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# Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR

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**Abstract** DNA marker-assisted selection was used to pyramid four bacterial blight resistance genes, *Xa-4*, *xa-5*, *xa-13* and *Xa-21*. Breeding lines with two, three and four resistance genes were developed and tested for resistance to the bacterial blight pathogen (*Xanthomonas oryzae* pv. *oryzae*). The pyramid lines showed a wider spectrum and a higher level of resistance than lines with only a single gene. To speed up the gene pyramiding process and to facilitate future marker-aided selection, we developed PCR markers for the two recessive genes, *xa-5* and *xa-13*, and used these to survey a range of rice germplasm. The results of the germplasm survey will be useful for the selection of parents in breeding programs aimed at transferring these bacterial blight resistance genes from one varietal background to another.

**Key words** Rice · Bacterial blight · Resistance genes · Pyramiding · MAS

## Introduction

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is one of the most destructive diseases of rice throughout the world, and in some areas of Asia it can reduce crop yield by up to 50%. The most effective approach to combat BB is the use of resistant varieties (Khush et al. 1989). So far, 19 resistance genes have been identified (Kinoshita 1995), and some of these have been incorporated into modern rice varieties. The exploitation of gene *Xa-4* resulted in the development of many BB-resistant rice varieties that have played an important role in protecting rice from *Xoo* (Khush et al. 1989). However, the large-scale and long-term cultivation of varieties carrying *Xa-4* has resulted in significant shifts in the race frequency of *Xoo* (Mew et al. 1992). In many areas of Indonesia, India, China and Phillippines, rice varieties with only *Xa-4* for defense against *Xoo* have become susceptible to the pathogen. One way to delay such a breakdown of BB resistance is to pyramid multiple resistance genes into rice varieties. This approach can however be very difficult or impossible using conventional breeding methods due to epistasis and/or the masking effect of genes, particularly when a breeding line already has a gene; for example, *Xa-21*, which shows resistance to all known BB races. With the conventional approach, breeding lines with *Xa-21* alone cannot be distinguished from breeding lines with *Xa-21* plus other genes. However, if DNA markers were available for each resistance gene, the identification of plants with multiple genes would become easy.

Several BB resistance genes have been mapped with restriction fragment length polymorphism (RFLP) markers (Ronald et al. 1992; Yoshimura et al. 1995; Zhang et al. 1996). Yoshimura et al. (1995) combined resistance genes in pairs (*Xa-4*/*xa-5*, and *xa-5*/*Xa-10*) and showed that plants with two genes can have a higher level of resistance to *Xoo* than would be expected from the sum of the parental levels. Zhang et al. (1996) identified *xa-13* via linkage to RFLP markers in three $F_4$ populations in which the *xa-13* gene would otherwise have been masked by *Xa-21*. In this paper, we report the pyramiding of four BB resistance genes, namely, *Xa-4*, *xa-5*, *xa-13* and *Xa-21*. To facilitate marker-assisted selection (MAS) in the future, we developed polymerase chain reaction (PCR) markers for the two recessive genes *xa-5* and *xa-13* and used the

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PCR markers to survey a range of rice germplasm for PCR polymorphism.

## Materials and methods

### Plant materials

The rice breeding lines used to pyramid genes for resistance to BB are listed in Table 1. These lines include four near-isogenic lines (NILs) and their recurrent parents, 'IR24'. IRBB50 with *Xa-4* and *xa-5* genes was provided by Atsushi Yoshimura, Kyushu University, Japan. IRBB52 and IRBB53 are breeding lines developed in this study (Table 2) and then used in a second round of pyramiding. Listed also are nine $F_2$ populations from which pyramid lines were selected. $F_3$ and $F_4$ populations developed from the selected pyramid lines were used to confirm the presence of identified genes. The selected and confirmed pyramid lines (Table 2) were used for overall resistance evaluation.

One hundred and eighty-seven rice accessions were used for the PCR polymorphism survey. These accessions were obtained from the Genetic Resource Center (GRC) at IRRI and represent a wide range of rice germplasm and IRRI rice varieties. The plants were grown in the field for DNA isolation.

### DNA markers

DNA markers linked to BB resistance genes were based on published results (Table 3). The RFLP markers were kindly provided by Steve Tanksley, Cornell University, and the Rice Genome Project, NIAR/STAFF, Tsukuba, Japan. Primer sequences for a PCR marker linked to *Xa-21* were from Chunwongse et al. (1993). Additional PCR primers are based on DNA sequences derived by manual sequencing from both ends of RFLP clones RG556 and RG136 (Table 4). In the early stage of this study, Southern analysis with RFLP markers was used for MAS because polymorphic PCR markers were not yet available. The standard procedure of Southern analysis was followed (Sambrook et al. 1989).

