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Identification of Bacterial Leaf Blight Resistance (*Xanthomonas oryzae* pv. *oryzae*) Genes in Advance Breeding Lines of Rice (*Oryza sativa* L.) through Molecular Markers

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ABSTRACT

The present study was conducted *kharif* (July–October, 2023) at Department of Genetics and Plant Breeding, IGKV, Raipur, Chhatisgarh, India to determine the presence of three Bacterial leaf Blight genes (*xa5, xa13* and *Xa21*) in rice by utilizing the gene specific markers in the advance breeding lines. This experiment utilized 37 advance breeding lines from a cross having suitable donor parent (for resistant BLB genes), and the developed lines were further utilized to determine the presence of resistance genes. MAS for three BLB genes was done using gene specific markers namely viz., pTA248, xa13 prom, *xa13*, xa5R, Xa21, RM13, RM31 and RM122. Among these eight gene specific markers three markers pTA248 for the *Xa21* gene, xa13 prom for the *xa13* gene and xa5R marker for the *xa5* gene, were shown to be polymorphic across donor and recurrent parents. These three markers were utilized to detect the presence of resistance genes in the population. The results revealed that eight plants have been present all the three genes while rest plants have been found two or single gene. 13 plants were found positive for two gene combination (*Xa21+xa13*) while 12 plants were found for gene combination (*Xa21+xa5*) and 10 plants were found gene combination of (*xa13+xa5*). These two gene combination and 3 gene combination plants will be further utilize as breeding materials for development of BLB resistant cultivars.

KEYWORDS: Rice, bacterial leaf blight, MAS, pyramiding

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

Rice is the world's principal food source and feeds more than half of the world's population (Tanvi et al., 2018), mostly in tropical and subtropical Asia (Danaisilichaichon et al., 2023). It ranked second in global agriculture; it generates employment and revenue in rural areas while accounting for more than one-fifth of the calories consumed by 3 billion people. Rice is farmed over an area of 165.04 mha with a global production of 776.46 mt of paddy. Asia produces and consumes about 90% of the world's rice (Fiyaz et al., 2022),with India contributing 22% to the global rice production (Annonymous 2021). With 46.3 mha under cultivation and an annual yield of 130.29 mt of paddy in 2022 (Annonymous, 2023a), India leads the world in both area and production (Dileep Kumar et al., 2023).

Chhattisgarh has 43.48 lakh hectares, yielding 13.23 mt with productivity of 3045 kg ha^{-1} (Anonymous, 2023b). Rice is also the main food crop of the Chhattisgarh state, and most of its economy depends on rice production and procurement (Sao et al., 2024a).

To fulfill the demands of a growing population and rising incomes, the world's supply of staple cereal grains, such as wheat, maize, and rice, will have to double over the next three decades (Sao et al., 2024b). However, the impending threats of human-caused climate change, which increases biotic and abiotic stressors on agriculture (Desari et al., 2022), make this critical aim much more difficult to attain. Plant breeders and farmers are now dealing with the immediate consequences of climate uncertainty.

However, a large number of diseaseslike bacterial, viral, and fungal origins limit the amount of rice that can be produced. One of the most destructive diseases that affects entire rice acreages is Bacterial Leaf Blight (BLB) (Fiyaz et al., 2016), which is caused by the gram-negative proteobacterium, *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) Dixit et al. (2020). Depending on the crop's stage, cultivar susceptibility, and environmental factors, BLB can result in severe yield losses of 20-100% (Kumar et al., 2023; Das et al., 2022).

Rice disease, characterized by Kresek and leaf blight (Fiyaz et al., 2022), affects rice growth stages and results in significant yield losses and grain quality issues. The leaf blight phase is the most distinctive and frequently seen, affecting photosynthetic area (Balakrishanan et al., 2022), output, and incomplete grain filling (Baliyan et al., 2016; Sombunjitt et al., 2017).

