manonmani[4], C Gopalakrishnan[4], M Raveendran[1] & J Ramalingam[1*]

[1]Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore 641 003, Tamil Nadu, India
[2]Department of Genetics and Plant Breeding, Tamil Nadu Agricultural University, Coimbatore 641 003, Tamil Nadu, India
[3]Department of Plant Breeding and Genetics, Tamil Nadu Agricultural University, Madurai 625 104, Tamil Nadu, India
[4]Department of Rice, Tamil Nadu Agricultural University, Coimbatore 641 003, Tamil Nadu, India

*Correspondence email - ramalingam.j@tnau.ac.in

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Abstract

Rice is an important food crop worldwide, especially in Asia. Rice growers face serious challenges due to major diseases such as blast and bacterial blight (BB), which result in significant yield losses and substantial economic hardship. To mitigate the occurrence of these two diseases, this study used MAS by pyramiding four resistance genes, namely *Pi54* for blast resistance and *Xa21*, *xa13* along with *xa5* for bac BB resistance, from a resistant donor Improved CO 51 into the aromatic medicinal rice of northeastern India. Chakhao Amubi was crossed with the donor and true F_1 plants were advanced to the F_2 generation. F_2 generation's molecular screening identified individuals homozygous for the blast-resistant gene *Pi54*, along with one or two blight-resistance genes. The goodness of fit had been also tested utilizing χ^2. The selected plants were then screened in the F_3 generation for these genes. Eleven F_3 families were identified and evaluated under disease screening for their resistance against blast as well as BB. In the field screening, the pyramided lines exhibited greater resistance to BB and blast as well as a wider range. These lines can be further advanced to develop superior lines that could be utilized as donors for disease resistance or potentially released as a variety following yield evaluation.

Keywords: bacterial blight; blast; Chakho Amubi; marker-assisted selection; rice

Introduction

Rice is the most widely grown cereal crop globally, alongside wheat and maize. In India, demand for rice has been projected to reach 130 MT by the year 2030 and 160 MT by 2050, driven by a daily per capita consumption of 189 g and a population growth rate of 1.8 % annually. To meet this increasing demand, enhancing rice productivity and production are crucial (1).

However, rice yields are often severely impacted by both abiotic and biotic stresses, with BB being one of the most destructive biotic stresses. This disease, caused by *Xanthomonas oryzae* pv *oryzae* (Xoo) can result in severe yield loss (2). Earlier reports have assessed the impact of BB and found that it could lead to yield reductions of up to 50 %, influenced by factors such as variety, growth stage, geographical location and environmental conditions that promote the spread of the infection (3). To combat this, researchers have identified 48 resistance genes from various rice sources (3,4). Among them, the *Xa21* gene derived from wild African rice species *Oryza longistaminata* is particularly notable. The *Xa21* gene is effective in conferring broad-spectrum resistance against BB (5, 6).

Rice blast disease, which is caused by *Magnaporthe oryzae* is another major danger to rice yields, with potential losses of 70 - 80 % in extreme cases. A total of approximately 146 resistance genes (R-genes) have been identified, each playing a vital role in the disease resistance (7). The *Pi54* gene, derived from the Tetep variety, has demonstrated significant resistance to Indian strains of rice blast (8). Moreover, the long-term effects of climate change are altering the responses of pathogens to changes in temperature and humidity that affect their growth, reproduction, spread and the degree of damage they cause (9).

Controlling BB and rice blasts through cultural, biological and technological methods often faces challenges due to compatibility issues with existing crop production systems or regulations (10). Additionally, the extensive use of broad-spectrum pesticides not only harms the environment but also encourages the development of resistance (11). As a result, the cultivation of disease-resistant rice varieties emerges as the most practical and cost-effective solution.