### DNA isolation

Two DNA isolation protocols were used. The procedure by Dellaporta et al. (1983) was followed to isolate large amounts of pure DNA for Southern analysis. To obtain crude DNA suitable for PCR analysis, we used a simplified procedure. A single piece of young rice shoot or leaf (2 cm long) was harvested and placed in a labeled 1.5-ml centrifuge tube on ice. The leaf was transferred to a well of a Spot Test Plate (Thomas Scientific). After the addition of 400 µl of extraction buffer (50 m*M* TRIS-HCl, pH 8.0, 25 m*M* EDTA, 300 m*M* NaCl and 1% SDS), the sample was ground with a polished glass rod (1 cm diameter). The tissue was ground until the DNA extraction buffer turned green (a sign of cell breakage and release of chloroplasts). After grinding, another 400 µl of DNA extraction buffer was added to the well and mixed by pipetting. From the well, 400 µl of lysate was transferred to the original tube for the rice tissue. The lysate was extracted with 400 µl of chloroform. The aqueous supernatant was transferred to another 1.5-ml tube, and DNA was precipitated with ethanol. After drying in air, the DNA was resuspended in 50 µl of TE. Aliquots of 1 µl were taken for PCR analysis, and the remaining DNA was stored at −20°C for later use.

### PCR analysis for MAS

The PCR reaction mixture contained 50 ng template DNA, 50 ng of each primer, 0.05 m*M* dNTPs, 1 × PCR buffer (10 m*M* TRIS, pH 8.4, 50 m*M* KCl, 1.8 m*M* $MgCl_2$ and 0.01 mg/ml gelatin) and 1 unit *Taq* DNA polymerase in a volume of 20 µl. Template DNA was initially denatured at 94°C for 5 min followed by 30 cycles of PCR amplification with the following parameters: 30 sec of denaturation at 94°C, 30 sec of primer annealing at 55°C and 1 min of primer extension at 72°C. A final 5-min incubation at 72°C was allowed for completion of primer extension. The amplified products were electrophoretically resolved on a 1% agarose gel in 1 × TAE buffer.

We initially used 10 µl of PCR product for gel electrophoresis to determine if PCR amplification was successful; the remaining 10 µl of PCR products was used for restriction digestion. The total reaction volume was usually 15 µl. The reaction mixture consisted of 3.2 µl sterile distilled water, 1.5 µl restriction buffer (10 ×), 0.3 µl restriction enzyme (10 U/µl) and 10 µl of PCR products. The

**Table 1** List of isogenic lines and $F_2$ population used in this study

| Line/population | Cross | Genes involved | Population size[a] |
|---|---|---|---|
| IR24 | | None | |
| IRBB4 | | *Xa-4* | |
| IRBB5 | | *xa-5* | |
| IRBB13 | | *xa-13* | |
| IRBB21 | | *Xa-21* | |
| IRBB50 | | *Xa-4*/*xa-5* | |
| IRBB52 | | *Xa-4*/*Xa-21* | |
| IRBB53 | | *xa-5*/*xa-13* | |
| $F_2$ populations | | | |
| NH8 | IRBB4/IRBB13 | *Xa-4*/*xa-13* | 157/51 |
| NH9 | IRBB4/IRBB21 | *Xa-4*/*Xa-21* | 238/94 |
| NH11 | IRBB5/IRBB13 | *xa-5*/*xa-13* | 298/85 |
| NH12 | IRBB5/IRBB21 | *xa-5*/*Xa-21* | 268/164 |
| NH15 | IRBB13/IRBB21 | *xa-13*/*Xa-21* | 112/72 |
| NH18 | IRBB21/IRBB13 | *xa-13*/*Xa-21* | 181/90 |
| NH21 | IRBB50/IRBB13 | *Xa-4*/*xa-5*/*xa-13* | 306/84 |
| NH24 | IRBB50/IRBB13 | *Xa-4*/*xa-5*/*xa-13* | 134/94 |
| NH56 | IRBB53/IRBB52 | *Xa-4*/*xa-5*/*xa-13*/*Xa-21* | 299/124 |

[a] Number of plants analyzed by BB inoculation/no. of plants analyzed by DNA marker

