

#### **Research Article**

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# Tibet Development and Breeding Application of the Fluorescence Molecular Marker of Rice Bacterial Blight Resistance Gene Xa7

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**Abstract** In this study, the closely linked fluorescence molecular marker PM-Xa7 of Xa7 gene was developed on the basis of sequence difference between the resistant variety 'IRBB7' containing Xa7 and other nine bacterial blight susceptible rice varieties, and combine with PARMS technology (Penta-primer amplification refractory mutation system). Genotypes of Xa7 were identified in 72 rice germplasm and the bacterial blight resistance level of 'Mei B' was improved by backcross and screening with this marker. The results showed that two rice germplasms containing Xa7 were screened out from 72 rice germplasm; By introducing the resistant Xa7 gene from 'IRBB7' into 'Mei B' with PM-Xa7, a stable new maintainer with resistance to bacterial blight was successfully obtained. The bacterial blight resistant fluorescence signal was detected in 289 plants and the bacterial blight susceptible fluorescence signal was detected in 93 plants of BC<sub>2</sub>F<sub>2</sub> population generated from 'IRBB7' and 'Mei B', and the genotypes were consistent with the phenotypes of Xao inoculation. These research results indicated that PM-Xa7 can be effectively used for the marker-assisted breeding of bacterial blight resistant rice variety with the resistant  $_Xa7$  gene.

Keywords Rice; Bacterial blight; Xa7; Fluorescence molecular marker

Rice bacterial blight is a bacterial disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The disease was first detected in Fukuoka, Japan in 1884 (Li et al., 2017). Subsequently, the disease was found in every major rice-growing area in the world. In China, rice bacterial blight was first discovered in the suburbs of Nanjing in 1950 (Shen, 2001). At present, the disease has occurred in all rice areas in China, especially in the southern coastal rice areas (Yu et al., 2012). The outbreak of bacterial blight not only resulted in severe rice yield, but also reduced rice quality. Practice shows that breeding resistant varieties is the most economical and effective way to control bacterial blight. However, many resistant varieties in production lose their resistance after 3~5 years (Shi et al., 2008). Therefore, it is of great significance to breed rice varieties with long-lasting and broad-spectrum resistance to bacterial blight.

In order to develop rice varieties with long-lasting and broad-spectrum resistance to bacterial blight, the key lies in the discovery and utilization of genes with long-lasting, broad spectrum and high resistance to bacterial blight. Among the resistance genes to bacterial blight, *Xa7* gene from the rice variety 'DV85' from Bangladesh is a gene with high resistance, broad-spectrum and persistent resistance to bacterial blight (Ma et al., 2013), which has not been cloned so far. Studies have shown that *Xa7* gene shows high resistance to 52 different bacterial blight strains from China and Japan (Liu et al., 2016), and *Xa7* is relatively stronger in high temperature environment (Webb et al., 2009), which makes it suitable for utilization in rice areas of South China. At present, some scholars have carried out molecular marker-assisted breeding for *Xa7* gene. The 'DV85' *Xa7* genes were incorporated into the 'Minghui63' and selected by markers STSP3 and M5 to improve the resistance of 'Minghui63' to bacterial blight (http://www.cropscience.org.au/icsc2004/poster/3/4/4/1041\_heyuqing.htm). Lan et al. (2011) improved the resistance of 'Hua 201S' to bacterial blight by selecting the linkage marker STSP3 of *Xa7* and combining with



conventional breeding methods. Yan et al. (2013) used linkage markers to polymerize the anti-bacterial blight genes Xa7 and Xa21 and anti-borer gene  $cry1C^*$  into 'Xianhui 207', and improved the resistance of 'Xianhui 207' to the blight and the borers. In the above studies, the markers selected for Xa7 gene were all linkage markers that were detected by electrophoresis after PCR amplification, and the detection process was relatively complicated, which was not conducive to the detection of large-scale samples. However, the fluorescent molecular markers developed by PARMS technology do not need electrophoresis detection, but can read the genotype signal directly by fluorescence scanning, and then the genotype can be obtained by combining with software data analysis, which can perform Xa7 genotyping on a large number of rice samples at the same time.

