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Molecular analysis of bacterial blight resistance gene *XA7* in advance population of rice using STS markers

Irfan Ullah*, Hamid Ali, Muhammad Islam, Wazi Ullah, Muhammad Haris, Muhammad Qasim Khan, Shafiq-ur-Rehman, Kamran Khan, Kabir Khan, Bushra Ghani

Department of Genetics, Hazara University Mansehra, Pakistan

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Abstract

Rice plays a vital role in achieving world food security. However, it is affected by several biological factors. Among these factors, Bacterial blight (BB) negatively effect rice efficiency and reduce rice production through out the world. In this study a molecular survey was conducted to identify BB resistance gene *Xa7* in an advance population of rice using STS markers. An amplicon of 207bp in size was observed in IRBB7 confirming the presence of *Xa7* gene. An amplicon of the same size was also observed in 11 other genotypes. The selected genotypes were then evaluated against BB local isolates in the field. Significant differences were recorded for mean lesion length developed by these isolates. However, all the selected genotypes along with the parental variety IRBB7 showed resistant reactions. Therefore, the presence of *Xa7* gene in rice will confer durable resistance against different isolates of bacterial blight.

* **Corresponding Author:** Irfan Ullah ✉ irfan.qasami@gmail.com

Introduction

Bacterial blight is one of the most destructive diseases of rice and is endemic in most of the rice growing regions throughout the world (Swings *et al.,* 1990). Under favorable condition, bacterial blight (BB) causes outbreaks, loss in respect of yield (Mew *et al.,* 1993; Ou, 1985). In Pakistan, BB was first reported in 1977; since then, it has slowly spread throughout the country and became a threat for rice cultivation (Jabeen *et al.,* 2012; Bashir *et al.,* 2010; Ali *et al.,* 2009; Akhtar *et al.,* 2003; Mew and Majid, 1977). Bacterial blight distresses plant vessels which manifest itself either in the seedling resulting in severe wilting of the plantlet or in leaves as leaf blight ultimately resulting in drying of leaves. In case of severe infection, plant either fails to produce panicles or produce sterile panicles that contain immature grains of no value (Khoa *et al.,* 2017; Akhtar *et al.,* 2008; Ou, 1985). Bacterial blight epidemics have occurred several times in Pakistan. The susceptible varieties include all of the highly aromatic and valuable basmati genotypes (Ali *et al*., 2009; Khan *et al*., 2009; Waheed *et al.,* 2009; Akhtar *et al.,* 2008). The disease can be managed in several ways including chemical application (Islam *et al.,* 2017; Rehman *et al.,* 2013), cultural practices (Islam, 2017) and use of resistant varieties (Noman *et al.,* 2016). Among all these, the adaptability of the resistant genotype is considered to be the best option (Islam, 2017; Ali *et al.,* 2016a; Ashraf *et al.,* 2013). Unfortunately, no variety of rice has yet been reported to be completely tolerant or resistant to the extant of strains of the pathogen (Samiullah *et al.,* 2015). More than 42 BB resistance (R) genes have been identified in different rice cultivars, related wild species, and mutated populations showing resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) the casual pathogen of bacterial blight (Busungu *et al.,* 2016). There are nine genes *Xa1, Xa3/Xa26, xa5, Xa10, xa13, Xa21, Xa23, xa25*, and *Xa27* that have been studied at the molecular level which code for a variety of proteins, suggesting the existence of multiple mechanisms of R genes-mediated *Xoo* resistance (Tian *et al.,* 2014; Chu *et al.,* 2006; Gu *et al.,* 2005; Sun *et al.,* 2004; Song *et al.,* 1995). So far, Twelve R-genes including *Xa4, Xa7, Xa22, Xa30, Xa31, Xa33, xa34, Xa35, Xa39, Xa40, xa42*, and *Xa42* have been mapped on the basis of their morphological and molecular markers (Liang *et al.,* 2017; Busungu *et al*., 2016; Zhang *et al.,* 2015). Further, sixteen of the R-genes including *xa5, xa8, xa9, xa13, xa15, xa19, xa20, xa24, xa25, xa26b, xa28, xa31, xa32, xa33, xa34*, and *xa42* have been identified as recessive (Liang *et al.,* 2017; Vikal and Bhatia, 2017; Chen *et al.,* 2011). Several resistant genes such as *Xa4, xa5, Xa7, xa13*, and *Xa21* have already been incorporated into rice cultivars for development of new resistance varieties which give fruitful results against the pathogen (Perumalsamy *et al.,* 2010; Sanchez *et al.,* 2000; Huang *et al.,* 1997).