**Table 2** Selected $F_2$ plants containing two or more BB resistance genes

| Code | Lines | Gene combinations | Verification through $F_3$ analysis[a] | | | | Reaction to races of BB[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Xa-4* | *xa-5* | *xa-13* | *xa-21* | 1 | 2 | 3 | 4 | 5 | 6 |
| | IRBB4 | *Xa-4* | | | | | R | S | S | S | R | S |
| | IRBB5 | *xa-5* | | | | | R | R | R | MS | R | S |
| | IRBB13 | *xa-13* | | | | | S | S | S | S | S | R |
| | IRBB21 | *Xa-21* | | | | | R | R | R | R | R | R |
| | IR24 | | | | | | S | S | S | S | S | S |
| 1 | IRBB50 | *Xa-4/xa-5* | | | | | R | R | R | R | R | S |
| 2 | IRBB51-1 | *Xa-4/xa-13* | x | | x | | R | S | S | R | R | R |
| 3 | IRBB51-2 | *Xa-4/xa-13* | x | | x | | R | S | S | R | R | R |
| 4 | IRBB51-3 | *Xa-4/xa-13* | x | | x | | R | S | S | R | R | R |
| 5 | IRBB51-4 | *Xa-4/xa-13* | x | | x | | R | S | S | R | R | R |
| 6 | IRBB52 | *Xa-4/Xa-21* | x | | | x | $R^+$ | R | R | R | $R^+$ | R |
| 7 | IRBB53-1 | *xa-5/xa-13* | | x | x | | R | R | R | R | R | R |
| 8 | IRBB53-2 | *xa-5/xa-13* | | x | x | | R | R | R | R | R | R |
| 9 | IRBB53-3 | *xa-5/xa-13* | | x | x | | R | R | R | R | R | R |
| 10 | IRBB53-4 | *xa-5/xa-13* | | x | x | | R | R | R | R | R | R |
| 11 | IRBB54-1 | *xa-5/xa-21* | | x | | x | $R^+$ | $R^+$ | $R^+$ | R | $R^+$ | R |
| 12 | IRBB54-2 | *xa-5/Xa-21* | | x | | x | $R^+$ | $R^+$ | $R^+$ | R | $R^+$ | R |
| 13 | IRBB54-3 | *xa-5/Xa-21* | | x | | x | $R^+$ | $R^+$ | $R^+$ | R | $R^+$ | R |
| 14 | IRBB55-1 | *xa-13/Xa-21* | | | x | x | R | R | R | R | R | R |
| 15 | IRBB55-2 | *xa-13/Xa-21* | | | x | x | R | R | R | R | R | R |
| 16 | IRBB55-3 | *xa-13/Xa-21* | | | x | x | R | R | R | R | R | R |
| 17 | IRBB55-4 | *xa-13/Xa-21* | | | x | x | R | R | R | R | R | R |
| 18 | IRBB56-1 | *Xa-4/xa-5/xa-13* | x | x | x | | $R^+$ | R | R | $R^+$ | $R^+$ | R |
| 19 | IRBB56-2 | *Xa-4/xa-5/xa-13* | x | x | x | | $R^+$ | R | R | $R^+$ | $R^+$ | R |
| 20 | IRBB57-1 | *Xa-4/xa-5/Xa-21* | x | x | | x | $R^+$ | $R^+$ | $R^+$ | $R^+$ | $R^+$ | R |
| 21 | IRBB57-2 | *Xa-4/xa-5/Xa-21* | x | x | | x | $R^+$ | $R^+$ | $R^+$ | $R^+$ | $R^+$ | R |
| 22 | IRBB57-3 | *Xa-4/xa-5/Xa-21* | x | x | | x | $R^+$ | $R^+$ | $R^+$ | $R^+$ | $R^+$ | R |
| 23 | IRBB58-1 | *Xa-4/xa-13/Xa-21* | x | | x | x | $R^+$ | R | R | $R^+$ | $R^+$ | R |
| 24 | IRBB58-2 | *Xa-4/xa-13/Xa-21* | x | | x | x | $R^+$ | R | R | $R^+$ | $R^+$ | R |
| 25 | IRBB58-3 | *Xa-4/xa-13/Xa-21* | x | | x | x | $R^+$ | R | R | $R^+$ | $R^+$ | R |
| 26 | IRBB59-1 | *Xa-5/xa-13/Xa-21* | | x | x | x | $R^+$ | $R^+$ | $R^+$ | $R^+$ | $R^+$ | R |
| 27 | IRBB59-2 | *Xa-5/xa-13/Xa-21* | | x | x | x | $R^+$ | $R^+$ | $R^+$ | $R^+$ | $R^+$ | R |
| 28 | IRBB59-3 | *Xa-5/xa-13/Xa-21* | | x | x | x | $R^+$ | $R^+$ | $R^+$ | $R^+$ | $R^+$ | R |
| 29 | IRBB60-1 | *Xa-4/xa-5/xa-13/Xa-21* | x | x | x | x | $R^+$ | $R^+$ | $R^+$ | $R^+$ | $R^+$ | R |
| 30 | IRBB60-2 | *Xa-4/xa-5/xa-13/Xa-21* | x | x | x | x | $R^+$ | $R^+$ | $R^+$ | $R^+$ | $R^+$ | R |

[a] x indicates presence of the gene
[b] + indicates shorter lesion than their parents

**Table 3** Bacterial blight resistance genes used for gene pyramid

| Gene | Isoline | Donor | Chromosome | Linked marker and distance | | Reference |
|---|---|---|---|---|---|---|
| *Xa-4* | IRBB4 | TKM6 | 11 | Npb181<br>Npb78 | 1.7 cM<br>1.7 cM | Yoshimura et al. (1995) |
| *xa-5* | IRBB5 | DZ192 | 5 | RG556<br>RZ207 | 0–1 cM<br>0–1 cM | Yoshimura et al. (1995) |
| *xa-13* | IRBB13 | Long grain | 8 | RZ28<br>RG136 | 5.1 cM<br>3.8 cM | Zhang et al. (1996) |
| *Xa-21* | IRBB21 | *O. longistaminata* | 11 | pTA248<br>pTA818<br>RG103 | 0–1 cM | Ronald et al. (1992) |

reaction mixture was incubated from 2 h to overnight at 37°C. The DNA fragments produced by restriction digestion were separated through gel electrophoresis (1.5% agarose) and visualized under UV light after staining with ethidium bromide.

