RESEARCH ARTICLE

GENE PYRAMIDING OF FOUR BLB RESISTANT GENES *(XA4, XA7, XA13* AND *XA21)* FROM IRBB65 INTO MAHAMAYA USING MAS.

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Abstract: Bacterial blight (BB) of rice (*Oryza sativa*) caused by *Xanthomonas oryzae pv. oryzae* (Xoo) is currently one of the most important diseases limiting rice production and it has become widespread in India. This disease was first noticed by the farmers of Japan in 1884 (Tagami and Mizukami 1962). Enhancing genetic resistance has proven to be the most effective control method for controlling the disease. Four bacterial blight (BB) resistance genes, *Xa4, Xa7, xa13 and Xa21*, were introgressed into an elite rice cultivar. Marker assisted selection was done using linked molecular markers for genes *Xa4, Xa7, xa13 and Xa21*. The ability to quickly and reliably select desirable material and to eliminate individuals that contain deleterious alleles is critical to the success of the plant breeding program (Dubcovsky 2004). We report here in two gene pyramids *Xa7+ Xa21* in 5 lines and *Xa4+Xa7* in 1 line. Genes in combinations were found to provide high levels of resistance. Besides pyramids a set of 8 genotypes with only *Xa7*, 2 genotypes with only *Xa13* and 5 genotypes for *Xa21* were also found in this study. High resolution maps generated *in-silico* around *Xa4, Xa7, xa13 and Xa21* can be useful for the precise placement of a gene of interest,analysis of regional and sub regional rates of recombination and appropriate combinations of markers for marker assisted selection.

Keywords: Gene Pyramiding, Bacterial blight, Resistance genes, Polymerase chain reaction, Marker Assisted Selection *Xanthomonas oryzae* pv. *oryzae*, MAS, Pyramiding

INTRODUCTION

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most destructive diseases active in the major rice growing countries of Asia. In the northern plains of India, including the State of Chhattisgarh, the disease is a serious problem, as rice is grown under irrigated and high fertilizer input conditions that are conducive to disease development. In severe epidemics, yield losses ranging from 20% to 40% have been reported (Sonti 1998). Breeding for disease resistance is the most effective and economical method for control of BB that has a neutral impact on the environment. To date, at least 39 BLB resistance genes conferring host resistance against various strains of Xoo have been identified and characterized (Verdier et al. 2012; Zhang et al. 2014). Several of these genes have already been incorporated into rice cultivars, which are now widely cultivated in many countries. Mahamaya is a long bold grain, high yielding, medium duration, lowland variety is very popular amongst the farmers and is well suited for cultivation in rainfed condition. Expresses moderate resistance to leaf blast and Brown spot. The disease, Bacterial blight has become a major production constraint for var. Mahamaya in particular. Marker assisted selection (MAS) for pyramiding important genes along with rapid background recovery of the recurrent parent, while maintaining the exquisite quality characteristics of rice, could be an effective approach for rice improvement. Molecular markers closely linked to each of the resistance genes make the identification of plants with two and three genes possible (Shanti et al. 2010; Singh et al. 2001; Sundaram et al. 2008). The combination of genes provided a wider spectrum of resistance to the pathogen population prevalent in the region; gene *Xa4* are resistance to bacterial blight at all stages of plant growth. Gene *Xa7*, which provides dominant resistance against the pathogen with avirulence (Avr) gene AvrXa7, has proved to be durably resistant to BB. The *xa13* gene is fully recessive, conferring resistance only in the homozygous status. It interacts strongly with other R genes such as *xa5, xa4* and *xa21*. The rice *Xa21* gene confers resistance to *Xanthomonas oryzae* pv *oryzae* in a race-specific manner. Gene *Xa21*, encoding a receptor-like kinase, is a member of a multigene family**.**

MATERIAL AND METHOD

Plant Material

Experimental materials consisted of parental lines and F_2 segregating population derived from a cross between Mahamaya (susceptible and high yielding variety) x IRBB65 (resistance variety) was used for genetic analysis and validation of selected markers for *Xa4* and *Xa7* genes.

