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Detection of Bacterial Blight Resistance Gene in Some Cultivated Rice Varieties Using Gene Based Molecular Markers

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Abstract

Bacterial leaf blight (BLB), caused by Xanthomonas oryzae pv. Oryzae, known as Xoo in short, stands as one of the oldest and most destructive diseases affecting rice in Bangladesh. Despite this longstanding challenge, developing highly resistant rice varieties against this disease remains elusive. To address this gap, we conducted a study examining 15 cultivated rice varieties to assess their disease reaction status against a highly virulent strain of Xoo. Using five sequence-tagged site (STS) markers that correspond to the Xa4, Xa5, Xa13, Xa21, and Xa23 genes, our investigation aimed to identify potential candidate resistant (R) genes that may be responsible for the resistant reaction. After the pathogenicity test, the results revealed 1 cultivated rice variety (BRRI dhan101) showed resistant reaction, 1 as moderately resistant, 2 as moderately susceptible, 8 as susceptible, and 3 as highly susceptible. Further molecular analysis of the 15 rice varieties revealed that 9 carried the Xa4 gene, 1 carried the Xa5 gene, and 1 carried the Xa21 gene. Interestingly, various gene combinations, ranging from 1 to 2 genes, were observed among the rice varieties, with 2 varieties carrying 2-gene combinations and 13 carrying single genes. Notably, the BRRI dhan101 rice variety, which exhibited resistance, harbored the most effective combination of Xa4 and Xa21 genes against the Xoo strain. The findings of this study hold promise in enriching and diversifying the rice gene pool, offering potential candidates for the development of durable resistant varieties against BLB in Bangladesh.

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Introduction

Rice (*Oryza sativa* L.) stands as the second most crucial global field crop, serving as a staple food for nearly half of Asia's population (Singh *et al*., 2020). In Bangladesh, where rice production is pivotal for food security and economic development, the crop contributes significantly to the country's GDP (BER, 2019; Jamal *et al*., 2023). Despite ranking third globally in rice production, Bangladesh faces challenges in meeting escalating demand due to limited arable land (BBS, 2022). Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* known as *Xoo* in short, emerges as a major threat to rice production in Bangladesh. Recent epidemics have led to substantial yield losses, ranging from 5.8% to 30.4% (Ansari *et al*., 2020). Current control measures, including cultural practices, chemical and biological controls, and disease forecasting, exhibit limitations in effectively managing BLB (Bharani *et al*., 2010). Developing BLB-resistant rice varieties has become imperative, and identifying resistant genes is a crucial step in this process. Over 46

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resistant genes have been identified, with notable ones like Xa4, Xa5, Xa7, Xa13, Xa21, Xa23, and Xa33 widely utilized in breeding programs (Chen *et al*., 2020). Breeding lines incorporating multiple resistant genes offer a broader spectrum and durable resistance to BLB (Panwar *et al*., 2018). Molecular marker technologies play a pivotal role in this context, aiding conventional breeding efforts and facilitating the identification of desirable germplasms. These technologies contribute to the development of resilient rice cultivars with enhanced resistance to BLB (Wang *et al*., 2015). In summary, addressing the challenges posed by BLB in Bangladesh's rice production requires innovative strategies, with molecular marker technologies providing essential tools for the development of resistant rice varieties (Akter *et al*., 2022). These advancements align with the goals outlined in initiatives like 'Rice Vision 2050' to ensure a resilient rice system in Bangladesh (BDP, 2018).

Materials and Methods

Collection of germplasm

A total of 15 cultivated rice varieties (Table 1) were collected from Bangladesh Rice Research Institute (BRRI), Bangladesh Institute of Nuclear Agriculture (BINA), Bangladesh Agricultural University (BAU). From the International Rice Research Institute (IRRI), Philippines, two susceptible lines, IR24 and TN1, and one pyramided resistant line, IRBB60 (*Xa4, Xa5, Xa13,* and *Xa21*), as well as five monogenic lines viz., IRBB4 (*Xa4*), IRBB5 (*Xa5*), IRBB13 (*Xa13*), IRBB21 (*Xa21*), IRBB23 (*Xa23*) resistant to bacterial leaf blight and containing the R gene were also obtained.

