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Introgression of bacterial leaf blight resistance gene (*xa5*) in rice (*Oryza sativa* l.) cultivar ir64 through marker assisted selection

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Abstract
Bacterial leaf blight is a severe rice disease throughout the world that is controlled primarily through use of resistant cultivars. Marker assisted selection (MAS) is essential for improvement of resistance in breeding programs because of they are co-dominant, based on PCR amplification, represent single loci and detect high levels of polymorphism. As on date, 30 major genes have been reported to confer resistance against *Xanthomonas oryzae pv oryzae* (Xoo), which included 21 dominant and 9 recessive R genes. In the present investigation the behavior of R genes in population indicated that faster the response rate is more likely a resistant phenotype is to arise. The recessive R genes i.e *xa5* (chromosome 5) which specifically confers resistance to BLB of rice only in recessive homozygous condition. The PCR based screening of selected 40 F_4 individuals, each plants selected which derived from cross between IRBB5xIR64.The F_4 progeny were tested and inoculated artificially by *Xanthomonas pv. Oryzae pv oryzae* (Dhamtari) isolate. In this study microsatellite and sequence-tagged site(STS)were most practical markers used for marker assisted selection of the targeted BLB resistant genes *xa5*.Tagged rice line #1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19, 22, 24, 25, 26, 27, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 derived from IRBB5xIR64 were resistant phenotypically and also show co-segregation with linked SSR marker RM610. These selected lines will further be phenotyped under field condition for resistance to BLB, further purification and for the restoration of the background.

Keywords: Bacterial leaf blight, Markers assisted selection, *Oryza sativa* L.

Introduction
Rice, the world's most important cereal crop, is the primary source of food and calories for about half of mankind. Bacterial leaf blight of rice, caused by *Xanthomonas oryzae pv. Oryzae*, is a serious disease of rice in tropical lowland rice environments (Mew *et al*. 1993; Gnanamanickam *et al*. 1999) and has become a model system for the study of diseases caused by bacterial pathogens on monocotyledonous hosts (Ronald 1997). It is one of the major diseases causes poor development and lowers quality of grain, and increases the number of underdeveloped grains, reduces weight and results in poor maturing and a high proportion of broken rice (Ou 1985). For control it in the field, use of seed from uninfected plants, resistant varieties and careful attention to crop management (for example, by water control, avoidance of damage to seedlings) are most important. Therefore, it is necessary to screen rice varieties (lines) which resist the disease. The advent of molecular markers tagged to resistance genes enabled convergence breeding. More than 30 resistance genes have been identified and designated in a series from *Xa1* to *xa32* till now (Khush and Angeles, 1999; Chen *et al*., 2001; Lee *et al*., 2003). DNA fingerprinting studies and pathotype analysis have indicated a significant diversity in the *Xoo* populations in India and other rice-growing countries. The effectiveness of resistance genes varies over locations due to geographical structuring of the pathogen. Knowledge of the pathogen population structure and virulence characteristics is therefore essential for a successful breeding program aimed at durable resistance. The *xa5* gene is fully recessive, conferring resistance only in the homozygous status (Khush and Angeles 1999). This gene specifically confers resistance to the Philippine *Xoo* race 6; the most virulent race and one not overcome by most reported R genes. The *xa5* gene is one of the recessively inherited resistance gene that provide race-specific resistance to bacterial leaf blight. This gene is found at the end of the short arm of chromosome 5 and several researchers have located or developed molecular markers that are closely linked to it (Sanchez *et al.*, 2000; Singh *et al.*, 2001) or constructed physical contigs around the gene (Saji *et al.*, 1996). *Xa5* is an important race-specific recessive gene in rice breeding due to its broad resistance spectrum to most *Xoo* strains. Pyramid lines with *xa5* gene and other R-genes have a higher and wider

spectrum of resistance than each of the single gene lines. A reliable marker for identifying *xa5* would reduce the amount of time and expense involved in transferring this important trait to new cultivars. Molecular markers offer great scope for improving the efficiency of conventional plant breeding. Availability of tightly linked genetic markers for resistance genes to be useful in identifying plant carrying these genes simultaneously without subjecting them to pathogen attack in early generation.