## 1 Results and Analysis

## 1.1 A fluorescent molecular marker for Xa7 gene selection

Referring to the locus of co-dominant marker M5 of Xa7 gene developed by Porter et al. (2003), the sequence was compared with that of 9 susceptible cultivars (Ce253, Gui582, Guanghui998, R128, Ce138, Guanghui308, Shen08S, 186B, II-32B). It was found that there were differences in A/C and T/A between 'IRBB7' and the 9 susceptible cultivars at the location of marker M5 (Figure 1). Based on this, primers Xa7-FaT and Xa7-FCA were designed to match the fluorescence primers of FAM and HEX in PARMS master mix, respectively. Primers Xa7-FaT, Xa7-FCA and general primers Xa7-R jointly constituted the fluorescent molecular marker PM-Xa7 of Xa7 gene (Table 1). The genotype of the Xa7 gene in the sample can be determined by analyzing the different signals obtained from the PCR amplification product scan of the marker PM-Xa7, so the marker can be used for selection of the Xa7 gene.

Ce253	gaaatctaatcaatttcagag	attactgtattcatctttc	gtgtatattcgtctgggt
Gui582	gaaatctaatcaatttcagag	attactgtattcatctttc	a gigialaticgicigggi
GuangHui998	gaaatctaatcaatttcagag	attactgtattcatctttc	a gtgtatattcgtctgggt
R128	gaaatctaatcaatttcagag	attactgtattcatctttc	gtgtatattcgtctgggt
Ce138	gaaatctaatcaatttcagag	attactgtattcatctttc	a gtgtatattcgtctgggt
GuangHui308	gaaatctaatcaatttcagag	attactgtattcatctttc	n gtgtatattcgtctgggt
Shen08S	gaaatctaatcaatttcagag	attactgtattcatctttc	a gtgtatattcgtctgggt
186B	gaaatctaatcaatttcagag	attactgtattcatctttc	a gtgtatattcgtctgggt
II-32B	gaaatctaatcaatttcagag		
IRBB7	gaaatctaatcaatttcagag	attactgtattcatctttc	gtgtatattcgtctgggt

Figure 1 Allelic analysis of Xa7 gene

Table 1 Information of developed primers

	1	1		
Marker name	Primer name	Primer sequence (5'-3')	Fluorescence signal	Allelic genotype
PM-Xa7	Xa7-FaT	GAAGGTGACCAAGTTCATGCTTTCAGAG	FAM	AT
		A ATTACTGTATTCATCTTTCT		
	Xa7-FCA	GAAGGTCGGAGTCAACGGATTCAGAGCA TTACTGTATTCATCTTTCA	HEX	CA
	Xa7-R	ATCAGCACTACCCAGACGAATATAC		

### 1.2 Two germplasms containing Xa7 gene were identified by PM-XA7 marker from 72 rice germplasms

72 rice germplasms were inoculated with the predominant species IV of Blight from Guangxi, and many of them showed resistance to bacterial blight. Furthermore, the Xa7 gene in 72 rice germplasm was identified by fluorescence molecular marker PM-Xa7. The results showed that two rice cultivars, 'IRBB7' and '82B', contained Xa7 allele. Other rice varieties, such as' IRBB5 'containing resistance gene Xa5 and' CBB23 'containing resistance gene Xa23, were all susceptible to Xa7 alleles (Figure 2). The results of inoculation identification and marker detection indicated that the fluorescent molecular marker PM-Xa7 could specifically and effectively detect Xa7 gene in the 72 rice germplasm resources, which would be helpful to explore new germplasm carrying Xa7 gene and improve the utilization rate of Xa7 gene in breeding work.





