Omonrice 13: 18-25 (2005)

STS AND MICROSATELLITE MARKER-ASSISTED SELECTION FOR BACTERIAL BLIGHT RESISTANCE IN RICE, *Oryza sativa* L.

Nguyen Vinh Phuc[*], Nguyen Thi Lang, Bui Chi Buu
([*] MSc scholar in Vinh Long)

ABSTRACT

DNA marker-assisted selection was employed to select xa-13, xa-5 and Xa-4 bacterial blight resistance genes. Genotypes with the genes were selected from four BC_2F_2 populations involving indica × indica crosses. With the assistance of PCR-based markers, 60 true breeding lines were identified from CLRRI and 100 BC_4F_4s were also subjected to marker-assisted selection for xa-13 locus. Plants were analyzed through STS marker RG136 that showed polymorphism between IR24 and Base having resistant and susceptible bands. PCR analysis using oligo RG136 in an BC_4F_4 population segregating for xa-13 locus. OMCS2000 showed banding pattern identical to that of its xa-13 parent. These plants were, therefore, assumed to carry xa-13 gene in homozygous state. The homozygotes and heterozygotes were scored, then goodness of fit was tested. One hundred plants were raised from each selected BC_4F_4. All the 60 lines raised from each selected lines were tested against 11 races of bacterial blight pathogen. All the plants showed resistance to BB pathogen. The resistance reaction was also confirmed by markers for SSRs and STSs to detect xa-13, xa-5 and Xa-4 genes. Sixty lines tested with marker superior plants carrying genes for resistance were selected. We detected OM2517 and OM5636 carrying xa-5, and OM2718, AS996, OM2514 , OMCS2000 and DS2002 carrying Xa-4.

Key words: bacterial blight (BB), marker-assisted selection (MAS), polymorphism, *Xanthomonas oryzae* pv *oryzae*

INTRODUCTION

Bacterial blight (BB) caused by Xanthomonas oryzae pv oryzae is found world-wide and causes substantial damage to rice yield. The rice yield losses of up to 10– 20% were recorded under moderate infection while they were as high as 50% in fields severely affected by bacterial blight (Mew 1989). Pathogenic specialization had been confirmed in this causal organism (Mew and Vera-Cruz 1979). Host plant resistance is yet to be proven as an effective and economical measure for controlling this disease. So far, 24 genes conferring resistance to specific races or clusters of races of Xoo have been identified through classical genetic analysis. So far, 10 major genes have been mapped using RFLP, RAPD and microsatellite markers (McCouch et al. 1991; Ronald et al. 1992; Yoshimura et al. 1992; Borines et al. 2000. Lang et al. 2005). The advent of molecular markers tagged to different resistant genes enabled convergence breeding and pyramiding of more than two different genes into an agronomic variety. Some of BLB have been transferred to modern rice cultivars and released in Japan and other Asian countries. Recently, two new genes Xa-22 in rice variety Zha-Chang-long (Gao et al. 1999) and Xa-23 in Oryza rufipogon were identified and mapped on different chromosomes. Due to continuous evolution of pathogenic races, breakdown of resistance has occurred in many improved varieties. Incorporating resistance genes can be very difficult using conventional methods of breeding due to epistasis or masking effects of other genes. The Xa-21 gene shows resistance to all known races of BB. The selection of lines having this gene would have been difficult or impossible by conventional screening methods. However, selection of plants with homozygous resistance loci will be possible at any growth stage if DNA markers linked with the target genes are available. MAS has been successfully used to transfer resistance genes

into seed parents (Borines et al. 2000, Lang et al 2001, 2003, 2004) and restorer lines (Chen et al. 2000). Molecular breeding approach has been successfully utilized in the Philippines and China for the improvement of bacterial blight resistance in modern rice cultivars. In this review, we highlight the markers, which can be employed in MAS for breeding varieties and hybrids with durable bacterial blight resistance

MATERIALS AND METHODS

Plant materials

Two genotypes IR24 (susceptible) and Base (resistant local genotype), and a set of 100 BC4F4 derived from these parents were used in the present study. Likewise, some of the F1's were backcrossed to the recurrent parents. Starting at BC3F3 generation, molecular markers were used to select plants with genes for resistance to BB. Phenotypic assessment was done to select plants with 11 races including 10 from IRRI and 1 from CLRRI. Sixty genotypes used in this study were selected from CLRRI Gene bank.