MAS has revolutionized plant breeding by facilitating the precise integration of resistance genes into high-yielding rice varieties. Resistance genes such as *Pi54, Pi1, Pi2, Pi9* (for rice blast) and *xa5, xa13, Xa21* (for BB) have been successfully incorporated to enhance disease resistance (12, 13). The strategy of pyramiding multiple resistance genes further strengthens disease management and ensures long-term effectiveness (14). Moreover, resistant lines can be easily identified in early generations, which reduce both time and costs by narrowing down the lines that proceed to the next breeding phase. Consequently, this study aims to identify rice lines with disease resistance using molecular marker-assisted technology at an early stage of the breeding cycle, focusing on developing multiple gene-pyramided rice varieties for sustainable agriculture.

Material and Methods

Genetic information / Plant material

The schematic flow of the work is shown in Fig. 1. Crosses were made between the improved Chakhao Amubi and CO 51 varieties. True hybrids in the F_1 generation were identified using polymorphic markers and then advanced to the F_2 generation. These selected hybrids were self-pollinated to produce the F_2 generation, where MAS was applied to identify resistance alleles. A total of 188 F_2 plants were analyzed through MAS and 165 plants were genotyped in the F_3 generation. The pyramided lines, confirmed in both F_2 and F_3 generations, were evaluated for resistance to *Xoo* and *Magnaporthe grisea* isolates. Taichung Native 1 (TN1) was used as the susceptible control for both blast along with BB, while Aduthurai 55 (ADT 55) had been utilized as the resistant control. The experiment was conducted and all generations were raised at the Department of Rice, Tamil Nadu Agricultural University (TNAU), Coimbatore.

Molecular screening

The DNA of all F_2 individuals and F_3 progenies was isolated from young plant tissues using the CTAB protocol. The molecular markers that were employed to screen the F_2 and F_3 lines through PCR are listed in Table 1. The PCR cycle was programmed as follows: 94 °C temperature kept for 5 min, after that 35 cycles of 94 °C temperature for 45 sec time period, annealing at the temperature specified in the table for the respective marker for 1 min, 72 °C temperature for 1.5 min, a final extension had been done at 72 °C temperature for 7 min, then cooling at 4 °C temperature. Electrophoresis of amplified PCR products had been performed utilizing a 3 % agarose gel that is stained with ethidium bromide. The stained gel was visualized under a UV transilluminator and the distinct, well-resolved amplicons were scored visually.

Screening for BB and blast

The parental line and selected F_3 families, carrying resistance genes in various combinations from the cross between improved Chakhao Amubi and improved CO 51, were evaluated for resistance against BB and blast utilizing standard protocols. For BB screening, plants were inoculated at the maximum tillering stage using the leaf clip method (15),

Table 1. Target gene and linked/ functional markers used for selection

Gene	Chromosome	Marker	Primer sequence		AT (°C)	Size (bp)	Reference
xa5	5	*xa5*	xa5FM-SF xa5FM-SR xa5FM-RF xa5FM-RR	GTCTGGAATTTGCTCGCGTTCG TGGTAAAGTAGATACCTTATCAAACTGGA AGCTCGCCATTCAAGTTCTTGAG TGACTTGGTTCTCCAAGGCTT	56 °C	424	(26)
xa13	8	xa13-prom	F R	GAGCTCCAGCTCTCCAAATG GGCCATGGCTCAGTGTTTAT	59 °C	500	(27)
Xa21	11	pTA248	F R	ATAGCTAGTTCATAGAGG ACATCCGTCACTCTGCCA	65 °C	925	(28)
Pi54	11	Pi54-MAS	F R	CAATCTCCAAAGTTTTCAGG TCCTTCATACGCAACAATCT	56 °C	216	(29)