Inoculum preparation

One major highly virulent strain of *Xanthomonas oryzae* pv. *oryzae*, was collected from Plant Pathology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh and used for varietal resistance screening based on lesion length developed on inoculated leaves. Bacterial (Xoo) inoculums were cultured for 48 hours at 28°C on Nutrient Broth Yeast Extract (NBY) agar medium. Distilled water was used to dilute the inoculum, and the absorbance was adjusted to roughly OD600 = 1. This absorbance value equates to a concentration of about 108 colony-forming units per milliliter (CFU/mL), which typically gives the host best possible infection with *Xoo*. (Haque *et al*., 2022).

Resistance screening based on lesion length

To facilitate evaluation of bacterial leaf blight resistance of 15 cultivated rice varieties, rice leaves were inoculated with the inoculum of *Xoo* strain according to Haque *et al*. (2021). In short, in a seedling nursery, seeds of cultivated rice varieties were sown alongside control varieties like IR24, TN1, and IRBB60 (shown in Table 1) during the rainfed season (Aman) 2023. Twenty-one days old rice seedlings were transplanted to experimental fields at Plant Pathology Division, BINA, Mymensingh (Longitude: 24.7232° N, Latitude: 90.4316° E) using randomized complete block design (RCBD). Applying the leaf clipping method, rice plants were inoculated at the maximum tillering stage (Kauffman *et al*., 1973). Following inoculation, plants were observed every 24 hours to record the emergence of disease. 21 days following inoculation, data on disease severity (lesion length) were collected

Table 1. Name of cultivated rice varieties

Sl No.	Name	Sl No.	Name	Sl No.	Name
1	BAU dhan1	6	BRRI dhan49	11	BRRI dhan89
2	BAU dhan3	7	BRRI dhan50	12	BRRI dhan101
3	BRRI dhan28	8	BRRI dhan58	13	BINA dhan17
4	BRRI dhan29	9	BRRI dhan64	14	BINA dhan20
5	BRRI dhan33	10	BRRI dhan74	15	BINA dhan23

from 15 leaves per entry. Highly resistant >1 cm (Score 0), resistant 1.1-3 cm (Score 1), moderately resistant 3.1-5 cm (Score 3), moderately susceptible 5.1-12 cm (Score 5), susceptible 12.1-20 cm (Score 7) and highly susceptible <20 cm (Score 9) were the categories assigned to the entries based on the severity of the disease (Huang *et al*., 2012; Xu *et al*., 2012). Using a scale, the lesion length covering the entire infected area of a leaf was determined.

Molecular screening for bacterial blight resistance genes

Young, fresh leaves were collected from each of the cultivated rice varieties and kept at -20°C in 50 mL falcon tubes. In this study, the modified cetyltrimethylammonium bromide (CTAB) method was used to extract genomic DNA from leaf samples (Hasan *et al*., 2012). The nanodrop spectrophotometer (Jenova Nano, UK) was used to measure the concentration and quality of DNA. Ultimately, the stock DNA solution was diluted using 1X TE buffer to achieve a 100 ng/µL DNA concentration for the working DNA solution, which was then kept at 4°C. To find the resistance gene(s) in the chosen germplasm, polymerase chain reactions (PCR) were used (Anik *et al*., 2022). Five STS markers that are closely linked to the genes *Xa4, Xa5, Xa13, Xa21,* and *Xa23* were used to identify the gene(s) that are resistant to bacterial leaf blight (Table 2).