Bacterial blight screening

Parents and progenies of all selected BC4F4 individuals from IR24/ Base were grown in the greenhouse. One hundred plants were raised in each selected BC4F4 lines. The rice plants were inoculated with 11 races at maximum tillering stage. Scissors clipping at 5 cm below the leaf tips inoculated the leaf blades. At 20 days after inoculation, the plants were scored as resistant or susceptible by measuring the length of lesion or by visual scoring. The selected 60 traditional cultivars were also inoculated with 11 races and confirmed their phenotypic reaction to the Xoo pathogen.

DNA markers

DNA markers linked to bacterial blight resistance genes were based on previous research paper (Lang and Buu 2004)

DNA isolation

A crude DNA preparation suitable for PCR analysis was prepared using a simplified miniscale procedure (Lang 2002). A single piece of healthy young leaf was harvested and placed in a labelled 1.5 ml centrifuge tube in ice. The leaf sample was treated using thick glass rod after adding 400 µl of extraction buffer (50 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 300 mM NaCl and 1% SDS). The leaf was grounded until the buffer turned into green colour. After grinding, another 400 µl of extraction buffer was added and mixed by pipetting. The contents were centrifuged at 13,000 rpm in micro centrifuge for 5 min. Nearly 400 µl of lysate was extracted with 400 µl of chloroform. The top aqueous supernatant was transferred to another 1.5 ml tube and DNA was precipitated with absolute ethanol. The contents were centrifuged for 3 min at full speed and the supernatants were discarded. The pellet was washed with 70% ethanol. The DNA was air dried and re-suspended in 50 µl of TE buffer (10mMTris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). One ml of aliquot was used for PCR analysis and the remaining solution was stored at –200C for any further use.

Polymerase chain reaction (PCR amplification)

A series of optimization experiments using parents and isogenic samples was carried out in which concentrations of template DNA, primers, dNTPs and Taq polymerase were varied to determine which conditions gave the strongest patterns. The PCR reaction mixture of 20 µl contained 25–50 ng template DNA, 50 ng of each primer obtained from CLRRI Genome Analysis Lab I, 0.05 mM dNTPs, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2 and 0.01% gelatin) and 1 unit of Taq polymerase. The template DNA was initially denatured at 940C for 5 min followed by 35 cycles of PCR amplification under the following parameters: 1 min denaturation at 940C, 1 min primer annealing at 550C and 1.5–2.0 min primer extension at 720C. A final 7 min incubation at 720C was allowed for completion of primer extension on Thermalcycler. Following amplification, the samples were run on a 1% agarose in 1X TBE buffer stained with 0.1 µg/ml of ethidium bromide and visualized under ultraviolet light. For PCR-RFLP, initially 5 µl of PCR product was used for gel electrophoresis to determine successful amplification. Seven to eight µl of the PCR product was then used for restriction enzyme digestion if the amplification was not successful. The total reaction volume was usually 20 µl. The reaction mixture consisted of 11.4 µl of double distilled water, 2 µl of 10 X restriction buffer, 0.6-µl restriction enzyme

(10 U/µl) and 6–8 µl of PCR products. The reaction mixture was incubated at 370C for 6–8 hrs. The DNA fragments digested by restriction enzymes were separated on 1.5% agarose gel and visualized under UV light after staining with ethidium bromide.

For microsatellite markers, the PCR amplified products were resolved on a 3% polyacrylamide denaturing gel for 4–6 h at 200W and silver stained (0.1% $AgNO_3$) for 10 minutes. After washing two times with double distilled water for 3–5 min the gel was transferred to a developing solution (6% NaOH) and the bands were viewed.

RESULTS AND DISCUSSION

Reaction of germplasm to bacterial blight pathogen

Reaction of short duration genotypes to eleven *Xoo* was presented in table 1. OM5239 resisted to 10 races (except PXO339). OM1490, AS996 and OM2517 exhibited to be resistant to 9 races.

