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Molecular Marker-based Screening for Bacterial Leaf Blight Resistance Genes in Landraces and Cultivars of Rice in Gujarat

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Bacterial Leaf Blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the most devastating disease and limiting factor for rice productivity worldwide. Since BLB pathogens are difficult to manage through chemical and other available cultural practices, development of host plant resistance is the most effective means of disease management. So far survey for resistance donor at molecular level in rice is scanty and the landraces of Gujarat state of India have received little attention. Therefore, a molecular screening was conducted to identify the presence of major BLB resistance genes *Xa4*, *xa5* and *Xa21* in landraces and local cultivars of Gujarat. In order to confirm the presence of BLB resistance genes, 25 genotypes were screened using PCR analysis. 10 genotypes (IR20, IR64, IR72, NWGR1095, NWGR2014, IET14726, GR7, GR102, Pankhali 203 and Ratna) were found to be *Xa4* positive. In contrast, only IET18483 genotype was found to be *Xa21* positive. Interestingly, not a single genotype was found to be positive for *xa5* BLB resistance gene. The status of BLB resistance genes in landraces and cultivars of Gujarat will help design BLB-resistance breeding programs.

Key Words: Bacterial Leaf Blight, PCR Screening, Rice Cultivars and Landraces, *Xa* Gene

Introduction

Rice is the oldest domesticated grain (~10,000 years). It is grown on every part of the globe and is the staple food for 2.6 billion people worldwide. The productivity of rice is severely affected by several biotic and abiotic factors. Among biotic factors, bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a devastating disease in the rice-growing countries including India. There are no effective chemical and biological bactericides available for controlling this pathogen. However, for BLB resistance, host plant resistance strategies are the most appropriate to manage the pathogen.

Till date, a total of 42 BLB resistance genes (R genes) have been identified in rice, including *Xa1*, *Xa2*, *Xa3/Xa26*, *Xa4*, *xa5*, *Xa6*, *Xa7*, *xa8*, *xa9*, *Xa10*, *Xa11*, *Xa12*, *xa13*, *Xa14*, *xa15*, *Xa16*, *Xa17*, *Xa18*, *xa19*, *xa20*, *Xa21*, *Xa22(t)*, *Xa23*, *xa24(t)*, *xa25/Xa25(t)*, *Xa25*, *xa26(t)*, *Xa27*, *xa28(t)*, *Xa29(t)*, *Xa30 (t)*, *xa31(t)*, *Xa32(t)*, *Xa33(t)*, *xa34(t)*, *Xa35(t)*, *Xa36(t)*, *Xa37, Xa38, Xa39, Xa40, xa41(t), Xa42.* The recessive resistance genes include *xa5*, *xa8*, *xa9*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa25/Xa25(t)*, *xa26(t)*, *xa28(t)*, *xa31(t)*, *xa33(t)*, and *xa34(t)*. Recently, integrated rice science database (available at https://shigen.nig.ac.jp) release the physical map of all the identified BLB resistance genes (Zhang *et al*., 2015). These R genes are known to act in a gene-for-gene interaction manner and are the main sources for genetic improvement of rice for resistance to *Xoo* (McDowell and Woffenden, 2003). Ten of the recessive R genes; *xa5* (Petpisit *et al.,* 1977), *xa8* (Singh *et al*., 2002), *xa13* (Ogawa *et al*., 1987), *xa24* (Kush *et al*., 1999), *xa26, xa28* (Lee *et al*., 2003) and *xa32* (Ruan *et al*., 2008) confer race-specific resistance.

BLB resistance gene *Xa4* is one of the most widely exploited resistance genes in many rice breeding programs and it confers durable resistance in many commercial rice cultivars. However, widespread cultivation of *Xa4* resistance genes containing varieties has led to the development of *Xa4* resistance *Xoo* races in India and most part of south east Asia (Ma *et al*., 1999, Mew *et al*., 1992; Sun *et al*., 2003).Similarly, the *Xa21* gene was identified in the wild species *Oryza longistaminata* and is highly effective against BLB races of South and Southeast Asia (Khush *et al*.,1990). The *xa5* gene, which is naturally found only within the *Aus* subpopulation of rice, provides recessive

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resistance to several *Xoo* races of the Philippines (Garris *et al*., 2003). The information regarding this context is very limited in respect of cultivars and landraces of rice in Gujarat. Therefore, in the current investigation, we performed PCR-based survey for *Xa4*, *xa5* and *Xa21* BLB resistance gene in landraces and cultivars of Gujarat. For the screening of *Xa4* gene, linked marker developed by Ma *et al*. (1999) was used. For *xa5* genes, CAPS markers designed by Iyer *et al*. (2007) were exploited. *Xa21* gene was detected using the STS marker developed by Chunwongse *et al.* (1993).