Control of Bacterial Leaf Blight through Chemicals is ineffective (Yen at al., 2021) thus; the best, most affordable, and ecologically safe way to manage BLB is through host-plant resistance (Sombunjitt et al., 2017). Researchers are working on creating resistant cultivars and finding genes to protect against BLB. A total of 46 bacterial leaf blight resistance genes have been discovered, and some of them have been introduced into popular high-yielding rice varieties (Hsu et al., 2020; Chukwu et al., 2019).

Marker-assisted selection (MAS) is a revolutionary approach to plant breeding that uses gene pyramiding to improve broad-spectrum resistance. The marker-assisted selection (MAS) has many advantages, such as identifying the true plants and producing no false positive results compared to the conventional methods.

Many prominent Indian rice cultivars are experiencing issues owing to biotic and abiotic stressors (Chen et al., 2020). To achieve durable resistance, many genes must be pyramided together. It is therefore, important to develop long durable BLB resistant rice cultivars (Swathi et al., 2019). This study emphasizes current research on the determination of major resistance genes in advance breeding lines and their application in rice breeding programs.

2. MATERIALS AND METHODS

The experiment was conducted from *kharif* (July–October) 2023 in the experimental field of the Department of Genetics and Plant Breeding, and all the molecular analysis work was done at the NQ LAB, Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidalaya Raipur (C.G.), India.

The present experiment consisted of 37 advance breeding lines (G_1-G_{37}) to study the presence of BLB resistance genes. The elite line R1853-105-1-82-1 had good agronomic characters along with good grain quality but is susceptible to BLB and was selected as a susceptible check, while Improved Samba Mahsuri (ISM) having three genes for resistance to BLB (*xa5, xa13,* and *Xa21*) was used as a resistance check.

2.1. DNA extraction

DNA samples were extracted from rice plants using the CTAB (CetylTrimethyl Ammonium Bromide) method as described by Doyle and Doyle, 1990. The quantity of the DNA samples was determined by using a nanodrop (spectrometer), and the quality of the DNA was checked by electrophoresis analysis under 0.8% agarose gel in 1X TAE solution. A DNA sample with high purity was stored at -20°C.

2.2. Molecular analysis

Eight gene-specific markers were used to identify advanced rice breeding lines carrying the *xa5*, *xa13*, and *Xa21* resistance genes. Of these markers, four (50%) showed polymorphism between susceptible and resistant checks. The pTA248, xa13 prom, and xa5R markers were successfully used to identify positive plants. The results revealed that eight plants

possessed all three genes, while others exhibited various two-gene combinations, demonstrating their potential for breeding BLB-resistant cultivars (Table 1).

Table 1: List of SSR markers used for studying polymorphism in rice genotypes

Sl. No.	Primer	Forward sequence	Reverse sequence	Ch.no
1.	xa5R	AGCTCGCCATTCAAGTTCTTGAG	TGACTTGGTTCTCCAAGGCTT	5
2.	RM13	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	5
3.	RM122	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGA	5
4.	RM31	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	5
5.	xa13Pro	GGCCATGGCTCAGTGTTTAT	GAGCTCCAGCTCTCCAAATG	8
6.	Xa13	GGCCATGGCTCAGTGTTTAT	GAGCTCCAGCTCTCCAAATG	8
7.	pT248	AGACGCGGAAGGGTGGTTCCCGGA	AGACGCGGTAATCGAAGATGAAA	11
8.	RM21	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	11

Table 2: PCR mix for one reaction (Volume 10 µl)

Reagent	Concentration	Quantity
Template DNA	40 ng μl^{-1}	1.5 µl
PCR Master mix (Taq DNA polymerase, dNTPs, $MgCl_2$, optimized buffer, gel loading dye (green), and a density reagent)	-	5.0 µl
Forward primer	10 µM	0.5 µl
Reverse primer	10 µM	0.5 µl
Sterile water	-	2.5 µl
Total volume		10 µl

conducted using the following settings: 30 seconds of 94°C denaturation, 30 seconds of primer extension at 72°C, and 30 seconds of annealing at 55°C to 58°C, depending on the primer. A final extension took place for seven minutes at 72°C. The following thermal regimes were used for the polymerase chain reaction (PCR) in a thermal cycler (Applied Boisystems and Thermo Fischer Scientific, USA) (Table 3).