Preparation of inoculum

The isolate was derived from the stock maintained at 4°c. Culture was revived and grown on Walkimoto's medium for 3 days at 30°C. For inoculating the plants bacterial suspension was prepared by mixing bacterial culture in sterilized distilled water to a concentration of 10^9 cells/ml. This suspension was immediately used for inoculating the plants which

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are at maximum tillering stage following the clip inoculation technique (Kauffman et al. 1973)

Evaluation of F_2 segregating population

Individual plants of 80 lines of Mahamaya X IRBB65 derived F_2 breeding segregating population and two parents were evaluated for field infections to bacterial blight. Observations were recorded by physical measurement of lesion and percent leaf area was worked out. Scoring for the disease incidence was evaluated 21 days after inoculation. The lesion length and total leaf length was recorded on five leaves and were further categorized based on 0-9 (IRRI,1991) which is as follows: 0=No lesion length, 1=lesion restricted to 0.5 to 1.0mm, 3= lesion elongated but less than ¼ of leaf blade, 5= lesion extended to ½ of the leaf blade, 7= lesion extended to more than ½ of the leaf blade, 9= lesion completely destroyed the leaf blade and sheath. The disease score were rated as HR, R, MR, S, and HS.

DNA extraction

DNA extraction was carried out by using modified CTAB protocol (Keb Llanes).The DNA samples were quantified using Nanodrop Spectrophotometer (ND1000). Further, concentration of DNA was adjusted to 30 ng/µl with TE buffer and stored at 4°C. The diluted DNA was subsequently used in PCR amplification.

Primers for DNA amplification

The marker RM224, Xa7M5, xa13pro and RM21 were used for *Xa4, Xa7 xa13* and *Xa21* respectively.

Table 1. SSR primers used for developing genotypic data for *Xa4, Xa7 xa13* and *Xa21.*

S.No	Primer	Type of marker	Forward/ Reverse	Sequence 5'→ 3'
1.	RM224	SSR	Forward	ATCGATCGATCTTCACGAGG
		SSR	Reverse	TCGTATAAAAGGCATTCGGG
2.	Xa7M5	SSR	Forward	CGATCTTACTGGCTCTGCAACTCTGT
		SSR	Reverse	GCATGTCTGTGTCGATTCGTCCGAGA
3.	xa13pro	SSR	Forward	GGCCATGGCTCAGTGTTTAT
		SSR	Reverse	GAGCTCCAGCTCTCCAAATG
4.	RM 21	SSR	Forward	ACAGTATTCCGTAGGCACGG
		SSR	Reverse	GCTCCATGAGGGTGGTAGAG

PCR Amplification

It was performed in a thermocycler with 1.5 µl of Genomic DNA. Autoclaved distilled water 5.25 µl, 1.0 µl of 10 X Buffer,1.0µl of dNTP mix, 1.0µl of Forward and Reverse Primer , 0.25µl of Taq polymerase. An initial denaturation was performed at 94^{o}C for 2minutes prior to 30 cyles of denaturation at 94^{o}C (1 minute), annealing at 55^{o}C (1 minute), and extension at 72^{o}C (2 minutes). A final extension for 5minutes at 72^{o}C will be performed. Polymorphisms in the PCR products will be detected after electrophoresis on 40% Polyacrylamide gel for SSR marker products for microsatellite before ethidium bromide staining.

RESULT AND DISCUSSION

Thirteen hundred F2 individuals were artificially inoculated and phenotyped for disease reactions. Out of 1300 plant population, 763 plants were scored resistant and 310 plants were susceptible and 187 plants were remained un-inoculated. Forty highly resistant (HR reaction) and 40 highly susceptible individuals were chosen from the F2 population and were used for selective genotyping to track the presence of genes (*Xa4, Xa7*) individually and/or in combinations.

Table 2. Reaction of F2 segregating population (MahamayaXIRBB65) a**g**ainst *Xanthomonas oryvzae pv.oryzae* (Dhamtari isolate)

Cross	Total Population	Resistance lines	Susceptible Lines	Highly Resistance Lines	Uninoculated Lines
Mahamaya X IRBB65	1300	763	310	40	187

Earlier reported PCR based markers for RM224, Xa7M5, xa13pro and RM21 were screened for parental polymorphism. Marker RM224 linked to gene *Xa4* resolved 160bp and 150bp band representing resistant and susceptible parent. Marker Xa7M5 linked to *Xa7* gene has resolved amplicon of 310bp representing resistant parent and no band was seen susceptible parent. xa13pro marker produced 530bp band and 280bp band representing resistant and susceptible parents respectively. Marker RM21 linked to *Xa21* gene resolved amplicon of 900bp representing susceptible parent and 1100bp size of band representing resistant parent.

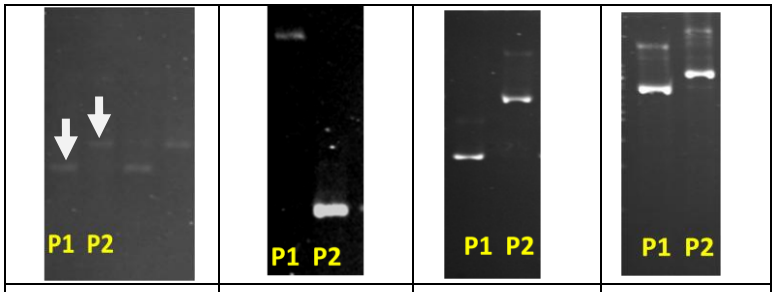

Fig 1. Image showing the pattern of segregation of molecular marker with P1 (Susceptible parent) and P2 (Resistant Parent).