Figure 2 Genotyping of 72 rice germplasm using PM-Xa7

#### 1.3 A new maintainer material against bacterial blight was created by labeling PM-Xa7

By using the marker PM-Xa7 for selection and phenotypic identification, a new mainstay material resistant to bacterial blight was created (Figure 3). In this process, the hybrid rice breeding parent 'MeiB' was cross-bred with the resistant parent 'IRBB7' and backcrossed in succession. 382 single plants of its  $BC_2F_2$  generation were detected by using the marker PM-Xa7. The detection results showed (Figure 4) that the blue dots were 92 homozygous single plants of *Xa7* gene with FAM fluorescence signal detected. Green dots represent 93 *Xa7* homozygous plants with HEX fluorescence signals detected, red dots represent 197 heterozygous plants with both FAM and HEX fluorescence signals detected, and gray dots represent negative controls. Field inoculation and identification (Figure 5) showed that 289 single plants were resistant, while the remaining 93 were all susceptible, which was consistent with the results of marker detection genotype. The separation ratio of resistant and susceptible single plants was close to 3:1 (chi-square 0.09). These results indicate that PM-Xa7 can accurately track *Xa7* gene and can be used for molecular marker-assisted breeding of *Xa7* gene.



Figure 3 A new line of resistance to bacterial blight created by PM-Xa7 was identified





Figure 4 Genotype identification of 382 F2 generation plants by PM-Xa7



Figure 5 Inoculation identified 382 plants of BC2F2 generation of 'Mei B' and resistant parent 'IRBB7'

## **2** Discussion

Molecular marker-assisted selection is not limited by environmental conditions or affected by physiological species of pathogenic bacteria, and can accelerate the selection of rice varieties resistant to bacterial blight (Li et al., 2011). At present, most of the molecular markers used in molecular marker-assisted breeding are those detected by polyacrylamide gel or agarose gel electrophoresis after PCR amplification. However, with the development of molecular marker technology, a fluorescent molecular marker technology based on PARMS established by PCR amplification of blocked mutation system has been applied in rice molecular marker-assisted breeding (Qing et al., 2018). The molecular markers developed by this technology do not need electrophoresis detection, and the genotype signal can be read directly by fluorescence scanning. Combined with software analysis, the genotype can be quickly realized. Compared with the traditional molecular markers, it has the advantages of convenience, fast, pollution-free, high detection efficiency and low detection cost, and is more suitable for large-scale genotyping. Therefore, the development of fluorescent molecular markers of rice resistance genes and their application in production practice will further promote the effective utilization of rice resistance genes and accelerate the breeding of resistant varieties.

Porter et al. (2003) developed a linkage marker of Xa7 gene, M5, which was used to screen bacterial blight resistant varieties. The marker was only 0.5 cM away from Xa7 gene, and AFLP31-10 (3 cM) was closer to Xa7 gene than the marker G1091 (6.0 cM). In this study, a fluorescent marker PM-Xa7 was designed based on the sequence contrast between the resistant variety 'IRBB7' and 9 susceptible varieties by referring to the locus of marker M5. This marker is closely linked to Xa7 gene and has good stability. Compared with previous M5



markers, it does not need to detect PCR products by electrophoresis. The operation is simpler and faster, which is more suitable for the detection of large-scale samples. In this study, the PM-Xa7 marker was further used to identify the Xa7 gene in the rice germplasm resources, and the Xa7 gene was selected from the hybrid offspring of 'IRBB7' and the susceptible variety 'Mei B', and the combination inoculation identification confirmed the effectiveness and stability of the marker. It is believed that the further utilization of PM-Xa7 will improve the utilization of Xa7 gene in rice breeding against bacterial blight.

Xa7 is a highly resistant, broad-spectrum and persistent bacterial blight resistant gene. Many researchers have used different linkage markers to identify this gene in rice germplasm resources and carry out molecular marker breeding application. Wu et al. (2019) identified one disease-resistant material containing Xa7 gene from 148 weedy rice samples using RM20582 marker. Liu et al. (2016) analyzed some rice germplasm resources in Guangdong Province with linkage markers and identified two germplasm containing Xa7 gene. He (2012) detected Xa7 gene in 72 rice parental materials through inoculation identification and M5 labeling, and found several rice parents containing Xa7 gene. In this study, fluorescence marker PM-Xa7 was used to detect 72 rice germplasm resources and inoculation identification. Only two varieties' IRBB7 'and' 82B 'were found to contain Xa7 gene, which indicated that Xa7 gene had low utilization rate in rice breeding for resistance to bacterial blight, which may be related to the easier expression of resistance under high temperature conditions and the expression of non-whole growth period. In order to take advantage of the stronger resistance of Xa7 gene under high temperature conditions, this study used 'IRBB7' to improve the resistance of susceptible parent 'Mei B' to bacterial blight, and combined with the marker PM-Xa7 for selection, a new mainstay material with stable resistance was obtained. Predecessors' (http://www.cropscience.org.au/icsc2004/poster/3/4/4/1041 heyuqing.htm) using Xa7 gene to rice bacterial leaf blight resistant effect of improvement is consistent. It was confirmed that this gene is an excellent disease resistance gene which can play a role in production. In this study, only Xa7 resistance genes were polymerized into the varieties to be improved, but studies have shown that rice varieties with multiple resistance genes to bacterial blight can show more durable, stronger and broader resistance (Huang et al., 2012). The next step is to combine other resistance genes with existing materials to develop rice varieties with long duration, broad-spectrum and high resistance to bacterial blight.