For analyzing markers, preparation of PCR reaction was performed. Preparation of PCR reaction included 2µL of 100 ng DNA template, 5 µL PCR master mix (GoTaq® G2 Green Master Mix which contains green buffer, dNTPs and 4 mM $MgCl_2$ from Promega Company), 1µL of primer, 2 µL Nuclease free water for making 10µL PCR reactions mixture. In the next step, PCR was run. In this experiment, the PCR machine was operated using the Touch Down protocol, which has three stages. The temperature was brought down to 94°C for two minutes prior to the first phase. Following that, 94°C was set for 45 seconds as denaturation temperature, 60 seconds of annealing at each primer's Tm, and 90 seconds of elongation at 72°C. The procedure was continued for up to 35 cycles. Then the PCR products were stored at 4ºC for further use. Using 1X TBE buffer, the PCR products were resolved in 1.5% agarose gel at 80 V–120 minutes. In order to identify the resistant genes, the monogenic resistant line of the corresponding gene was used as a resistant check, and two susceptible lines, IR24 and TN1, were used as susceptible checks. The gels were visualized under a transilluminator (Bio-Rad, Hercules, CA, USA) and the desired bands (related to resistant and susceptible genes) were viewed on a monitor and saved on the computer memory drive for taking photographs

Table 2. List of five STS markers, resistant genes, and their details

Resistant gene	Primer name	Primer sequences (5′-3′)	Annealing temp.	Resistant band (bp)	Susceptible band (bp)	Reference
Xa4	MP1-F	ATCGATCGATCTTCACGAGG	50˚C	150	120	Sun *et al*., 2003
	MP2-R	TGCTATAAAAGGCATTCGGG				
Xa5	RM122-F	GAGTCGATGTAATGTCATCAGTGC	56˚C	240	230	Chen *et al*., 1997
	RM122-R	GAAGGAGGTATCGCTTTGTTGGA				
Xa13	Xa13prom-F	GGCCATGGCTCAGTGTTTAT	55˚C	560	250	Hajira *et al*., 2016
	Xa13prom-R	GAGCTCCAGCTCTCCAAATG				
Xa21	pTA248-F	ATAGCAACTGATTGCTTGG	55˚C	950	780, 650	Sundaram *et al*., 2014
	pTA248-R	CGATCGGTATAACAGCAAAAC				
Xa23	03STS1-F	CTCGGTTTCCGTCTTCTCAG	55˚C	1200	1350	Wang *et al*., 2015
	03STS1-R	GACTTTGCGTGCTTTCCAGC				

Data analysis

Statistical analysis was performed using Statis.tix 10 software.

Results

Identification of germplasm having bacterial leaf blight resistance

On the basis of the disease reaction pattern (Table 3) of 15 cultivated rice varieties against the virulent strain of *Xoo*, only 1 cultivated rice variety was found resistant (score 1), 1 variety was found moderately resistant (score 3), 2 varieties were showed moderately susceptible (score 5), 8 varieties were showed susceptible (score 7) and 3 varieties were showed highly susceptible (score 9) reaction against bacterial blight disease causing pathogen of rice, *Xoo* (Table 3).

Table 3. Disease incidence, lesion length and disease reaction of *Xoo* inoculated cultivated rice varieties

Sl. No.	Cultivated rice varieties	7 DAI			14 DAI			21 DAI		
		DI (%)	LL (cm)	DR	DI (%)	LL (cm)	DR	DI (%)	LL (cm)	DR
1	BAU dhan1	50de	1.91	R	90b	2.85	R	100	4.83	MR
2	BAU dhan3	60cd	4.07	MR	83.33bc	6.47	MS	100	10.83	MS
3	BRRI dhan28	53.33cde	10.86	MS	86.67bc	15.27	S	100	19.65	S
4	BRRI dhan29	40f	9.87	MS	73.33d	14.35	S	100	19.79	S
5	BRRI dhan33	60cd	4.07	MR	83.33bc	6.47	MS	100	10.83	MS
6	BRRI dhan49	100a	12.01	MS	100a	15.58	S	100	22.78	HS
7	BRRI dhan50	100a	12.01	MS	100a	15.57	S	100	22.78	HS
8	BRRI dhan58	63.33bc	8.34	MS	80cd	12.85	S	100	18.55	S
9	BRRI dhan64	73.33b	8.94	MS	90b	11.1	MS	100	19.68	S
10	BRRI dhan74	100a	11.01	MS	100a	16.57	S	100	23.78	HS
11	BRRI dhan89	46.67ef	8.51	MS	90b	11	MS	100	19.51	S
12	BRRI dhan101	60cd	0.91	HR	73.33d	1.64	R	100	1.97	R
13	BINA dhan17	60cd	8.49	MS	86.67bc	14.29	S	100	19.41	S
14	BINA dhan20	46.67ef	3.22	MR	73.33d	10.93	MS	100	19.75	S
15	BINA dhan23	53.33cde	4.65	MR	90b	11.03	MS	100	19.99	S
Level of Significance		***	-	-	***	-	-	-	-	-
CV (%)		10.4	-	-	6.78	-	-	-	-	-

