https://doi.org/10.17221/55/2021-CJGPB

Disease resistance of improved MR220 lines against *Pyricularia oryzae* Cavara and their preliminary agronomic performance

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Citation: Mohamad S.N.A., Ab Ghaffar M.B., Othman A.S., Misman S.N., Abd Rahman Z., Ab Razak M.S.F., Seman Z.A., Mohd Yusof M.F., Ahmad K., Yazid S.N., Hashim H. (2022): Disease resistance of improved MR220 lines against *Pyricularia oryzae* Cavara and their preliminary agronomic performance. Czech J. Genet. Plant Breed.

Abstract: Blast disease caused by *Pyricularia oryzae* is one of the most destructive fungal diseases of rice in Malaysia. Utilisation of resistant varieties is the most efficient management approach towards reducing yield losses. The line IRTP21683 with the *Pi9* gene has shown strong resistance against the isolate MPO988.3 of pathotype $P_{0.0}$, the most prevalent *P. oryzae* pathotype in Malaysia. Crossing of IRTP21683 was undertaken with the recurrent parent MR220, a susceptible elite Malaysian rice variety, using a marker assisted backcrossing technique with two simple sequence repeat markers, RM19776 and RM7311, as the tag for the *Pi9* gene. Twenty BC_3F_4 lines with the *Pi9* gene were resistant when challenged with MPO 988.3. The cluster analysis based on seven agronomic parameters showed that the resistant BC_3F_4 lines could be divided into four groups, of which the members in group 1 and 2 have shown comparable or better performance than MR220. Five lines in group 1, B220PI9-3-48, B220PI9-3-76, B220PI9-3-77, B220PI9-3-79 and B220PI9-3-82 showed outstanding yield performance with early maturation.

Keywords: blast disease; marker assisted backcrossing (MAB); *Pyricularia oryzae*; *Pi9* gene; rice

Rice blast disease caused by *Pyricularia oryzae* Cavara is recognised as one of the most serious threats to rice production in Malaysia. This fungal pathogen is capable of infecting rice crops at any growth stage (Bonman 1992). Loss of the total yield could occur in the field if the environmental conditions are favourable for the disease dispersion and outbreak. Serious disease severity causes plant death or results

Supported by Malaysian Agricultural Research and Development Institute (MARDI) and Malaysian Ministry of Energy, Science, Technology, Environment and Climate Change (MESTECC), Malaysia, Project No. FP0214B055 (DSTIN).

https://doi.org/10.17221/55/2021-CJGPB

in empty grains. For instance, in 2015, more than 4 000 ha of the rice areas in Malaysia were affected by this disease which resulted in a 50% to 70% yield loss (Latiffah & Norsuha 2018). Various methods of control have been adopted including fungicides, management of cultural practices and utilisation of resistant varieties to control the disease in the field (Ribot et al. 2008). However, utilisation of resistant rice varieties is regarded as one of the best methods of blast disease management in Malaysia. The disease severity and symptoms can vary amongst rice varieties depending on the weather conditions, the operational resistance genes in the plants and pathotypes of the pathogen. Virulent pathotypes will inflict higher disease incidence and severity and, thus, will cause severe yield loss. Therefore, knowledge on functional genes in the rice plants and pathological variability of *P. oryzae* within an ecosystem is very crucial in the development of improved varieties with durable disease resistance (Chen et al. 2005).

Approximately, 100 *R*-genes controlling blast resistance have been mapped and 35 of them have been cloned (Wang et al. 2017). The *Pi9* gene, identified from a wild species, *Oryza minuta* (Amante-Bordeos et al. 1992) has been introgressed into an indica rice line 75-1-127 (Liu et al. 2002). This *Pi9* gene has a wide ranging disease resistance against many *P. oryzae* isolates from the Philippines and thirteen other countries (Liu et al. 2002). The *Pi9* locus is finely mapped on rice chromosome 6 at the 76-kb region of the nucleotide-binding site and leucine-rich repeat protein (NBS-LRR) (Qu et al. 2006). The NBS-LRR is a member of a multigene family in rice which plays an important role in specific pathogen recognition corresponding to an avirulence gene (Liu et al. 2002; Rairdan et al. 2008). The *Pi9* gene was tightly linked to two microsatellite markers, RM7311 and RM19776 (Jiang et al. 2012; Yadav et al. 2019).