Materials and Methods

Seeds of the 36 rice genotypes/lines (Table 1) were obtained from Main Rice Research Station, Nawagam, Anand Agricultural University (AAU), Gujarat. The 36 genotypes include six monogenic differentials for BLB resistance genes in the background of IR24, 4 pyramided lines for BLB resistance genes and 5 varieties developed by IRRI. Pyramided lines and IR24 were selected as a positive control and negative control, respectively. Five lines and five advanced breeding lines generated by Main Rice Research Station, Nawagam, along with 8 varieties and 4 landraces released by the station were also incorporated in this study.

Fourteen-day old seedlings grown in petri-plates were used for DNA isolation. Fresh seedlings from ten individuals were bulked together and DNA was extracted

Table 1. List of rice genotypes/lines included in current investigation

S. No.	Genotypes/lines	Descriptions
1	IRBB1	Monogenic differentials in background of IR24 carrying Xa1 gene
2	IRBB4	Monogenic differentials in background of IR24 carrying Xa4 gene
3	IRBB7	Monogenic differentials in background of IR24 carrying Xa7 gene
4	IRBB8	Monogenic differentials in background of IR24 carrying xa8 gene
5	IRBB13	Monogenic differentials in background of IR24 carrying xa13 gene
6	IRBB21	Monogenic differentials in background of IR24 carrying Xa21 gene
7	IRBB57	Pyramided lines carrying Xa4, xa5,xa13 gene (Background IR24)
8	IRBB58	Pyramided lines carrying Xa4, xa13, Xa21 gene (Background IR24)
9	IRBB59	Pyramided lines carrying xa5, xa13, Xa21 gene (Background IR24)
10	IRBB60	Pyramided lines carrying Xa4, xa5,xa13, Xa21 gene
11	IR8	Varieties released by IRRI
12	IR20	
13	IR24	
14	IR64	
15	IR72	
16	NWGR1068	Cross between GR11 X (GR101 X GR3)
17	NWGR1095	Cross between GR11 X IET11763
18	NWGR2014	Cross between Sathi 34-36 X Ratna
19	NWGR2035	Cross between GR11 X Pusa Basmati
20	NWGR99123	Cross between GR4 X IR64
21	IET14726	Advanced breeding lines
22	IET16626	
23	IET17909	
24	IET18483	
25	GR6	Varieties released by Main Rice Research Station, Nawagam
26	GR7	
27	GR9	Upland rice variety released by Nawagam
28	GR11	Varieties released by Main Rice Research Station, Nawagam
29	GR12	
30	GR102	
31	GR104	
32	GAR13	
33	Pankhali203	Aromatic land cultivar
34	Ratna	Released rice variety
35	Sathi34-36	Released upland rice variety of Gujarat
36	SK-20	

using a modified CTAB method as described by Zhang *et al*. (2004). The concentration of extracted genomic DNA was quantified using a spectrophotometer. In order to estimate the quality of isolated DNA, DNA was checked on 0.8% agarose in 0.5x TBE buffer (45mM Tris-borate and 1mM EDTA). For pre-staining, ethidium bromide was added to the gel at a concentration of 10µg/ml before the gel was poured. The samples were run on the gel at 60V until the bromophenol blue dye migrated almost to the end of the gel. Thereafter, electrophoresis gel documentation of stained gels was made using G-BOX from Syngene. Concentrated DNA was diluted to 100ng/ µl using TE (10mM Tris-HCl and 1mM EDTA) buffer and stored at -20ºC till further use.

The genomic DNA was amplified using primers selected on the basis of literature survey. The details of primers, linked genes, sequences and corresponding references are given in Table 2. PCR reactions for SSR/ CAPS were carried out in a reaction volume of 25µl including 2.5 µl of 10 X Taq buffer, 1.0 µl of 50 mM MgCl2 solution, 2.5 µl of 2.5 mM dNTPs mixture, and 0.5 µl of each of the forward and reverse primer at a concentration of 10 pmole/µl, 1.25 µl of 3U/µl Taq DNA polymerase, 3 µl of purified genomic DNA (100 ng/ µl), 1.25µl of glycerol and 13.5 µl of nuclease free water. The PCR components were ordered from Bangalore Genei Pvt. Ldt. The polymerase chain reactions were performed in a thermocycler (Eppendorf) with the following cycle: the initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min., annealing at 55°C for 45 sec. and extension at 72°C for 2 min. and 15 min. at 72°C for the final extension. In order to determine polymorphism, PCR products were checked on 2.5% agarose in 0.5x TBE buffer (45 mM Tris-borate and 1 mM EDTA). For pre-staining, ethidium bromide was added to the gel at a concentration of 10µg/ml before the gel was poured. The samples were run on the gel at 100V until the bromophenol blue dye migrated almost to the end of the gel. Thereafter, electrophoresis gel documentation of stained gels was made using G-BOX from Syngene.

