Evaluation of bacterial blight resistance in rice lines carrying multiple resistance genes and *Xa21* transgenic lines

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Bacterial blight (BB), a major disease of rice, is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo). Six distinct Indian isolates of *Xanthomonas oryzae* were tested on rice genetic backgrounds carrying single or multiple BB resistance genes. Three BB resistance genes were introduced into a susceptible line, MH2R, by marker-assisted selection, which resulted in a gain of resistance. In addition, Pusa Basmati 1 (PB1) transgenic lines carrying the BB resistance gene, *Xa21*, and IR72-*Xa21* transgenic lines were evaluated after inoculation with the six Xoo isolates. It was found that PB1 lines expressing the *Xa21* gene were susceptible to the six Xoo isolates. These results indicate that introducing multiple BB resistance genes by marker-assisted selection may be a more effective strategy in breeding for BB resistance against Indian isolates.

Keywords: Bacterial blight, marker-assisted selection, resistance genes, rice, transformation.

BACTERIAL blight (BB) is a serious disease of rice caused by the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo), resulting in significant crop yield[1] of around 20 to 40%. Studies on the genetics of BB resistance have resulted in the identification of more than 20 resistance (*R*) genes, and the development of donor lines carrying major *R* genes. A number of these donor lines have been used in rice breeding programmes around the world. The *Xa21* gene was transferred from the wild species *Oryza longistaminata* into IR24, resulting[2,3] in the near-isogenic line, IRBB21. In tests for disease resistance, IRBB21 has been reported to be resistant to many Xoo strains from the Philippines and India[3]. Thus far, two BB resistance genes *Xa21* and *Xa1* have been isolated using map-based cloning strategies[4,5]. Both encode proteins bearing nucleotide binding sites (NBS) and leucine-rich repeats (LRR), and bear significant similarity to other resistance genes such as *RPS2*, *RPM1* and *RPP5* from *Arabidopsis*, *N* from tobacco and *L6* from flax[6].

Breeding for durable resistance against BB by pyramiding multiple *R* genes is the most effective strategy for controlling the disease. This has been achieved by a number of groups using marker-assisted selection. Singh *et al.*[7] used molecular markers to introduce *xa5*, *xa13* and *Xa21* into PR106, a BB susceptible line extensively grown in Punjab. A three-gene combination appeared to be the most effective, with *Xa21* contributing the largest component of resistance. *Xa21* was reported to confer resistance to pathotypes from the Philippines and India at post-seedling growth stages[2], and has been reported to be the single most effective gene against 17 Xoo isolates from Punjab[7]. However, Goel *et al.*[8] reported several isolates of the pathogen, again from Punjab, which were virulent to IRBB21.

Using a transgenic approach, agronomically important cultivars such as IR64 and IR72 have been transformed with the *Xa21* gene and successful field trials of selected lines undertaken in China[9,10]. Further, the *Xa21* gene has been introduced into a widely used restorer of hybrid rice in China, Minghui 63, in order to produce BB-resistant hybrid rice with elite agronomic characters[11].

In this study, we compared the degree of resistance of lines bearing single or multiple BB resistance loci using six genetically diverse Indian Xoo isolates. Using marker-assisted selection, up to three resistance genes were introduced into a hybrid rice parental line and the resulting progeny lines were evaluated against the pathogen. In addition, a large number of transgenic lines carrying *Xa21* were generated to test the efficacy of the transgene against the six isolates, and compared in terms of resistance to transgenic IR72 containing the *Xa21* gene. We describe here the results on lines found to be most effective against the isolates tested, and discuss the possible optimum combination of resistance genes to provide durable resistance against the Indian isolates.

