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Molecular screening of rice (*Oryza sativa* L.) germplasm for *Xa4, xa5 and Xa21* bacterial leaf blight (BLB) resistant genes using linked marker approach

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Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) has now become one of major rice diseases. During recent years, its occurrence has increased in Pakistan, particularly in 'Kallar tract' which is well-known for quality rice cultivation. Development of new resistant varieties by utilizing the available resistant gene-pool is most reliable breeding technique for the plant breeders. The present study was conducted to explore the genetic resources regarding presence and absence of BLB resistance genes *Xa4*, *xa5* and *Xa21* through DNA marker technology. Effectiveness of *Xa21* followed by *xa5* and *Xa7* genes has been reported to confer more resistance level for BLB in traditional rice belt of Pakistan. For this purpose, eighty (80) rice genotypes comprising of diverse origin including three isogenic lines viz., IRBB4 (carrying *Xa4*), IRBB5 (carrying *xa5*) and IRBB21 (carrying *Xa21*) as positive resistant gene checks and IR24 (carrying none of these) as negative gene check were genotyped. DNA fingerprinting results indicated the presence of *Xa4* gene in 41 entries, while 14 lines were positive for *xa5* gene. Only one local line was carrying *Xa21* gene along with *Xa4*. Thus, the present study will not only be helpful for rice breeders to develop new rice varieties carrying disease resistant genes, but will also provide the donors to incorporate / pyramid disease resistant genes in popular rice varieties like Super Basmati and Basmati 515 which are susceptible to BLB disease.

Key words: Molecular screening, rice germplasm, Xa4, xa5 and Xa21 genes, bacterial leaf blight.

INTRODUCTION

Millions of people in the world take rice as a staple food (Chakravarti et al., 2012; Deepti et al., 2013). The usage of rice is more than 90% in Asian countries, therefore it is consumed and produced more in Asia. In Pakistan, rice is a major cash crop as well as staple food. It is cultivated on 6.82 million acres occupying about 11% of total cultivated area during 2014 to 2015 and produces 6.73 million metric tons of milled rice (Economic survey of Pak., 2015). Among rice producing countries, Pakistan was ranked as third exporter of Basmati rice.

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Approximately, 2/3 of the produce is consumed at domestic level and the rest is surplus for export, which is around 10% of the total rice trade in the exporting country in the world after Thailand, Vietnam and India.

Rice is considered as the most grown food crop world-wide (Tang et al., 2001). The major constraint to rice production includes diseases caused by pathogenic fungus, bacteria and viruses; most drastic is bacterial leaf blight (BLB) which is caused by *Xanthomonas oryzae* pv. *oryzae* (Nagaraju et al., 2007; Das et al., 2014). In 1884, the farmers of Japan, growing rice noticed the disease first (Onasanya et al., 2010). After that the disease was also noticed from various parts of Asia, northern Australia, Africa and USA (Shanti et al., 2001; Wang et al., 2009). In various parts of the world, the disease is now becoming epidemic with losses of rice crop up to 50% with extreme severity. It is known to occur, more in "Kallar" belt of Pakistan (Khan et al., 2000). It was further reported by Agrawal et al. (2005) that loss of yield in more than 100% in Basmati rice due to bacterial blight.

BLB causes serious losses of rice production in Asia, Australia, Latin America, Africa and the United States (Chu et al., 2006). The Xanthomonas stain enters through hydathodes, stomata and wounds on the roots or leaves (Nino-Lui et al., 2006) which causes leaf wilting and affects photosynthesis that results in yield loss upto 20 to 50% (Adhikari et al., 1995). Such infected plant bring into being immature grains due to which more broken rice produced during milling (Khan et al., 2014). The loss of crop shows that the severity of disease depends upon many factors including susceptibility of the host, growth stages, and favorable environmental condition for the disease to occur. Strategies are required to prevent the damage as a result of infection and to reduce disease incident to become epidemic (Srinivasan and Gnanamanickam, 2005).

The chemical control of bacterial leaf blight is not efficient. Therefore, varietal resistance is the preferred approach for disease management that is more economic and environment friendly (Khush et al., 1989). In rice, the primary way to control bacterial leaf blight is single-gene resistance but with the passage of time, the virulence pattern of the pathogens has been changed which devastate the host resistance (Mew et al., 1992). It can be delayed by combining multiple genes (gene pyramiding) which provide resistance against a variety of pathogens (Babujee and Gnanamickam, 2000). The gene pyramiding shows more durable resistance in genotypes as compared to ordinary gene action (Ogawa et al., 1987; Yoshimura et al., 1995) which indicates a type of complimentary gene action among R genes (Narayanan et al., 2002; Swamy et al., 2006).