## **3** Materials and Methods

## **3.1 Experimental materials**

The experimental materials were 72 rice parents including 'IRBB7' containing Xa7 gene, 'IRBB5' containing Xa5 and 'CBB23' containing Xa23 collected by Guangxi Crop Genetic Improvement and Biotechnology Laboratory (Table 2), and 382 single plants of BC<sub>2</sub>F<sub>2</sub> generation of 'IRBB7' and susceptible maintainer line 'Mei B'.

### 3.2 Development of fluorescent molecular marker for Xa7 gene

According to the locus of co-dominant marker M5 of *Xa7* gene in rice variety 'IRBB7', the 200 kb sequence near was selected. According to the difference between 'IRBB7' and 9 susceptible cultivars, the sequence was compared with 9 susceptible cultivars (Ce253, Gui582, Guanghui998, R128, R138, Guanghui308, Shen08S, 186B, II-32B). Primers Xa7-FaT and Xa7-FCA, which can match with the fluorescence primers of FAM and HEX in PARMS Master Mix, were designed and combined with the universal primer Xa7-R to form the fluorescence molecular marker PM-Xa7 of *Xa7* gene.

### 3.3 DNA extraction and PCR amplification

Rice genomic DNA was extracted from individual leaves two weeks after transplanting according to the CTAB method used by Murray and Thompson (1980). The *Xa7* gene of the test material was amplified by PCR amplification system of 10  $\mu$ L. The system composition was as follows: 2×PARMS master mix 5  $\mu$ L, 10 mmol/L forward labeled primer (Xa7-FaT) 0.15  $\mu$ L, 10 mmol/L forward labeled primer (Xa7-FaT) 0.15  $\mu$ L, 10 mmol/L forward labeled primer (Xa7-FCA) 0.15  $\mu$ L, 10 mmol/L general reverse marker primer (Xa7-R) 0.4  $\mu$ L, template DNA 1.0  $\mu$ L, ddH<sub>2</sub>O 3.55  $\mu$ L. PCR amplification procedure: 95°C for 4 min, 94°C for 20 s, 65°C (decrease 0.8°C per cycle) for 1 min, 10 cycles. Then 94°C for 20 s, 56°C for 1 min, 32 cycles.