For Restriction Fragment Length Polymorphism (RFLP) analysis of PCR product, amplified product was digested with different restriction enzymes (Table 3). The PCR reaction were digested by following manufacturer instruction followed by gel electrophoresis on 2% agarose gel contain 10 µg/ml ethidium bromide. Gel documentation was performed as described earlier. The amplified fragments of all the rice genotypes/ lines were observed and compared with positive and

Table 2. List of gene-specific primers used for screening of BLB resistance genes

Gene	Primer Name	Sequence (5'-3')	References
Xa4	RM224	F-TCTCCCTCCTCCTCCTCCTACG	Ma *et al.* (1999)
		R-GATTCAGCACAGCGATTGTTGC	
	MP12	F-ATCGATCGATCTTCACGAGG	
		R-TGGTATAAAAGGCATTCGGG	
xa5	xa5_1F	F-CTCTACCGGAGGTCCACCATT	Iyer *et al.* (2007)
	xa5_2F	F-ACGCTCGACGAGATGGTCTC	
	xa5_4F	F-CTGGAAGAAGCTCTTAATTT	
	xa5_5F	F-CGGATAGCAGCATTTCCAAGAG	
	xa5_6F	F-GATAGCAGCATTTCCAAGAG	
	xa5_1R	R-AGGAACAGCAACATTGCAAC	
	xa5_4R	R-GATTCCTTTAGCAAGGTGTG	
Xa21	PTA248	F-AGACGCGGAAGGGTGGTTCCCGGA	Chunwongse *et al.* (1993)
		R-AGACGCGGTAATCGAAAGATGAAA	

Table 3. Restriction enzymes used in CAPS assay

S. No.	Restriction Enzyme	Reaction composition	Incubation
1	*Bsr*I	5–10 µl PCR product, 2 µl 10X buffer recommended by the manufacturer, and 5 U of enzyme.	65^{o}C
2	*Sml*I	5–10 µl PCR product, 2 µl 10X buffer recommended by the manufacturer, and 5 U of enzyme and 0.2 µl of 100X BSA	55^{o}C
3	*Xho*I and *Hpa*II	10 µl PCR product, 18 µl nuclease free water, 2 µl 10X buffer recommended by the manufacture, 2 µl of restriction enzyme	37^{o}C

negative controls, and for the presence and absence of BLB resistance gene plus and minus sign were assigned respectively.

Results and Discussion

DNA analysis of *Xa4* resistance genes in all the selected rice germplasm exhibited the presence of two different sizes of bands with marker MP12 and three with RM224. With marker MP12 and RM224, the banding pattern of all the genotypes was either similar to the IRBB4, IRBB57, IRBB58, IRBB60 and IR64 (positive control) and IR24, and IR8 (negative control). With marker MP12, the size of the band was 165bp which corresponds to the IRBB4 whereas, the band which corresponds to the IR24 was 144bp in size while with marker RM224 the size of the band was 163 bp corresponding to the IRBB4 and with IR24 was 143 bp. During the gene survey using MP12 marker, out of 36 rice genotypes, 14 genotypes (IRBB4, IRBB57, IRBB58, IRBB60, IR20, IR64, IR72, NWGR1095, NWGR2014, IET14726, GR7, GR102, Pankhali 203 and Ratna) along with a positive control amplified a 165 bp size fragments which indicated the presence of *Xa4* gene. While the remaining 22 genotypes amplified 144bp DNA fragments which showed the absence of *Xa4* gene (Fig. 1a). Ma *et al.* (1999) identified and synthesized MP12 primers based on the sequence of a DNA marker tightly linked to the rice BLB resistance gene *Xa4* for the survey of hybrid rice germplasm. Wang *et al*. (2000) used the same set of primers for the fine mapping of the *Xa4* gene. They analyzed the F2 population of a cross between IR24 and IRBB4 using the same primers and found that *Xa4* is tightly linked to this primer. Arif *et al.* (2008) reported the polymorphism between positive and negative controls with 150bp DNA fragments corresponds to the positive control (IRBB4 and IR64) and 120bp fragments with negative control (IR24) but in present studies the presence of 165bp fragment corresponds to the positive control and 144bp fragments to the negative control were observed. With marker RM224, 14 genotypes amplified