Materials and methods

Plant material

Mahyco experimental hybrid parental lines were tested for the presence of BB resistance genes (Table 1). The paren-

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tal line types used were – A-lines: MH1A, MH2A, MH3A, MH4A; B-lines: MH1B, MH2B, MH3B and MH4B, and R-lines: MH1R, MH2R, MH3R, MH4R, MH5R and MH6R. Near-isogenic IRBB lines containing combinations of *Xa* genes were used as controls for BB resistance testing. Taichung Native 1 (TN1) was used as a susceptible control, IR72 and IR72 carrying the *Xa21* gene were tested for BB resistance. These lines and the BB resistance genes they contain are summarized in Table 1. The IRBB and IR72 lines, originally developed at IRRI, Manila were obtained from the Mahyco Research Foundation, Hyderabad.

BB isolates and their characterization

BB isolates were collected from different locations in India, as described in Table 3. Genetic characterization of BB isolates was performed using standard AFLP techniques and kits from Applied Biosystems. The data generated by primers were analysed[12] by NTSYS-pc: *Eco*RI-ACT with *Mse*I-CTC or *Mse*I-CTG.

DNA isolation and markers used

Genomic DNA was isolated from young leaves of six-week-old seedlings by the method of Dellaporta *et al.*[13]. The extracted DNA was analysed on agarose gels for quality. The markers used for determining the presence of various *Xa* genes were *xa5*: RM122 (ref. 14), *xa13*: RG136 (ref. 15), and *Xa21*: pTA248 (ref. 16).

Marker analysis

Typical PCR reactions contained 50 ng genomic DNA, 0.5 µM of each forward and reverse primer, 200 µM dNTPs, 1X PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mm $MgCl_2$ and 0.01 mg ml^{-1} gelatin), and 1U of Taq polymerase in a reaction volume of 20 µl. The genomic DNA was denatured by heating to 95°C for 3 min, followed by 35 cycles of PCR amplification with the following parameters: 94°C, 30 s, 55°C, 1 min and 72°C, 1 min. This was followed by one cycle of 72°C for 5 min. One half of each of the PCR samples was resolved on 1.4% agarose gels and visualized by ethidium bromide staining. In RG136 reactions, for the detection of *xa13*, the remainder of the reaction was subjected to digestion with *Hinf*I in a total volume of 20 µl for 1h at 37°C.

Table 1. Rice lines tested for bacterial blight reaction against Mxo isolates

Line	*R* gene(s) present*
IRBB4	*Xa4*
IRBB5	*xa5*
IRBB21	*Xa21*
IRBB56	*Xa4*, *xa5*, *xa13*
IRBB57	*Xa4*, *xa5*, *Xa21*
IRBB58	*Xa4*, *xa13*, *Xa21*
IRBB59	*xa5*, *xa13*, *Xa21*
IRBB60	*Xa4*, *xa5*, *xa13*, *Xa21*
MH2R	*xa5*
MH3R	*xa5*
IRBB60 X MH2600 F2	*Xa4*, *xa5*, *xa13*, *Xa21*

*Presence of *Xa4* could not be determined; presence of *xa5*, *xa13* and *Xa21* was confirmed in this study. Lines negative for *R* genes tested: MH1A, MH2A, MH3A, MH4A, MH1B, MH2B, MH3B, MH4B, MH1R, MH4R, MH5R, MH6R and TN1.

Inoculation of BB isolates and scoring

All plant lines were raised in the greenhouse and inoculated at the maximum tillering stage by leaf-clipping[17]. TN1 was used as a susceptible check. Plant reaction to the inoculation was recorded 21 days after infection. Plants were characterized as resistant or susceptible based on lesion lengths: 0–3 cm, resistant; 3–6 cm, moderately resistant; > 6 cm, susceptible.

Generation of transgenic Pusa Basmati lines carrying Xa21

Mature rice seeds (*Oryza sativa* L.) of variety Pusa Basmati 1 were used as source for callus induction. Induction of embryogenic calli, selection of transgenic calli and regeneration of transgenic plants were carried out as described by Azhakanandam *et al.*[18]. Plasmid pWRG1515 bears hygromycin phosphotransferase (*hpt*) and *GUS* genes, both driven by the CaMV 35S promoter and binary plasmid pAHA*Xa21* (ref. 4) carries the *Xa21* gene driven by the maize ubiquitin promoter; these were co-bombarded into rice calluses in a 1 : 3 ratio. Preparation of gold particles and microprojectile bombardment were performed using standard protocols[19,20]. Expression of *GUS* gene was assayed histochemically[21].