The conventional approach in developing resistant rice varieties is time consuming and difficult to achieve. However, recent advancements in DNA technology have led to the utilisation of marker-assisted selection (MAS) or marker-assisted backcrossing (MAB) in breeding programmes. Both rely on DNA polymorphism rather than on phenotypic observation for conducting the selection (Collard & Mackill 2008). Therefore, potential resistant lines can be selected in early segregating generations and at early stages of plant development, making breeding programmes much less time consuming (Chen et al. 2005). The selection method is free from other gene effects or environmental influences, thus, making the results of the genotype selection more reliable. The markers used were usually tightly linked to the targeted loci where the genetic distance could be less than 5 cM (Collard & Mackill 2008). Several studies have shown that MAS can efficiently monitor the introgression of blast resistance genes both into the recipient plants and improved varieties. For example, the *Pi9*/*Pi92* gene was introgressed into the rice restorer line Hui 316 by Tian et al. (2019). In the present study, we demonstrate the application of MAB in breeding to improve the blast resistance of an elite Malaysian rice variety, MR220 by transferring the *Pi9* gene from a donor parent into the recipient variety.

MATERIAL AND METHODS

Plant material and backcrossing breeding scheme. Seeds of the rice varieties MR220, MR211 and MR219 were obtained from the MARDI Research Station, Malaysia while the seeds of IRTP21683 harbouring the blast resistant gene *Pi9* were obtained from International Rice Research Institute (IRRI). A series of backcrossing was initiated using IRTP21683 as the donor parent, while a popular Malaysian cultivar MR220 was used as the recurrent parent (Figure S1 in the Electronic Supplementary Material (ESM)). Marker-assisted selection was conducted in each generation from BC_1F_1 to BC_3F_4 to ensure that the target gene was present in the selected plants. The two tightly linked markers, RM7311 and RM19776 (Jiang et al. 2012; Yadav et al. 2019) were used in the selection programme.

DNA isolation and PCR protocol. Genomic DNA was extracted from fresh healthy young leaves from each individual plant using a taco TM Plant DNA/RNA Extraction Kit (www.tacomag.com. 2011). Two primers RM7311 (F: AGTGGTCGTTGAACTCGGAG; R: TCGTGGCGCCTTTAATCTC) and RM19776 (F: ACCTGCTCCATCCATCTCTACGG; R: AGCAACGTGGTACAGATTACAGAAGC) were used. The polymerase chain reaction (PCR) mixture with a final volume of 20 µL was prepared containing approximately 30 ng/µL of plant genomic DNA, 1.0 mM of forward and reverse primers, 0.2 mM of dNTP, 1.5 mM of $MgCl_2$, 1× PCR buffer and 1 unit of polymerase. The PCR reaction was performed using a thermocycler (Master Cycler Gradient® Nexus, Eppendorf, Germany) with the following conditions: pre-denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 30 s,

https://doi.org/10.17221/55/2021-CJGPB

annealing at 54.9 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. The PCR products were resolved using a 2% agarose gel under a BioRad Gel Imager transilluminator (Bio-Rad, USA). A 50 bp ladder was used to confirm the allele sizes observed. The allele size of the resistant IRTP21683 was scored as a homozygous 'R' allele, while the allele of the susceptible MR220 was scored as the homozygous 'S' allele (Figure S2 in the ESM).

Evaluation of blast resistance. The isolate MPO 988.3 of *P. oryzae* representing $P_{0.0}$, the predominant pathotype of the blast pathogen in Malaysia (Misman & Zakaria 2019) was obtained from the Culture Collection Unit, MARDI. The isolate was used to confirm its virulence and the resistance status of the parents used in the backcrossing programme. Two rice varieties, namely MR219 and MR211, were used as the controls. The seeds of the four varieties were sown in a trough of 40 cm^2 plots (based on the seed ratio of 120 kg/ha) in a randomised complete block design (RCBD) with three replicates. MR211 seeds of were also sown at the border of each trough to serve as the inoculum source spreader. Seedlings at 21 days-old were then inoculated using a hand sprayer with a conidial suspension of 1.5×10^5 spores/mL containing 0.05% Tween-20 (Hayashi et al. 2009). The inoculated seedlings were maintained under glasshouse conditions for eight to nine days. Disease evaluation scores were carried out following a 0–9 scale based on the standard evaluation system (SES) of the International Rice Research Institute (IRRI 2013) (Table S1 in the ESM). The presence of blast disease lesions as well as the percentage of the diseased leaf area (DLA) was assessed using the method as described by Notteghem et al. (1981). The disease severity (DSe) was calculated based on the formula shown below:

