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Developing rice restorer lines resistant to bacterial blight, blast and brown plant hopper by molecular pyramiding

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Abstract

Bacterial blight, blast and brown plant hopper resistance (BB, BL and BPH respectively) genes *Xa23, Pi-9, Bph14* and *Bph15* were pyramided into an elite restorer line R1005 by using marker assisted selection backcrossing (MABC) and simple sequence repeats (SSR). PCR markers facilitated and accelerated the process of pyramiding of *Xa23, Pi-9, Bph14* and *Bph15* genes. In this study *75-1-127* harboring *Pi9*, JYQ9008 harboring *Xa23*, and *B5* harboring *Bph14* and *Bph15* against resistant bacterial blight, blast and brown planthopper were used as *R*-genes donor respectively. The pyramided genes and evaluation of agronomic traits related to bacterial blight, blast and brown plant hopper represent best ways in which resistance can be studied. Largest resistance levels were observed against the bacterial blight followed by blast and lastly brown plant hopper pyramid lines. PCR markers for these four genes, *Xa23, Pi-9, Bph14, 15* were made available. DNA marker technology was used to identify plants that contained resistant genes to BB, BL and BPH. Restorer lines are known to determine success of pyramided genes. Hence careful background searches should be done before settling on one. Resistant lines can really be the answer to environmental problems that hamper growth of food crops particularly rice and ensure food security for the vulnerable people in society. Pyramiding is considered an ethical way of creating better varieties. The newly variety we created that was resistant to all the three factors had a higher yield than the control. These traits were carried through successive generations as was the case in the hybrid variety.

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Introduction

Bacterial blight, Blast and Brown Planthopper are the most devastating diseases that affect rice growth and development particularly in countries where hybrid rice is largely grown. Previously, researchers have largely focused on pyramiding genes for resistance against bacterial blight and blast (J, *et al.,* 2006; W *et. al.,* 2013). Our research went a step further by including Brown Planthopper so as to develop a variety that can withstand detrimental effects of the two diseases and the pest. Rice is an essential source of food for many people in the world. Its production and consumption is concentrated in Asia. It is the staple food in major parts of Asia that includes China and the Pacific, North and South America and Africa (Shaoqing Li *et al.,* 2007). Millions of hectares planted throughout the world produce millions of metric tons of paddy rice every year which are exposed to pests and diseases including BB (*Xanthomonas Oryzae Pv.oryzae (Xoo*), BL (*Pyricularia grisae*) and BPH (*Nilaparvata lugens*). The diseases and pests affect rice production, causing annual yield losses throughout the world. Efforts have been made to limit yield losses caused by diseases and insect pests by pyramiding gene using molecular markers for plant resistance (Ghulam *et al.,* 2013). It should be noted that it is not easy to use conventional breeding methods due to dominance and epistatic effects of genes governing disease resistance by gene pyramiding. Recent developments that employ DNA markers offer new ways to fight diseases and pests by using resistant genes and molecular pyramiding. The dominant gene *Xa21* conferring multiple resistance against bacterial disease and a fused Bt gene *cry1Ab/cry1Ac* conferring resistance to lepidopteran insects have been individually introduced simultaneously into the same genetic background of an elite Indica cytoplasm male sterile (CMS) restorer line (Minghui 63) by marker-assisted selection (MAS) (Chen *et al.,* 2000). However, the results obtained from these lines and their hybrids showed high resistance to both disease and insect pests without reducing yield in field tests. Paddy fields are infested by some species of insects and most of them are considered as serious pests because they cause significant damages and loss of the rice crop. Rice diseases caused by Bacterial blight (BB) *Xanthomonas Oryzae PV. Oryzae (Xoo),* blast caused by *Pyricularia grisae* is considered to be the most devastating diseases in most rice-growing regions (Amy & Struedee, 2010; Jung *et al.,* 2013). Also, the brown planthopper (BPH) caused by *Nilaparvata lugens* is one of the most devastating pests in Asian countries where rice (*Oryza sativa L.)* is widely grown. In recent years, rice has been recognized as a genetic model for molecular biology research aimed toward understanding mechanisms for growth, development and stress tolerance as well as disease resistance. Resistant cultivars and application of pesticides have been used for disease control. Resistance gets eroded by highly pathogenic pests over time. Breeding for disease and pest resistance can contribute to improved quality of yield in rice plants by carrying out indirect selection through molecular markers linked to the traits of interest (Fujino *et al.,* 2008). Gene pyramiding holds better prospects in achieving durable resistance against biotic and abiotic stresses in crops. The creation of resistant varieties by backcrossing assisted by markers is a method that is economical and presents no risk to ecosystems in terms of ethics because genetic resources have been used since the beginning of selection. The use of resistant varieties is an essential component in majority of breeding programs and a means of effectively controlling effects of pests and diseases by minimizing negative effects on our environment. Thus, there is a need to develop strategies providing durable resistance in a broad geographic area. The R1005, one of the elite restorer lines of the hybrid rice program of China, was used as the recipient parent crossed with *Xa23* (JYQ9008), *B5 (Bph14, 15*) and *Pi-9(75-1-127).* MAS were carried out in each generation so as to confirm the target genes. We made multiple crosses to pyramid different genes. Thus, the objectives of this recent study was to pyramid *Xa23, Pi-9, Bph14, Bph15* into R1005 for developing several new rice restorer lines which will be resistant to bacterial blight and blast diseases and the brown plant hopper and thereby limit the use of pesticides and