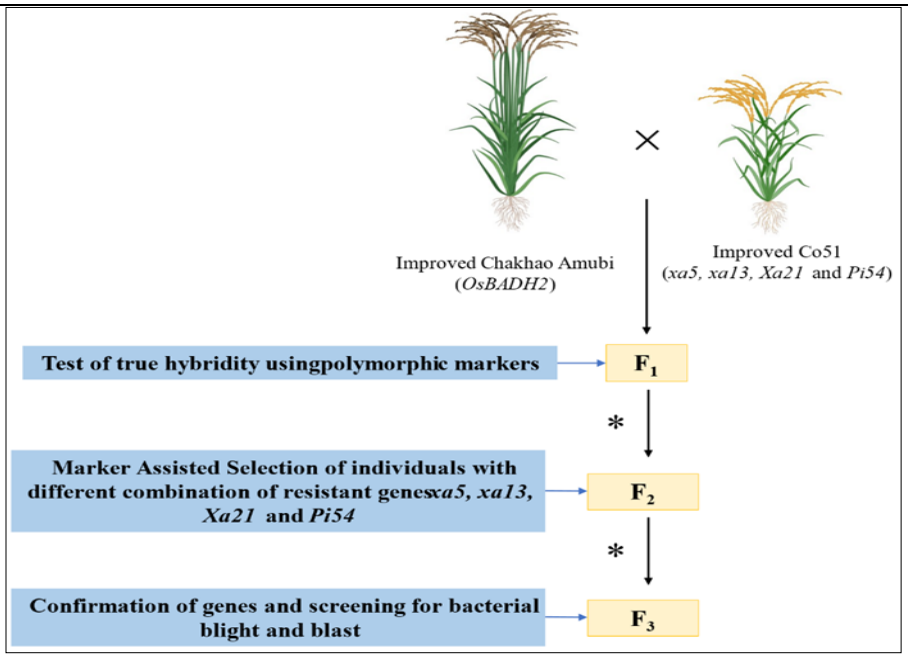

Fig. 1. Schematic representation of work flow used in the study.

a widely adopted technique for evaluating disease resistance. Disease evaluation followed the Standard Evaluation System (SES) for the rice provided by International Rice Research Institute (IRRI) (16). Xoo had been cultured in Peptone Sucrose Agar (PSA) under optimal growth conditions. The bacterial concentration was standardized by measuring the optical density at 600 nm (OD_{600}), where an OD_{600} of 1.0 corresponded to approximately 10^8 CFU/mL. This standardization ensured consistency in bacterial inoculum across all experiments (17).

Inoculated leaves were assessed 18 days post-inoculation using a six-class scoring system (0, 1, 3, 5, 7, 9). Plants having scores of 0 to 1 were classified as the resistant (R), 3 to 5 were identified as moderately resistant (MR), whereas 7 to 9 were regarded as susceptible (S) as described in the SES for rice by IRRI (16). Blast screening was conducted in a sick plot under nursery conditions at the Hybrid Rice Evaluation Centre (HREC), Gudalur. The F_3 families were raised in the nursery along with infector rows planted as borders to facilitate disease spread.

Results

The F_1 hybrid combination of improved Chakhao Amubi and the improved CO 51 variety was cultivated alongside their respective parent lines to confirm the presence of true hybrids. The heterozygosity of polymorphic markers further confirmed the true hybrids. The confirmed F_1 plants were self-pollinated as well as advanced to the F_2 generation.

Segregation analysis of F_2 generation

F_2 generation (Improved Chakhao Amubi x Improved CO 51), consisting of 188 individuals, was cultivated in field as well as subjected to MAS to identify homozygous plants carrying resistance alleles for targeted resistance genes. All F_2 plants were analyzed for four DNA markers associated with resistance genes; *xa5, xa13*, *Xa21*, which confer resistance to BB and *Pi54*, which provides resistance to blast. Observed along with expected frequencies of homozygotes and heterozygotes for each molecular marker were analyzed using the chi-square test to evaluate segregation patterns. The chi-square analysis showed no significant difference at both the 5 % and 1 % levels, as the computed chi-square values were greater than the critical values of 5.99 (5 %), 9.21 (1 %) for all markers (Table 2). For the *Pi54* marker, 30 homozygous plants with functional resistant alleles corresponding to the donor parent were identified along with 86 heterozygotes and 72 homozygous plants with non-functional alleles corresponding to the susceptible parent. Similarly, for the *xa5* marker, 109 plants were homozygous resistant, 68 were heterozygous and 11 were homozygous susceptible, with segregation patterns also showing non-significance.

