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Full Length Research Paper

Detection of bacterial blight resistant gene xa5 using linked marker approaches

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Rice is the primary source of food for 57% of the world's population. Genetic resistance is important to control many kinds of pathogenic diseases. Bacterial blight caused by *Xanthomonas oryzae pv oryzae (Xoo)* decreases rice production by 20 – 30% and up to about 90% loss of grain weight. *xa5* is an important recessive bacterial blight resistant gene, which is effective and important in Asian rice breeding program. It was also used in combination by incorporation with various recessive and dominant BB resistant genes. The purpose of our study was to identify the bacterial blight resistant genes *xa5* in Pakistani rice germplasm including Basmati varieties. The seeds were collected from different research institute and then sowed in the National Institute for Biotechnology and Genetic Engineering (NIBGE) in pots. DNAs were extracted and surveyed for polymorphism by using DNA marker linked to *xa*5 gene. During this polymorphic survey, out of 88 germplasm lines, 45 lines showed the presence of *xa*5 gene like MB 2, MB 33 MB 57 and MB 66. All these lines showed the amplification of 240 bp corresponding to resistant source IRBB-5 line, while 43 germplasm lines showed no such fragment and elucidated same bands as susceptible source IR-24 having fragment of about 230 base pair. The 10 Pakistani Basmati varieties were also surveyed for *xa*5 gene. It was observed that none of our cultivated basmati varieties exhibited the presence of *xa*5 gene. The purpose of screening of *xa*5 gene in Pakistani rice germplasm is to utilize the local source of *xa*5 gene for elite molecular breeding program being carried out at NIBGE in future including pyramiding of different disease resistant gene in Basmati varieties.

Key words: Rice, *xa5*, bacterial blight, linked marker.

INTRODUCTION

Rice (*Oryza sativa* L.), a member of the family Poaceae is widely grown in tropical and subtropical regions (Ezuka and Kaku, 2000). Rice plays a major role as it is the staple food for over 2.7 billion people worldwide. It also provides employment for over one billion, who either work directly in rice production or in related supported activities (Dat, 2004). Rice production and consumption is concentrated in Asia, where more than 90% of all rice is consumed. Out of 15 of the rice growing countries only 2 countries are outside of Asia (Khush and Brar, 2002). The current rice production is standing at approximately 560 million tons and cultivated area is 360 million acres (Ronald et al., 1992). Thus, 350 million tons of more rice will have to be produced by the year 2020 (Khush, 1995). Pakistan is one of the rice growing countries and third largest Basmati exporter. During the year 2007 - 2008, the cultivated rice area of Pakistan was 2.51 million hectares and the production was 5.48 million tons (Anonymous, 2007, 2008).

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Abbreviations: BLB, Bacterial leaf blight; **CR,** complete resistance; **PR,** partial resistance; **LRR,** leucine-rich repeat; **NBS,** nucleotide-binding site; **RFLP,** restriction fragment length polymorphism; **BAC,** bacterial artificial chromosome; **PCR,** polymerase chain reaction.

Unfortunately, such an important crop is under the threat of abiotic and biotic stresses. Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae pv oryzae (Xoo)* is one of the major biotic destructive diseases throughout the world (Khan, 1996). The disease is known to occur in epidemic proportions in many parts of the world, incurring severe crop losses of up to fifty percent. Crop loss assessment studies have revealed that this disease reduces grain yield to varying levels. (Gnanamanickam et al., 1999). The yield losses in severely infected fields generally range from 20 to 30% but may reach up to 80% (Singh et al., 1977; Ou, 1985). The options for the control of the disease include cultural practices, chemical control or resistant varieties. The chemical control of BB in the monsoon climate of Asia is impractical. Additionally, no effective bactericide is commercially available for disease control. Therefore, the preferred strategy for disease management is through varietal resistance.