BB screening

The parents and $F_2$ generations were grown in an IRRI screenhouse. At the seedling stage, the rice plants were inoculated with race

**Table 4** Distribution of alleles of three PCR marker loci linked to resistance genes in 187 accessions of rice germplasm

| Marker | Primers 5′ → 3′ | Linked gene | Alleles[a] | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | Null |
| RG556 | F TAGCTGCTGCCGTGCTGTGC<br>R AATATTTCAGTGTGCATCTC | *xa-5* | 48 | 22 | 103 | 15 |
| RG136 | F TCCCAGAAAGCTACTACAGC<br>R GCAGACTCCAGTTTGACTTC | *xa-13* | 4 | 181 | – | 4 |
| pTA248 | F AGACGCGGAAGGGTGGTTCCCGGA[b]<br>R AGACGCGGTAATCGAAAGATGAAA | *Xa-21* | 1 | 96 | 89 | 5 |

[a] A few lines are mixtures of genotypes and carry more than 1 allele so the total number of alleles is greater than the number of accessions, 187, of germplasm surveyed
[b] Primer sequences are from Chunwongse et al. (1993)

6 (isolate PXO 99) of *Xanthomonas oryzae* using the leaf clipping method (Kauffman et al. 1973). Plant reaction to the disease was recorded 14 days after inoculation. Susceptible plants were discarded and resistant plants were analyzed with DNA markers to select plants carrying multiple resistance genes. The selected plants were further tested in the $F_3$ and $F_4$ generations to confirm the presence of resistance genes.

To examine the disease reaction of gene pyramids, we inoculated, all 6 races of *Xoo* present in the Philippines to all pyramid lines in the $F_4$ generation.

## Results

### Pairwise combination of bacterial blight resistance genes

To pyramid all four BB resistance genes into 1 breeding line, we first produced all six pairwise combinations of the genes. One of these combinations (*Xa-4* and *xa-5*) was provided by Atsushi Yoshimura of Kyushu University, Japan. The other five pairwise gene combinations were developed at IRRI (Table 1). To reduce the population size for DNA marker analysis, we inoculated the $F_2$ populations with race 6 of BB pathogen and susceptible plants were discarded. This procedure removed those plants lacking all resistance genes (Table 1).

DNA markers were then used to identify homozygotes for each of the genes in the five combinations. Southern analysis was used in the early stage of the project. Figure 1 shows the identification of homozygotes for resistance gene *xa-5* with linked marker RG556 as probe. $F_2$ plants (lanes 2, 3, 5, 6, 10, 11, 12) with a banding pattern identical to that in IRBB5 were determined to be containing *xa-5*. Plants homozygous for markers linked to *Xa-4*, *xa-13* and *Xa-21* resistance genes were similarly identified. Plants homozygous for the resistant allele at two marker loci were retained (Table 2). For each of the five combinations, 1–6 true-breeding lines were selected (Table 2). Four lines were selected for the *Xa-4*/*xa-13* combination and the *xa-5*/*xa-13* combination. Only 1 line was

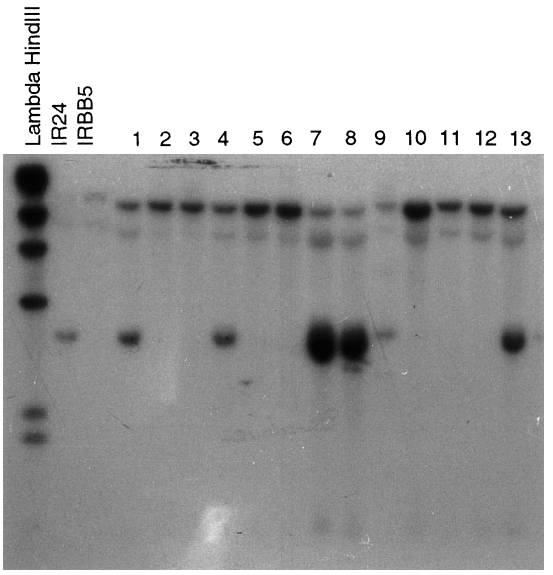


**Fig. 1** Southern analysis of $F_2$ population derived from cross between IRBB5 and IRBB13. The RFLP probe is RG556. DNA was digested with *Hin*dIII

selected for *Xa-4*/*Xa-21*. We were able to select 3 lines for *xa-5*/*Xa-21* and 4 lines for *xa-13*/*Xa-21*. To ensure that the selection was accurate, we conducted DNA-based progeny testing in the $F_3$ generation. As shown in Fig. 2, all $F_3$ progenies from selected $F_2$ individuals thought to have *xa-13* were found to contain the expected band, indicating the presence of *xa-13*. All other selected lines were also confirmed as correct, except for 1 line from the *xa-5*/*xa-13* gene combination, which was discarded.