Resolution of PCR products is most commonly achieved by agarose gel electrophoresis. Agarose gel concentration is selected based on product size. A 2.5% agarose gel was made in 1XTBE. After cooling the solution, 5 µl 100 ml^{-1} of ethidium bromide was added. The amplified PCR products were loaded onto a gel electrophoresis along with a standard low-range DNA ruler (100–1000 bp) to determine the expected size of the DNA. The DNA profile was recorded using a geldocumentation unit (Model Alpha Imager 1200, Alpha InfoTech Corp., USA) following the completion of the electrophoresis.

3. RESULTS AND DISCUSSION

The PCR reaction mixtures were made in accordance with Table 2. After five minutes of initial denatured template DNA at 94°C, 35 cycles of PCR amplification were

Table 3: Temperature profile used for PCR amplification using SSR primers

Profile	Activity	Temperature (°C)	Duration (min.)	Cycles
1.	Initial denaturation	95	5	1
2.	Denaturation	94	1	35
3.	Annealing	55 & 58	1	35
4.	Extension	72	1	35
5.	Final extension	72	7	1
6.	Storage	4	-	1

3.1. Validation and parental polymorphism survey

Marker-assisted selection requires co-segregation of gene with molecular marker, polymorphic marker between parents, and confirmation of disease resistance before crossover programme begins. The investigation involved testing a donor parent in *kharif* 2021 for target resistance genes using previously published molecular markers, providing detailed information on linkage group and allele size on Table 4. The three primers, pTA248 for the *Xa21* gene, xa13 prom for the *xa13* gene and xa5R marker for the *xa5* gene, were shown to be polymorphic across donor and recurrent parents. These markers were all used for the determination of positive plants.

The primer pair Xa13 prom produced a 490 bp fragment in the resistant check (ISM) and a 290 bp fragment in the susceptible check (R1853-105-1-82-1). The primer pair pTA248 amplified segments of 950 bp in the resistant check (ISM), but only 700 bp in the susceptible check (R1853-105-1-82-1). The primer pair xa5R produced a 150 bp fragment only in the resistant parent ISM and no fragment in the susceptible check R1853-105-1-82-1 (Figure 1).

Table 4: Details of the molecular markers used for determination of positive plants

Sl. No.	Gene	Marker	Chr. No	Amplicon size (bp)	Trait	Reference
1.	*Xa21*	pTA248	11	950	BLB resistance	Huang et al. (1997)
2.	*xa13*	Xa13 prom	8	490	BLB resistance	Hajira et al., 2016
3.	*xa5*	xa5R	5	150	BLB resistance	Sundaram et al., 2014

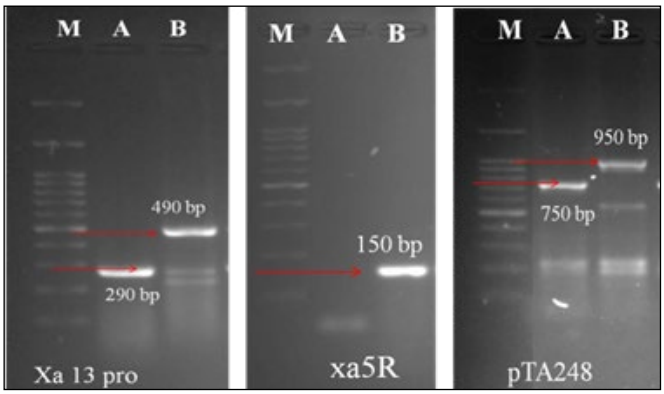

Figure 1: Marker validation in the parents for target genes with gene specific markers; M=100 bp, A-R1853-105-1-82-1, B-ISM

The study found that gene-based markers accurately identified gene-positive plants at all phases of MABB, distinguishing between resistant and susceptible lines and separating resistance alleles in homozygous or heterozygous conditions.