Selection of plants with BB resistance gene combinations in F_2 and F_3 generation

The pairwise combinations of BB and blast resistance genes identified in the F_2 and F_3 generations are given in Table 3. Overall, 188 F_2 plants had been screened molecularly and 33 of these plants exhibited pairwise combinations of the BB along with blast resistance genes. Plants homozygous for resistant alleles at two marker loci had been identified, while the remaining plants were susceptible at other loci. Specifically, molecular analysis identified 19 plants homozygous for *Pi54* + *xa5*, two plants homozygous for *Pi54* + *xa13*, 11 plants homozygous for *Pi54* + *Xa21* and one plant homozygous for *Pi54* + (*xa5* + *Xa21*). Agarose gel electrophoresis image illustrating the presence of *Pi54, xa13, xa5 and Xa21* in resistant genotypes compared to resistant and susceptible checks are depicted in Fig. 2. These selected plants were self-pollinated and advanced to the next generation for phenotypic evaluation. In the F_3 generation, eleven families were selected from the advanced families with various gene combinations and these families were further used for disease screening.

Phenotypic evaluation for BB and blast resistance

Tests for BB and blast resistance were executed on the parental lines, check varieties and 11 chosen F_3 families that carried resistance genes in distinct combinations. Among these 11 families, six were homozygous for *Pi54* + *xa5*, three were homozygous for *Pi54* + *xa13*, one was homozygous for *Pi54* + *Xa21*, and one was homozygous for *Pi54* + (*xa5* + *Xa21*). Phenotypic evaluation for BB was performed by scoring 10 individuals per family through artificial inoculation and the mean along with the standard deviation (SD) had been computed for each family. Results revealed that families with combinations of resistance genes were significantly more effective against the pathogens compared to those carrying a single resistance gene. Notably, family number #171-6 carrying *Pi54* + (*xa5* + *Xa21*), exhibited a low mean BB score

Table 2. Segregation ratio of the marker genotypes in the F_2 population of Improved Chakhao Amubi and Improved CO 51

Markers	Observed frequency			Total	X^2 (1:2:1)
Pi54- MAS(*Pi54*)	30	86	72	188	20.12
xa13-Prom (*xa13*)	15	16	157	188	343.95
pTA 248 (*Xa21*)	50	7	131	188	230.84
Xa5 (*xa5*)	109	68	11	188	116.55

Table 3. Combination of BB resistant and blast resistant genes identified in the F_2 and F_3 population of Improved Chakhao Amubi × Improved CO 51 cross

Sl. NO.	Generation	Pairwise combination of BB and blast resistance genes	
		Number of lines identified	Gene Combination
1	F_2	19	Homozygote for *Pi54* + *xa5*
2	F_2	2	Homozygote for *Pi54* + *xa13*
3	F_2	11	Homozygote for *Pi54* + *Xa21*
4	F_2	1	Homozygote for *Pi54* + (*xa5* + *Xa21*)
5	F_3	7	Homozygote for *Pi54* + *xa5*
6	F_3	3	Homozygote for *Pi54* + *xa13*
7	F_3	2	Homozygote for *Pi54* + *Xa21*
8	F_3	1	Homozygote for *Pi54* + (*xa5* + *Xa21*)