insecticides in rice cultivation so as to conserve the environment.

Materials and methods

Plant material and molecular pyramiding

R1005 is an elite restorer line, which was widely used as male parent of CMS-system rice hybrids in Southern China. Five hybrids in which R1005 was used as male parent have been released in the past ten years. But, these hybrids were not resistant to blast, bacterial blight and brown planthopper. In this study, *75-1-127* harboring *Pi9* and resistant against blast (BL), JYQ9008 harboring *Xa23* and resistant against bacterial blight (BB), and *B5* harboring *Bph14* and *Bph15* and resistant against brown planthopper (BPH), were used as *R*-donor genes. R1005 was used as recipient parent for improving its resistances to BL, BB and BPH. The process of population development was as shown in (Fig. 1).

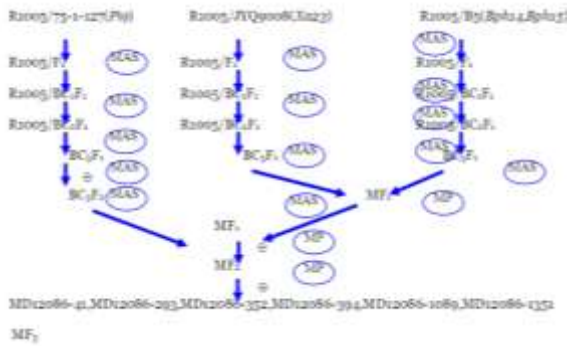

Fig. 1. Scheme showing the development of resistant materials using Marker Assisted Selection (MAS).

Bacterial blight inoculation and evaluation

The experiments were conducted in the experimental station since 2012 and 2013 in Huazhong Agricultural University city of Wuhan and Hainan Island Rice breeding station (Lingshui County, Hainan-China). All materials were germinated under 30℃ in the incubator. After that, the seeds are sown in plastic pots on May 10th 2013. 25 days old seedlings were then transplanted as single plant per hill in the main field. They were divided by putting them into paper bags and soaked in water for 48 hours. Each line comprised of 8 plants in one row planted with a spacing of 16.7×26.7cm. Plants were inoculated with the bacterial suspension at a density of 10^9cells/ml at maximum tillering stage of plant development. *Xoo* strains ZHE173 (from Zhejiang province) and GD1358 (from Guangdong province) were inoculated on three leaves per plant in replications 1 and 2 successively. Three leaves per plant in each line were inoculated. The lesion length was carefully measured in cm after three weeks in all leaves that were inoculated. The results of bacterial blight resistance showed that all the breeding lines and their derived hybrid were highly resistant to the two epidemic strains ZHE173 and GD1358. Plant reaction to bacterium was scored in accordance to the standard method for scoring rice varieties' reaction to BB in the China National Rice Trial program.