PCR analysis of transformants

The presence of transgenes in the putative transgenic lines was confirmed by PCR analysis. Plant genomic DNA was extracted by the method of Edwards *et al.*[22]. Approximately 100 ng of the genomic DNA was amplified by 10 pM of reverse and forward primer, 2.5 units of Taq polymerase and 100 µM of dNTPs in 25 µl of reaction mixture each. PCR analysis was carried out for *Xa21* gene at different generations. A part of *Xa21* gene sequence was amplified using the oligonucleotide primers with following sequences: forward primer 5′-ACTGTATAGCA CAATCATACC-3′, reverse primer 5′-CCAAATGTTTG AACGATCGG-3′. Thermal cycling conditions used for *Xa21* gene amplification were denaturation at 95°C for

Table 2. BB reactions of rice lines inoculated with Mxo isolates Disease reactions are characterized as resistant (R, 0–3 cm lesion length), moderately resistant (MR, 3–6 cm lesion length) and susceptible (> 6 cm lesion length). Each value is the mean of 7–10 inoculated leaves, by the method of Kauffman *et al.*[17]

Line	Isolate					
	Mxo1	Mxo2	Mxo3	Mxo4	Mxo5	Mxo6
IRBB4	R	MR	R	S	S	S
IRBB12	R	R	R	MR	R	MR
IRBB56	R	R	R	R	R	R
IRBB57	R	S	R	S	S	S
IRBB58	R	R	R	R	R	R
IRBB59	R	R	R	R	R	R
IRBB60	R	R	R	R	R	R
IRBB60 × MH2R F2	R	R	R	R	R	R
TN1	S	S	S	S	S	S

35 s, annealing at 55°C for 1 min and polymerization at 72°C for 1 min.

Southern blot analysis

Genomic DNA from young leaf tissues of T1 transgenic as well as non-transgenic plants was isolated following Dellaporta *et al.*[13]. Ten µg of DNA was digested with *Eco*RV enzyme and run on a 1.2% agarose gel electrophoresis, and then blotted overnight onto nylon membranes (Roche). The DNAs were hybridized against a DIG-labelled probe specific for the *Nos* terminator. The probe was generated by PCR amplification of the *Nos* terminator. Hybridization was carried out at 60°C in a rotary hybridization oven, and washes performed according to the kit manufacturers' instructions.

RT–PCR analysis

Total RNA was extracted from approximately 10 mg leaf tissue using RNeasy Plant Minikits (Qiagen). Total RNA was treated with RNAase-free DNAase followed by a heat-inactivation step (75°C for 5 min). Reverse transcription for each sample was carried out in a 20 µl reaction using a first strand cDNA synthesis kit (Roche, Germany). Second-strand synthesis was carried out with the *Xa21* forward primer, and PCR was performed on 100 ng of each cDNA using *Xa21*-specific primers (forward primer: 5′-ACT GTATAGCACAATCATACC-3′ and reverse primer: 5′-CTCGATGGCAATTCCTGAG-3′). PCR conditions were as described earlier for confirmation of the presence of the *Xa21* transgene.

Results

Survey of Xa genes with molecular markers

Fourteen MH parental lines were screened for the presence of *xa5*, *xa13* and *Xa21* using linked DNA markers RM122 for *xa5* (0.4 cM distant), RG136 for *xa13* (5.5 cM) and pTA248 for *Xa21* (0 cM) respectively. Two lines, MH2R and MH3R, showed presence of the *xa5* gene, whereas all other lines evaluated were negative for the presence for the three *R* genes (Table 1). MH2R was chosen for further breeding due to its preferred agronomic characters. We also surveyed a number of other rice lines for the presence of the *xa5*, *xa13* and *Xa21* BB resistance genes. Surveyed lines were IRBB5, IRBB21, IRBB56, IRBB57, IRBB58, IRBB59 and IRBB60, which have variable numbers of *Xa* genes (Table 1). A susceptible variety, TN1, was also tested. Transgenic lines carrying *Xa21* were also evaluated along with their near-isogenic non-transgenic lines. The lines surveyed were IR72 and transgenic IR72-*Xa21* (ref. 10), Pusa Basmati 1 and transgenic Pusa Basmati-*Xa21*. The presence of the appropriate Xa genes was confirmed in the IRBB lines, with the exception of *Xa4*, which we were unable to detect. IR72 carries an endogenous *Xa4* gene, while Pusa Basmati and TN1 were negative for *xa5*, *xa13* and *Xa21* genes (Table 1).