Materials and methods

Plant materials

IRBB5 x IR64 derived F_4 Segregating population used for validation of *in-silico* generated markers of 50 individual resistance plants and 50 susceptible plants. Among those 100 plants only 40 plant was selected for phenotyping and genotyping of infection *Xanthomonas oryzae pv. Oryzae.*

Xanthomonas oryzae pv. *Oryzae* (*Xoo*) inoculums preparation and inoculation

Xanthomonas oryzae pv. *oryzae* (*Xoo*) Dhamtari isolate was chosen for this study.The inoculum was grown on Wakimoto solid medium (potato 300 g, sucrose 20 g, $Na_2HPO_4.12H_2O$ 2 g, $Ca(NO_3)_2.4H_2O$ 0.5 g, agar 25 g, H_2O 1L) at 25°C for 72 hours (Ou 4 1985), and then preserved at 4° (Chien and Shieh 1989; Hsieh *et al*. 2005). Single colony was subcultured in Wakimoto medium at room temperature for 72 hours, and then suspended with distilled water into 10^7-10^8 cells/ml.

Disease evaluation

At the maximum tillering stage, uppermost fully expanded leaves of each plant were inoculated with *Xoo* isolate (collected from Dhamtari) by the leaf-clipping method (Kauffman *et al*. 1973). The bacterial inoculum was prepared by the following procedures as developed by Dr.A.S. Kotasthane (Personal communication),Reactions of individual F_4 plants to the pathogen were evaluated 14–21 days after inoculation by measuring the lesion length scored as follows: very resistant (0, HR, < 3 cm), resistant (1, R, 3-5 cm), moderate resistant (3, MR, 5-8 cm), moderate susceptible (5, MS, 8-13 cm), susceptible (7, S, 13-22 cm), and very susceptible (9, HS, >22 cm).

DNA isolation

Miniscale DNA isolation was carried out for F_4 segregating population along with two parents using modified CTAB protocol. The DNA was quantified by Nano Drop system (ND1000), further concentration of DNA was adjusted to 30 ng/µl with TE buffer. The diluted DNA was subsequently used for PCR amplification (stored at 4 ºC).

PCR amplification and Sample resolution

A series of optimization experiments using parents and segregating F_4 population samples was carried out in which concentrations of template DNA, primers, d NTPs and *Taq* polymerase were varied to determine which conditions gave the strongest patterns. The PCR reaction mixture of 20 µl contained 30 ng/µl template DNA, 1µM of each primer, 10 MM d NTPs, 10X PCR buffer. And 1unit/µl of *Taq* polymerase. The template DNA was initially denatured at 94 ºC for 5 min followed by 35 cycles of PCR amplification under the following parameters: 1 min denaturation at 94 ºC, 1 min primer annealing at 55ºC and 1.5–2.0 min primer extension at 72 ºC. A final 7 min incubation at 72 ºC was Allowed for completion of primer extension on Thermal cycler. Following amplification, the samples were resolve on 6% Urea PAGE gel and stained with silver nitrate.

Results and discussion

The *xa5* gene is one of the recessively inherited resistance genes that provide race-specific resistance to bacterial leaf blight. This gene is found at the end of the short arm of chromosome 5 and several researchers have located or developed molecular markers that are closely linked to it (McCouch 1997; Huang *et al*., 1997; Sanchez *et al.,* 2000; Singh *et al*., 2001) or constructed physical contigs around the gene (Saji *et al*., 1996). *Xa5* is unlinked to any other known genes for resistance to diseases or insects, but to-date the gene has not been isolated. Isolation of the *xa5* gene is of interest in order to understand how this recessive gene functions in pathogen recognition and plant defense responses. Recessive bacterial blight resistance genes are of particular interest because they are likely to be different in nature from their dominant counterparts. Recessive resistance genes generally do not cluster with other resistance genes in rice (Ronald 1998; Richter and Ronald 2000) nor do they map to the same location as resistance gene analogs and other candidate genes as many of the dominant genes do (Ilag *et al*.,2000; Wang *et al*., 2001). The markers developed following bioinformatics approach which allowed us to narrow-down the area of interest around *xa5*, define the frequency of recombination occurring in regions flanking the gene and identify candidate genes. They can also be used to select appropriate combinations of markers for marker assisted selection in plant-breeding programs. The development of molecular markers diagnostic for the selection of resistance genes is a goal of many rice breeding programs. In the present investigation seventeen previously known markers will be used for validation with a view to identify a set of markers of diagnostic importance for the selection of *xa5* resistance critical to the success of a plant breeding program.