Resistance gene confirmation by DNA markers

Polymerase chain reaction (PCR) analysis confirmed the homozygous target genes. Markers assisted selection (MAS) and simple sequence repeat (SSR) marker, were carried out in each generation to confirm the location of resistance genes that we aimed to target. M-Xa23 was used in the presence of *Xa23,* Ms5 and MRG239 in presence of *Bph14* and *Bph15* respectively, and PB9-1 for presence of *Pi-9*. The total genomic DNA from young leaves was collected 15 days after transplanting of progenies and parental lines. They were extracted and amplified by PCR reaction using SSR markers. CTAB concentration of 1.5 was used to get the DNA sample. A total volume of 20ul of PCR reaction system was used, wherein the template was 2ul, sterilized water 12.8ul, 2ul buffer with Mg2 + (25mm / L), 1.8ul mM dNTP (5mM / L) and 0.2ul each for the forward and reverse primer. Before putting the mixture into the PCR instrument, add 20 ul of mineral oil to protect the reaction system. PCR amplification was performed and thermo cycler profile was: initial preheating 10min at 94℃, denaturation 1min at 94℃, annealing 45s at 50℃, 55℃, 60℃; extension for 1min at 72℃ and final extension 10 min at 12℃ for a total of 30 cycles.PCR products were stored at -20℃.The PCR amplification products were resolved on 6% polyacrylamide gels and visualized by silver staining as shown in table 1.

Table 1. Genetic testing used to verify M-Xa23, Ms5, MRG2329, PB9-1 primers for resistance genes.

genes	Primers	Forward primers	Reverse primers	Annealing temperature
Xa23	M-Xa23	5'-TTGCTCAAGGC TAGGAAAATG-3'	5'-CCCCATCAAC GAACTACAGG-3'	55℃
Bph15	Ms5	5'-TTGTGGGTCC TCATCTCCTC-3'	5'-TGACAACTTTG TGCAAGATCA-3'	55℃
Bph14	MRG2329	5'-GCACATACAG AAATGGTGAA-3'	5'-GGCAAGGGAC ATGTAGTAAC-3'	55℃
Pi-9	PB9-1	5'-TAGACTCCTTC CAAGTTTGACT-3'	5'-TGTGATTTTC AGAATTTTCGT-3'	55℃

Rice blast resistance evaluation

The seeds were prepared for each entry, by putting them in paper bags and soaking them in water for 48 hours. All materials were germinated under 30°C in the incubator and sown in the experimental site of Huazhong Agricultural University. After 25 days, the seedlings were taken to Yuanan and Enshi, for transplanting and evaluation of the grades of resistance to leaf and neck blast. Blast resistance evaluation results under natural conditions showed that leaf blast score and panicle blast incidence of the breeding lines and their derived hybrids were lower than their recipients as shown in table (2).

Table 2. Identification of rice blast resistance for breed lines at Enshi and Yuanan, Hubei.

Breeding lines	R-genes	S	LBS	PBI(%)
MD12086-41	*Xa23,Pi-9,Bph14,Bph15*	Yuanan	3	11.0
	Xa23,Pi-9,Bph14,Bph15	Enshi	3	8.0
MD12086-293	*Xa23,Pi-9,Bph14,Bph15*	Yuanan	3	11.0
	Xa23,Pi-9,Bph14,Bph15	Enshi	2	6.0
MD12086-352	*Xa23,Pi-9,Bph14,Bph15*	Yuanan	3	8.0
	Xa23,Pi-9,Bph14,Bph15	Enshi	2	6.0
MD12086-394	*Xa23,Pi-9,Bph14,Bph15*	Yuanan	3	8.0
	Xa23,Pi-9,Bph14,Bph15	Enshi	2	7.0
MD12086-1089	*Xa23,Pi-9,Bph14,Bph15*	Yuanan	2	11.0
	Xa23,Pi-9,Bph14,Bph15	Enshi	2	9.0
MD12086-1351	*Xa23,Pi-9,Bph14,Bph15*	Yuanan	2	8.0
	Xa23,Pi-9,Bph14,Bph15	Enshi	4	12.0
75-1-127(donor parent)	*Pi-9*	Yuanan	2	6.0
	Pi-9	Enshi	2	7.0
R1005(recipient parent)	-	Yuanan	4	25.0
	-	Enshi	4	30.0

R-genes =resistance genes,S=sites,LBS=leaf blast score, PBI(%)=panicle blast incidence.

Greenhouse evaluation of brown planthopper resistance

The restorer line R1005 with high quality yield were used as females for crossing with *75-1-127 (Pi-9)* and *B5 (*carrying *Bph14 and Bph15)*. All materials were germinated under 30°C in incubator and individual lines were sown in plastic pots. Seedlings at the three-leaf stage were infected with second- or third-in star nymphs at a density of 10-12 nymphs per seedling. Rathu Heenati (RH) and Taichung Native (TN1) susceptible varieties were used as controls. When all the seedlings of TN1 died, the plants of each line were examined and each seedling was given a score of 0, 1, 3, 5, 7 or 9, according to the criteria of standardization. Evaluation of brown plant hopper resistance showed that all of the breeding lines and their derived hybrids were highly resistant to brown planthopper.