### Selection of plants with 3 and 4 genes

Three $F_2$ populations were developed to select plants with three or four genes (Table 1). Stringent phenotypic selection was again conducted to reduce the population

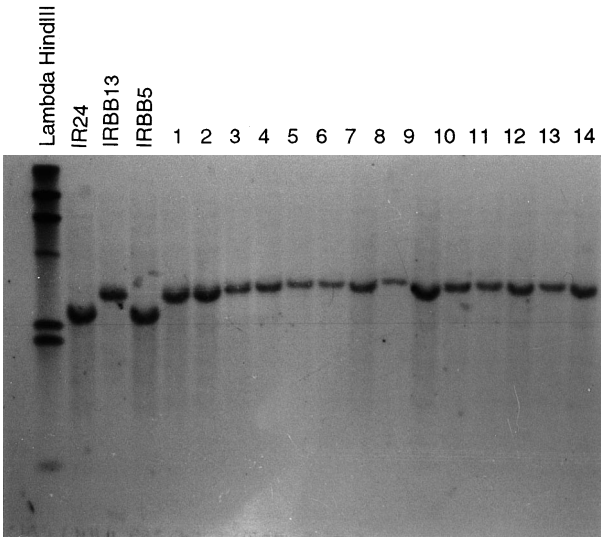


**Fig. 2** Southern analysis of $F_3$ plants from selected pyramid lines carrying *xa-13*. The blot was probed with RG136. The DNA was digested with *Dra*I

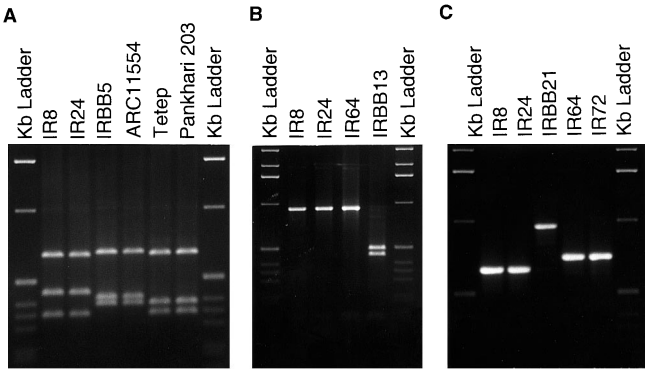


**Fig. 4A–C** PCR analysis of three marker loci. **A** DNA amplified with RG556 primers and digested with *Dra*I, **B** DNA amplified with RG136 primers and digested with *Hin*fI, **C** DNA amplified with pTA248 primers (Chungwongse et al. 1993)

size (Table 1). Because MAS based on RFLP using Southern analysis is laborious, time-consuming and costly and involves the use of radiochemicals, we have tried to develop and use polymorphic PCR markers derived from the RFLP markers.

The first PCR marker used was from Chunwongse et al. (1993). The PCR marker linked to *Xa-21* allowed efficient screening of $F_2$ populations (Fig. 3). The $F_2$ plant in lane 4 of Fig. 3 showed a banding pattern identical to that of its resistance parent, and this plant was therefore, assumed to contain *Xa-21*.

PCR markers were developed for other genes by sequencing both ends of RFLP clones RG556 (linked to *xa-5*) and RG136 (linked to *xa-13*), and then designing PCR primers (Table 4). Amplification of DNA from IR24 and IRBB5 with RG556 primers did not produce any polymorphism. The PCR products were therefore digested with 30 restriction enzymes and examined for specific amplicon polymorphism (SAP). *Dra*I was effective in this respect (Fig. 4A). The $F_2$ populations were therefore analyzed with the PCR approach instead of Southern analysis. Figure 5 shows PCR analysis of the RG556 locus in an $F_2$ population segregating for *xa-5*. Plants in lanes 2, 13 and 20 were selected because their banding pattern was the same as that of IRBB5. Similarly, the amplification of DNA from 'IR24' and IRBB13 with RG136 primers did not produce any polymorphism. The PCR products were therefore digested with any restriction enzymes until SAP was generated using *Hin*fI (Fig. 4B). We tried to obtain a PCR marker for *Xa-4* at markers Npb181 and Npb78 but were not successful in obtaining SAP with primers for these markers (data not shown).

With the assistance of Southern analysis and PCR markers, true breeding lines carrying three or four genes were quickly identified. We obtained 2 lines for *Xa-4*/*xa-5*/*xa-13*, 3 lines for *Xa-4*/*xa-5*/*xa-21*, 3 lines for *Xa-4*/*xa-13*/*Xa-21*, 3 lines for *xa-5*/*xa-13*/*Xa-21* and 2 lines with all four genes (*Xa-4*/*xa-5*/*xa-13*/*Xa-21*). These lines were confirmed through $F_3$ analysis (Table 2). The overall marker patterns for all selected lines and their parents are shown in Fig. 6.