Xa7 is a dominant gene, located on chromosome 6 with the genetic distance of 0.07 cM between GDSSR02 and RM20591 (Fig.1) and provides resistance against various *Xoo* isolates (Chen *et al.,* 2008). Many researchers have reported the use of polymerase chain reaction (PCR) markers for probing resistance genes in rice cultivars, such as the study of *Xa*4 and *Xa*7 resistance genes in rice germplasm (Arif *et al*., 2008; Muhammad *et al*., 2015) and *Xa*4, *xa*5, *Xa*7 and *xa*13 resistance genes in basmati rice (Ullah *et al*., 2012). Genetic resistance is an important sought after trait by the rice growers and producers, thus detection of *Xa7* associated STS markers coupled with phenotypic resistance shown by the selected genotypes are important leaping points for further evaluation and eventually release of BB resistant elite lines.

The present study was therefore design with aims to analyze selected F7 genotypes of rice along with their parental varieties for the presence of *Xa*7 gene and their responses to BB local isolates (*Xoo*).

Materials and methods

Plant material

Experimental materials consisted of 29 homozygous lines of an advance (F7) population of rice derived from the Basmati-385/IRBB7 cross. These lines were planted in Hazara University Mansehra Pakistan during rice growing season 2018.

Extraction of genomic DNA from the fresh seeds

The gDNA was extracted by applying DNA extraction protocol, reported by Ali *et al.* (2016) with minor modifications. For DNA extraction three half fresh seeds of each sample were taken in 2 ml eppendorf tube and 700µl of heated (60° C) 2x CTAB buffer having 50mM Tris-HCl, pH 8.0, 25mM EDTA, 300mM NaCl and 2% CTAB were added to each sample. After an hour the samples were crushed with the help of a glass rod to make a homogenous solution. The samples were then incubated at 56° C overnight and again crushed with the help of a glass rod. Then 700µl Chloroform: Isoamyl alcohol (24: 1) solution was added and the samples were kept at room temperature for 30 minutes. The samples were then centrifuged at 9000 rpm for 20 minutes and a clear supernatant was formed, of which 500µl was transferred to a newely labeled eppendorf tube to which 500µl ice cold isopropanol and 40µl sodium acetate were added and incubated at -20° C overnight. The samples were again centrifuged at 9000 rpm for 20 minutes to make DNA pellet. The supernatant was discarded and the pellet was washed with 70% ethanol and dried at room temperature. After this, 40µl TE buffer was added to the sample to dissolve DNA pellet. For RNA degradation 1µl RNAase was added to each tube and incubated at 37° C for one hour. The quality and quantity of DNA was checked on 1% agarose gel stained with ethidium bromide. The concentration of DNA was adjusted from 20 to 50 ng/µl by using double distilled water and stored the sample at 4°C for further use.

Amplification of Xa7 gene through PCR

Sequence tag site (STS) primers were used for amplification of *Xa7* gene (Table 1). PCR reactions were carried out in 50µl reaction volumes having 1-2 µl genomic DNA, 1µl of each of forward and reverse primers (10µM/µl), and 25µl 2x green master mix (Thermo Scientific).

Amplification of *Xa7* gene was carried out in DNA thermal Cycler (Applied Bio System). The conditions set were 94°C for 6 minutes as initial denaturation, 36 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes, followed by a final extension of 72°C for 7 minutes. The PCR products were run on 2% agarose gel in TAE buffer. The gel was stained with ethidium bromide (10 ug/ml) and observed under UV light. The data was scored for the presence of *Xa7* linked fragments.