In this study seventeen previously reported PCR based markers were screened (Table1) for parental polymorphism, out of which eight SSRs markers (RM601, RM602, RM607, RM609, RM610, RM611, RM122, and RM13) resolved parental polymorphism and were further used for selective genotyping with selected set of resistant lines. When analyzed for parental polymorphism nine SSR markers out of 17 (RM605, RA603, RM604, RM608, RA558, RZ390, RM390, STS556,RM606,) were resolved as monomorphic and could not be genetically mapped Blair *et al.* 1997 were also unable to genetically map RM602, RM604, RM605, RM606, owing to poor amplification. Recombinant F_4 individuals were also selected by a sequential screening process using seven SSR loci three at the flanking end of RM607 (i.e. RM122, RM601, RM602)one on the flanking end of RM611 (i.e. RM13) and one in between RM609 and RM611 (i. e. RM610). In the order of their occurrence on the BAC clones seven markers, RM601, RM602 and RM607**,** RM609, RM610, RM611, RM13 anchored to BAC clones OSJNBb0035J08 and OSJNBa0068N01 were used to map *xa5* using F_4 populations using forty selected resistant individual. Markers RM601, RM602, RM607**,** RM609, RM610, RM611 and RM13 detected 33, 17, 23, 9, 5, 14 and 34 recombination events, in the selected 40 resistant lines respectively placing *xa5* at 0.825, 0.425, 0.575, 0.225, 0.125, 0.175, 0.85 cM away.

Table 1: Genotype of selected resistant lines for SSR loci RM601, RM602, RM607, RM609, RM610, RM611, RM13 ,RM122 covering the *xa5* region flanked by RM601 and RM13 accounting for a physical distance covering 16,53,733 bases (1653 kb region)

PlantNo.	Field Reaction	RM122	RM601	RM602	RM607	RM609	RM610	RM611	RM13
1	R	A	A	A	A	A	A	B	A
2	R	B	B	B	A	B	A	A	B
3	R	B	A	B	B	B	A	B	B
4	R	A	B	A	A	-	A	B	A
5	R	A	B	-	A	A	A	A	H
6	R	B	A	B	B	B	A	A	H
7	R	A	A	A	A	A	A	B	H
8	R	A	B	A	B	A	A	A	H
9	R	B	A	A	A	B	A	A	A
10	R	B	A	B	B	B	A	-	H
11	R	B	B	B	A	B	A	B	H
12	R	B	B	B	A	-	A	A	H
13	R	B	B	A	A	-	A	-	H
14	R	B	B	B	A	B	A	A	A
15	R	B	B	A	A	B	A	A	-
16	R	B	B	B	B	B	A	A	H
17	R	B	B	A	A	A	A	B	H
18	R	B	B	B	B	A	A	B	H
19	R	B	B	B	B	A	A	B	H
20	R	B	B	A	A	A	B	B	A
21	R	B	B	A	A	A	B	A	H
22	R	B	B	A	B	A	A	A	H
23	R	A	B	A	B	A	B	A	B
24	R	B	A	-	A	A	A	A	B
25	R	B	B	B	B	A	A	A	B
26	R	B	B	B	B	A	A	B	B
27	R	B	B	B	B	A	A	B	B
28	R	B	B	B	B	A	A	-	B
29	R	B	B	A	B	A	B	B	B
30	R	B	B	A	B	A	A	B	B
31	R	B	B	A	B	A	A	-	B
32	R	B	B	A	B	A	A	B	B
33	R	B	B	-	B	A	A	A	B
34	R	B	B	-	B	A	A	A	B
35	R	B	B	A	B	A	A	A	B
36	R	B	B	B	A	A	A	A	B
37	R	B	B	B	B	A	A	A	B
38	R	B	B	B	A	A	A	A	B
39	R	B	B	-	B	A	A	A	B
40	R	B	B	A	B	-	B	A	B
IRBB5(A)		6	7	18	17	27	35	27	5
IR64 (B)		34	33	17	23	9	5	7	34
NoBand(-)		NIL	NIL	5	NIL	4	NIL	NIL	1
Recombinant		34	33	17	23	9	5	6	34
cM		0.85	0.825	0.425	0.575	0.225	0.125	0.175	0.85

A=*xa5/xa5;* H=*Xa5/xa5;* B=*Xa5/Xa5;* - = no amplification

Eight amplicons out of which RM610, completely Co-segregated with 35 tagged resistant lines but the SSR loci RM609 and RM611, 9 and 12 recombinants respectively placing *xa5* 0, 26, and 0.34 cM away. RM610 covering the *xa5* region accounts for a physical distance of 24369 bases was identified as linked marker in our present investigation useful for MAS.Tagged rice lines #1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 24, 25,26, 27, 28, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, phenotyped as resistance through artificial inoculation also showed co segregating with the linked SSR marker RM610.