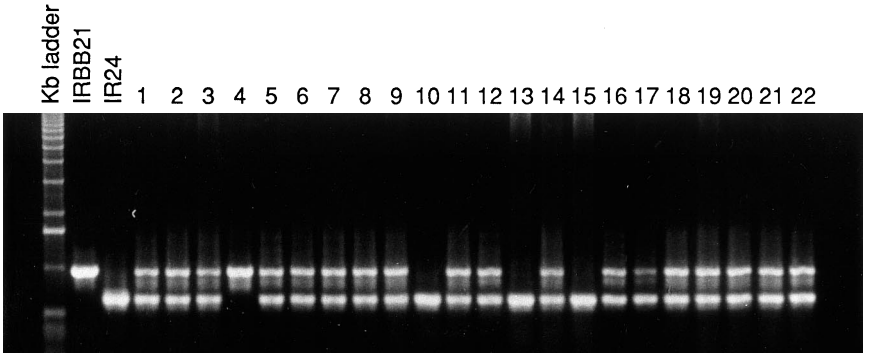


**Fig. 3** PCR analysis of $F_2$ population derived from cross between IRBB13 and IRBB21 carrying *Xa-21*. PCR primers were from Chungwongse et al. (1993)

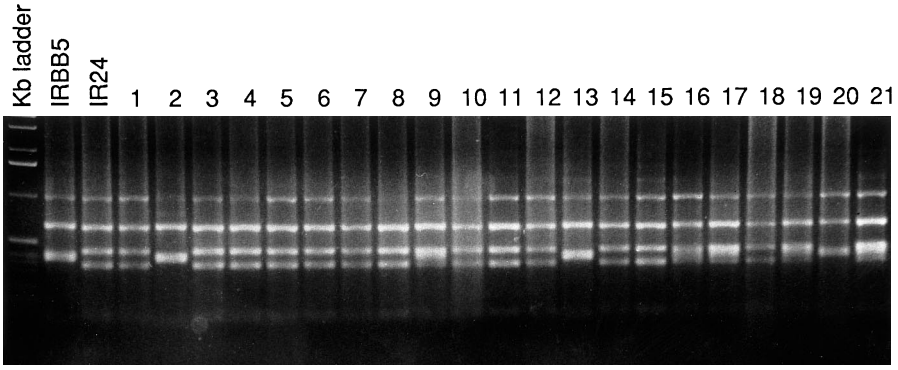


**Fig. 5** PCR analysis of $F_2$ population with RG556 primers and PCR products digested with *Dra*I

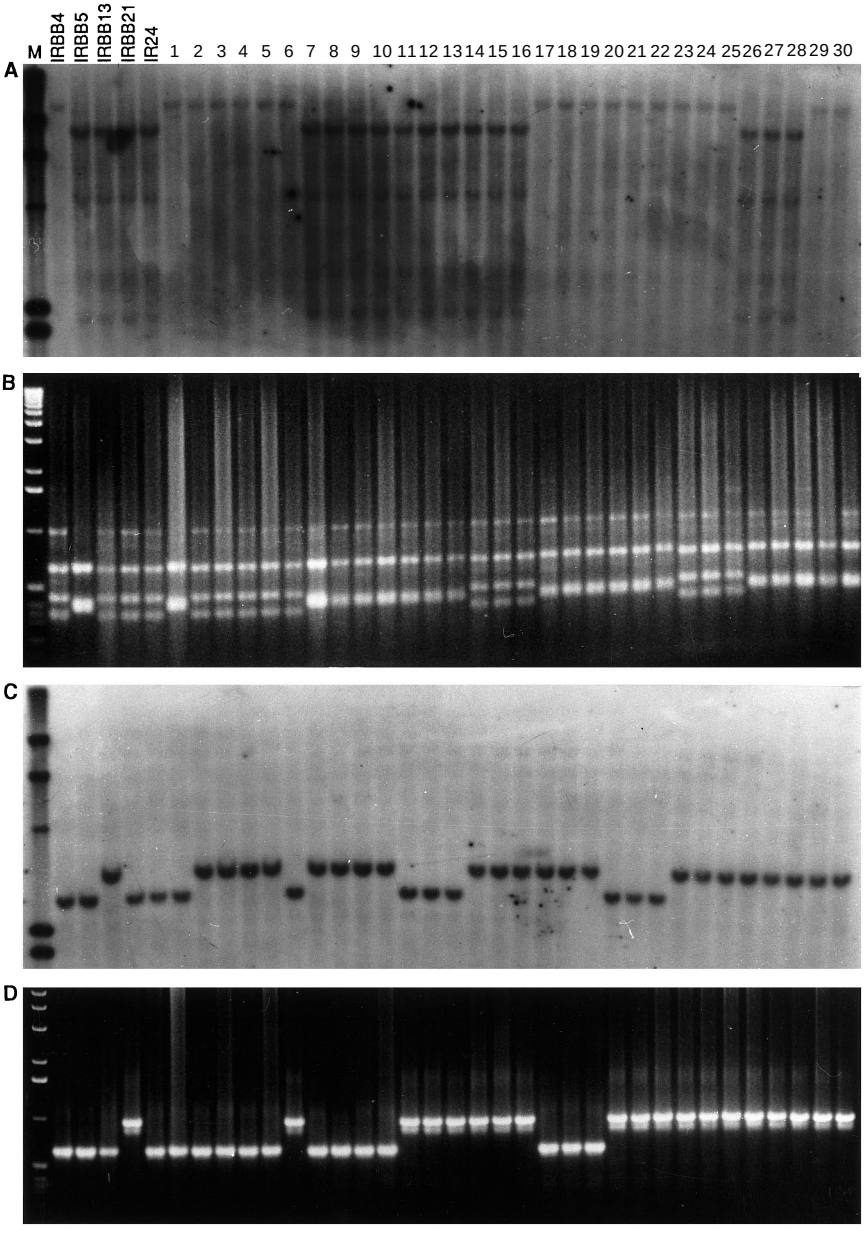


**Fig. 6A–D** Marker banding patterns of selected pyramid lines along with their parents. The numbers for each lane correspond to the code in **Table 3**. **A** Blot probed with DNA marker, g181, **B** PCR analysis with primers derived from RG556, **C** blot probed with RFLP marker, RG136, **D** PCR analysis with primers derived from pTA248. *M* Molecular weight markers, either lambda/*Hin*dIII or kb lader

### Reaction of gene pyramids to BB pathogen

The resistance of pyramid lines to the BB pathogen was confirmed up to the $F_4$ generation via single-race (race 6) inoculation. In the $F_4$ generation, the reaction of all of the gene pyramid lines was tested for all six Philippine races of BB pathogen (Table 2). A wider spectrum and a higher level of resistance to the BB pathogen were observed for the pyramid lines than for the parental lines. For example, IRBB4, with *Xa-4*, is resistant to races 1 and 5 but susceptible to other races. On the other hand, IRBB13, with *xa-13*, is only resistant to race 6. The pyramid lines of *Xa-4* and *xa-13* showed resistance not only to races 1, 5 and 6, but also to race 4, which can infect both parents (IRBB4 and IRBB13). The lengths of the lesions in many pyramid lines following inoculation with specific races were much shorter than those in the parents, indicating an increased level of resistance to the BB pathogen (Table 2).

### Germplasm survey of polymorphic PCR markers

Following the development of a PCR marker for *Xa-21*, PCR markers were available for three BB genes towards the end of the gene pyramid project. The use of PCR markers increased significantly the efficiency of the MAS process. One limitation of the PCR marker approach is the time needed to generate SAP between parents. To facilitate future PCR-based marker-aided transfer of BB resistance genes to new elite breeding lines, we surveyed a range of rice germplasm for PCR polymorphism (Table 4). This collection of germplasm consists of cultivars representing all six isozyme groups and many IRRI rice varieties ('IR5'–'IR74').