The genes for resistance against many pathogens (Xoo) are well indentified in rice. Thirty four genes (23 dominant and 11 recessive) have been identified till now that confer resistance to various strains of Xoo (Chen et al., 2011) and characterized in series from *Xa1* to *Xa29* (Kinoshita, 1995; Zhang et al., 1998; Lin et al., 1996; Chen et al., 2002; Gu et al., 2004; Chu et al., 2006). The characterization of major genes has contributed in the accomplishment for breeding resistant cultivars (Gu et al., 2004). The newly emerging genes have been inserted into new rice varieties so they show broad spectrum of resistance against a number of pathogens (Sanchez et al., 2000; Perumalsamy et al., 2010; Rajpurohit et al., 2010).

To avert the breakdown of resistance, pyramiding more than one resistance genes in a single genotype is recommended strategy (Babujee and Gnanamanickam, 2000). Presence of two or more effective genes in a rice variety conferred more resistance and the possibility of pathogen mutations to overcome the resistance is much lower than a single gene (Mundt, 1990). It is complicated to recognize plants having multiple resistance genes in presence of a major dominant gene (*Xa21*), which gives resistance against many races of pathogen in Pakistan. For the purpose, normally tightly linked molecular markers are exploited in order to identify genotypes with multiple resistance genes. Many Several SSR markers specific to BLB resistance genes have been previously studied (Davierwala et al., 2001; Blair et al., 2003; Portera et al., 2003; Rao et al., 2003; Sun et al., 2003; He et al., 2006). The pyramided rice lines used in this study, possess R genes viz, *Xa4*, *xa5* and *Xa21*.

The older methods for identification of resistance genes are also being utilized (Lee et al., 2003; Kihupi et al., 2001), but it will cost more to inoculate all the lines with all pathotypes that makes it laborious and time consuming. Marker aided selection (MAS) approach has proved its efficiency in breeding program to improve rice genotypes against disease (Mohan et al., 1997; Abbasi et al., 2010) which allows to introgress/pyramid single/multiple resistance genes in a genotype with desirable traits.

Xa4 gene was identified by Petpisit et al. (1977) which conferred durable resistance at all stages of plant growth in many commercial rice cultivars, that is, Pusa Bas 1, Pusa Bas 834, Punjab Mehran Basmati and Punjab Mehak (Mew et al., 1992). The *xa5* gene is an important race-specific recessive gene located on chromosome 5 which is widely used in breeding programs owing to its broad spectrum resistance against many Xoo strains. More recently *Xa21* (locus for BLB resistance) was identified in *Oryza longistaminata* that is a wild species. *Xa*21 encodes a leucine-rich repeat (LRR) receptor proteins and it is also the first cloned R gene for BLB (Song et al., 1997). The genes *Xa4*, *xa5* and *Xa21* were transferred into rice variety IR24 through repeated backcross (BC) breeding, results in the production of a near isogenic lines IRBB4 (carrying *Xa4*). IRBB5 (carrying *xa5*) and IRBB21 (carrying *Xa21*) respectively (Ogawa et al., 1991).

Gene pyramiding has been known to be the most effective, where *Xa21* contributes the largest portion of

resistance. It has further been reported that *Xa21* is the single gene to confer most effective resistance against 17 Xoo pathotypes from Punjab. Polymerase chain reaction (PCR) based DNA markers MP1 for *Xa4* (Ma et al., 1999), RM 122 for *xa5* (Chen et al., 1997) and STS marker pTa-248 (Ronald et al., 1992) have been developed. They are used to identify genotypes which contain these resistance genes to develop rice cultivars possessing multiple resistance genes (Perumalsamy et al., 2010; Rajpurohit et al., 2010).

In this research, we screened basmati varieties and rice lines of diverse origin for the status of BLB resistance genes (*Xa4, xa5* and *Xa21*) using specific primers. We believe that our findings may accelerate the breeding efforts to develop BLB resistant basmati varieties through gene pyramiding using Marker Assisted Selection (MAS).