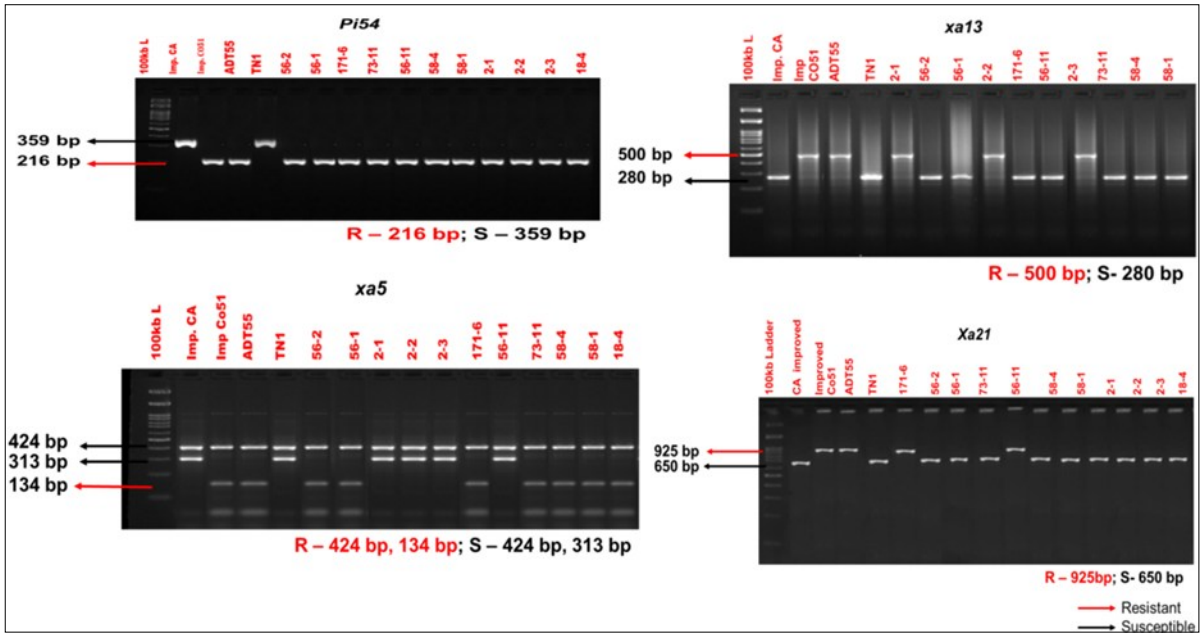

Fig. 2. Blast and BB screening using linked markers *Pi54* (for blast) and *xa13*, *xa5* and *Xa21* (for blight).

(1.60) compared to other progenies. For blast resistance, scoring was conducted at the nursery stage, considering the entire family (Fig. 3). Plants #58-4 and #58-1 recorded a score of 1 for blast resistance and displayed resistance to BB through the *xa5* gene (Table 4). However, the highly resistant family for BB, #171-6, recorded a blast score of 3, indicating comparatively lower resistance to blast (Fig. 4). As expected, the parental lines, Improved CO 51 (donor) and the positive check (ADT 55) showed resistance to both blast and BB. In contrast, susceptible check (TN1) and Improved Chakhao Amubi were both recorded as susceptible for both blast and BB

Discussion

Molecular markers have become essential tools for genetic analysis and crop improvement. The foundation of plant breeding lies in selecting specific plants with desirable traits and gene combinations to develop improved varieties. In cereal crops like rice, frequently utilized pedigree breeding approach focuses on selecting desirable plants for traits with higher heritability starting in early generations. Molecular marker techniques significantly aid breeders in selecting individuals at early stages, reducing the labour and costs associated with advancing large populations.

Molecular MAS in rice is efficiently employed in numerous breeding programs (5,18,19) using linked DNA markers. At every stage of plant growth, segregating populations might be utilized to identify the target genes. Gene pyramiding is a powerful approach, that leverages existing genetic resources to combine multiple beneficial genes, enhancing resistance and improving overall crop performance (20). Multiple resistance genes including *Xa21* and *Pi54*, have been successfully combined into a single genetic background through gene pyramiding; demonstrating the enhanced disease resistance. This approach provided broad-spectrum and durable resistance, making it highly effective for developing rice cultivars capable of withstanding severe disease pressures. These insights from marker-assisted breeding studies underscore the importance of molecular markers as a sustainable strategy for crop improvement. Present study focused on selecting for resistance against BB (Xoo) and blast (*M. grisea)* in the F_2 and F_3 generations of rice using molecular markers connected with the *xa5*, *xa13*, *Xa21*, *Pi54* genes. These genes are well-known for their broad-spectrum resistance against the pathogens (21-24).