Isolation of Xanthomonas oryzae pv. oryzae strains

The infected leaves having clear BB lesions were collected from different rice fields of Districts Mansehra and kept in refrigerator at 4°C.

The samples were washed with tap water, air dried and then cut into small pieces of about 2 to 4 cm. Sterilized with 70% ethanol and washed with sterile ddH_2O. These pieces were then put into eppendorf tube having 1 ml sterile water and crushed with a glass rod and kept for one hour at room temperature. The bacteria ooze out into the water and was streaked into the petri dishes having nutrient agar medium. The petri dishes were then kept in incubator at 28°C for 4 days. Single round, smooth, golden yellow and mucous colonies were selected and streaked on new petri dishes to get pure cultures.

Preparation of inoculum from pure culture

For preparation of inoculum 15 ml distialled water was taken in 30 ml tubes. Pure culture of *Xoo* was made by suspending freshly grown Xoo on a plate making an inoculum of approximately 10^8 cfu/ml. Rice seedlings were inoculated at seedling stages using clip method as reported by Kauffman *et al.* (1973). After inoculation disease symptoms were observed on daily basis. Sixteen days after inoculation, the final data was recorded and disease incidence was calculated.

The responses of rice genotypes against the *Xoo* was measured using the scale, presented in (Table 2).

Results

In the present investigation, initially genomic DNA was extracted from fresh seeds of selected genotype by using CTAB method (Fig. 2). The concentration of DNA was adjusted from 20 to 50 ng/ul.

Table 1. Primer sequence of *Xa7* gene.

Primer name	Primer Sequence (5' to 3')	Reference	Linked gene
GDSSR02	(F) TGCCCACCGTCGAACTCGTGG	(Chen *et al.*, 2008)	*Xa7*
	(R) AGCTAGCAATTCGCATGATTGC		

The PCR was used for amplification of *Xa7* linked fragment (207bp) and IRBB7 was used as a positive control for the presence of *Xa7* gene (Fig. 3). Among the selected genotypes, 11 genotypes along with IRBB7 showed 207 bp bands, thereby, possessing *Xa7* gene. These positive genotypes include lines-1, 3, 8, 10, 11-14, 19, 20 and 29 while the rest of selected genotypes such as lines-2, 4-7, 9, 15-18, 21-28 and Basmati-385 lacking *Xa7* gene. The data was scored using "1" sign for presence of gene (*Xa7*) and "0" sign for absence of gene (*Xa7*) (Table 3).

Table 2. Bacterial blight disease rating scale.

Groups	Lesion percentage	Disease Rating Scale
HR (Highly Resistant)	0-3	1
R (Resistant)	4-12	3
MR (Moderately Resistant)	12-25	4
MS (Moderately Susceptible)	25-50	5
S (Susceptible)	51-87	7
HS (Highly Susceptible)	87-100	9

Responses of rice genotypes to bacterial blight isolates

Three local isolates of bacterial blight were used to evaluate the responses of selected genotypes. Analysis of varience showed significant differences among the genotypes for mean lession length developed by *Xoo*1, *Xoo*2, and *Xoo*3, respectively. Lesion size of selected genotypes on inoculation with *Xoo*-1 ranged as 2.93 – 42.73%. Minimum value was recorded for line 14 and maximum for basmati-385. Lesion size of selected genotypes on inoculation with *Xoo*-2 ranged as 0.733 – 37.733%. Minimum value was manifested by basmati-385 while manimum by line 19. Lesion size of selected genotypes on inoculation with *Xoo*-3 ranged as 1.033 – 53.00%. Minimum value was observed on line 21 while maximum on Basmati-385.