3.2. Determination of the presence of xa5 gene

The xa5R marker was used to identify the *xa5* resistance gene in the rice population. Before application, the marker was validated against resistant and susceptible checks. Amplification of the xa5R marker revealed only the resistant allele in the population. Parental polymorphism confirmed the presence of the resistant allele at 150 bp, which was absent in susceptible lines (Figure 2). The resistant allele was further confirmed in ISM at 150 bp.

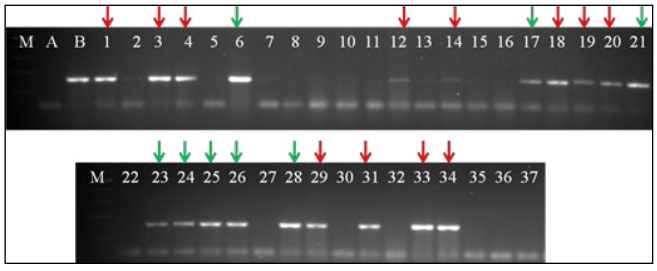

Figure 2: Determination of *xa5* gene using gene specific marker *xa5R*; M=100 bp, A: Recipient line, B: Donor line, Arrow indicates a 'Positive' plant for *xa5* gene, (Green Arrow indicates a 'Positive' plant for all three genes)

The presence of the bands for xa5R markers revealed the resistant alleles on them, while no bands show the absence of the resistant allele. Only 18 plants from the 37 advanced populations confirmed positive for the target genes (Table 5). These 18 plants were verified to contain the *xa5* gene and were recognized as authentic representatives of the parental plants.

The similar results were reported by Sundaram et al., 2011, and Pradhan et al., 2023; they reported presence of a resistant allele at 150 bp when xa5R marker was applied. The resistant gene was located on chromosome 5 of rice.

3.3. Determination of the presence of xa13 gene

The xa13 prom marker has been previously used to identify the *xa13* resistance gene in rice populations. In our study, PCR amplification of the xa13 prom marker revealed two alleles; a resistant allele of 490 bp and a susceptible allele of 290 bp (Figure 3). The resistant allele was validated in the resistant check ISM, and its size was confirmed using a 100-bp ladder. The resistant allele was reported on the chromosome 8 of the rice plant. Similar results have been reported by Sundaram et al., 2008 and Chukwu et al., 2019.

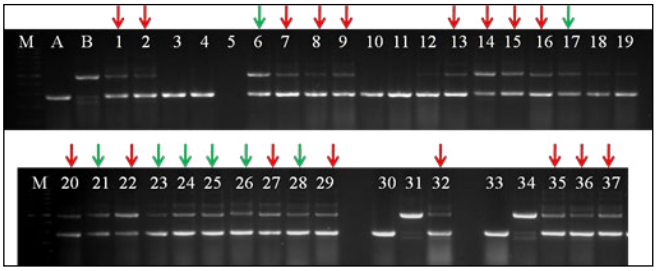

Figure 3: Determination of *xa13* gene using gene specific marker xa13 prom; M=100 bp, A-Recipient line, B-Donor line, Arrow indicates a 'Positive' plant for *xa13* gene, (Green Arrow indicates a 'Positive' plant for all three genes)

Out of the 37 advanced populations, only 24 plants tested positive for the presence of the target genes (Table 5). These 24 plants were confirmed to carry the *xa13* gene and were identified as true representatives of the parental plants. The plants containing the resistance genes remained genetically consistent with the parental lines, while the remaining plants either underwent selfing or lacked the resistance genes. The absence of these genes can be attributed to allelic segregation.