Agronomic traits and grain quality evaluation

The Introgression Lines (ILs) and their parent were planted in Huazhong Agricultural University (HZAU)-Wuhan in the spring season of 2013. The ILs and F_1 test cross were planted in plots. Each plot consisted of 5 rows that had 50 plants with spacing of 16.7×26.7 cm. The materials were arranged following randomized complete blocks with 2 replications and evaluated for agronomic traits in the experimental

plot. 10 plants from each line were used to determine Plant height (PH/cm); heading date(HD/day), Panicle length (PL/cm); Spikelet/Panicle; Number of spikelet per plant (NSP); Grain filling percentage; Grain yield per plant performance; 1000 grain weight; seed setting rate (SR) and filled grain number per plant(G/P) .

Results

Evaluation of BB, BL and BBH resistance

To pyramid all of the four resistance genes, we produced a combination of the three genes i.e. *Xa23, Pi-9* and *Bph14, Bph15* and introduced them into our breeding line. To reduce the population size for DNA marker analysis, we inoculated the F_3 generation with four of the resistant genes and susceptible plants were discarded. This method removed those plants lacking all resistance genes. In fact, DNA markers were used to identify homozygotes for each of the genes in the different combinations. Marker analysis for the F_3 population that was derived from selected pyramid lines was carried out. Fig. 2 shows the identification of homozygotes on gels (BDFH) and heterozygotes on gels (ACEG) for resistant genes to BB,BL and BPH with markers M-Xa23, Ms5, MRG2329 and PB-1 closely linked to *Xa23, Pi-9* and *Bph14, Bph15* resistance genes. The F_3 population on gel of fig. 2 determines the presence of the different genes. Plants homozygous for markers linked to *Xa23, Pi-9, Bph14* and *Bph15* resistances were identified. Plants homozygous for resistant allele at four markers loci were retained. Nineteen lines with *Xa23* and *Pi-9* were selected as BB and BL resistant. Thirty-eight lines with *Pi-9* and *Bph14* or *Bph15* were selected as BL and BPH resistant. Thirteen lines with *Xa23* and *Bph14 or Bph15* were selected as BB and BPH resistant. Thirty lines with *Xa23, Pi-9, Bph14* and *Bph15* were selected as BB, BL and BPH resistant. To ensure better selection, DNA based progeny testing was employed as illustrated in (Fig. 2).

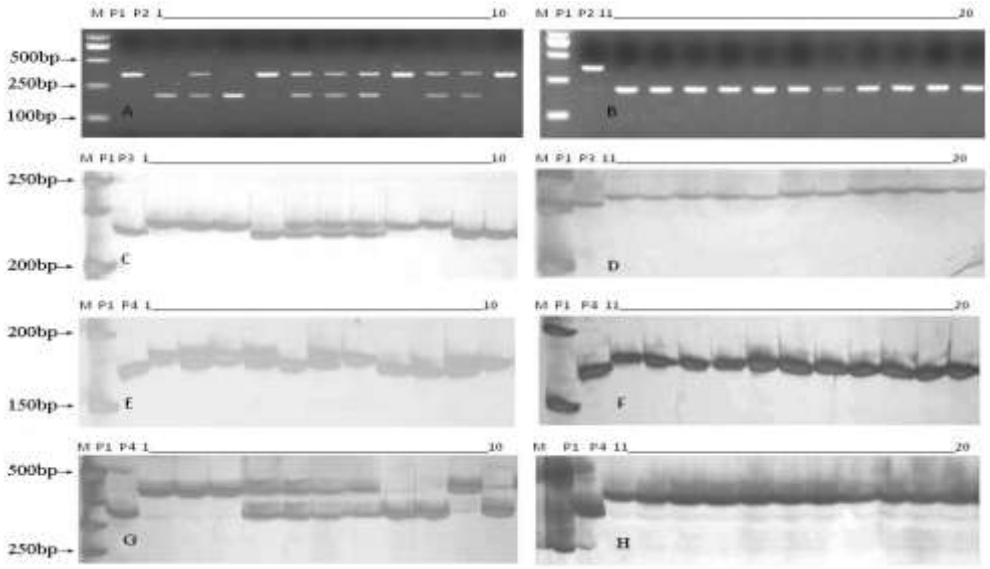

Fig. 2. PCR detection of *Xa23* (A and B), *Pi9* (C and D), *Bph14* (E and F) and *Bph15* (G and H) for pyramiding four resistant genes. M: DNA marker, P1: R1005 (recipient parent), P2: JYQ9008 (donor parent of *Xa23*), P3: 75-1-127 (donor parent of *Pi9*), P4: B5 (donor parent of *Bph14 and BBph15*). A, C, E and G: the PCR detection results of heterozygous generations for *Xa23*, *Pi9*, *Bph14* and *Bph15*, respectively. B, D, F and H: the PCR detection results of homozygous generations for *Xa23*, *Pi9*, *Bph14* and *Bph15*, respectively.