MATERIALS AND METHODS

Plant materials

Eighty rice genotypes comprising of local and exotic origin three isogenic lines viz., IRBB4 (carrying *Xa4*), IRBB5 (carrying *xa5*) and IRBB21 (carrying *Xa21*) as positive resistant gene checks andIR24 as negative gene check were in this study (Table 1). For this purpose, leaf samples were taken from rice seedling grown in the growth chamber of MAS laboratory of Rice Research Institute, Kala Shah Kaku, Pakistan and used for isolating genomic DNA.

Genomic DNA extraction

Rice seeds of all the genotypes were grown in germination jars under controlled environment (temperature-35°C and humidity 80 to 85%) of growth chamber. Young leaves were taken for DNA extraction at seedling stage using Miniprep protocol given by Dellaporta et al. (1983). Few leaves (10 to 15 days old) were ground in liquid nitrogen and then added 800 ul extraction buffer. To prepare 100 ml of DNA extraction buffer, 1 M Tris HCL (10 ml), 0.25 M EDTA (20 ml), NaCl (2.9 g), sodium bisulphate (0.38 g), 20% SDS (6.25 ml) and dH_2O (63.75 ml) were mixed to form a homogeneous solution. The samples were incubated for 20 min at 65°C in water bath. Then equal volume of Chloroform:Isoamyl alcohol (24:1) was added. Centrifugation was carried out it for 8 min at 11,000 rpm. In a new eppendorf tube (1.5 ml), 500 ul of supernatant was taken and 1000 ul of chilled iso-propanol (-20°C) added. The samples were now centrifuged at 10,000 rpm for 10 min. Aqueous solution was discarded carefully so that pellet does not get damaged/lost. Pellet was then washed with 1000 ul of 70% ethanol and resuspended in 100 µl of Tris EDTA (TE) buffer.

To check the DNA quality of isolated genomic DNA, 3 ul of stock DNA mixed with 2 ul of loading dye was loaded in 0.8% agarose gel prepared in 1X TAE buffer (pH8.5). After 35 min run at 80 V, the genomic DNA concentration was observed using gel documentation system. Lambda DNA was used for estimation of DNA concentration of stock solution. Finally, the working dilutions were prepared with 30 to 50 ng/ul DNA concentration and stored at 4°C.

PCR amplification

DNA fragment carrying *Xa4, xa5* and *Xa21* was amplified using tightly linked and co-segregated primers “MP1” (Ma et al., 1999), RM122 (Chen et al., 1997) and “pTa 248” (Ronald et al., 1992) respectively. The sequences (forward and reverse) of these markers are given at Table 2. A reaction volume of 10 µl was used for PCR amplification, containing 2 µl DNA (30 ng/µl), 0.75 µl of forward Primerand 0.75 µl of reverse primer (10 p moles/µl), 1.5 µl of double distilled water and 5 µl of green master mix (Thermo, USA). Amplification was performed in a programmed thermocycler (Kyratech, Australia) with initial denaturation at 95°C for 5 min, followed by 35 denaturation cycles at 95°C for 1 min, annealing at 54°C for 1 min, elongation at 72°C for 2 min, and final extension at 72°C for 5 min. Amplified products of *Xa4, xa5* and *Xa21* were subjected to electrophoresis in 3.5% agarose gel run in 0.5X TAE buffer. Ethidium bromide (10 µg/mL) was used to stain the gel, followed by gel documentation (UV Tech, USA) and identified for the presence (++), absence (--) of BLB gene linked DNA fragment.

Data analysis

The amplified fragments of all the rice genotypes were scored by comparing with respective resistance (IRBB near-isogenic lines) and susceptible (IR24) bands. The data was scored using “++/--” signs for presence/absence of target gene respectively.

RESULTS

The exotic and indigenous advanced and approved lines (80) were screened for the presence and absence of three bacterial leaf blight resistance genes viz. *Xa4*, *xa5* and *Xa21* using PCR based gene linked markers MP1, RM122 and pTA248, respectively. The susceptible line IR24 and its 3 near-isogenic lines (NILs), IRBB4 (*Xa4*), IRBB5 (*xa5*) and IRBB21 (*Xa21*) were included as check for respective genes. Amplification of marker MP1which is a sequence-tagged site (STS) marker linked to *Xa4*, revealed a 150 bp fragment specific for *Xa4* mediated BLB resistance in NIL IRBB4, and a 120 bp DNA fragment that corresponds to a susceptible variety IR24 (Figure 1). Microsatellite marker RM122 was used for the screening of *xa5* resistance gene, which showed resistant and susceptible amplicons of 240 and 230 bp, respectively.