3.4. Determination of the presence of Xa21 gene

Marker pTA248 was utilized to identify the BLB-resistant plants for the presence of *Xa21*. 950 bp was the amplified PCR product size for the resistant allele of ISM, while the

Table 5: Estimation of different gene combinations

Sl. No.	Gene combinations	No. of resistant plant	Plant with resistant gene
1.	*xa5*	18	1, 3, 4, 6, 12, 14, 17, 18, 19, 20, 21, 23, 24, 25, 26, 28, 29, 31
2.	*xa13*	25	1, 2, 6, 7, 8, 9, 13, 14, 15, 16, 17, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 32, 35, 36, 37
3.	*Xa21*	18	3, 4, 6, 13, 15, 16, 17, 18, 21, 22, 23, 24, 25, 26, 27, 28, 31, 34
4.	*Xa21+xa13*	13	6, 13, 15, 16, 17, 21, 22, 23, 24, 25, 26, 27, 28
5.	*Xa21+xa5*	12	3, 4, 6, 17, 18, 21, 23, 24, 25, 26, 28, 31
6.	*xa13+xa5*	10	1, 6, 17, 21, 23, 24, 25, 26, 28, 29
7.	*Xa21+xa13 +xa5*	8	6, 17, 21, 23, 24, 25, 26, 28

susceptible allele was found at 750 bp when it was observed under the 2.5% agarose gel electrophoresis.

Out of the 37 advanced breeding lines, 18 were identified as having the *Xa21* gene (Table 5). These positive plants displayed double bands in the gel images (Figure 4), indicating the presence of both alleles of the *Xa21* gene. In contrast, single bands suggested the absence of these genes. The positive plants were chosen for further development and utilization in breeding programs. Previous studies by Sundaram et al. (2008), Pradhan et al. (2016), Chukwu et al. (2019), and Dileep Kumar et al. (2023) also reported the presence of a resistant allele of the *Xa21* gene at a size of 900-950 bp.

Analysis of the 37 advanced breeding lines identified eight plants possessing all three genes (*Xa21, xa13*, and *xa5*).

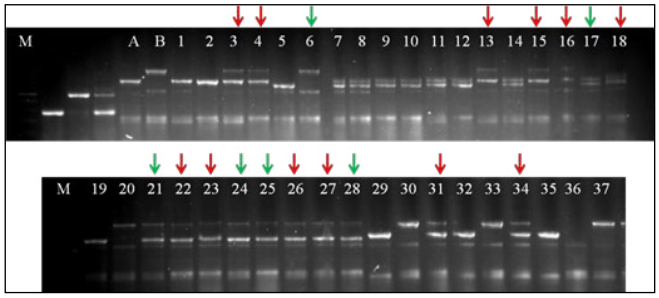

Figure 4: Determination of *Xa21* gene using gene specific marker pTA248; M=100 bp, A-Recipient line, B-Donor line, Arrow indicates a 'Positive' plant for *Xa21* gene, (Green Arrow indicates a 'Positive' plant for all three genes)

Additionally, 13 plants were found to have the *Xa21* and *xa13* genes, while 12 plants exhibited the *Xa21* and *xa5* genes, and 10 plants had the *xa13* and *xa5* genes (Table 5). These plants with two or three gene combinations were promising candidates for further breeding efforts to develop cultivars resistant to bacterial leaf blight.

4. CONCLUSION

This study investigated the presence of three bacterial leaf blight (BLB) resistance genes (*xa5*, *xa13*, and *Xa21*) in a population of rice plants. While eight plants possessed all three genes, a significant number exhibited two-gene combinations (*Xa21+xa13*, *Xa21+xa5*, and *xa13+xa5*). The identification of these plants with multiple resistance genes highlights their potential as valuable breeding resources for developing BLB-resistant rice cultivars.

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