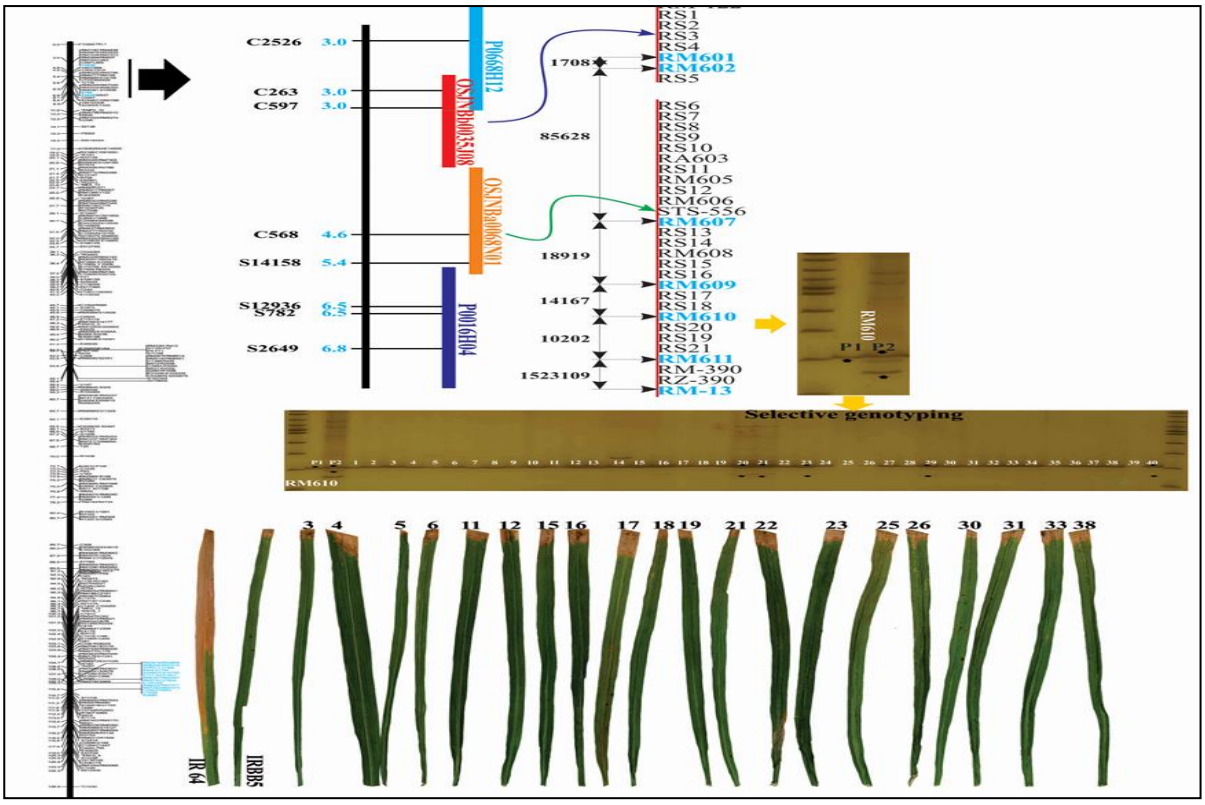

Fig1: Screening of parental polymorphism and selective genotyping with SSR marker(s) RM610 completely co-segregating as per the phenotype in the selected F_4 plants

A contig map covering the *xa5* region is flanked by RM609 and RM611 accounting for a physical contig map covering 43kb region (33086 bases (33.1kb) between RM609 and RM610 and 24369 bases (24.4kb) between RM610 and RM611. Selected 35 lines through MAS will be further rescreened through artificial inoculation and further backcrossing for the restoration of the background of IR64.