Screening for the dominant *Xa21* resistance gene by the amplification of STS marker pTA-248 was carried out which employed to track the resistant and susceptible amplicons of 1040/925 and 730 bp respectively. STS marker pTA 248 located within 1 cM of *Xa21* and originally obtained by sequencing the genomic clone of the RAPD 248 fragment using the same primer (Chunwongse et al., 1993; Ronald et al., 1992). The dominant gene *Xa21* is the most effective individually against many pathovars of *Xanthomonas oryzae* in Asian sub-continent in combination with *xa5 and xa13*. Ramalingam et al. (2001), Lee et al. (2003) and Kihupi et al. (2001) also conducted similar type of polymorphic survey for the presence of *xa5, xa13* and *Xa21* gene in rice germplasm.

During different polymorphic studies, it was revealed that 41 rice lines out of eighty including IRBB-4 amplified 150 bp size fragments indicating the presence of *Xa4* gene while the remaining 39 rice lines were found to be

Table 1. Screening of Rice lines/varieties for *Xa4, xa5* and *Xa21* gene using *MP1, RM 122* and *pTA 248.*

S/N	Designation	Origin	Type	Gene status			S/N	Designation	Origin	Type	Gene status		
				Xa4	*xa5*	*Xa21*					*Xa4*	*xa5*	*Xa21*
1	154	Pak	C	--	--	--	41	50189	Pak	F	--	++	--
2	4320	Pak	C	++	--	--	42	C622-2	Pak	F	--	--	--
3	4321	Pak	C	++	--	++	43	Mesva	Pak	F	++	--	--
4	33897-1	Pak	C	++	--	--	44	DD - 17	Pak	F	++	--	--
5	Mushkan	Pak	C	--	--	--	45	PK BB 4	Pak	F	--	--	--
6	Musa Tarmee	Pak	C	++	--	--	46	FL 478	Pak	C	--	--	--
7	Shahdab	Pak	C	--	--	--	47	LUKE 3			--	--	--
8	310	Pak	C	++	--	--	48	Sadri dumsiah	Iran	J	--	--	--
9	Mani Pur Black Rice 1	India	C	++	--	--	49	Super Bas/Bas515 (OL82)	Pak	F	--	--	--
10	RathaJhana Red Rice	India	C	--	--	--	50	RRI-7	Pak	F	--	--	--
11	LalMati Rice	India	C	--	--	--	51	8431	Pak	F	++	--	--
12	DR 82	Pak	C	--	--	--	52	DGWG			--	++	--
13	IR-9	IRRI	C	--	--	--	53	CSY-26			++	--	--
14	IR-26	IRRI	C	++	++	--	54	8685	Pak	F	--	--	--
15	IR-28	IRRI	C	++	--	--	55	Giza 172	Egypt	C	--	++	--
16	IR-74	IRRI	C	++	++	--	56	RustamDhan			++	++	--
17	NARC-10-2	Pak	C	++	++	--	57	Rexoro	USA	C	++	++	--
18	Sathra	Thailand	C	++	--	--	58	Indian Bas	India	F	--	--	--
19	TKM 6	India	C	--	++	--	59	Kashmir Bas	India	F	--	--	--
20	T N (1)	Taiwan	C	--	--	--	60	Pusa Bas 1	Pak	F	++	--	--
21	WP 153	India	C	++	--	--	61	Pusa Bas 834	Pak	F	--	--	--
22	Shua 92	Pak	C	++	--	--	62	Punjab Mehran Basmati	Pak	F	++	--	--
23	NIA-625	Pak	C	++	--	--	63	Punjab Mehak	India	F	++	++	--
24	Pk 7797-1-2-1-1	Pak	C	--	--	--	64	IRANI RICE	Iran	F	--	--	--
25	MOROBEREKAN	Pak	C	--	--	--	65	Sadri dumsufaid	Iran	F	--	--	--
26	KSK 418	Pak	C	++	--	--	66	Super Himalaya	Pak	F	--	--	--
27	KSK 427	Pak	C	++	--	--	67	Giza 171	Pak	F	--	--	--
28	KSK 462	Pak	C	++	--	--	68	SPR 7484-57-5	Pak	F	++	--	--
29	KSK 463	Pak	C	--	++	--	69	Sadri dumzard	Iran	F	--	--	--
30	KSK 466	Pak	C	++	--	--	70	SUWEON 300	Pak	F	--	--	--
31	KSK 476	Pak	C	++	--	--	71	Koshihikari	Pak	F	--	--	--
32	KSK 133	Pak	C	++	--	--	72	Tetop	India	F	++	--	--
33	IR 22	IRRI	C	++	--	--	73	Zenith	USA	F	++	--	--
34	RC 222	IRRI	C	++	--	--	74	NPT early	Pak	F	++	++	--
35	IRBB 58	IRRI	C	++	--	--	75	Swarna Sub1	IRRI	J	++	--	--