The exploitation of host resistance has been shown to be the only reliable method to control the disease. Investigations carried out on the genetics of the host resistance to causal organism *Xoo* showed two types of host resistances, complete resistance (CR), and partial resistance (PR). Several BB resistance genes including 14 dominant *Xa1, Xa2, Xa3, Xa4, Xa7, Xa10, Xa11, Xa12, Xa14, Xa16, Xa17, Xa18, Xa21* and *Xa22* and 6 recessive *xa5, xa8, xa13, xa15, xa19 and xa20* have been identified so far (Khush and Kinoshita, 1991; Lin et al., 1996; Chun et al., 2007). The resistant genes *xa5, xa8, xa13, xa24, xa26* and *xa28* occur naturally and confer race-specific resistance. The other three*, xa15, xa19 and xa20*, have been created by mutagenesis and each confers a wide spectrum of resistance to *Xoo* (Ogawa, 1996; Lee et al., 2003). Li et al. (2001) reported that *xa5* was partially dominant, as F1 individuals from an IR24 × IRBB5 cross had lesion lengths intermediate between the two parents for all *Xoo* races except Philippines four races. To date, only two dominant R genes for BB resistance, *Xa1* and *Xa21*, have been cloned. *Xa21* encode leucine-rich repeat (LRR) receptor kinase-like proteins, whereas, *Xa1* encodes a nucleotide-binding site (NBS)-LRR protein (Song et al., 1995; Yoshimura et al., 1998; Sun et al., 2004). BB resistant gene *xa5* has been positionally cloned and encodes the gamma subunit of transcription factor IIA (TFIIAγ). Sequencing of TFIIAγ in resistant and susceptible isolines revealed two nucleotide substitutions resulting in an amino acid change between resistant and susceptible cultivars. This association was conserved across 27 resistant and nine susceptible rice lines in the Aus-Boro group (Iyer and McCouch, 2004). The bacterial blight resistance gene *xa*5 has been mapped on chromosome 5 with restriction fragment length polymorphism (RFLP) markers RG556 and RZ390 and microsatellite markers RM122 and RM390 (Blair and McCouch, 1997). Later on, *xa*5 region on chromosome 5 was cloned successfully using bacterial artificial chromosome (BAC) clones 9E8 and 28N22 containing *xa*5 locus. *xa5* is genetically defined as recessive; however, con-troversy exists regarding its recessive action. Li et al. (2001) BB resistance genes of rice varieties are distributed in almost all of the Asian countries but the percentages vary from country to country from a high 17.2% for Indonesia to a low 0.3% for India. The distribution of *xa5* gene showed area specificity in Bangladesh and Nepal, the percentage of varieties with *xa5* was 25.9 and 13.3%, respectively, but in other countries such as Thailand and Indonesia the percentage was less than 1.0%. Only a few varieties with *Xa10* and *Xa14* were found resistant (Busto et al., 1990).

Bacterial blight is considered to be an important disease in various parts of rice growing areas of Pakistan. In the subcontinent, bacterial blight is a major biotic constraint in the irrigated rice belt comprising Punjab and the adjoining north-western States of India (Goel et al., 2002).

Waheed et al. (2009) reported the loss in yield by bacterial leaf blight of 11 rice genotypes viz., PARC-291, PARC-292, PARC-293, PARC-294, PARC-295, PARC-296, PARC-297, PARC-298, PARC-299, PARC-300 and PARC-301 studied under natural field. Significant differences were observed for yield and yield components. Lowest infection was observed in the lines PARC-301 and PARC-298, PARC-299 whereas PARC-301 showed resistance to bacterial leaf blight. In Asian Rice Research Programs, *xa5* is a major source of resistance and has been used in breeding programmes. The present research has been conducted for the detection of the presence of BB resistant gene *xa5* using polymerase chain reaction (PCR) technology in Basmati varieties and in rice germplasm available in Pakistan.

MATERIALS AND METHODS

Plant Materials

Hundred rice genotypes/lines (list given in Table 1 along with their accession # and local names) obtained from the Institute of Agricultural Biotechnology and Genetic Resources (IABGR), National Agriculture Research Council (NARC), Islamabad, along with 19 basmati breeding lines (collected from Rice Research Institute Kala Shah Kaku), 8 commercial Basmati varieties viz., Basmati 370, Super Basmati, Basmati 385, Basmati Pak., Basmati 2000, Basmati 198, Kashmir Basmati and Shaheen Basmati and 2 IRRI varieties IRBB-5, (having *xa5* gene) and IR-24 (with no *xa5* gene) were grown in pots at the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad.