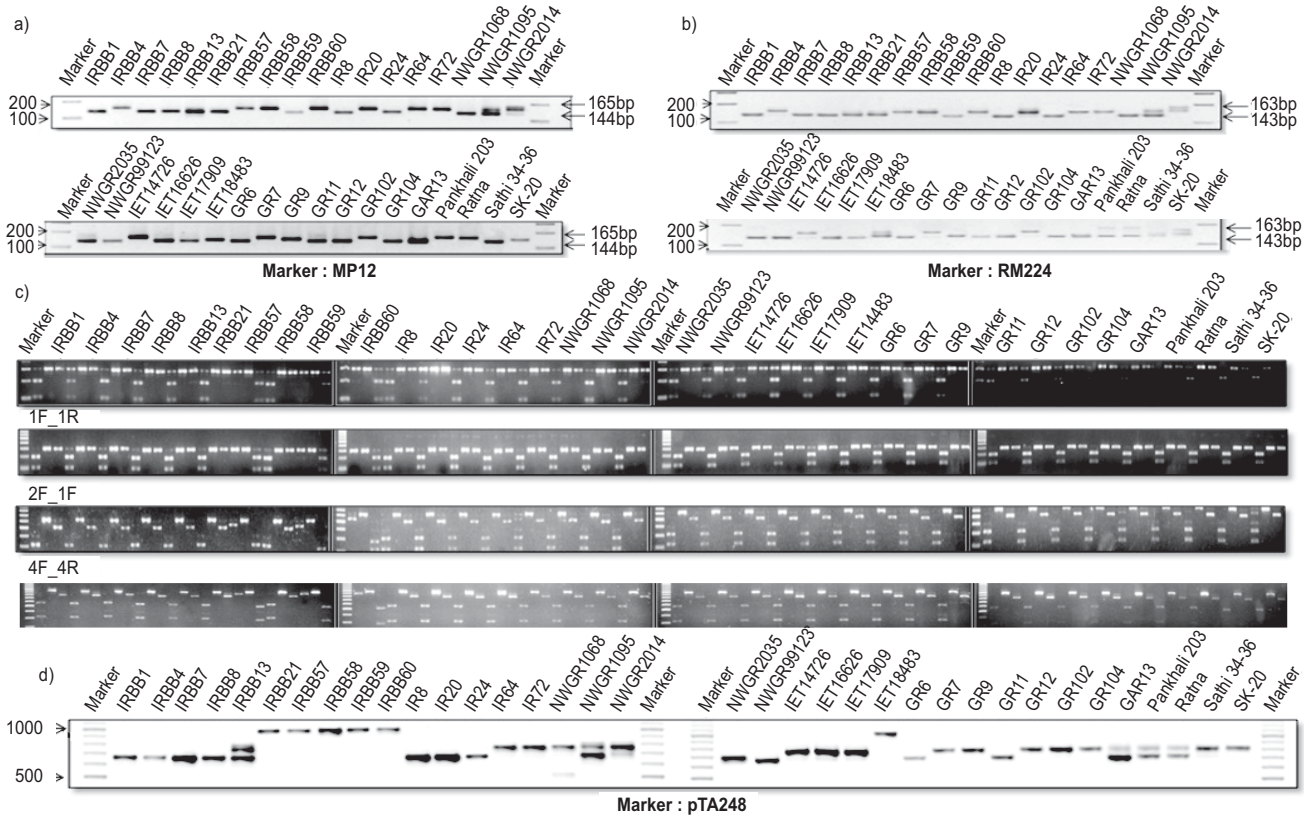

Fig. 1. PCR based genotyping of rice genotypes for BLB resistance gene *Xa4* and *xa5* a) Genotyping of genotypes using *Xa4* linked marker MP12 b) Genotyping of rice genotypes for *Xa4* BLB resistance genes using SSR marker RM224 c) Analysis of rice genotypes for the presence of *xa5* BLB resistance gene using CAPS marker. Each genotype was represented by 3 well, first well contains PCR product digested by *Bsr*I enzyme, second well contain PCR product and third well contain PCR product digested by *Sml*I enzyme d) STS maker pTA248 based genotyping of rice genotypes to screen the presence of *Xa21* BLB resistance gene

Table 4. Primers used for the amplification of CAPS marker

Primers	Distance to FNP	Primer Pair	Product size	Approximate digested product size
xa5_1F	100	1F_1R	299	100, 199
xa5_1R	199			
xa5_2F	61	2F_1R	260	61, 199
xa5_1R	199			
xa5_4F	225	4F_4R	625	225, 400
xa5_4R	400			
xa5_5F	551	5F_4R	951	551,400
xa5_4R	400			
xa5_6F	549	6F_4R	949	549,400
xa5_4R	400			