Table 1. Contd.

36	1	Pak	F	--	--	--	76	IRBB 66	IRRI	F	++	++	++
37	436-3	Pak	F	++	--	--	77	IRBB 5	IRRI	F	--	++	--
38	1021-5	Pak	F	--	--	--	78	IRBB 4	IRRI	F	++	--	--
39	4029-B	Pak	F	--	--	--	88	IRBB 21	IRRI	F	--	--	++
40	C622	Pak	F	--	--	--	80	IRBB 57	IRRI	F	++	++	++

++, Presence of gene; -, indicate absence of gene; C = coarse, F = Fine, J = Japonica.

Table 2. Sequence tagged site (STS) and microsatellite (SSR) markers used for detection of bacterial blight genes.

Gene	Located on chromosome	Marker	Type of marker	Primer sequence (5'-3')	Resistant band (bp)	Susceptible band (bp)	References
Xa4	4	MP1	STS	ATCGATCGATCTTCACGAGG TCGTATAAAAGGCATTCGGG	150	120	Ma et al., 1999
xa5	5	RM122	STS	GAGTCGATGTAATGTCATCAGTGC GAAGGAGGTATCGCTTTGTTGGAC	240	230	Chen et al., 1997
Xa21	11	pTA 248	STS	AGACGCGAAGGGTGGTTCCCGA AGACGCGGTAATCGAAGATGAAA	925 or 1100	730	Ronald et al., 1992

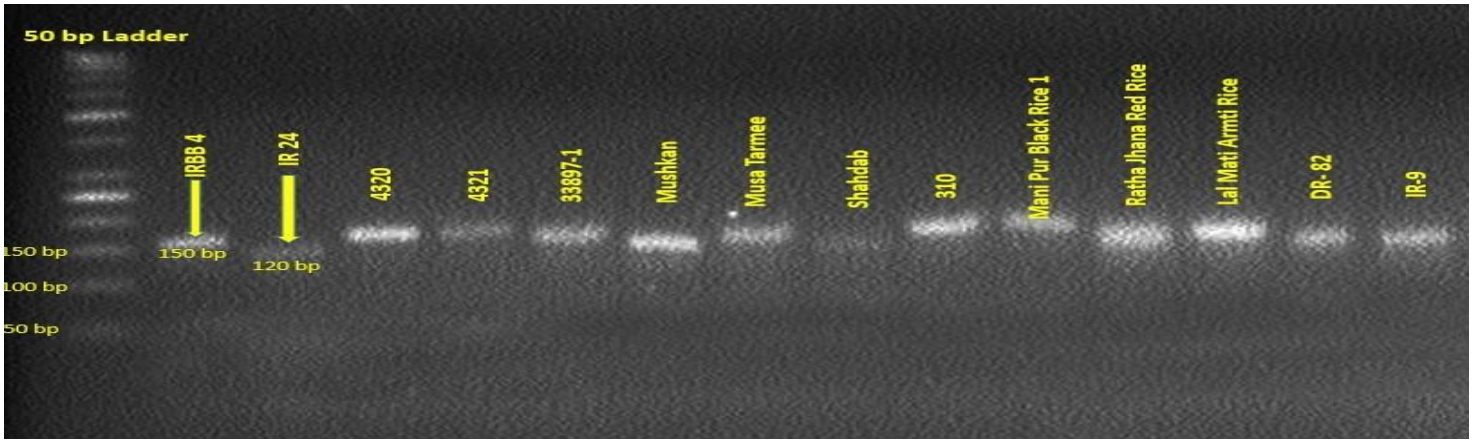

Figure 1. Banding patterns showing the presence and absence of *Xa4* gene in rice germplasm amplified 150 and 120 bp, size fragments respectively.