Reaction to mid-duration genotypes was presented in table 2. OM1346 resisted to 11 races, IR42 to 10 races (except PX0339), OM1351-2, OM2417 and OM2496 9 races

Introgression at *xa-13* locus

BC_4F_4s were generated from IR24 / Base, a near isogenic line carrying the *xa-13* gene with different genotypes. PCR-based markers were produced based on the sequences of RFLPs related. In order to confirm polymorphism between resistant and susceptible genotypes, DNA markers were used to discriminate different near isogenic lines carrying specific resistant genes from recurrent parent IR24. The true hybrids in BC_4F_4 combinations were also fixed based on the presence of heterozygous bands. Those plants carrying bands from both parents when subjected to PCR analysis alone were selfed and advanced to BC_4F_4 generation. Stringent phenotypic selection was conducted to reduce population size. These lines were subjected to PCR analysis using the linked marker RG136 and thus allow efficient surveying of BC_4F_4 for *xa-13*. The 800 bp band corresponds to an allele from susceptible parent (IR24), whereas the 1,120 bp band represents resistant parent (Base). Sixteen plants from the IR24 / Base cross showed a banding pattern identical to that of the resistant parent, *xa-13*, and these plants, therefore, were determined to contain *xa-1* (Figure 1). The heterozygote and homozygous susceptible plants also were scored using the marker, then goodness of fit was tested .

Table 1: Reaction of short duration genotypes to BLB in 2005 wet season

Designation	BACTERIAL LEAF BLIGHT RACES										
	PXO 61	PXO 86	PXO 340	PXO 71	PXO 112	PXO 99	PXO 145	PXO 280	PXO 339	PXO 341	Race 11
OM4498	S	S	S	S	R	S	R	S	S	R	R
OM4495	**S**	R	R	**S**	S	**R**	R	S	R	**R**	**R**
OM2717	R	**R**	S	S	**R**	**R**	R	R	S	R	R
OM2718	S	**R**	R	R	**R**	R	S	R	**S**	**R**	R
AS996	R	R	R	R	S	R	R	R	R	R	S
OM2517	R	**R**	R	R	R	R	R	**R**	**S**	S	R
OM2514	**R**	R	S	**R**	S	S	R	S	R	R	R
OM2492	R	R	**R**	**S**	**R**	R	R	R	S	R	**S**
OM5239	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**S**	**R**	**R**
OM3419	R	R	R	R	S	R	R	R	R	R	R
OM1346	R	**R**	R	R	**R**	R	**R**	R	R	R	R
OMCS2000	R	**R**	R	**S**	**R**	R	**S**	S	S	R	R
OM1490	R	**R**	R	R	**S**	R	**S**	R	R	R	R
OM5636	R	**S**	R	R	**S**	S	**R**	S	S	R	R
OM5637	R	**S**	R	R	**S**	R	**R**	R	R	S	R
DS2002	S	**S**	R	S	**S**	S	**S**	S	R	R	S

Table 2: Reaction of mid-duration genotypes to BLB in 2005 wet season

Designation	BACTERIAL LEAF BLIGHT RACES										
	PXO 61	PXO 86	PXO 340	PXO 71	PXO 112	PXO 99	PXO 145	PXO 280	PXO 339	PXO 341	Race 11
OM1352-5	R	R	R	R	R	S	S	S	S	R	R
OM3674	**R**	R	R	**S**	S	**R**	R	S	R	**R**	**R**
OM1351-2	R	**R**	R	S	**R**	**R**	R	R	S	R	R
OM1348-9	S	**R**	R	S	**S**	S	K	K	**S**	**R**	R
OM1350-7	R	R	R	R	S	R	R	R	S	R	R
OM2417	R	**S**	R	R	S	R	R	**R**	**R**	R	R
OM1337	**R**	S	R	**S**	S	R	R	R	R	S	R
OM2496	R	R	**R**	**R**	**R**	S	R	R	R	R	**S**
OM1346	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
IR42	R	R	R	R	S	R	R	R	S	R	R