Table 3. Frequency distribution of phenotyped lines in which the markers Co-segregated as per the phenotype.

S.no.	Gene	Marker	Phenotype		Total
			Resistance P_2type band	Susceptible P_1 type band	
1	*Xa4*	RM224	1	0	1
2	*Xa7*	Xa7M5	13	1	14
3	*xa13*	xa13pro	2	3	5
4	*Xa21*	RM21	10	11	21

P1 type band = Mahamaya (Susceptible); **P2 type band** = IRBB65 (Resistant); **H** = Presence of P1 and P2 type band.

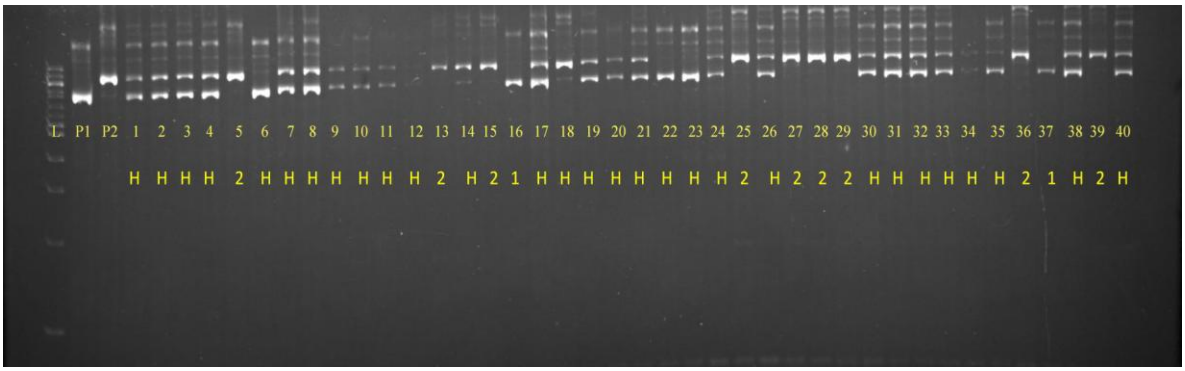

Fig 2. PCR amplification of population derived from cross Mahamaya X IRBB65 with primer RM21 (Xa21 gene specific primer).

Table 4. One gene lines identified through Linked markers.

Introgressed line(s) with one gene	**Line number**
Co-segregating marker for *Xa7* :Xa7M5	Lines #1, 8,11,12,14,17,19,37.
Co-segregating marker for *Xa13*:xa13pro	Lines # 7,40.
Co-segregating marker for *Xa21*:RM21	Lines #15,16,25,28,29.

Table 5. Two gene Pyramids identified through linked markers.

Introgressed lines with two genes ***Xa7 + Xa21***	**Introgressed lines with two genes** ***Xa4 + Xa7***
Line # 5,13,27,36,39	Line # 2

The IRBB 65 (a near isogenic line in the background of IR24) was crossed with Mahamaya, former as the male parent. A subset of selected 40 phenotypically superior plants carrying genes for resistance and susceptible F_2 segregants were selected for genotyping using RM224, Xa7M5, xa13pro and RM21markers which are linked to *Xa4*, *Xa7*, *xa13*, *Xa21* genes respectively. Plants showed banding

pattern identical to that of its resistant parent IRBB65 were, therefore, assumed to carry the gene for resistance in homozygous state. Eight lines in the population which confirmed presence of *Xa7* loci, xa13pro confirmed the presence xa13 loci in two and similarly RM21 identified the presence of *Xa21* loci in 5 lines (Table 4).Besides single gene carrying lines, superior lines carrying two gene pyramids for resistance were also identified. 5 lines (#5,13,27,36,39) carrying two gene pyramids of *Xa7+Xa21* and one line (#2) carrying *Xa4+Xa7* were found in this investigation**(**Table 5).

CONCLUSION

Identified two gene pyramids *Xa7 + Xa21* in 5 lines, and *Xa4 + Xa7* in line 1 line. Besides pyramid lines, single gene containing lines with *Xa4, xa13, Xa21* were also found in 8, 2, 5 number of lines respectively. Physical map was developed by identifying BAC or PAC clones that simultaneously contained a hit from the marker. Such map will help is to develop more markers for fine mapping of the genes , foreground selection and a panel of selection markers for MAS / MAB.

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