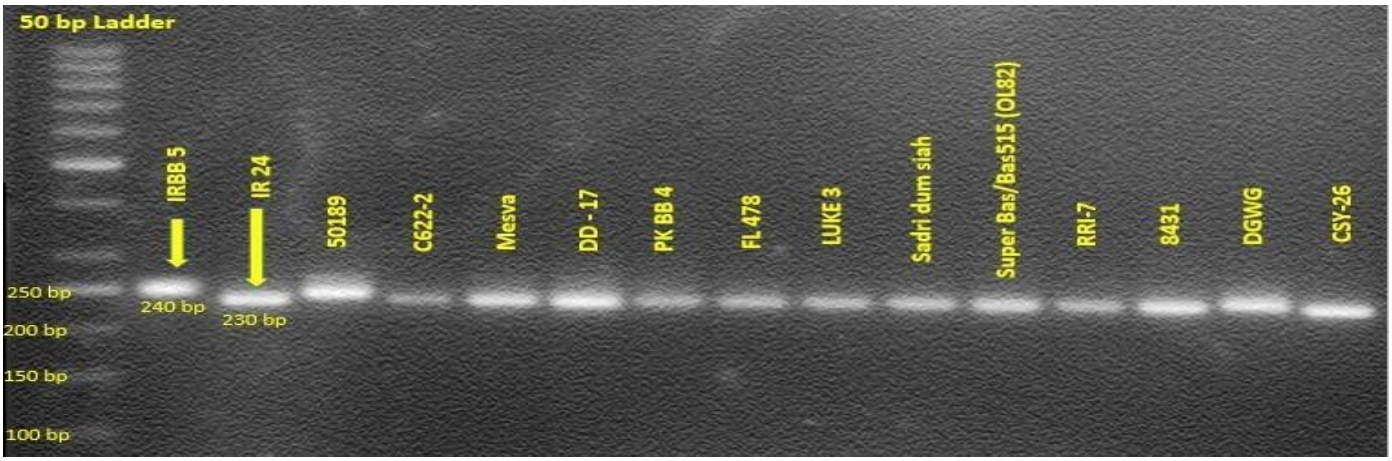

Figure 2. Banding patterns showing the presence and absence of *xa5* gene in rice germplasm amplified 240 and 230 bp size fragments, respectively.

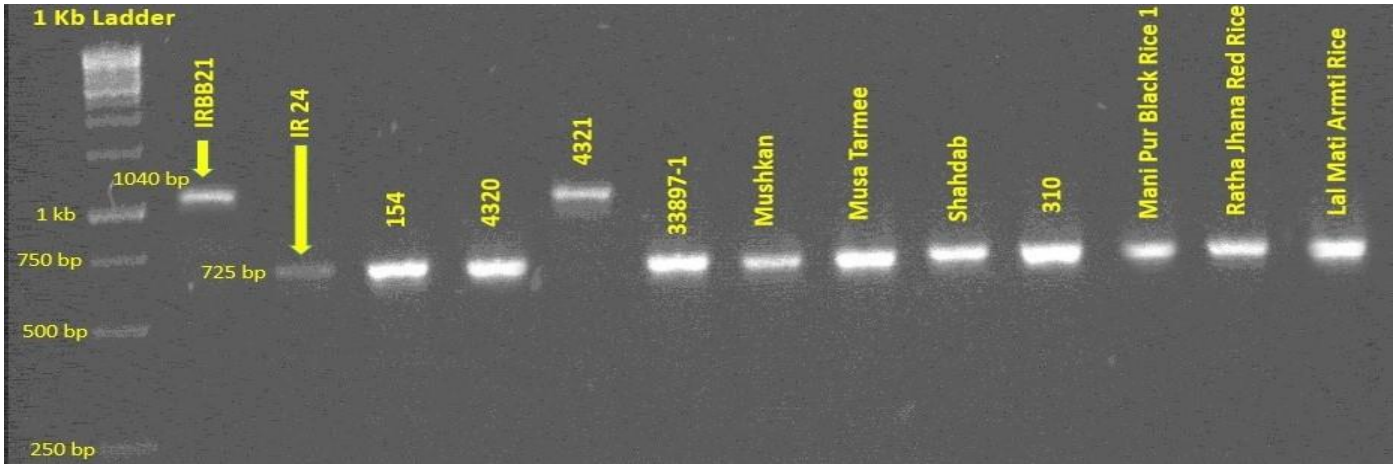

Figure 3. Banding patterns showing the presence and absence of *Xa21* gene in rice germplasm amplified 1040 and 725 bp size fragments, respectively.

