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IDENTIFICATION OF BACTERIAL LEAF BLIGHT RESISTANCE GENES IN RICE (*ORYZA SATIVA* L.)

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ABSTRACT
An experiment was conducted during *Kharif 2013* to screen the thirty four rice cultivars against *Xanthomonas oryzae* pv. *oryzae*, and identify the presence of bacterial leaf blight resistance genes *Xa21*, *xa13* and *xa5*. On the basis of mean lesion length at 15 days after inoculation, seven cultivars showed resistance, 4 moderately resistance, 6 moderately susceptible and 17 cultivars were susceptible to bacterial leaf blight. During the polymorphic survey of thirty four rice cultivars, no amplicons specific to *Xa21* and *xa13* allele were detected, showing the absence of these two genes in all the cultivars evaluated, while twenty cultivars along with resistant checks amplified 219 bp size fragments indicating the presence of *xa5*. Therefore, the resistant cultivars could be used for the transfer of bacterial leaf blight resistance gene to well adapted high yielding rice cultivars.

KEY WORDS: Bacterial leaf blight, lesion length, SSR markers, resistance and rice cultivar.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the oldest domesticated crops, which provides food for more than half of the world's population and constitutes a major source of calories for urban and rural inhabitants (Khush, 2005), unfortunately, whose production is constrained by considerable number diseases of fungal, bacterial and viral origin. Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most devastating diseases affecting entire rice acreages and causes severe yield losses of up to 80% depending on the stage of the crop, cultivar susceptibility and the environmental conditions (Srinivasan & Gnanamanickam, 2005). Exploitation of host plant resistance is considered the most effective, economical and environmentally safe measure for controlling BLB in combination with management practices. To tackle this problem, several attempts have been made to identify and characterize BLB resistance genes. Till date, approximately 34 genes (23 dominant and 11 recessive) conferring resistance against various strains of *X. oryzae*, have been identified (Chen *et al.*, 2011). Major resistance genes, including *Xa4*, *xa5*, *Xa7*, *xa13* and *Xa21* have been incorporated into rice cultivars, in order to develop new resistant varieties (Perumalsamy *et al.*, 2010). However, the cultivars containing a single major resistance gene proved susceptible due to pathogen mutation. Recently, pyramiding of more than one major resistance gene has been proven to deliver durable resistance against *Xoo* (Rajpurohit *et al.*, 2010). Conventional breeding tools are inefficient for gene pyramiding, particularly in the case of recessively inherited resistance genes, such as *xa5* and *xa13*. These limitations can be addressed by marker-assisted selection (MAS), which enables the evaluation of the expression status of resistance gene(s) and allows for pyramiding of multiple resistance genes in a desirable genetic background. Polymerase chain reaction based DNA markers for some of these genes have been identified and employed to identify germplasm containing these genes (Blair & Mc-Couch, 1997), and develop rice cultivars with single and multiple resistance genes (Perumalsamy *et al.*, 2010; Rajpurohit *et al.*, 2010). In the present study, we screened rice cultivars to identify the resistance genes *Xa21*, *xa13*and *xa5*; which accelerate the breeding efforts for the development of BLB resistant rice cultivars through pyramiding approaches using marker assisted selection.

MATERIALS & METHODS

Plant material and experimental design

The experimental materials comprised of thirty four rice cultivars along with three resistance check (RP Bio-226, CRMAS 2231-37 and CRMAS 2232-71). Twenty five cultivars were received from Networking Project National Research Centre on Plant Biotechnology, New Delhi, India and nine cultivars from Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. Twenty one days old single seedlings were transplanted with spacing 20 × 15 cm apart in 3.0 × 1.5 m plot size in randomized block design with three replications at Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi during *Kharif-2013*. The recommended packages of practices were followed to raise a healthy crop.

Strain revival, pathogenicity test and observation recorded

The culture of *Xanthomonas oryzae* pv. *oryzae* (strain *BX043* wild type) was obtained from Directorate of Rice Research, Hyderabad, India and sub cultured on peptone sucrose agar (PSA) medium (Distilled water: 1 L, Sucrose: 20 g, Peptone: 5 g, K_2HPO_4: 0.5 g, $MgSO_4.7H_2O$: 0.25 g and Agar: 15 g) and maintained it at pH 7.2 – 7.4 (Fahy

and Persley, 1983). For pathogenicity test, clipping method was used for inoculation in the rice plants with *Xanthomonas oryzae* pv. *oryzae*. The test was conducted on fully developed leaves at the age of 45 days old rice plants after transplanting. The top 1 – 3 inches of completely developed leaves were clipped off one by one by sterilised scissor dipped in bacterial suspension containing 10^8 cfu/ml. Following inoculation, the plants were observed after every 24 hours time interval to note the appearance of disease symptoms. On the basis of lesion length these cultivars were categorized by using standard IRRI (1988) procedure as resistant 1-5 cm, moderately resistant 5-10 cm, moderately susceptible 10-15 cm and susceptible > 15 cm. The average lesion length at 15 days after inoculation (DAI) was recorded to identify the degree of pathogenecity on 0 – 4 rating scale, Standard Evaluation System IRRI (1988).