Marker-assisted selection for evaluation of BB resistance

An F_2 population derived from a cross between MH2R and IRBB60 was subjected to PCR analysis using linked markers RM122 for *xa5*, RG136 for *xa13* and pTA248 for *Xa21*. Genomic DNA was extracted from 384 F_2 plants and used for PCR amplification to test for the presence of the *Xa21* gene, using the marker pTA248. Out of the 384 individuals tested, 71 were found to be homozygous for the *Xa21* locus, which was not different than expected as determined by chi-square analysis (data not shown). The 71 plants were checked for the presence of *xa13* using the marker RG136, which was found to be in homozygous condition in 17 plants. Presence of the *xa5* gene was confirmed with marker RM122 in these 17 plants as expected, as the gene is present in both MH2R and IRBB60 in the homozygous condition.

Generation of transgenic Pusa Basmati-Xa21

Transgenic Pusa Basmati1 rice plants were generated via particle bombardment of rice calluses with the plasmid pAH*Xa21*. Twenty-four independent transgenic events were obtained and analysed by PCR to determine the presence of the transgene. Among these, *Xa21* PCR-positive T1 generation plants from ten lines were subjected to Southern blot analysis (Figure 1). Due to the presence of multiple *Xa21*-like genes in the rice genome, the *Nos* terminator sequence was used as a probe. Digestion with *Eco*RV, a unique restriction site within the gene cassette, revealed distinct patterns of hybridization in the ten lines that were analysed. The copy number of integrations varied from 1 to 4. RT–PCR analysis of individual plants of six Pusa-Basmati-*Xa21* lines in the T1 generation revealed that all lines tested expressed the *Xa21* gene at the transcriptional level (Figure 2).

Genetic characterization of BB isolates

The six Mxo isolates tested on different rice lines were subjected to AFLP analysis[23] using two different *Eco*RI and *Mse*I primer combination pairs. The isolates were distinguishable from each other on the basis of polymorphic DNA bands, suggesting that they are genetically distinct (data not shown). Cluster analysis of AFLP data using 200 polymorphic bands revealed that Mxo1 and Mxo6, though collected from different geographical locations, are the most closely related amongst isolates with a correlation coefficient of 0.7 (Figure 3). Mxo2 was found to be the most genetically distinct isolate included in this study.

Inoculation of rice line with BB isolates

The rice lines listed in Table 1 were inoculated with Mxo isolates (Table 3) at the stage of maximum tillering. The 17 F_2 plants derived from a cross between IRBB60 and MH2R, and homozygous for 3 *Xa* genes, were also individually inoculated with the same isolates. Lesion length recordings were taken at 21 days after inoculation (d.a.i.). Plants containing multiple genes were highly resistant. Further, F_2 plants that were homozygous for *xa5*, *xa13* and *Xa21* were as resistant as the parental line IRBB60 used in the cross, demonstrating the effectiveness of resistance gene pyramiding (Table 2). Near-isogenic lines containing single *R* genes *Xa4* or *Xa21* were less resistant than those containing multiple *R* genes. IRBB4 was susceptible to Mxo2, Mxo4, Mxo5 and Mxo6. IRBB21 was moderately resistant to Mxo4 and Mxo6 and resistant to the other isolates. IRBB56, IRBB58, IRBB59 and IRBB60 were resistant to all six isolates, with the latter two lines displaying the highest overall degree of resistance (Table 2). Interestingly, IRBB57, which lacks *xa13*, was susceptible to Mxo2, Mxo4, Mxo5 and Mxo6, suggesting an important role for this *R* gene against the Xoo isolates tested.