R: Resistance; S: Susceptible

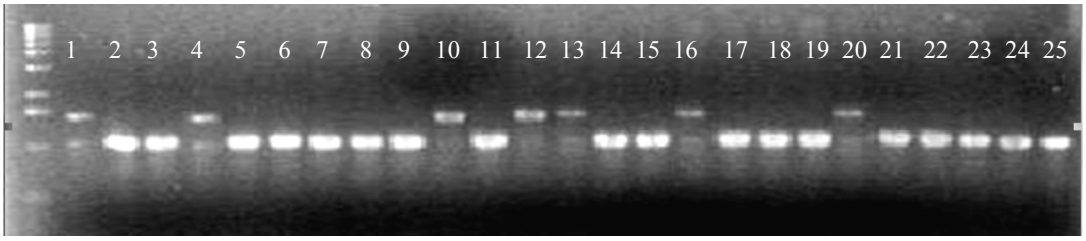

Figure 1: STS marker detected gene *xa*-13 at locus RG136. Lane 1: Base (R), lane 2: IR24 (S), lane 3-23: BC_4F_4 individuals of IR24 / Base.

DNA survey by microsatelites

Diversity of alleles in BLB resistant genotypes was measured with four microsatellite markers. For *xa-5* gene, 40 genotypes were surveyed, RM13 exhibited the highest degree of polymorphism with 6 alleles ranging in size 220 bp and 1,400bp (fig. 3) and RM122 on chromosome 5 indicated more informative feature than RM13 (fig. 2). with 3 alleles ranging in size 231 bp and 250 bp.

RM390 was less polymorphic among tested microsatellites with only two alleles detected. RM21 failed to detect polymorphism for all genotypes.

For STS markers, no alleles were identified among the genotypes surveyed with RG556. Marker-assisted selection with RM13 helped develop OM2517 and OM5636.

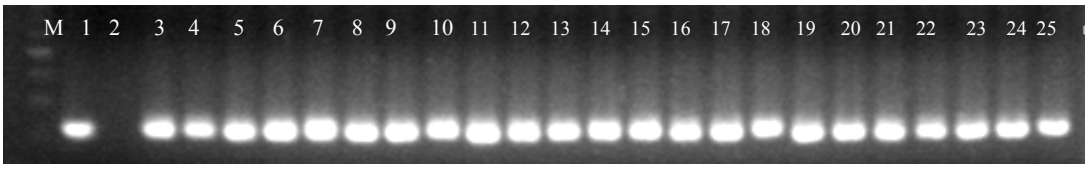

Figure 2: PCR products of different genotypes generated by DNA amplification with primer at locus RM122.

1: IBB4; 2: IBB5; 3: IBB13; 4: IBB21; 5: IR36; 6: OM4498; 7: OM 4495; 8: OM2717; 9: OM 2718; 10: AS996; 11: OM2517; 12: OM2514-343; 13: OM2008; 14: OM2492; 15: OM 5239; 16: OM4872; 17: OM5240; 18: OM3428; 19: OM2490; 20: OMCS 2000; 21: OM1490; 22: DS20; 23: DS2002; 24: OM5636; 25: OM 5637.

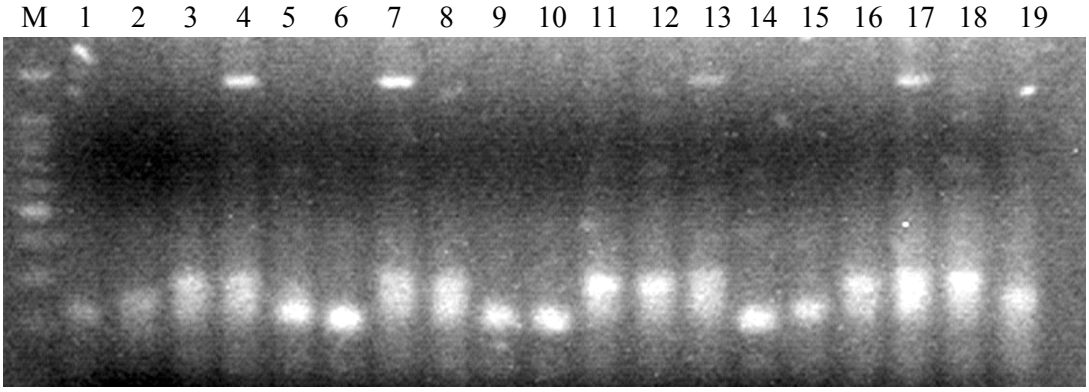

Figure 3: PCR products of different genotypes generated by DNA amplification with primer at locus RM13.