No.	Variety name	Fluorescent type	Genotype	No.	Variety name	Fluorescent type	Genotype
1	IRBB7	FAM	AT	37	9802S	HEX	CA
2	IRBB5	HEX	CA	38	Jing S	HEX	CA
3	112B	HEX	CA	39	Long S	HEX	CA
1	186B	HEX	CA	40	838R(luo)	HEX	CA
5	9311B	HEX	CA	41	Fuhui 838	HEX	CA
6	II-32B	HEX	CA	42	GR076	HEX	CA
7	82B	FAM	AT	43	GS2	HEX	CA
3	Bo B	HEX	CA	44	CBB23	HEX	CA
)	Bo IIB	HEX	CA	45	Mei B	HEX	CA
10	Bo IIIB	HEX	CA	46	R128	HEX	CA
1		HEX	CA	47	R138	HEX	CA
12	Gang 46B	HEX	CA	48	R1439	HEX	CA
13	Gufeng B	HEX	CA	49	R273	HEX	CA
4	Guang 8B	HEX	CA	50	Molixiang No.2	HEX	CA
5	Hengfeng B	HEX	CA	51	Guanghui 308	HEX	CA
6	Hua 2048B	HEX	CA	52	R318	HEX	CA
7	Jing 23B	HEX	CA	53	R319	HEX	CA
18	Liangfeng B	HEX	CA	54	R402	HEX	CA
19	Shan B	HEX	CA	55	R463	HEX	CA
20	Shen 95B	HEX	CA	56	R8	HEX	CA
21	Taifeng B	HEX	CA	57	R527	HEX	CA
22	Te B	HEX	CA	58	Gui 582	HEX	CA
23	Tianfeng B	HEX	CA	59	R5867	HEX	CA
24	Wufeng B	HEX	CA	60	Gui 7571	HEX	CA
25	Xifei B	HEX	CA	61	R774	HEX	CA
26	Yexiang B	HEX	CA	62	R789	HEX	CA
27	Yixiang B	HEX	CA	63	R795	HEX	CA
28	Yu 19B	HEX	CA	64	R9516	HEX	CA
29	Yu 4B	HEX	CA	65	R087	HEX	CA
30	Zhenfeng B	HEX	CA	66	R07-1	HEX	CA
31	Zhongjiu B	HEX	CA	67	Bing 4114	HEX	CA
32	Zhongzhe B	HEX	CA	68	Ce 253	HEX	CA
33	Zhun S	HEX	CA	69	Duoxi No.1	HEX	CA
34	H068S	HEX	CA	70	Guanghui 998	HEX	CA
35	1518	HEX	CA	71	696B	HEX	CA
36	3428	HEX	CA	72	Sheng B	HEX	CA

Table 2 72 rice variety and genotypes of marker PM-Xa7

# 3.2 Development of fluorescent molecular marker for Xa7 gene

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## 3.4 Phenotypic identification of the tested material

The tested pathogen was the bacterial blight (IV) strain of rice in Guangxi, which had the highest occurrence frequency and the most extensive damage distribution in Guangxi, and was considered as the dominant pathogenic bacteria in Guangxi (Sun et al., 1993). According to the bacterial culture and inoculation methods of Wu et al. (2019), the disease was investigated about 20 days after inoculation. The disease was graded according to the percentage (%) of disordered spots in the total leaf length (Qin et al., 2014): 0~3.0% of high resistance (HR), 4%~12.0% of resistance (R) and 13%~25.0% of moderate resistance (MR), 26%~50.0% of moderate susceptible (MS), 51%~85.0% of susceptible (S), 86%~100.0% of high susceptible (HS).

## 3.5 Xa7 allele type

The fluorescence signal intensity of PCR amplification products was detected by using the enzyme label analyzer ROX fluorescence detection channels. of FAM, HEX and By SNP decoder (http://www.snpway.com/snpdecoder01/) software analysis each sample PCR amplification of FAM and HEX fluorescent signal values, and the corresponding scatter plot format output, genotyping of amplification products. If the fluorescence signal of FAM is obtained by scanning, it is the Xa7 allele containing A and T bases, which is divided into green dots on the scatter map. If the HEX fluorescence signal is obtained by scanning, it is the Xa7 allele containing C and A bases, which is the blue dot on the typing map. If the fluorescence signals of both FAM and HEX are obtained by scanning, the genotype contains heterozygous A/C and T/A base alleles, which are represented by red dots on the typing map.

### **3.6 Breeding application**

Using Xa7 gene 'IRBB7' as the donor, cross with Guangxi high quality hybrid rice susceptible parent 'Mei B' and backcross to BC<sub>2</sub>F<sub>1</sub> generation. After three successive self-crossing generations, each generation was selected by using the marker PM-Xa7 and combined with phenotypic identification to create a new maintenance line material resistant to bacterial blight. In this process, in order to verify the accuracy of Xa7 gene selection by marker PM-Xa7, 382 BC<sub>2</sub>F<sub>2</sub> generation single strains were detected by marker PM-Xa7, and 2 negative controls were added.

### Authors' contributions

WH is the experimental designer and the executor of this study. DGF, GLJ, DXX and HJ completed the data analysis and the writing of the first draft of the paper. GH, QDJ, CWW and YSY participated in the experimental design and analysis of the experimental results. LHF was the architect and the person in charge of the project, guiding the experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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