Here, DAI- Days after inoculation; DI- Disease Incidence; LL- Lesion Length; DR-Disease Reaction; R-Resistance; MR- Moderately Resistance; MS-Moderately Susceptible; S-Susceptible; Highly Susceptible

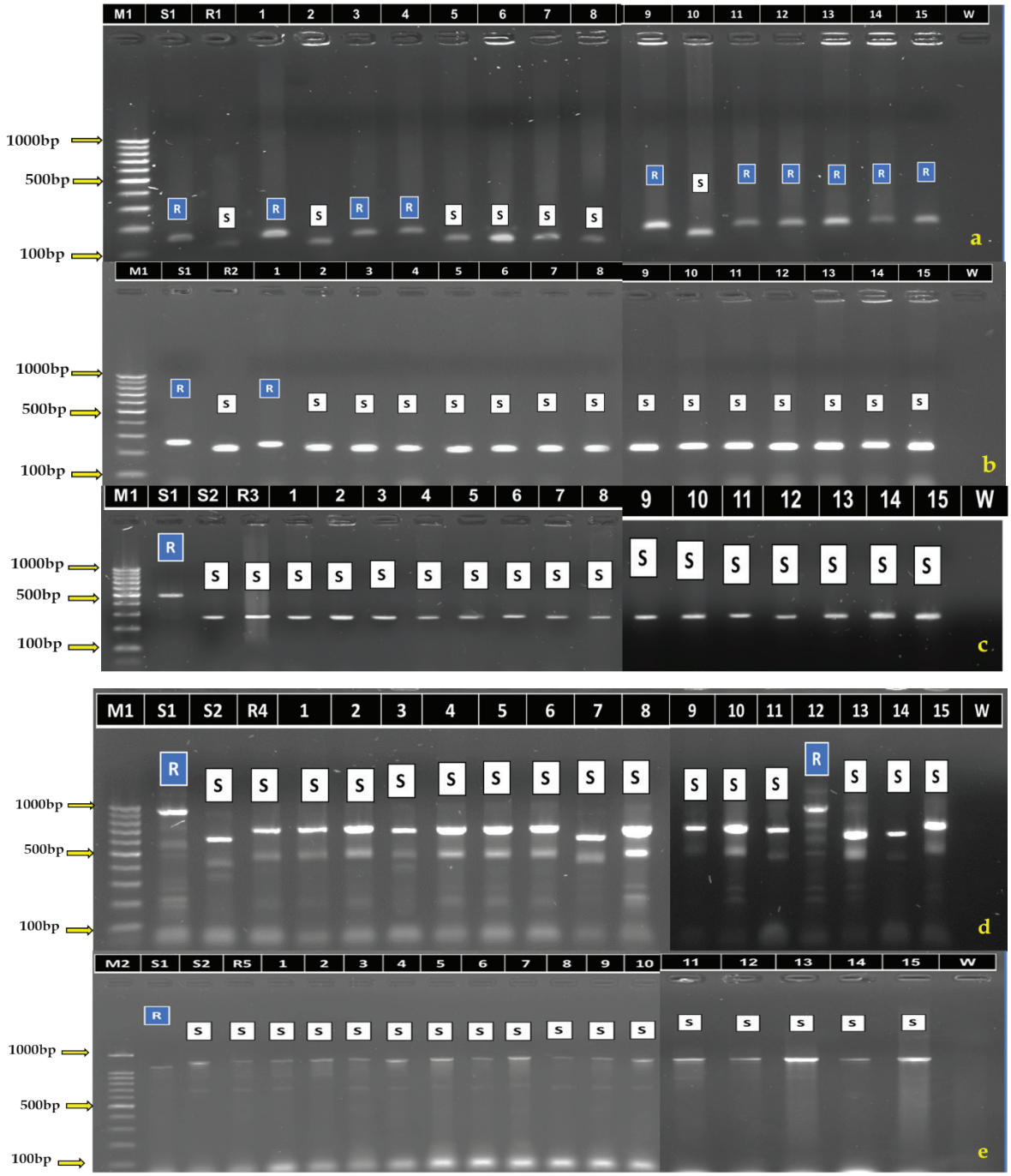

Figure 1. Sample gel images displaying the amplification patterns produced by the various STS markers employed in the study: a. MP1-2 primer (linked to the *Xa4* gene), b RM122 primer (linked to the *Xa5* gene), c. Xa13-prom primer (linked to the *Xa13* gene), d. pTA248 primer (linked to the *Xa21* gene), e 03STS primer (linked to the *Xa23* gene). S1 and S2 is susceptible check IR24 and TN1, R1-IRBB4, R2-IRBB5, R3-IRBB13, R4-IRBB21 and R5-IRBB23. M1/M2 stands for a 100 bp DNA ladder, respectively. The 15 cultivated rice varieties that were studied and listed in Table 1 are represented by the numbers 1 through 15

Identification of the bacterial leaf blight resistant genes

MP1-2 (*Xa4* gene linked) primer detected in 9 rice varieties (60%) as *Xa4* positive and 6 rice varieties (40%) as *Xa4* negative (Figure 1a). On the other hand, RM122 primer (*Xa5* linked) was identified in 1 rice variety (6.6%) harboring *Xa5* allele while 14 rice varieties (93.4%) were found negative (Figure 1b). However, pTA248 primer (*Xa21* gene linked) was detected in 1 rice variety (6.6%) carrying *Xa21* allele while 14 rice varieties (93.4%) as *Xa21* negative (Figure 1d). Notably, Xa13-prom primer (*Xa13* gene linked) and 03STS primer (*Xa23* gene linked) identified no bacterial resistant gene among the studied cultivated rice varieties (Figure 1c and 1e).

Table 4. Presence of bacterial blight resistance gene and disease reaction of *Xoo* inoculated cultivated rice varieties

Sl. No.	Cultivated rice varieties	Resistance gene					Disease reaction at 21 DAI
		Xa4	*Xa5*	*Xa13*	Xa21	Xa23	
1	BAU dhan1	1	1	0	0	0	MR
2	BAU dhan3	0	0	0	0	0	MS
3	BRRI dhan28	1	0	0	0	0	S
4	BRRI dhan29	1	0	0	0	0	S
5	BRRI dhan33	0	0	0	0	0	MS
6	BRRI dhan49	0	0	0	0	0	HS
7	BRRI dhan50	0	0	0	0	0	HS
8	BRRI dhan58	0	0	0	0	0	S
9	BRRI dhan64	1	0	0	0	0	S
10	BRRI dhan74	0	0	0	0	0	HS
11	BRRI dhan89	1	0	0	0	0	S
12	BRRI dhan101	1	0	0	1	0	R
13	BINA dhan17	1	0	0	0	0	S
14	BINA dhan20	1	0	0	0	0	S
15	BINA dhan23	1	0	0	0	0	S

Here, the BB resistant genes are scored according to the amplicon's presence (1) and absence (0) linked to five allele-specific molecular markers

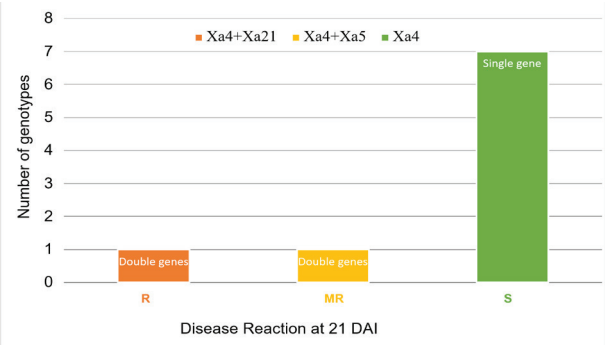

Figure 2. Resistance profiles of 15 cultivated rice varieties containing one or more *R* genes for BB disease.