1: IRBB5 2: IR24 3: OM4285; 4: OM3406 5: OM3238 6: OM4196
7: OM1238 8: OM3243 9: OM2403 10: OM4214 11: OM3837-31 12: OM3432
13: OM3834 14: OM4218 15: OM4096 16: OM4749 17: OM2768 18: OM4325
19: DT70

For *xa-13*, two alleles were identified among the genotypes surveyed with RG136 and the PCR products were digested by *Hin*fI. The allele in IRBB 13 was designated as allele 1, 5, 15 and 16 lines carrying this allele. MAS cannot be practised in this group as they possess similar allele as that of IRBB13. The marker allele in the susceptible line IRBB13 was not designated the same size of *xa-13*. PCR products were not detected in 25 rice lines after repeating. This suggested that these lines carry one allele and digested by *Hin*fI.

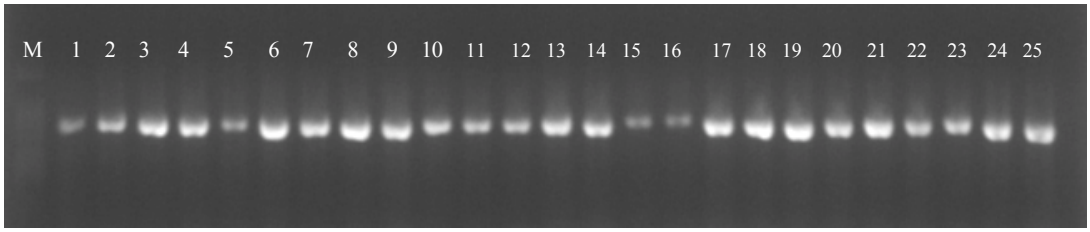

Figure 4: PCR products of different genotypes generated by DNA amplification with primer RM136 digested by *Hin*fI

1: IRBB13 2: OM 5929; 3: OM4498 4: OM4495 5: OM2717 6: OM 2718
7: AS 996 8: OM 2008 9:OM 2492 10: OM4872 11: OM3428 12: OM 2490
13: OM1490 14: OM4285 15: OM4196 16:OM1238 17:OM 3243 18:OM2403
19:OM 4214 20:OM 3837-*31 21:OM3834 22:OM4218 23:OM4096 24:OM4799
25:DT 70

Similarly, plants were also selected from *Xa-4* .Using RM 144 and RM 244 microsatellite marker on chromosome 11 . For RM 144 three alleles were detected. Allele 1 was detected in five lines , Allele 2 in 1,3,4,6 ,7,8 , allele 3 in 9,10,12,17,20,23 lines and allele 3 in IRBB 4 , 9, 10, 12, 20 and 23 lines . The PCR polymorphism can be generated between these accessions and IRBB 4 thus allowing MAS among these lines (OM 2718, AS 996, OM 2514 , OMCS 2000 and DS 2002).

For RM 244 , all allele scored codominant markers and tested

Fig 5: PCR products of different genotypes generated by DNA amplification with primer RM144

1: IRBB4;	2: IRBB5	3: IRBB13	4: IBB21	5: IR36	6: OM4498
7: OM4495	8:OM2717	9: OM2718	10:AS996	11: OM2517	12:OM2514
13: OM 2008	14:OM2492	15:OM5239	16: OM4872	17: OM5240	18: OM3428
19: OM2490	20:OMCS2000	21: OM 1490	22: DS20	23: DS2002	24: OM5636
25: OM5637					

CONCLUSION

In this study, marker-aided selection was employed to expedite the transfer of bacterial leaf blight resistance to improve rice varieties. Three BB resistance genes, *Xa-4*, *xa-5* and *xa-13* were identified to links to suitable STS markers and microsatelliles. Genes *Xa-4*, *xa-13,* and *xa-5* closely linked to RM144, RG136 and RM122, respectively. RM13 was also linked to *xa-5* while RG566 (STS marker) located on chromosome 5 failed to amplify consistently in the segregating population.