$$\text{DSe (\%)} = \frac{(\text{sum of all numerical rating on plants}) \times 100}{(\text{No. of plants observed} \times 9)}$$

A similar procedure was also conducted in accessing the blast resistant status of the 20 BC_3F_4 lines generated from the backcrossing, while MR211 was included as the susceptible check variety.

Field evaluation on the agronomic performance of BC_3F_4 lines. A field trial was conducted to evaluate the agronomic performance on the selected BC_3F_4 lines. The test lines were planted in a 4 × 4 m planting block and the planting distance between plants was 0.25 m. The field layout followed an RCBD with three replicates. Throughout the experiment, the field was maintained with regular hand-weeding while pesticide and fertiliser applications were applied as recommended. All the morphological and agronomic traits associated with the yield and yield components were measured and recorded following the IRRI standard of procedures (IRRI 2013). These included the number of tillers per plant (TN), plant height (PH), days of maturation (MAT), length of the panicle (LOP), filled spikelet % per panicle (FSPP), weight of 1000 spikelets (WOS) and crop cutting test yield (CCT).

Statistical analysis. All the relevant data were analysed using SAS Ver. 9.3 computer statistical package for an analysis of variance (ANOVA) to determine the significant differences amongst the parental and improved lines. Data at a 5% probability level were considered as statistically significant. The means were compared using Duncan's multiple range test (DMRT) and Ward's (1963) cluster analysis was used to evaluate the similarity between the lines.

RESULTS AND DISCUSSION

Parental reactions to blast inoculation. The identification and validation of the resistance expression of a resistant gene is one of the most important steps in the development of blast resistant varieties. In this study, the inoculation was undertaken using MPO 988.3 representing the $P_{0.0}$ blast pathotype. The results from blast screening showed that IRTP21683, the donor for *Pi9* gene had a '0' value for all the disease score measurements, which demonstrated that IRTP21683 was highly resistance against the disease (Table 1). In contrast, the recurrent parent MR220, as well as the other two control varieties, namely MR211 and MR219, showed significantly higher values of susceptibility scores above '4', which demonstrated that they are highly susceptible against the predominant blast pathogen pathotype in Malaysia. These results justified the classification of IRTP21683 as highly resistant (HR) and the other three varieties including the recurrent parent as being susceptible, which was in line with the earlier findings of Misman and Zakaria (2019). These results also suggested that the suitability of using MPO 988.3 in assessing the resistance of the generated BC_3F_4 lines.

Resistance of BC_3F_4 lines to blast disease infection. The successful introgression of the *Pi9* gene into the genome of the BC_3F_4 lines (Figure S2 in the ESM) led to an increase in the disease resistance after being challenged with MPO 988.3. All twenty introgressed BC_3F_4 lines showed strong resistance (HR)

https://doi.org/10.17221/55/2021-CJGPB

Table 1. Resistance reaction of the parents and control varieties following inoculation with isolate MPO 988.3 representing the prevalent *Pyricularia oryzae* pathotype $P_{0.0}$ in Malaysia

Variety/lines	Resistance reaction			
	DLA (%)	DS	DSe (%)	LR
IRTP21683 (donor parent)	0[b]	0[b]	0[b]	HR
MR211 (susceptible control)	10.1[a]	5.0[a]	51.9[a]	MS
MR220 (recurrent parent)	8.2[a]	4.0[a]	45.9[a]	MS
MR219 (susceptible control)	8.1[a]	4.0[a]	43.7[a]	MS