without *Xa4* geneas 120 bp DNA fragment amplified in all these lines and also in IR-24 (Figure 1). 14 entries carried *xa5* gene as 240 bp DNA fragment amplified in them, indicate presence of *xa5* gene (Figure 2). No amplicons specific to *Xa21* allele were detected except 4321 (Figure 3) which is present in Rice germplasm of Kala Shah Kaku (KSK). Similar type of polymorphic survey was done by Arif et al. (2008) for the presence (++) and absence (--) of *Xa4* gene in Pakistan rice germplasm.

DISCUSSION

The present study provides molecular characterization for three resistance genes, that is, *Xa4*, *xa5* and *Xa21* in our germplasm including lines from different countries. It will facilitate rice breeders to insert the resistance genes from the known donor source to our local basmati varieties and advance lines during the crossing and marker assisted selection procedures. Huang et al. (1997) identified muliti-genic lines carrying different combinations of BB resistant genes through gene specific PCR markers. None of our germplasm carries *Xa21* gene in our findings except 4321 (line in germplasm). Sodhi et al. (2003) reported that combination of *Xa21* with xa13 and *Xa5*BB resistance gene are effective against the prevalent strains of Xanthomonas (X00).

The genes *Xa4*, *xa5* along with *Xa21* provide wide spectrum of bacterial blight resistance against many *X. oryzae* races (Perumalsamy et al., 2010). Eight entries possess combination of *Xa4* and *xa5*, which confer more durable resistance against different races of pathotypes. BLB resistance gene Xa21 is effective against the most prevalent pathotypes in Punjab followed by xa5 and *Xa7* (Sodhi et al., 2003). However, it has been reported that

pathogen populations of diffetent countries and different regions within countries are regionally different, which could be due to the slow movement or spreading of the pathogen or slow separation of host genotypes (Adhikari et al., 1995). Therefore, there is an urgent need to identify more BLB resistance genes in rice. Furthermore, efficacy of already identified genes against the prevalent Xoo strains needs to be categorized.

Rice is highly profitable cash crop of Pakistan and Bacterial leaf blight is among major obstacle in obtaining good yield since last few decades. Several strategies have been adopted to manage this problem but the most important and reliable method is to develop resistant rice varieties by introgression of resistant genes and subsequent marker assisted selection for other quality related traits which are crucial for development of resistant and high yield varieties. These results will be helpful for rice breeders to develop such kind of resistant varieties carrying disease resistant genes.

This information can be advantageously utilized to complement the bacterial leaf blight resistant gene pool available in Pakistan. This study indicates the presence of *Xa21* gene in rice cultivar (4321 line) may be used as a donor parent in hybridization breeding programmes, which will accelerate efforts to develop bacterial leaf blight resistance cultivar through MAS-based approach without compromising yield and grain quality.

Conclusion

This study indicates the presence of *Xa4* gene in forty one (41) rice genotypes, *xa5* in fourteen (14) genotypes, while *Xa21* in only two (2) genotypes including NILs among all the studied eighty (80) genotypes. Among these, eight entries were found to have combination of *Xa4* and *xa5* genes, while only one line 4321 has both *Xa4* and *Xa21* genes. On the other hand, two NILs IRBB57 and IRBB66 showed combination of all three genes. Results clearly show that both *Xa4* and *xa5* genes have been successfully pyramided in eight entries that can be further back-crossed with recurrent parent to recover desirable traits. Likewise, line 4321 possessing both *Xa4* and *Xa21* genes can be utilized as parent in breeding program as these two genes are known to show maximum resistance against wide spectrum of pathotypes to develop BLB resistance in rice varieties. These multi-genic lines in the germplasm, which contain the genes *Xa4, xa5* and *Xa21* in different combination will accelerate the efforts for developing broad spectrum BLB resistance in rice. The study further declares that gene pyramiding using MAS approach is an efficient and durable technique in rice breeding.

Conflict of Interests

The authors have not declared any conflict of interests.

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