The F_2 generation showed considerable variation in molecular markers for both BB and blast, indicating allele segregation at multiple loci consistent with monogenic inheritance for all markers. However, some markers did not conform to Mendelian segregation patterns possibly due to sampling error associated with the minimal number of

Table 4. Phenotypic screening of pyramided F_3 families against blight and blast

Sl. NO.	Plant number	*Pi54*	*xa13*	*xa5*	*Xa21*	BB Score										Mean	SD	Blast Score
						1	2	3	4	5	6	7	8	9	10			
1	56-2	R	S	R	S	7	5	5	7	5	7	7	5	5	7	6.00	1.00	3
2	56-1	R	S	R	S	5	5	7	5	5	3	5	5	5	5	5.00	0.89	3
3	171-6	R	S	R	R	1	1	3	1	1	3	1	1	3	1	1.60	0.92	3
4	73-11	R	S	R	S	5	5	3	3	1	5	3	3	3	3	3.40	1.20	5
5	56-11	R	S	S	R	3	5	3	5	5	3	3	3	3	3	3.60	0.92	3
6	58-4	R	S	R	S	5	3	5	3	3	5	5	5	3	5	4.20	0.98	1
7	58-1	R	S	R	S	5	3	3	5	3	5	5	5	5	3	4.20	0.98	1
8	2-1	R	R	S	S	3	5	5	5	3	3	3	5	5	5	4.20	0.98	5
9	2-2	R	R	S	S	3	3	3	3	5	5	3	3	5	3	3.60	0.92	5
10	2-3	R	R	S	S	3	5	3	5	5	3	3	3	3	3	3.60	0.92	5
11	18-4	R	S	R	S	5	5	3	3	5	3	5	3	5	5	4.20	0.98	5
12	Improved CO 51	R	R	R	R	3	1	1	3	1	3	3	1	1	3	2.00	1.00	1
13	Improved Chakhao Amubi	S	S	S	S	9	9	7	7	7	9	7	9	7	7	7.80	0.98	9

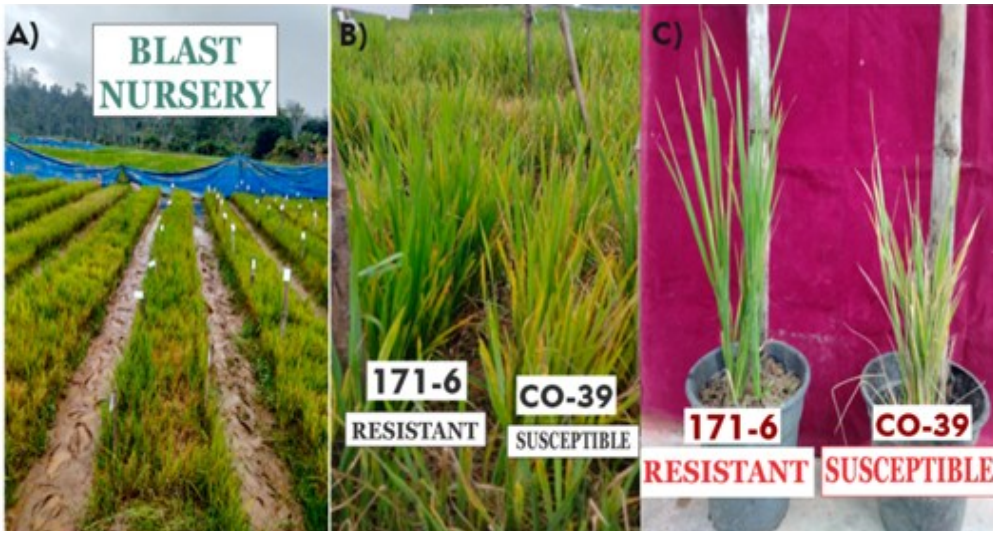

Fig. 3. Blast screening of F_3 families under natural epiphytotic conditions. (A) Blast nursery setup for screening rice genotypes in field conditions. (B) Field evaluation showing resistant (171-6) and susceptible (CO-39) responses to M. oryzae infection. (C) Pot experiment confirming the resistance of 171-6 and susceptibility of CO-39 under controlled conditions.