Total genomic DNA extraction

Total genomic DNA was extracted using young leaves at seedling stage as described by Dellaporta et al. (1983). Fresh leaves from 5 individuals of each line/variety were bulked together and the DNA was extracted. The concentration of extracted genomic DNA was measured by flourometer DyNA Quant^{TM}200 and the DNAs were diluted to 10 ng/uL using sterilized distilled water and stored in microfuge tubes at 4°C for further use.

Table 1. Rice genotypes/lines used in genetic analysis studies showing presence (+) and absence (-) of *xa5* gene.

S/N	Varieties/ Lines code	Acc. No.	Local Name	*xa5*	S/N	Varieties/ Lines code	Acc. No.	Local Name	*xa5*
1	MB-1	Pak 0244	Jhona 426-37	-	51	MB-57	Pak 0440	Ratua 69	+
2	MB-2	Pak 0253	Santhi sufaid	+	52	MB-58	Pak 0445	Dhan Munji 238	+
3	MB-3	Pak 0255	Jhoni 213	-	53	MB-59	Pak 0448	Bamla suffaid	-
4	MB-4	Pak 0257	Dhan 263	+	54	MB-60	Pak 0450	Sathra 338 A4	-
5	MB-5	Pak 0260	Dhan 400	+	55	MB-61	Pak 0452	Sathra surkh	+
6	MB-6	Pak 0262	TIRI 424-2	-	56	MB-62	Pak 0457	Son 15	+
7	MB-7	Pak 0263	TIRI 429-3	+	57	MB-63	Pak 0462	91 S2	+
8	MB-8	Pak 0264	1A	+	58	MB-65	Pak 0467	Munji sufaid	+
9	MB-10	Pak 0268	6	-	59	MB-66	Pak 0468	170	+
10	MB-11	Pak 0272	11	+	60	MB-68	Pak 0472	Dhan 300	-
11	MB-12	Pak 0279	18A	+	61	MB-69	Pak 0474	Sathra 343	-
12	MB-13	Pak 0282	20	-	62	MB-70	Pak 0475	345	-
13	MB-14	Pak 0287	24	-	63	MB-71	Pak 0476	368	+
14	MB-15	Pak 0289	24A-10	-	64	MB-72	Pak 0479	Santhi sufaid	-
15	MB-16	Pak 0292	29A-1	+	65	MB-73	Pak 0481	Santhi 232	-
16	MB-17	Pak 0297	31	-	66	MB-74	Pak 0482	Sathi Kalri 235	-
17	MB-18	Pak 0298	32	-	67	MB-75	Pak 0483	Santhi sufaid	-
18	MB-19	Pak 0305	38	+	68	MB-76	Pak 0484	Santhi 243	-
19	MB-20	Pak 0308	40	-	69	MB-77	Pak 0485	Santhi 256	-
20	MB-22	Pak 0312	43	-	70	MB-78	Pak 0487	Santhi 288	+
21	MB-23	Pak 0315	45	+	71	MB-79	Pak 0488	Santhi 290	+
22	MB-24	Pak 0317	52	+	72	MB-80	Pak 0489	Santhi 290A	+
23	MB-25	Pak 0318	70	+	73	MB-81	Pak 0490	Sathra 252A	-
24	MB-26	Pak 0319	71	+	74	MB-82	Pak 0498	Sathra 305	-
25	MB-27	Pak 0322	73	+	75	MB-84	Pak 1764	Cheeni	-
26	MB-28	Pak 0324	75	-	76	MB-85	Pak 1768	Khanduri	-
27	MB-29	Pak 0325	76	+	77	MB-86	Pak 1772	Cheeni	-
28	MB-31	Pak 0331	81B	-	78	MB-87	Pak 1775	Cheeni	-
29	MB-32	Pak 0342	93	+	79	MB-88	Pak 1777	Chingan	+
30	MB-33	Pak 0344	SM3-34	+	80	MB-90	Pak 2783	-	+
31	MB-34	Pak 0347	SM6-34	-	81	MB-92	Pak 2830	Brinj	-
32	MB-36	Pak 0350	SM12-34	+	82	MB-93	Pak 2836	Brinj	-
33	MB-37	Pak 0351	SM16-34	+	83	MB-94	Pak 2862	Murgi brinj	**+**
34	MB-39	Pak 0363	Dhan 247	+	84	MB-95	Pak 2873	Murgi brinj	**+**
35	MB-40	Pak 0365	Dhan 263	-	85	MB-97	Pak 2925	Chawal	-
36	MB-41	Pak 0366	Kharsu 295A	-	86	MB-98	Pak 2967	Kharay ganjay	-
37	MB-43	Pak 0379	Mushkan 56	-	87	MB-99	Pak 3375	Nali	-
38	MB-44	Pak 0380	Mushkan 73S	+	88	MB-100	Pak 3402	Chinese	-
39	MB-45	Pak 0382	Mushkan 77	+	89			IRBB5	**+**
40	MB-46	Pak 0383	Mushkan chahi	+	90			IR-24	**-**
41	MB-47	Pak 0394	Chambu 128	+	91			Basmati-370	**-**
42	MB-48	Pak 0395	Chahora 144	+	92			Basmati-2000	**-**
43	MB-49	Pak 0398	Patti 168	-	93			Basmati-385	**-**
44	MB-50	Pak 0409	Bara	+	94			Super Basmati	**-**
45	MB-51	Pak 0424	Palman 188	-	95			Shaheen Basmati	**-**
46	MB-52	Pak 0425	Sufaida 246	+	96			Basmati-198	**-**
47	MB-53	Pak 0428	Basmati 502	+	97			Basmati Pak	**-**
48	MB-54	Pak 0429	Mutant 11-9	+	98			Kashmir Basmati	**-**
49	MB-55	Pak 0432	Nc1 –536	+	99			Basmati 386	**-**
50	MB-56	Pak 0438	Ratua 3882	-	100			Indian Basmati	**-**