Most of the selected genotypes showed resistant reactions to *Xoo*-1, however Basmati-385, lines-6, 7, 23 and 24 showed susceptible reactions. Six genotypes including lines-1, 8, 10, 11, 13 and 14 along with IRBB7 were found highly resistant (HR), to *Xoo-1* (Table 4, Fig. 4). Similarly, most of the selected genotypes showed resistant reactions to *Xoo*-2, however, Basmati-385 and line-7 showed susceptible reactions. Eleven genotypes including lines-1, 3, 8, 10, 11, 12, 13, 14, 19, 20, and 29 along with IRBB7 were found highly resistant (HR) to *Xoo-2* (Table 4).

Table 3. Screening of selected genotypes of rice for the presence of bacterial blight resistance gene *Xa7*.

S/No	Genotypes	*Xa7* gene	S/No	Genotypes	*Xa7* gene
1	Basamti-385	0	17	Line-15	0
2	IRBB7	1	18	Line-16	0
3	Line-1	1	19	Line-17	0
4	Line-2	0	20	Line-18	0
5	Line-3	1	21	Line-19	1
6	Line-4	0	22	Line-20	1
7	Line-5	0	23	Line-21	0
8	Line-6	0	24	Line-22	0
9	Line-7	0	25	Line-23	0
10	Line-8	1	26	Line-24	0
11	Line-9	0	27	Line-25	0
12	Line-10	1	28	Line-26	0
13	Line-11	1	29	Line-27	0
14	Line-12	1	30	Line-28	0
15	Line-13	1	31	Line-29	1
16	Line-14	1			

Similarly most of the selected genotypes showed resistant reactions to *Xoo*-3, however, Basmati-385, lines-6, 7 and 24 showed susceptible reactions. Ten genotypes including lines-1, 3, 8, 10, 12, 13, 14, 19, 20, and 29 along with IRBB7 were found high resistant (HR) to *Xoo-3* (Table 4).

Table 4. Reactions of selected genotypes of rice in response to *Xoo* isolates.

Variety/Line	Lesion size (as percent of leaf length)			Responses		
	Xoo-1	Xoo-2	Xoo-3	Xoo-1	Xoo-2	Xoo-3
Basmati-385	42.73	37.733	53	MS	MS	S
IRBB7	3.5	1.610	1.967	HR	HR	HR
Line-1	3.33	2.833	2.233	HR	HR	HR
Line-2	7.2	2.367	1.767	R	HR	HR
Line-3	4.77	3.233	3.033	R	HR	HR
Line-4	16.33	2.367	4.433	MR	HR	R
Line-5	4.18	6.867	9.2	R	R	R
Line-6	36.043	1.6	43.433	MS	HR	MS
Line-7	33.4	25.433	45.633	MS	MS	MS
Line-8	3.5	1.633	3.33	HR	HR	HR
Line-9	14.213	2.767	9.1	MR	HR	R
Line-10	3.667	2.7	2.5	HR	HR	HR
Line-11	3.067	2.05	5.233	HR	HR	R
Line-12	3.267	1.567	3.433	HR	HR	HR
Line-13	3.567	2.4	2.567	HR	HR	HR
Line-14	2.933	2.9	3.5	HR	HR	HR
Line-15	6.967	3.18	4.267	R	HR	R
Line-16	9.967	6.943	6.9	R	R	R
Line-17	9.113	1.01	1.667	R	HR	HR
Line-18	4.267	3.367	7.615	R	HR	R
Line-19	4	0.733	3.667	R	HR	HR
Line-20	4	2.833	3.633	R	HR	HR
Line-21	3.887	13.33	1.033	HR	MR	HR
Line-22	23.133	3.1667	2.1	MR	HR	HR
Line-23	25.533	19	1.8	MS	MR	HR
Line-24	27	0.787	50.233	MS	HR	MS
Line-25	14.473	1.503	17.567	MR	HR	MR
Line-26	14.38	2.067	10.333	MR	HR	R
Line-27	16.9	3.733	3.100	MR	HR	HR
Line-28	18.783	1.767	3.433	MR	HR	HR
Line-29	4	1	3	R	HR	HR

HR = highly resistant, MR = moderately resistant, MS = moderately susceptible, S= susceptible.