DNA extraction

The young leaves were collected from three week of transplanted cultivar during *Kharif-2013*. Around 40 mg leaves were taken from each cultivar placed in 1.2 ml collection microtubes (Qiagen Tissue Lyser II, Qiagen, U.S.A.) and in each microtube 3 mm tungsten beads were dispensed by bead dispenser and kept at -80 ^oC for 4 hrs. Tissues were disrupted and homogenized by qiagen tissue lyser to a fine powder at frequency of 30 vibrations/seconds for 30 seconds. Fine powdered leaf samples were used for isolation of genomic DNA using CTAB (hexadecyl trimethyl ammonium bromide) method (Doyle & Doyle, 1987). The DNA was quantified spectrophotometrically (Perkin Elmer, Singapore) by measuring A260/A280, and DNA quality was checked by electrophoresis in 0.8% agarose gel.

SSR analysis

Three reported STS and SSR markers synthesized by Eurofins Genomics (Bangalore, India), were used to analyze the status of the bacterial leaf blight resistance genes (Table 1). The amplification was carried out in 15µl of reaction mixture containing 30 ng genomic DNA, 1.5 mM PCR buffer (MBI Fermentas, USA), 400 µM dNTPs (MBI Fermentas), 1U Taq DNA polymerase (MBI, Fermentas) and 0.4 µM primer using a thermal cycler (Mastercycler gradient, Eppendorf). Thermal cycling program involved an initial denaturation at 94 ^oC for 4 min, followed by 34 cycles of denaturation at 94 ^oC for 45 sec, annealing at 2 ^oC below Tm of respective primers for 30 sec, primer extension at 72 ^oC for 30 sec, followed by a final extension at 72 ^oC for 8 min. The amplified PCR products with a 100 bp DNA marker ladder (MBI, Fermentas) were size fractioned by electrophoresis in 2.5% agarose gel prepared in TAE buffer and visualized by staining with ethidium bromide (0.5 µg/ml) in a gel documentation system (BIO-RAD, USA).

TABLE 1. List of bacterial leaf blight resistance genes, STS and SSR markers and germplasm used as check

S.No.	Gene	Chromosome locus	Linked Marker	Linkage distance (cM)	Primer sequence	Reference
1	*Xa21*	11	pTA-248 (STS)	0.0	**F:**AGACGCGGAAGGGTGGTTCCCGGA **R:**AGACGCGGTAATCGAAGATGAAA	Ronald et al. (1992)
2	*xa13*	8	xa-13prom (SSR)	0.0	**F:**GGCCATGGCTCAGTGTTTAT **R:**GAGCTCCAGCTCTCCAAATG	Singh et al. (2011)
3	*xa5*	5	RM-13 (SSR)	17.9	**F:**TCCAACATGGCAAGAGAGAG **R:**GGTGGCATTCGATTCCAG	Mc-Couch et al. (1996)

RESULTS & DISCUSSIONS

Phenotypic screening for bacterial leaf blight resistance

Thirty four rice cultivars along with resistant checks (RP Bio-226, CRMAS 2231-37 and CRMAS 2232-71) were screened against *Xanthomonas oryzae* pv. *oryzae* under epiphytotic condition during *Kharif 2013*. The results of phenotypic screening are presented in table 2. On the basis of mean lesion length at 15 days after inoculation, seven cultivars showed resistance, 4 moderately resistance, 6 moderately susceptible and 17 cultivars were susceptible to bacterial leaf blight. Similarly, wide response of genotypes against the *Xanthomonas oryzae* pv. *oryzae* has been earlier recorded by various workers (Akhtar *et al.*, 2011; Madhavi *et al.*, 2011; Sharma & Pandey, 2012). The initial symptoms of bacterial leaf blight *viz.* linear yellow to straw coloured stripes with wavy margins, generally on both edges of leaf, rarely on one edge was observed with variable intensities. These symptoms were first appeared in cultivar Lumbini after 3 days of inoculation, while cultivars Tsao Wanching, Akita Komachi and M-202 showed these symptoms after 7 days as compared to resistance checks RP Bio-226, CRMAS 2231-37 and CRMAS 2232-71, which showed symptoms 10 days after inoculation. These finding were in agreement with earlier report by Singh *et al.* (2013), reported that first symptoms appeared after 7 days in moderately susceptible rice germplasm. Among these cultivars, Chanyngat and Lumbini showed lowest (3.33 cm) and highest (30.63 cm) mean lesion length, respectively compared with resistant check RP bio-226 (3.63 cm), CRMAS 2231-37 (3.90 cm) and CRMAS 2232-71 (3.87 cm). The resistance cultivars ranged the mean lesion length from 3.33 to 4.60 cm, moderately resistance from 7.57 to 8.60 cm, moderately susceptible from 11.60 to 14.97 cm and susceptible from 15.80 to 30.63 cm. These results are inconformity with the findings of Akhtar *et al.* (2011).