Accession # and local names are the same as mentioned in plant germplasm catalogue 1997, published by Institute of Agro. Biotechnology and Genetic Resources [previously Plant Genetic Resources Institute (PGRI)], NARC, Islamabad.

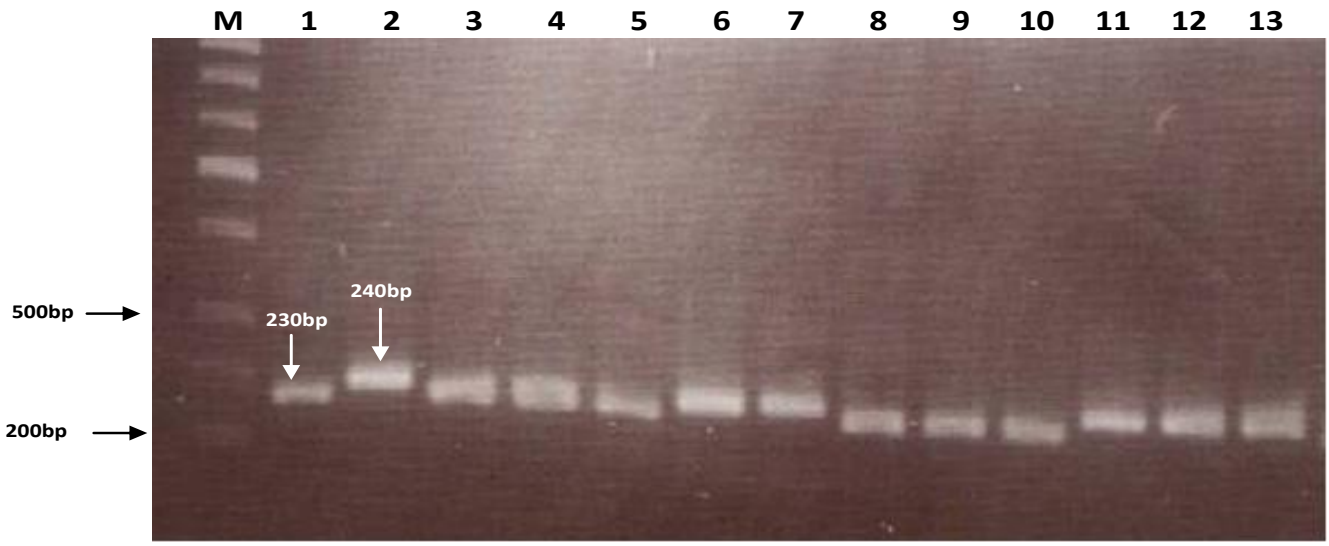

Figure 1a. Banding patterns showing the presence and absence of *xa-5* gene in germplasm of rice amplified 240 and 230 bp size fragments, respectively. Lane M = 100 bp DNA ladder, lane 1 = IR-24, lane 2 = IRBB-5, lane 3 = MB-1, lane 4 = MB-3, lane 5 = MB-6, lane 6 = MB-2, lane 7 = MB-4, lane 8 = MB-14, lane 9 = MB-20, lane 10 = MB-28, lane 11 = MB-16, lane 12 = MB-26, lane 13 = MB-33.