REFERENCES

Borines LM, CM VeraCruz, ED Redona, JF Hernandez, MP Natural, AD Raymundo, H Leung. 2000. Marker-aided pyramiding of bacterial blight resistance genes in maintainer lines for hybrid rice production. IRRI Conf Abstr 2000. 4(2):162.

Gao HP, XH Lin, GX Yu, DP Zhang, YF Xie. 1999. Inheritance of resistance to bacterial blight of four Yunan rice varieties. Rice Genet Newsl 12: 233–234.

Huang N, ER Angeles, J DOMingo, G Magpantay, AS Singh, G Zhang, N Kumaravadivel, J Bennett, GS Khush. 1997. Pyramiding of bacterial blight resistance genes in rice: marker assisted selection using RFLP and PCR. Theor Appl Genet 95: 313–320.

Lang NT, BC Buu. 2004. Molecular genetic analysis and marker assisted selection for restorer line and bacterial blight resistance in hybrid rice. Sabrao 36 (2): 83-93.

Lang NT. 2002. Biotechnology Protocols. Agriculture Publishing House, Ho chi Minh City, Vietnam [in Vietnamese]

McCouch SR, SD Tanksley. 1992. Genetic and physical analysis of the rice bacterial blight disease resistance locus *Xa 21*. Mol Gene Genet 236: 113–120.

Mew TW, CM VeraCruz. 1979. Variability of *XanthOMonas oryzae* in infection of rice differential. Phytopathol 69: 152–155.

Mew TW. 1989. An overview of the world bacterial bight situation. *In:* Bacterial Blight of Rice, pp. 7–12. International Rice Research Institute, Los Banos, Laguna, Philippines.

Ronald PC, B Albano, R Tabien, L Abenes, K Wu, SR McCouch, SD Tanksley. 1992. Genetic and physical analysis of the bacterial blight disease resistance locus, *Xa-21*. Mol Gen Genet 236:113–20.

Yoshita J, D Krishnaveni, APK Reddy, RV Sonti. 1997. Genetic diversity within the population of *XanthOMonas oryzae* pv *oryzae* in India. Phytopathology 87:760–5.

Áp dụng STS và microsatellite marker trong chọn giống lúa kháng bệnh bạc lá

Nguyen Vinh Phuc, Nguyen thi Lang, Bui Chi Buu

Áp dụng marker phân tử trong chọn giống lúa kháng bệnh bạc lá với gen mục tiêu xa-*13, xa-5* và *Xa-4* được thực hiện tại Viện Lúa ĐBSCL. Đánh giá kiểu gen được tiến hành trên bốn quần thể BC_2F_2 trong tổ hợp lai indica × indica. Với sự trợ giúp của PCR-based markers, 60 dòng con lai đã được phân lập, và 100 cá thể BC_4F_4s được chọn ra nhờ marker tạilocus *xa-13*. Cây lúa được phân lập nhờ STS marker RG136, cho thấy tính chất đa hình giữa IR24 và giống bản địa Ba se, thông qua băng nhiễm và băng kháng rất rõ. Phân tích PCR bằng cách sử dụng oligo RG136 trên quần thể BC_4F_4 tại locus *xa-13* cho thấy OMCS2000 có cùng một băng với kiểu gen kháng *xa-13*. Những cây này được xem như có mang gen *xa-13* ở trạng thái đồng hợp tử. Con lai đồng hợp tử và dị hợp tử được cho điểm và trắc nghiệm tỉ lệ phân ly tương ứng. Trong 100 cây được chọn từ BC_4F_4, có 60 dòng được xét nghiệm với 11 nhóm nòi vi khuẩn gây bệnh bạc lá tại Đông nam Á và Việt Nam. Tất cả những cây được lựa chọn ấy đều có phản ứng kháng với nguồn vi khẩun gây bệnh bạc lá. Phản ứng kháng như vậy được khẳng định lại bằngmarker SSRs và STSs để phát hiện cá thể mang gen *xa-13*, *xa-5* và *Xa-4*. Các dòng lúa có triển vọng trong sản xuất hiện nay như OM2517 và OM5636 mang gen *xa-5,* OM2718, AS996, OM2514 , OMCS2000 và DS2002 mang gen *Xa-4*.