Pusa Basmati-*Xa21* transgenic lines failed to show resistance against all the isolates tested (Table 4). These lines often had lesions covering the entire inoculated leaf, leading to plant death at 21 d.a.i. Transgenic IR72-*Xa21* displayed resistance against Mxo1 and Mxo5, moderate resistance to Mxo2, Mxo3and Mxo6 and was susceptible to Mxo4. This enhanced resistance against BB over the PB-*Xa21* lines may be due to the combined effect of *Xa4*

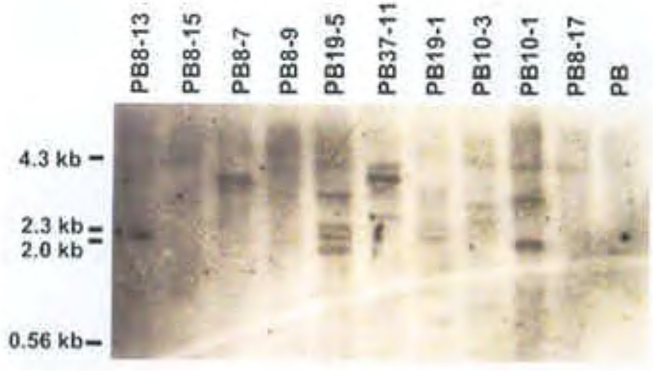

Figure 1. Southern blot of transgenic Pusa Basmati1 rice lines carrying the *Xa21* gene. The probe used was a PCR amplified *Nos* terminator fragment. Genomic DNA was digested with *Eco*RV, which is absent within the *Xa21* gene cassette. PB, Non-transformed Pusa Basmati1 DNA. 10 µg of DNA was loaded for each plant sample.

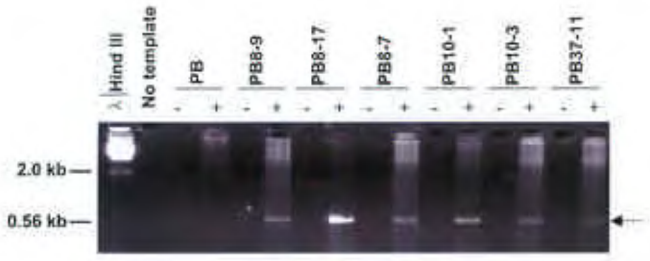

Figure 2. RT–PCR analysis of individual PCR-positive plants from six Pusa Basmati1 lines. Total RNA from lines of control and transgenic plants was analysed with specific *Xa21* primers. λ *Hin* dIII, Phage λ DNA digested with *Hin*dIII; No template, Control PCR reaction with neither RNA or DNA added; PB, Non-transformed Pusa Basmati 1 control; – and + above lanes indicate addition of total RNA or cDNA respectively, from each of the transgenic lines tested. A 573-bp band indicated by arrow is expected in plants expressing the *Xa21* transgene.

Table 3. *Xanthomonas oryzae* pv. *oryzae* isolates

Isolate	Location collected	Rice variety	Year of collection
Mxo1	Raipur, Madhya Pradesh	Mahyco hybrid	1997
Mxo2	Nellore, Andhra Pradesh (AP)	Nellore local	1997
Mxo3	Karnal, Haryana	Karnal local	1997
Mxo4	Hyderabad, AP	TN1	1995
Mxo5	Kallakal, AP	Mahyco hybrid	1998
Mxo6	Kallakal, AP	Mahyco parental line	1998

Table 4. BB reactions of transgenic rice lines and their corresponding controls inoculated with Mxo isolates. Disease reactions are characterized as resistant (R, 0–3 cm lesion length), moderately resistant (MR, 3–6 cm lesion length) and susceptible (>6 cm lesion length). Each value is the mean of 7–10 inoculated leaves, by the method of Kauffman *et al.*[17]