Genotypic screening for bacterial leaf blight resistance

Thirty four rice cultivars were screened for the presence/absence of three bacterial leaf blight resistance genes *viz. Xa21*, *xa13* and *xa5* using PCR based markers pTA248, xa-13prom and RM-13, respectively linked to these genes. The resistance check (RP Bio-226, CRMAS 2231-37 and CRMAS 2232-71) was included as gene differential lines. Estimation of PCR results for three bacterial leaf blight resistance genes *viz.*, *Xa21*, *xa13* and

xa5 were determined by visualization of amplicons on near 982 bp, 498 bp and 219 bp of positive fragments, respectively.

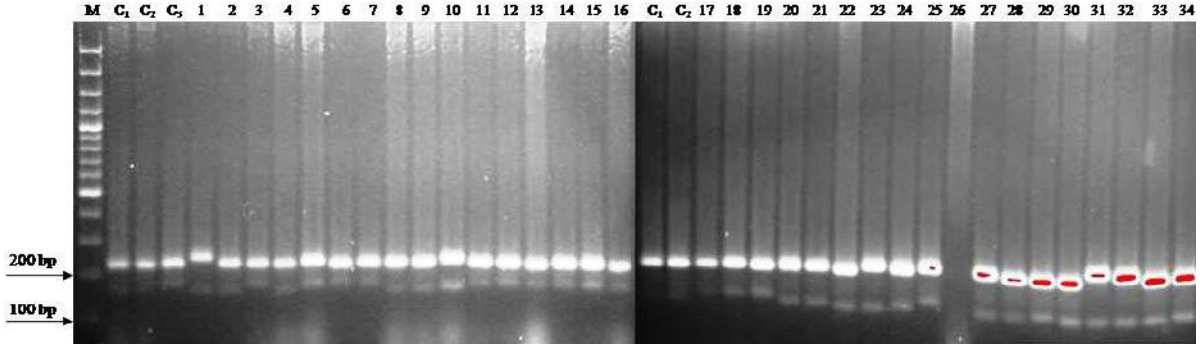

FIGURE 1. Agarose gel electrophoretic pattern of 34 rice cultivars generated by using SSR markers RM-13, where M is 100 bp DNA size marker, C_1, C_2 and C_3 are resistant check variety and numbers 1-34 represent rice cultivars as described in table 2

TABLE 2. Phenotypic and genotypic screening for bacterial leaf blight resistance in thirty four rice cultivars