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References

1. Blair MW, McCouch SR. Microsatellite and sequence tagged site markers diagnostic for the rice bacterial blight resistance gene *xa*5. *Theor Appl Genet.* 1997; 95:174-184.
2. Chen S, CG Xu, XH Lin, Q. Zhang. 2001. Improving bacterial blight resistance of '6087', an elite restorer line of hybrid rice, by molecular marker assisted selection. *Plant Breed.* 1997; 120:133-137.
3. Gnanamanickam SS, Priyadarisini VB, Narayanan NN, Vasudevan P. An overview of bacterial blight disease of rice and strategies for its management. Curr. Sci. 1999; 77:11.
4. Huang N, ER Anjeles, J Domingo, G Magpantay, S Singh, G Zhang. *Et al*. Pyramiding of bacterial blight resistance genes in rice: marker aided-selection using RFLP and PCR. Theor. Appl. Genet. 1997; 95:313-320.
5. I lag LL, Yadav RC, Huang N, Ronald PC, Ausubel FM. Isolation and characterization of disease resistance gene homologues from rice cultivar IR64. Gene. 2000; 19:245-55.
6. Kauffman HE, APD Reddy, SPY Hsieh, SD MERCA, An improved technique for evaluating resistance of rice varieties to *Xanthomonasoryzae*. Plant Disease Rep. 1973; 57:537-541.
7. Khush GS, ER Angeles. New gene for resistance to bacterial blight *Xanthomonas oryzae pv. Oryzae* in rice. *Rice Genet.Newsl.* 1999; 16:92-93.
8. Khush GS, ER Angeles. New gene for resistance to bacterial blight *Xanthomonas oryzae pv. Oryzae* in rice. *Rice Genet.Newsl.* 1999; 16:92-93.
9. Lee KS, S Rasabandith, ER Angeles, GS Khush. Inheritance of resistance to bacterial blight in 21 cultivars of rice. The Am. Phytopathol. Soc. 2003; 93(2):147-152.
10. Mc Couch S, Cho YG, Yano M, Paul E, Blinstrub M, Morishima H. *et al*. Report on QTL nomenclature. *Rice* Genet. Newsl. 1997; 14:11-13.
11. Mew TW, Alvarez AM, Leach JE, Swings J. Focus on bacterial blight of rice. Plant Dis. 1993; 77:5-12.
12. Ou SH. Rice Disease. IRRI, Los Banos, Laguna, the Philippines. 1972.
13. Richter TE, Ronald PC. The evolution of disease resistance genes. Plant Mol Biol. 2000; 42:195-204.
14. Ronald PC. Resistance gene evolution. CurrOpin Plant Biol. 1998; 1:294-298.
15. Ronald PC. Molecular basis of disease resistance. Plant Mol Biol. 1997; 35:179-86.
16. Saji S, Umehara Y, Kurata N, Ashikawa I, Sasaki T. Construction of YAC contigs on rice chromosome 5. DNA Res, 1996; 3:297-302.
17. Sanchez AC, Brar DS, Huang N, Li Z, Khush GS. Sequence tagged site marker-assisted selection for three bacterial blight resistance genes in rice. Crop Sci. 2000 40:792-797.
18. Sanchez AC, DS Brar, N Huang, Z Li, GS Khush. Sequence tagged site markerassisted selection for three bacterial blight resistance genes in rice. Crop Sci. 2000;

40:792-797.
19. Singh S, JS Sidhu, N Huang, Y Vikal, Z Li, DS Brar. *Et al*. Pyramiding three bacterial blight resistance genes (*xa5*, *xa13* and *Xa21*) using marker assisted selection into *indica* rice cultivar PR106. Theor. Appl. Genet. 2001; 102:1011-1013.
20. Singh S, JS Sidhu, N Huang, Y Vikal, Z Li, DS Brar. *Et al*. Pyramiding three bacterial blight resistance genes (*xa5*, *xa13* and *Xa21*) using markerassisted selection into *indica* rice cultivar PR106. Theor. Appl. Genet. 2001; 102:1011-1013.
21. Wang Z, Taramino G, Yang D, Liiu G, Tingey SV, Miano GH. *Et al*. Rice ESTs with disease-resistance gene or defense-response gene-like sequences mapped to regions containing major resistance genes or QTLs. *Mol Genet Genomics*. 2001; 265:302-310.