	Isolate					
Line	Mxo1	Mxo2	Mxo3	Mxo4	Mxo5	Mxo6
PB10-1	S	S	MR	S	S	S
PB10-3	S	S	MR	S	S	S
PB19-5	S	S	S	S	S	S
PB8-4	S	S	MR	S	S	S
PB8-5	S	S	MR	S	S	S
PB8-6	S	S	S	S	S	S
PB8-7	S	S	S	S	S	S
PB8-X	S	S	S	S	S	S
PB8-9	S	S	S	S	S	S
PB8-10	S	S	MR	S	S	S
PB8-13	S	S	S	S	S	S
PB8-15	S	S	MR	S	S	S
PB8-17	S	S	MR	S	S	S
Pusa Basmati 1	S	S	S	S	S	S
IR72	R	S	MR	S	S	S
IR72 + *Xa21*	R	MR	MR	S	R	MR
TN1	S	S	S	S	S	S

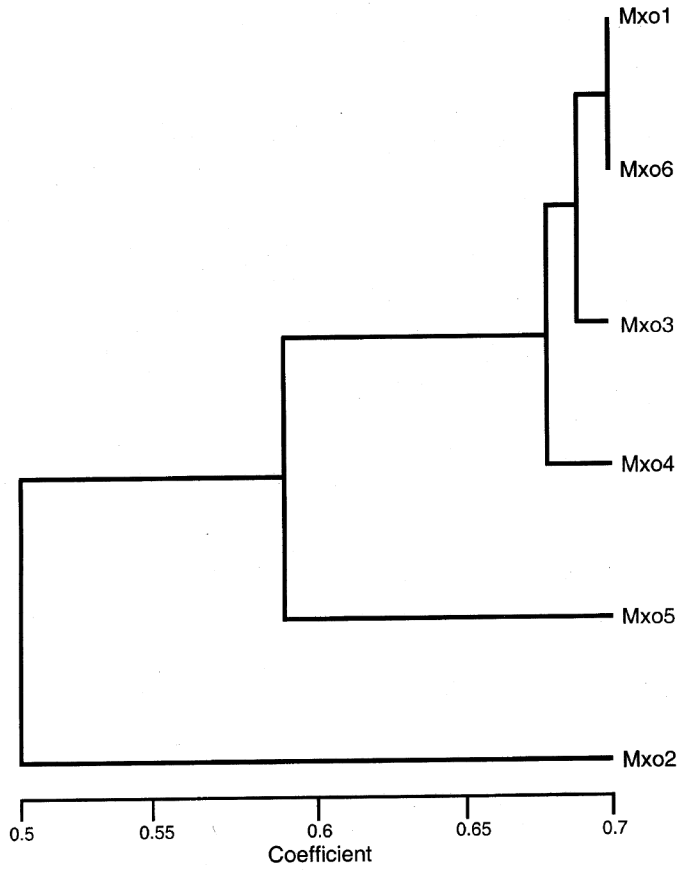

Figure 3. Cluster analysis of BB isolates Mxo1-6 used in this study. AFLP band data obtained from primer combinations *Eco*RI-ACT with *Mse*I-CTC and *Mse*I-CTG respectively, were used to generate a similarity matrix. Cluster analysis was performed using UPGMA, and the dendrogram was generated by NTSYS-pc (ref. 12). Bootstrap values are indicated at the nodes of branches, and were generated using WinBoot[24].

and *Xa21* genes. The parental line MH2R displayed resistance to Mxo1, but was susceptible to the other isolates.

Discussion

The main goal in breeding for BB resistance is to achieve durable resistance in the field. In this study, two approaches were taken to develop and evaluate BB resistance – stacking of multiple BB resistance genes by marker-assisted selection and a transgenic approach in which *Xa21*, one of two cloned BB resistance genes, was introduced into Pusa Basmati1, a genetic background lacking known major BB resistance genes. Plants were challenged with a number of genetically distinct BB isolates from the Indian subcontinent. A consistent finding was that lines which carried two or more BB resistance genes, showed a higher degree of resistance over lines, both non-transgenic and transgenic, containing single BB resistance genes. The single Xa gene containing line IRBB4 was susceptible to the more virulent Mxo isolates (Mxo4 and Mxo6), while IRBB21 was moderately resistant to Mxo4 and Mxo6. These results agree with those of studies done with BB isolates from Punjab in which it was observed that combinations of *R* genes provide a broader spectrum of resistance to the disease[7,8].