S.N.	Cultivars	Source	Lesion length at 15DAI ± SD	Host response	BLB resistance genes		
					Xa-21	*xa-13*	*Xa-5*
Resistant	RP Bio-226 (C_1)	BHU	03.63 ± 0.60	R	+	+	+
Check	CRMAS 2231-37 (C_2)	CRRI	03.90 ± 0.36	R	+	+	+
	CRMAS 2232-71 (C_3)	CRRI	03.87 ± 0.81	R	+	+	+
1	Tsao Wanching	IRRI	03.93 ± 1.69	R	-	-	-
2	JC-1	IRRI	26.97 ± 7.56	S	-	-	+
3	Lumbini	IRRI	30.63 ± 4.15	S	-	-	+
4	Madhukar	IRRI	08.40 ± 1.25	MR	-	-	+
5	N-22	IRRI	22.93 ± 2.10	S	-	-	-
6	Binulawan	IRRI	19.30 ± 2.46	S	-	-	+
7	Kala Rata 1-24	IRRI	29.90 ± 4.83	S	-	-	+
8	Tatan	IRRI	04.60 ± 0.89	R	-	-	+
9	Bapakribuna	IRRI	04.20 ± 0.66	R	-	-	+
10	Gota	IRRI	24.13 ± 2.65	S	-	-	-
11	Khaiyen	IRRI	14.97 ± 2.21	MS	-	-	+
12	Kamnan	IRRI	30.43 ± 6.96	S	-	-	+
13	Lemont	IRRI	11.60 ± 2.99	MS	-	-	+
14	Latsika	IRRI	07.57 ± 0.85	MR	-	-	+
15	Akita Komachi	IRRI	03.60 ± 0.66	R	-	-	+
16	M-202	IRRI	03.70 ± 0.72	R	-	-	-
17	Amaroo	IRRI	04.27 ± 0.81	R	-	-	+
18	Nipponbare	IRRI	12.87 ± 2.72	MS	-	-	+
19	Asami Dhan	IRRI	14.23 ± 2.46	MS	-	-	+
20	Chanyngat	IRRI	03.33 ± 0.76	R	-	-	+
21	Budkodi	IRRI	07.90 ± 1.21	MR	-	-	+
22	Dub Gelong	IRRI	24.53 ± 4.15	S	-	-	-
23	Sawitri	IRRI	08.60 ± 0.82	MR	-	-	+
24	Asthu Bhejna	IRRI	12.37 ± 2.07	MS	-	-	+
25	Tundaniya	IRRI	13.87 ± 1.93	MS	-	-	+
26	IR-64	BHU	20.80 ± 2.69	S	-	-	-
27	IR-64 sub-1	BHU	17.70 ± 2.75	S	-	-	-
28	Sarjoo-52	BHU	21.70 ± 5.43	S	-	-	-
29	MTU-7029	BHU	15.80 ± 3.26	S	-	-	-
30	HUR 2-1	BHU	18.53 ± 4.38	S	-	-	-
31	HUR-38	BHU	22.80 ± 3.11	S	-	-	-
32	HUR-105	BHU	20.03 ± 2.67	S	-	-	-
33	HUR-3022	BHU	23.60 ± 3.15	S	-	-	-
34	HUBR-40	BHU	20.67 ± 2.25	S	-	-	-
	Frequency (%)				00.00	00.00	58.82
	Approx. size (bp)				982	498	219

The rice bacterial leaf blight resistance gene scored as the presence (+) and absence (-) of amplicon linked to three of allele specific SSR markers

The results of genotypic screening of thirty four rice cultivars are presented in table 2, and electrophoretic patterns of SSR markers RM-13 for resistance genes *xa5* is shown in Figure 1. During this polymorphic survey, out of thirty four rice cultivars, no amplicons specific to *Xa21* and *xa13* allele were detected except resistant checks RP Bio-226, CRMAS 2231-37 and CRMAS 2232-71, showing the absence of these two genes in all the cultivars evaluated while twenty cultivars along with resistant checks amplified 219 bp size fragments indicating the presence of *xa5*. Similar results were also reported by Singh *et al.* (2012) in forty two landraces of rice, results showed that 29 landraces carried *Xa4* specific allele; whereas none of the landraces had *Xa21* and *xa13* genes. The resistance gene *xa13*, originally derived from a land race BJ1 from the Indian subcontinent, is effective individually as well as in combination with *Xa2*, *Xa4*, *xa5* and *Xa21* against many pathotypes of *Xanthomonas oryzae* pv. *oryzae* (Lore *et al.*, 2011). But it is significant that gene *xa13* was not detected in any one of the rice cultivars. Absence of *Xa21* in all the cultivars was also significant as this gene was introgressed from *Oryza longistaminata*, a wild relative of the cultivated species *Oryza sativa* (Khush & Angeles, 1999). The present findings are in agreement with the reports of Davierwala *et al.* (2001), surveyed rice genotypes popularly used in Indian breeding programme using markers closely linked to *xa5*, *xa13* and *Xa21*, and reported that eight lines carried *xa5*, only two had *Xa21* and none of them carried *xa13*. This calls for a further detailed genetic analysis for the presence of novel BLB resistance genes and their tagging and cloning in the rice cultivars. This information can be gainfully utilized to supplement the bacterial leaf blight resistant gene pool available in India. The consistent results showed with the selected SSR markers for respective genes was highly reliable and make them the marker of choice for molecular screening of rice bacterial leaf blight resistant genes among the rice cultivars. This study indicates the presence of *xa5* gene in rice cultivars, may be use of these cultivars as a donor parent in hybrid-ization programmes, which will expedite efforts to develop bacterial leaf blight resistance cultivar through MAS-based pyramiding approach without compromising yield and grain quality.

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