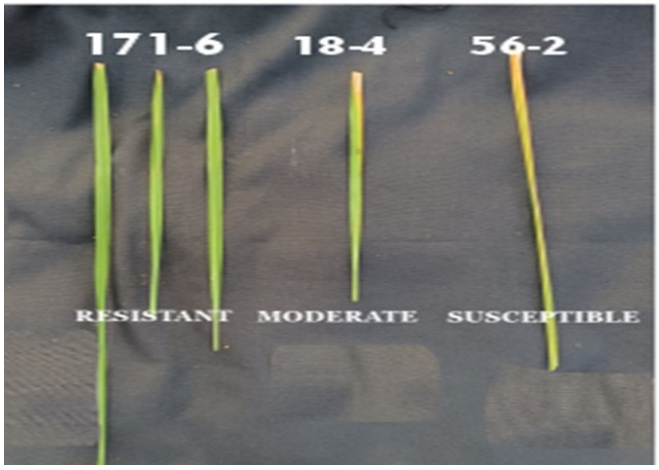

Fig. 4. BB scores in the F_3 population. Lines 171-6 (resistant), 18-4 (moderately resistant), and 56-2 (susceptible) show varying disease responses to *Xoo*.

individuals in the F_2 population. Despite this, individuals homozygous for both blast resistance and BB resistance genes had been detected in F_2 generation. The segregation ratios observed in the F_2 population reflect typical inheritance patterns of BB resistance genes including *Xa21* and *Xa4*, along with the blast-resistant gene *Pi54*, indicating stable gene expression and Mendelian inheritance. Molecular markers used in the study successfully identified homozygous resistant gene combinations in the early generation of F_2 and confirmed the presence of homozygosity in F_3 generation. These results emphasize the critical role of early-generation selection in achieving stable resistance to BB. A comparable pattern was observed for blast resistance, where targeted selection under appropriate disease pressure successfully fixed favourable alleles by the F_3 generation.

The phenotypic evaluation of F_3 families with various marker combinations revealed that families carrying more than one BB resistance gene (*xa5*+*Xa21*) along with *Pi54* in a homozygous dominant condition exhibited higher resistance to BB compared to families carrying only a single BB resistance gene (*xa5*, *xa13*, or else *Xa21*) along with *Pi54*. This finding suggests that the existence of multiple BB resistance genes in an individual provides significantly higher resistance and is more advantageous than the introgression of a single resistance gene. The pyramiding of multiple resistance genes provides enhanced protection against BB compared to single -gene combinations; however, variations were observed in blast resistance where all families even though possessed *Pi54* in a homozygous state, which is likely due to influence of the genetic background (25). This highlights need to comprehend the minor genes' role or the genetic background that affects the expression of blast-resistance genes. Such insights are crucial for the effective utilization of these genes in developing rice cultivars with the durable resistance.

Among eleven families screened in the F_3 generation, one family (#171-6) had been identified to be highly resistant to the BLB and moderately resistant to blast. Two families (#58-1, #58-4) exhibited moderate resistance to the BB and high resistance to blast, while one family (#73-11) showed moderate resistance to both BB and blast. These families can be advanced to the next generation to achieve phenotypic homozygosity, facilitating the selection of superior lines for yield improvement. Lines with both high yield and strong resistance can be considered for further breeding programs or even released as new varieties, contributing to improved crop performance. Conducting multi-location trials of the genotypes with distinct combinations of genes across multiple crop cycles would be crucial for identifying lines with durable resistance.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process : The author used the Turnitin AI language editing tool to check grammar. After using this tool, the author(s) reviewed and edited the content as needed and take full responsibility for the publication's content.

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