PCR amplification of *xa5* specific fragments

Amplification of *xa5* linked DNA fragment was carried out using tightly linked PCR based markers RM 122 (developed by Chen et al., 1997). Amplification reactions were carried out in 25 uL reaction volumes containing 50 ng genomic DNA, 1.0 µM each of primer RM122 forward and RM122 reverse, 100 µM each of dATP, dCTP, dGTP and dTTP, 1 unit of Taq DNA Polymerase (Fermentas), 1X Taq polymerase buffer and 2.5 mM $MgCl_2$. DNA amplification was performed in DNA thermal cycler (BioRad) programmed as follows: an initial denaturation of 5 min at 94°C; 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 2 min (extension). One additional cycle of 10 min at 72°C was used for final extension. Amplification products were resolved by electrophoresis on 3% agarose gels run in 0.5X TAE. The amplified products were observed under UV transilluminator after being stained with ethidium bromide (10µg/mL) and scored for the presence and absence of *xa5* linked DNA fragment.

Data analysis

The PCR product was measured as polymorphic bands pattern. The bands of *xa5* gene were standardized by the amplified DNAs of IRBB-5 and IR-24 used as control. IRBB-5 showed the amplification DNA fragment having a gene of about 240 bp, and IR-24 showing the DNA band of about 230 bp was considered as susceptible line. The data was scored using "+" sign for presence of gene and "–" sign for those having no *xa5* gene.

RESULTS AND DISCUSSION

DNA analysis of all the rice germplasm, basmati breeding lines and different basmati varieties exhibited two different sizes of band. The banding pattern of all the individuals were either identical with that of the IRBB-5 (having *xa5* gene) or with that of the IR-24 (without *xa5*) gene. The size of the band corresponding to IRBB-5 was 240 bp whereas the band corresponding to IR-24 was 230 bp in size. During this polymorphic survey, out of 88 rice lines, 45 rice lines along with IRBB-5 amplified 240 bp size fragments indicating the presence of *xa5* gene (Table 1), while the remaining 43 rice lines were found to be without *xa5* gene as 230 bp DNA fragment was found to be amplified in all these lines and also in IR-24 (Figures 1a and b).

Ramalingam et al. (2001) performed similar type of molecular survey for the presence of bacterial blight resistance genes *xa5, xa13* and *Xa21* in Chinese rice germplasm. They surveyed 56 germplasm, 23 were reported to carry allele 3, 30 had allele 2 and no allele was reported in one genotype. Arif et al. (2008) also conducted a molecular survey for the detection of *Xa*4 gene in Pakistan rice germplasm (*Xa*4 is the dominant resistant gene showed resistance against many bacterial strains). They screened more than 100 genotypes along with basmati lines for the presence and absence of *Xa*4 gene in Pakistani rice germplasm. The present study generate molecular information regarding presence and absence of resistance gene *xa*5 in our existing germplasm, which can facilitate rice breeder to incorporate the resistance genes from the known source to our cultivated basmati varieties through marker assisted selection. Huang et al., (1997) used gene specific PCR markers for the identification of pyramided line carrying different combinations of BB resistant genes. They obtained lines with different combination of genes, 2 lines for *Xa4*/*xa5*/*xa13*, 3 lines for *Xa4*/*xa5*/*xa21*, 3 lines for *Xa4*/*xa13*/*Xa21*, 3 lines for *xa5*/*xa13*/*Xa21* and 2 lines with all four genes