163bp DNA fragments which indicated the presence of *Xa4* resistance gene whereas 22 genotypes amplified 143bp DNA fragments which showed the absence of *Xa4* resistance gene. Out of 14 resistant genotypes three genotypes namely NWGR2014, Pankhali 203 and Ratna showed the presence of an additional allele of 190bp which was absent in both the positive and negative controls (IRBB4 and IR24 respectively). Sun *et al* (2003) used RM224 to map *Xa4* gene and found polymorphism between the parents of the F2 population. In our study this marker also showed polymorphism and this polymorphism was in synchronization with the polymorphism detected by MP12 except for genotype IET18483, which showed susceptible band with MP12 and heterozygous condition at RM224 loci (Fig. 1b).

The recent identification of the *xa5* gene demonstrated that it encodes the small subunit of transcription factor TFIIAγ. This gene differs in resistant and susceptible cultivars by two nucleotides. *Bsr*I and *Sml*I digest the susceptible and resistant alleles, respectively (Iyer *et al*, 2007). Iyer *et al.* developed seven primers, which were combined into five different sets of primer pairs and was used in the current investigation. Primers xa5_6F and xa5_5F are 96 and 98 base pairs upstream of the first nucleotide of the full-length xa5 cDNA respectively, while xa5_1F, 2F, and 4F are within exon2. xa5_1R and 4R are located in the third intron. Overall, 36 genotypes are screened for *xa5* resistance gene with CAPS markers shown in Table 4. Out of 36 genotypes only 3 genotypes i.e. IRBB57, IRBB59, and IRBB60 (positive control) showed the presence of *xa5* specific bands and rest of the genotypes showed the absence of *xa5* specific bands and banding patterns are identical to the IR24 (negative control) (Fig. 1c). The study conducted by Iyer *et al* (2007) showed a perfect correlation between the CAPS markers genotype and observed phenotype, therefore, the result generated by these markers are reliable to genotyped germplasm for BLB resistance gene *xa5*.

The presence of *Xa21* gene in germplasm was detected by the STS marker pTA248, developed by Chunwongse *et al* (1993). DNA analysis of the genotypes with pTA248 marker exhibited the presence of four alleles of 950bp, 780bp, 650bp and 500bp. Out of these four alleles, 950bp DNA fragments were present in positive controls (IRBB21, IRBB57, IRBB58, IRBB59, and IRBB60) showed resistant nature of the genotype and 650bp DNA fragment is present in negative control (IR24) while the presence of other variants also showed susceptibility of the germplasm. Out of 36 genotypes, six genotypes IRBB21, IRBB57, IRBB58, IRBB59, IRBB60, and IET18483 showed the amplicon of 950bp corresponding to the resistant allele and hence considered as resistant genotypes (further validated at Navagam rice research station, AAU, Nawagam, Data not shown). Eleven genotypes IRBB1, IRBB2, IRBB4, IRBB8, IR8, IR20, IR24, NWGR2035, NWGR99123, GR6, and GR11 showed the presence of 650bp amplicon. Twelve genotypes, IR64, IR72, NWGR2014, IET18483, IET14726, IET16626, IET17909, GR7, GR9, GR12, GR102, GR104, Sathi 34-36 and SK-20 have amplicon of 780bp. Six genotypes showed heterozygosity and out of these five genotypes IRBB13, NWGR1095, GAR13, Pankhali 203 and Ratna were found heterozygous for the allele with two fragments of 780bp and 650bp, and one genotype NWGR1068 was found heterozygous for the allele with two fragments of 780bp and 500bp each (Fig. 1d). Huang *et al.* (1997) reported 3 alleles for pTA248 but in our study, an additional allele of 500bp for pTA248 was found in NWGR1068.The presence of the *Xa21* gene was detected by the STS marker pTA248 located within 1cM of *Xa21* and was originally obtained by sequencing the genomic clone of the RAPD248

fragment using the same primer (Chunwongse *et al*., 1993). Ronald *et al.* (1992) genetically mapped it at 0.1cM distance from *Xa21* gene. pTA248 detected a band of approximately 1Kb in all resistant lines. In current investigation the same polymorphism was also detected between resistant and susceptible lines.

Conclusions

In conclusion, the *xa5* and *Xa21* gene is absent in landraces and cultivars of Gujarat. The use of rice accessions includes IRBB57, IRBB59, and IRBB60 as a donor parents in hybridization program with modern cultivar will accelerate efforts to develop BLB-resistant cultivars through MAS-based pyramiding approaches without compromising yield and grain quality.

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