In order to bring about durable BB resistance in elite rice lines, marker-assisted selection has proved to be invaluable. We were interested in bringing in multiple BB resistance genes into our proprietary hybrid parent lines. We found that the line MH2R, containing *xa5*, was susceptible to the isolates Mxo2, Mxo4, Mxo5 and Mxo6 (Table 2). However, upon marker-aided introduction of *xa13* and *Xa21* into MH2R using IRBB60 as the source of resistance genes, *xa5*-, *xa13*- and *Xa21*-homozygous F_2 individuals showed clear resistance against the same Mxo isolates (Table 2), providing an example of the value of this approach.

Another goal of this study was to evaluate the efficacy of the *Xa21* transgene against the *Mxo* isolates. From the results obtained here, it appears the *Xa21* gene alone is ineffective against some virulent isolates (Mxo4, Mxo5 and Mxo6) and thus must be deployed in genetic backgrounds that contain other BB resistance genes. This is supported by data obtained with transgenic IR72-*Xa21*, which contains an endogenous *Xa4* gene. IR72-*Xa21* proved resistant or moderately resistant to the pathogen isolates tested, except for isolate Mxo4, which showed virulence (Table 4). IR72 was susceptible against most isolates tested. Pusa Basmati1 and its transgenic derivative PB-*Xa21* appeared to be much more susceptible to the virulent Mxo isolates than IRBB21, suggesting a difference in either levels of expression of the *Xa21* gene or contributions from the IRBB genetic background. RT–PCR confirmed transcriptional activity of the *Xa21* transgene in a number of PB-*Xa21* lines.

With respect to earlier studies in which resistance reactions of individual *Xa* genes against various Xoo isolates were analysed, the results presented in the present study show differences in terms of individual gene performance, but broadly agree with the finding that multiple resistance gene combinations are most effective. When individual PR106 lines carrying *xa5*, *xa13* and *Xa21* respectively, were challenged with isolates from Punjab, it was found that *Xa21* was the most effective, followed by *xa5* and *xa13* (ref. 7). This report recommended combinations of *Xa21* with other BB resistance genes. In the present study, IRBB21 was used along with a number of transgenic lines expressing *Xa21* in the PB1 background and challenged with isolates comprising a diverse set from North, Central and South India. IRBB21 showed resistance or moderate resistance towards the isolates tested, whereas the transgenic lines were all susceptible, showing that the genetic background of the host plant and the Xoo isolate used are both crucial in determining susceptibility or resistance.

A major challenge is developing broad-spectrum resistance to BB in the Indian subcontinent due to the diverse agro-climatic zones where rice is cultivated, as well as the number of genetically distinct virulent Xoo strains from different geographical areas of the region. Based on the diverse though limited number of isolates used in this study we suggest that at a minimum, the use of a three-gene combination such as *xa5* with *xa13* and *Xa21* would be desirable to achieve durable and broad-spectrum resistance. Careful field evaluation of pyramided lines is needed to confirm these results. Further, marker-assisted backcrossing of IRBB60 × MH2R F_2 plants would be needed to select for the desired agronomic traits.

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ACKNOWLEDGEMENTS. We thank Drs George Thottappilly and V. V. Shenoy, Mahyco Research Foundation, Hyderabad for the IRBB, IR72 and IR72-*Xa21* lines; Dr P. Kadirvel for helpful comments on the manuscript; Mr Kafil Ahmed and Dr P. Srinivas for analysis of the AFLP data and Ms Y. Kokila and Mr Ratnapal Gandhi for help in preparation of figures.

Received 13 December 2004; revised accepted 24 November 2005