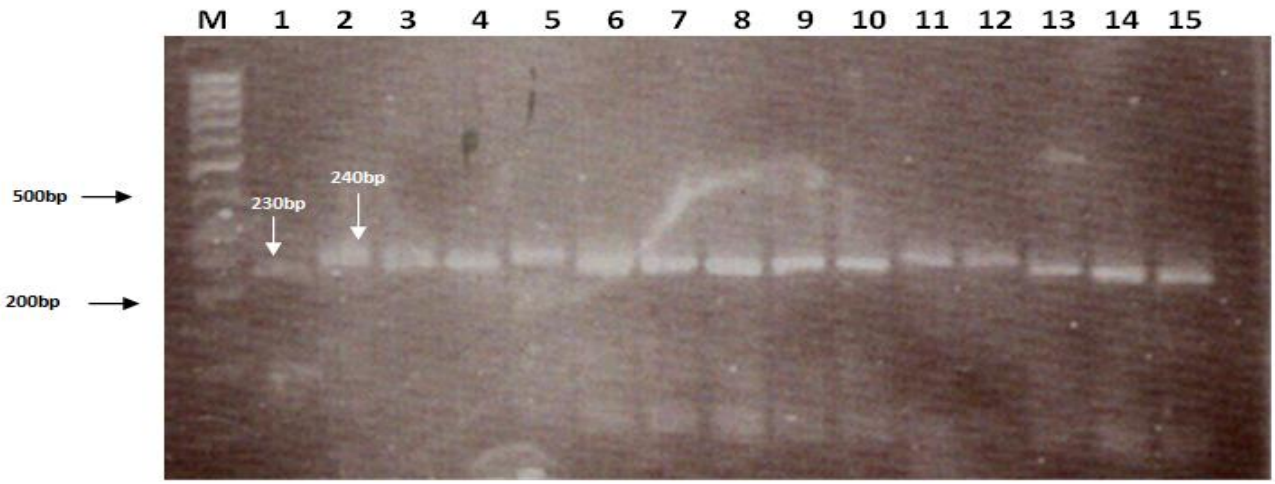

Figure 1b. Banding patterns showing the presence and absence of *xa-5* gene in germplasm of rice amplified 240 and 230 bp size fragments, respectively. Lane M = 100 bp DNA ladder, lane 1= IR-24, lane 2 = IRBB-5, lane 3 = MB-45, lane 4 = MB-54, lane 5 = MB-57, lane 6 = MB-31, lane 7 = MB-68, lane 8 = MB-69, lane 9 = MB-70, lane 10 = MB-72, lane 11 = MB-66, lane 12 = MB-71, lane 13 = MB-73, lane 14 = MB-74, lane 15 = MB-75.

(*Xa4*/*xa5*/*xa13*/*Xa21*). They also observed that *xa5* in any combination showed resistance against six BB Philippine races.

Khan et al. (2000) observed that BLB incidence is increasing in Pakistan especially in "Kaller" belt which is famous for rice cultivation. Although conventional approach for the identification of different resistance genes in rice germplasm is also being used (Lee et al., 2003; Kihupi et al., 2001), it is time consuming and need artificial inoculation of all the lines with different pathotypes of the pathogen. In our study, out of 88 germplasm lines 45 showed the presence of *xa5* gene in the existing gene pool in Pakistan. This implies that these lines can be used as source of *xa5* which could be transferred to different basmati varieties during the crossing and selection procedures. The most important result which came out of this screening was that none of our commercial basmati line possess *xa5* gene. Vera Cruz et al. (1996) observed in their study that different races of the same pathogen exist in the same field on the same cultivar. *Xoo* populations collected from different districts of Indian Punjab found high level of diversity in pathogen population. They also found that BB resistance gene *Xa*8 and *Xa*21 are effective against the prevalent isolates in Indian Punjab followed by *xa5* and *Xa*7 (Sodhi et al., 2003). The Basmati rice growing areas of Punjab in Pakistan is adjacent to Indian Punjab; it could be possible that the same genes effects are seen in Pakistani rice growing areas. However, studies on pathogen populations between countries and regions within countries have indicated that regionally defined pathogen populations are distinct, which could be attributed to the slow movement/ dispersal of the pathogen or slow partitioning of host genotypes (Adhikari et al., 1995; Leach et al., 1992; Nelson et al., 1994). Therefore, there is a need to identify other bacterial blight resistance genes in rice germplasm and Basmati breeding lines and also to check the effectiveness of identified bacterial blight resistance genes against the prevalent strain of Xoo in Pakistan. The knowledge of the effective resistance genes and the pathogen population structure would be helpful in deploying suitable resistance genes in different rice growing areas.

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