Interestingly, it was found that single to multiple genes were present in various combinations in phenotypically resistant rice cultivars. Two of the cultivated rice varieties under this study had 2 genes, while 7 varieties had just one gene (Figure 2). In particular, out of the 15 cultivated rice varieties tested for pathogenicity, only one, rice variety BRRI dhan101, which possesses *Xa4* and *Xa21*, proved resistant. Notably, in phenotypic resistance screening for bacterial blight disease, rice varieties carrying two genes were found to be resistant, whereas varieties with a single R gene were found to be moderately resistant to highly susceptible. (Table 4; Figure 2).

Discussion

The diversity of the *Xa4* gene is underscored by the study, revealing that 60% of rice varieties (9 out of 15) harbor this dominant resistant gene. Its, have contributed to its widespread use in rice breeding programs (Sabar *et al*., 2016; Sombunjitt *et al*., 2017; Panwar *et al*., 2018; Wang *et al*., 2020; Anik *et al*., 2022).

Conversely, investigating the recessive R genes *Xa5* and *Xa13*, it was found that Xa5 was present in 6.6% of rice cultivars and that its prevalence varied across countries. In contrast, *Xa13* was found in no rice varieties, underlining its rarity. The study emphasizes the race-specific features of *Xa5* and *Xa13*, particularly in the Indian subcontinent, and highlights their varied prevalence in different regions (Sabar *et al*., 2016; Sombunjitt *et al*., 2017; Jiang *et al*., 2020; Anik *et al*., 2022).

The presence of 6.6% of the *Xa21* gene, known for broad-spectrum resistance, in the studied rice varieties, suggests its lower prevalence compared to other *R* genes. Similarly, the newly cloned *Xa23* gene, recognized for providing broad-spectrum protection against *Xoo,* was not identified. This points to greater diversity in *Xa4, Xa5*, and *Xa13* genes compared to *Xa21* and *Xa23*, aligning with previous research findings (Panwar *et al*., 2018; Wang *et al*., 2020).

The study evaluates the efficacy of single and multiple gene combinations in conferring resistance. While the *Xa4* gene alone showed limited effectiveness, combinations like *Xa4+Xa5* and *Xa4+Xa21* demonstrated moderate to high resistance highlighting the importance of multiple gene combinations for enhanced and durable resistance in rice breeding programs (Rashid *et al*., 2021; Haque *et al*., 2021; Anik *et al*., 2022; Kanipriya *et al*., 2023).

This study contributes valuable insights into the diversity of resistance genes against bacterial leaf blight, providing a foundation for future research and bacterial blight-resistant rice varieties development.

Conclusions

A phenotypic resistance screening-based lesion length on inoculated leaves was conducted on 15 rice cultivars to assess their disease reactions, spanning from highly resistant to highly susceptible, upon exposure to a virulent *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain. Subsequent molecular evaluation unveiled various combinations of 1 to 2 resistance (*R*) genes within these cultivated rice varieties. Notably, among them, BRRI dhan101 emerged as exceptional, carrying both the *Xa4* and *Xa21* genes while displaying a highly resistant reaction to the disease.

Due to its remarkable resistance, BRRI dhan101 presents a strong contender for future breeding programs targeting the development of superior rice varieties resistant to bacterial leaf blight. These findings not only enrich and broaden the genetic resources within the rice gene pool but also hold promise for the creation of enduring varieties highly resistant to bacterial leaf blight.

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Declaration

No potential conflict of interest was reported by the authors.

Author's contribution

The first and fifth authors conceived the presented idea and design of the experiment. The first, second, third and fourth authors wrote the manuscript and carried out the research work.

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