Discussion

Bacterial blight (BB) has become a serious threat for rice cultivators in many Asian countries including Pakistan. The rate of BB incidence has rapidly increased in many rice growing areas of Pakistan (Ali *et al.,* 2016a). Several accessions of the world famous basmati varieties including Basmati-385, are at risk of being affected by BB, necessiating to control the disease urgently. Exploration and deployment of the BB-resistance host genes is the most effective and environment-friendly method to control this disease. Several resistance genes such as *Xa4, xa5, xa13*, and *Xa21* have already been incorporated into rice cultivars for development of new resistance varieties which give fruitful results against the pathogen (Perumalsamy *et al.,* 2010; Sanchez *et al.,* 2000; Huang *et al.,* 1997). In the present study a molecular survey was conducted for identification of *Xa7* gene in an advance population of rice. Eleven genotypes were identified having *Xa7* gene.

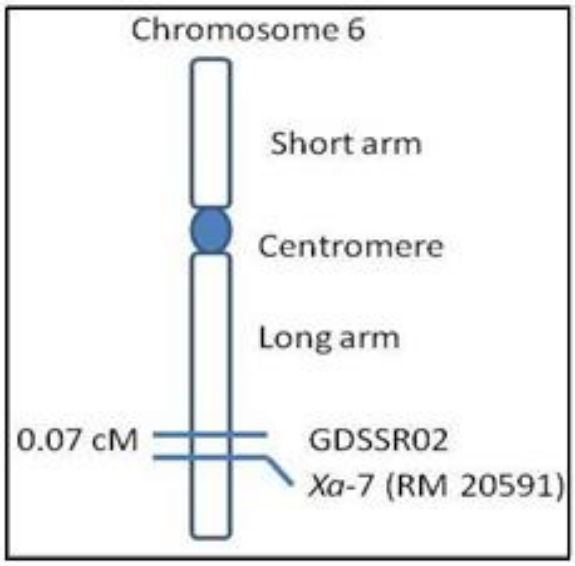

Fig. 1. Chromosome six of rice and approximate locus of *Xa-7*.

These genotypes showed high level of resistance to bacterial blight when artifically inoculated in the field. Therefore, the presence of *Xa7* gene in rice may confer durable resistance against different isolates of bacterial blight.

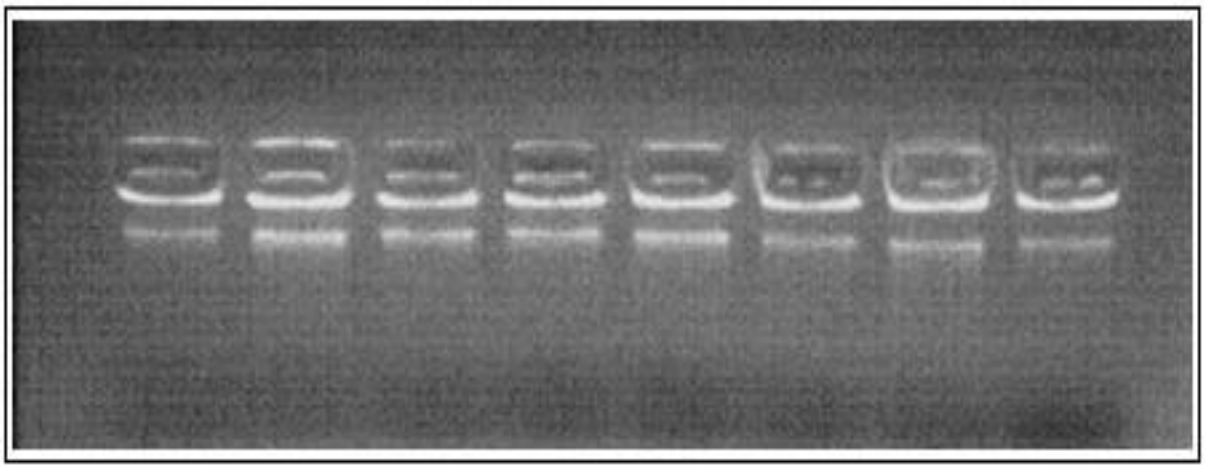

Fig. 2. Genomic DNA extracted from fresh seeds of selected genotypes of rice.