Three alleles were identified among the 187 accessions of rice germplasm surveyed with RG556 primers and the PCR products digested with *Dra*I (Fig. 4A). The marker allele in 'IR24' and 'IR28' was called allele 3, and the allele in 'Tetep' and Pankhari 203' was designated as allele 2. PCR products were not detected in 15 rice breeding lines after many repetitions, and it was assumed that these lines carried a null allele. Only 48 accessions of rice carried allele 1, and MAS cannot be practiced within this group as they possess the same allele as IRBB5. The majority of the rice germplasm tested had allele 2 or 3 or the null allele (140 accessions). It is expected that PCR polymorphism can be generated between these accessions and IRBB5 by permitting MAS between IRBB5 and germplasm carrying alleles 2 or 3 and the null allele.

The alleles for RG136 locus vary greatly in frequency in the germplasm collection. Only 4 accessions carried allele 1 (IRBB13, Jhona 349, Lambayaete-1 and C5444 (Fig. 3A, Table 4). This situation provides ample opportunity to perform MAS for this gene as most crosses between IRBB13 and other rice accessions would give PCR polymorphism. If there is an allelic association between the resistance and marker alleles, the other three lines, Jhona 349, Lambayaete-1 and C5444 might already possess *xa-13*.

Two alleles of locus pTA248 have been reported by Chunwongse et al. (1993). Allele 1 is present in IRBB21 while allele 2 is present in 'IR8' and 'IR24'. After surveying the germplasm, we found an additional allele (allele 3) in 'IR64' and 'IR72' (Fig. 4C), which subsequently turned out to be the most abundant (Table 4). Five rice accessions appeared to have a null allele of this locus. Among the 187 accessions, only the isoline with *Xa-21* (IRBB21) had allele 1. This is consistent with the hypothesis that *Xa-21*, having been introgressed from *O. longistaminata* does not exist in cultivated rice germplasm.

## Discussion

The concept of DNA marker-aided selection (see Tanksley et al. 1989 for a review) and some recent reports in rice (Abenes et al. 1993; Hittalmani et al. 1994; Yoshimura et al. 1995; Zhang et al. 1996) show that target genes can be identified in a segregating population at any plant growth stage based on linked DNA markers. We extended these preliminary studies to pyramid four BB resistance genes in all possible combinations. Starting with four near-isogenic lines, each with a different BB resistance gene (Table 1), we were able to generate the pyramid lines in a straightforward manner. Because the DNA markers were co-dominant, homozygous pyramid lines were readily selected from $F_2$ generations without the need for progeny testing.