DLA – disease leaf area; DS – disease score; DSe – percentage disease severity; LR – line response; HR – highly resistant; MS – moderately susceptible; means within a column with a common letter are not significantly different by Duncan's multiple range test at a level of $P \leq 0.05$

Table 2. Average resistance scores of the BC_3F_4 lines and their parents following the inoculation with isolate MPO 988.3 of the $P_{0.0}$ pathotype of *Pyricularia oryzae*

Variety/lines	Resistance reaction			
	DLA (%)	DS	DSe (%)	LR
BC3F4 lines with *Pi9* gene	0.99 [b]	0.7 [b]	7.2 [b]	HR
MR220 (recurrent parent)	56.33[a]	8.3[a]	90.37[a]	HS
MR211 (susceptible control)	56.167[a]	9[a]	100.0[a]	HS
IRTP21683 (donor parent)	0.33[b]	1.3[b]	11.86[b]	HR

DLA – disease leaf area; DS – disease score; DSe – percentage disease severity; LR – line response; HR – highly resistant; MS – moderately susceptible; means within a column with a common letter are not significantly different by Duncan's multiple range test at a level of $P \leq 0.05$

with low average DLA (0.99), DS (0.7) and DSe (5.0) values (Table 2). Out of these, B220PI9-3-12 was among the best rice lines, with a disease score and percentage of affected leaf area score of 0, followed by B220PI9-3-76 (Table S2 in the ESM). The scores of these two lines were not significantly different from the score of the donor parent, IRTP21683. On the other hand, the seedlings of the recurrent parent, MR220, and the control susceptible variety, MR211, showed high disease scores. Both susceptible varieties have above 56% DLA, 8.0 DS and 90% DSe. The severe symptom scores with blast lesions covering about 60% of the leaf area finally led to the wilting and drying of the leaves (Figure 1). The results clearly demonstrated the effectiveness of the MAB application in monitoring the transference of the candidate *Pi9* gene into the recurrent variety, MR220. Similar successful findings were also presented in detail by

Figure 1. Reaction of seedlings of different varieties and lines after nine days post-inoculation with isolate MPO 988.3 of the $P_{0.0}$ pathotype: MR211 (A), MR220 (B), IRTP21683 (C) and B220PI9-3-12 (D)

https://doi.org/10.17221/55/2021-CJGPB

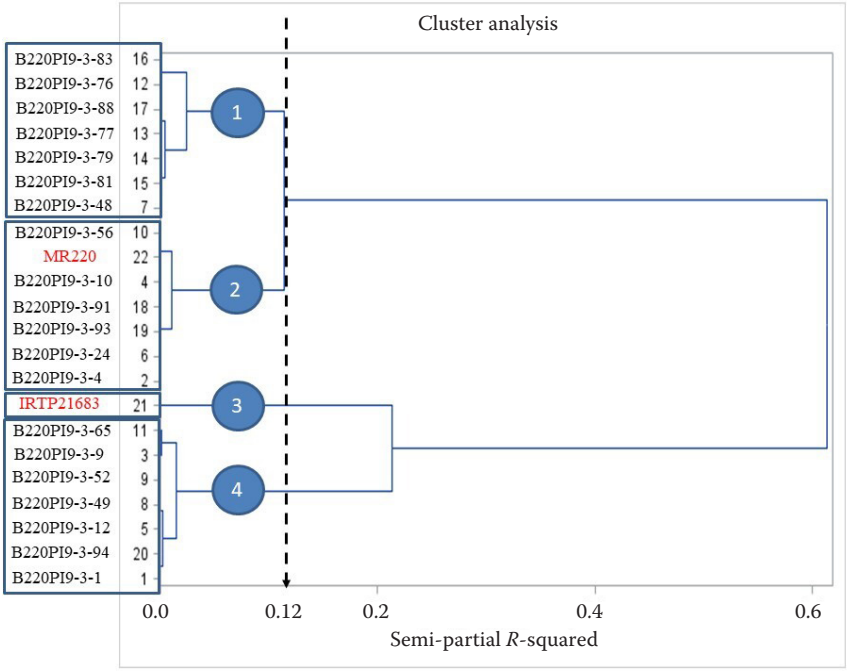

Figure 2. Dendrogram showing Ward's clustering of twenty BC_3F_4 lines and parents based on seven agronomic characteristics (SAS 2013)

Jain et al. (2017), which showed that all the improved Pusa Basmati 1 NIL population with the *Pi9* gene have a higher expression when the blast pathogen was introduced.