Similar survey was previously conducted by Ramalingam *et al.* (2001) for the presence of BB resistance genes *xa5*, *xa13* and *Xa21* in Chinese rice germplasm. Traditional approaches are also used for the identification of different resistance genes in rice germplasm but the drawback is that it takes longer period of time and needs artificial inoculation with different pathotypes (Abbasi *et al.,* 2011). Therefore, it is important to develop effective strategies for molecular identification of major bacterial blight resistance gene (s).

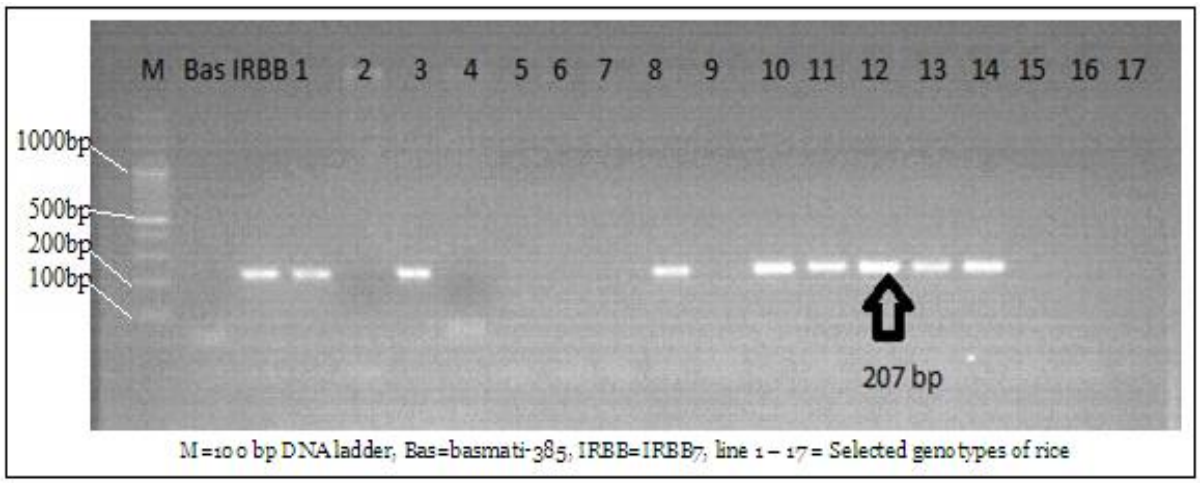

Fig. 3. Screening an advance population of rice for the presence of *Xa7* gene (Arrow showing 207 bp bands linked to *Xa7* gene).

Genetic resistance is an important sought after trait by the rice growers and producers, thus detection of *Xa7* associated STS markers coupled with phenotypic resistance shown by the selected genotypes are important leaping points for further evaluation and eventually release of BB resistant elite lines. However, the pathogen may overcome the single gene resistance rapidly due to considerable variations in pathogen population leading to the emergence of new strains (Singh *et al.,* 2001). Therefore, effective stratigies should be made to pyramid two or more than two resistance genes into a single rice cultivar to

attain a broad and durable range of resistance (Huang *et al.,* 1997).

Fig. 4. Lesion developed by *Xoo-1* on Basmati-385 and IRBB7.

Conclusion

In this study a molecular survey was conducted for identification of *Xa7* gene in an advance population of rice. Eleven genotypes were identified having *Xa7* gene. These genotypes showed high level of resistance to bacterial blight when artifically inoculated in the field. Therefore, the presence of *Xa7* gene in rice may confer durable resistance against different isolates of bacterial blight.

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