The pyramid lines generally showed a higher level of resistance and/or wider spectrum of resistance to the BB pathogen than was expected from the parental behavior. The pyramid lines with *Xa-4*/*xa-13* were resistant to race 4 to *Xoo* to which the parents of pyramid lines were susceptible (Table 2). Other pyramid lines showed shorter lesions than their parental lines. These wider spectra or higher levels of resistance in the pyramids might be due to interaction and/or complementation between the resistance genes. With the availability of gene pyramid lines, we can now conduct quantitative analysis on the effect of each gene and its interaction with other genes.

The linked DNA markers could be identified by either RFLP analysis using Southern hybridization or amplicon analysis using PCR. Practical application of MAS requires that markers be identified with a high level of accuracy and efficiency, be cost effective and be easy to use. PCR markers can offer these advantages (Williams et al. 1991; Ghareyazie et al. 1995). We developed two PCR markers in this study; one for *xa-5*, another for *xa-13*. Both required restriction digestion of the PCR products to obtain PCR polymorphism. In spite of this, the PCR approach is still much faster and

easier than Southern analysis. The main limitation of the PCR approach is that suitable PCR polymorphisms may not exist at some marker loci. To facilitate future PCR-based MAS, we generated a small database by surveying representative rice germplasm with the PCR markers (Table 4). This database can be used as a guide to select gene donor and recipients that are likely to be polymorphic at target loci.

DNA isolation for PCR analysis can be a limiting step in marker-assisted breeding because the population size in the breeding program is generally quite large. A technically simple, rapid and reproducible DNA isolation procedure is needed for marker-assisted breeding. Reproducible results can be obtained with large-scale procedures such as that of Dellaporta et al. (1983). These procedures are, however, generally time-consuming and labor-intensive and are therefore not suitable for large sample sizes. We used a new DNA isolation protocol which does not require liquid nitrogen, needs only a very small amount of tissue sample and can be done very rapidly. DNA isolated through this procedure is of low molecular weight but sufficient for PCR analysis. The total DNA yield is estimated to be about 1 μg from 2 cm of rice leaf. DNA has been isolated from different rice breeding lines and from different growth stages of rice. Because this extraction protocol was developed specifically for PCR analysis, the quality of DNA is judged by the amplification of PCR products (Figs. 2, 4 and 5). The PCR products amplified from the crude extract produced with the simple protocol are just as clear as those amplified from carefully purified DNA, indicating that crude DNA extracted by the simplified protocol is amplifiable and the results are reproducible. This protocol is now the standard procedure in our routine PCR-based MAS service.

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## References

Abenes MLP, Angeles ER, Khush GS, Huang N (1993) Selection of bacterial blight resistant rice plants in the $F_2$ generation via their linkage to molecular markers. Rice Genet Newsl 10:120–123

Chunwongse J, Martin GB, Tanksley SD (1993) Pregermination genotypic screening using PCR amplification of half seeds. Theor Appl Genet 86:694–698

Dellaporta SL, Woo J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Ghareyazie B, Huang N, Second G, Bennett J, Khush GS (1995) Classification of rice germplasm I. Analysis using ALP and PCR-based RFLP. Theor Appl Genet 91:218–227

Hittalmani S, Foolad M, Mew T, Rodrigues R, Huang N (1994) Identification of blast resistance gene, *Pi-2*(t) in rice plants by flanking DNA markers. Rice Genet Newsl 11:144–146

Kauffman HE, Reddy APK, Hsien SPY, Merca SD (1973) An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis Rep 57:537–541

Khush GS, Mackill DJ, Sidhu GS (1989) Breeding rice for resistance to bacterial blight. In: *Bacterial blight of rice*. International Rice Research Institute, Los, Banos, Manila, Philippines, pp 207–217

Kinoshita T (1995) Report of committee on gene symbolization, nomenclature and linkage groups. Rice Genet Newsl 12:9–153

Mew TW, Vera Cruz CM, Medalla ES (1992) Changes in race frequency of *Xanthomonas oryzae* pv. *oryzae* in response to the planting of rice cultivars in the Philippines. Plant Dis 76:1029–1032

Ronald PC, Albano B, Tabien R, Abenes L, Wu K, McCouch S, Tanksley SD (1992) Genetic and physical analysis of the rice bacterial blight disease resistance locus, *Xa21*. Mol Gen Genet 236:113–120

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. Cold Spring Harbor Laboratory Press, New York

Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for old sciences. Biotechnology 7:257–264

Williams MNV, Pande N, Nair S, Mohan M, Bennett J (1991) Restriction fragment length polymorphism analysis of polymerase chain reaction products amplified from mapped loci of rice (*Oryza sativa* L.) genomic DNA. Theor Appl Genet 82:489–498

Yoshimura S, Yoshimura A, Iwata N, McCouch SR, Abenes ML, Baraoidan MR, Mew TW, Nelson RJ (1995) Tagging and combining bacterial blight resistance genes in rice using RAPD and RFLP markers. Mol Breed 1:375–387

Zhang G, Angeles ER, Abenes MLP, Khush GS, Huang N (1996) RAPD and RFLP mapping for the bacterial blight resistance gene *xa-13* in rice. Theor Appl Genet 93:65–70