Agronomic performance of BC_3F_4 lines. The overall mean performance of twenty BC_3F_4 lines and their parents are presented in Table S3 in the ESM. A cluster analysis was used to summarise the overall agronomic data and classify the lines into groups based on their similarity distance. In this analysis, the twenty BC_3F_4 lines and their parents were divided into four main groups at a 0.12 semi partial R square value (Figure 2). Both Groups 1 and 2 consisted of seven lines, including the recurrent parent, MR220, as a member of Group 2. The donor parent IRTP21683 was the only member in Group 3. The seven remaining lines were in Group 4. Further analysis showed that segregation of the lines into the four groups were due to presence of significant variations in the four agronomic characteristics, namely, the maturation (MAT), length of panicle (LOP), weight of 1 000 spikelets (WOS) and crop cutting test yield (CCT) (Table S4 in the ESM). The position of IRTP21683 in a separate individual group was due to its significant variation from the other lines in the four parameters, especially the CCT value in which it only produced 1 600 kg/ha. The low CCT yield of the donor parent resulted from its shorter LOP (19.5 cm) and low WOS (19.3 g). However, this variety has a positive parameter, i.e., early MAT at 90.7 days which is good for early maturing rice varietal development. Group 1 has the best average yield of 5 331 kg/ha (Table 3) which is better than the yield of the recurrent parent MR220 (4 315 kg/ha).

In terms of individual performance, several lines with high yields in Group 1 were B220PI9-3-77, B220PI9-3-79, B220PI9-3-76, B220PI9-3-81 and B220PI9-3-48. These five BC_3F_4 lines also have

Table 3. Means of the agronomic and yield related characteristics of the four groups of the 22 tested lines as formed by Ward's cluster analysis

Group/characters	Total lines	MAT (days)	LOP (cm)	WOS (%)	CCT (kg/ha)
1	7	102.9[a]	25.2[a]	24.6[a]	5 330.7[a]
2	7	100.3[a]	24.7[a]	24.9[a]	4 556.1[b]
3	1	90.7[b]	19.5[b]	19.3[b]	1 600[d]
4	7	98.5[a]	25.0[a]	24.8[a]	3 719.6[c]

MAT – maturation days; LOP – length of panicle; WOS – weight of 1 000 spikelets; CCT – crop cutting test yield; means within a column with a common letter are not significantly different by Duncan's multiple range test at a level of $P \leq 0.05$

https://doi.org/10.17221/55/2021-CJGPB

a shorter maturation, less than 103 days. This shorter maturation trait would be highly suitable for the Malaysian dual rice cropping system per year practice, where the maturation of the most recent rice varieties is around 120 to 135 days, which are classified as medium maturation days (Elixon et al. 2017). Currently, the only Malaysian commercial variety with less than 100 days maturation is the herbicide tolerant variety MR220CL. Therefore, these five blast resistant lines have the potential for consideration in a future advance yield trial. The present study also indicated that utilisation of MAB for the improvement of the varietal resistance to pest and diseases, where the donor parents are usually of inferior agronomic traits, still has great potential. MAB may facilitate the development of improved lines with a good yield due to the minimised linkage drag effect from the donor parent (Shamsudin et al. 2016). A similar successful conclusion has also been documented on the improvement of the resistance of other commercial rice varieties, such as MR263 (Hasan et al. 2016) and MR219 (Miah et al. 2017).

CONCLUSION

This study showed the successful introgression of the blast resistant gene *Pi9* from IRTP21683, a donor parent with poor agronomic traits into twenty BC_3F_4 improved blast resistant lines having a high yielding potential comparable to their recurrent parent variety, MR220. The results obtained also support the potential advantage of using MAB as a supporting tool in backcross breeding, capable in minimising the linkage drag effect.

Acknowledgement: We thank the MARDI research assistants, Adham Azmi and Mohamad Ariff Asrofp Rahim for their valuable help.

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Received: June 13, 2021
Accepted: October 26, 2021
Published online: November 24, 2021