Molecular marker assisted selection of Xa23, Pi-9 and Bph14, Bph15 resistance genes

Four SSR markers M-Xa23, Ms5, MRG2329 and PB-1 closely linked to *Xa23, Pi-9* and *Bph14, Bph15* resistance genes which was reported previously were chosen for parental lines R1005. DNA markers were then used to identify homozygotes for each of the three gene combinations to ensure good results were

obtained. Thus, the PCR markers proved the pyramiding of the *Xa23, Pi-9, Bph14* and *Bph15* genes that are resistant against BB, BL, and BPH. The pyramided *Pi-9* and *Bph14* and *Bph15* genes are susceptible to BL and BPH. The resistance of pyramided lines for BB, BL pathogens and BPH pest confirmed our line as a good candidate for BC_2F_1 generation. The largest resistance level was observed against the bacterial blight, followed by blast and lastly for brown plant hopper in the pyramided lines. Development of PCR markers for these genes *Xa23, Pi-9, Bph14 and Bph15* was done after pyramiding. The used of PCR markers increased significantly the process of MAS. In this study we found 41 homozygote progenies for *Xa23*, 52 for *Pi-9*, 16 for *Bph14* and 22 for *Bph15* that were detected by PCR analysis with the markers M-Xa23, Ms5, MRG2329 and PB-1 respectively, which were tightly linked with the target genes. PCR analysis of parental lines and foreground selection derived from backcross progenies between R1005 and different pyramiding resistant genes *Xa23, Bph14, Bph15* and *Pi-9* with their primers M-Xa23, MRG2329, Ms5, PB9-1 respectively are in the table 1.

Agronomic traits and grain quality performance

The agronomic performance of the pyramided lines R005 carrying *Xa23, Pi-9, Bph14, 15* and its hybrids produced by crossing with three CMS lines RongfengA, Q2A and Hua1165S were examined and evaluated in Huazhong Agricultural University field experiment and laboratory in 2012 and 2013 with randomized complete blocks of 2 replications. Data that was collected from parental lines, and their derivative hybrids included; Plant height (PH/cm), Panicle length (PL/cm), Spikelet/Panicle, Heading date (HD/day), Number of spikelet per plant (NSP), Grain filling percentage, Grain yield per plant, 1000 grain weight, filled grain number per plant were as determined in the (table 7), Quality performance of different rice breeds and hybrid combinations are as shown in table 6 and 8 above. The grain quality, seed set rate, grain density, spikelet per panicle, plant height of selected lines were higher than their recipient parent. Chalky grain rate and chalkiness were opaque for all six breeding lines of the recipient parent in table (5). The Chalky grain rate and chalkiness of hybrid combinations of rice quality performance in table (8) shows they significantly improved. The gel consistency of Rongfeng A/MD12086-41 was reduced; its amylose content and the gel consistency for all thirteen hybrid combinations were improved. In order to determine more accurately the yield per plant, the sample remaining after plant harvesting was threshed, dried and weighed together with the corresponding sample to calculate the yield of 3 plants. Plant yield was then statistically analyzed. Each samples' traits were determined from the average of three agronomic traits by numerical data analysis. The result of our studies demonstrated that the yield of newly improved hybrid variety was higher than the control, and stably maintained the elite agronomic traits of the hybrid rice.

Table 3. Breed strains and hybrids of bacterial blight resistance 2013.

	Genes	ZHE173	GD1358
Combination name		Lesion and RL	Lesion and RL
MD12086-41	*Xa23, Pi-9, Bph14,15*	0.51±0.14(HR)	0.27±0.06(HR)
MD12086-293	*Xa23, Pi-9, Bph14,15*	0.48±0.13(HR)	0.24±0.06(HR)
MD12086-352	*Xa23, Pi-9, Bph14,15*	0.47±0.14(HR)	0.32±0.09(HR)
MD12086-394	*Xa23, Pi-9, Bph14,15*	0.49±0.14(HR)	0.34±0.06(HR)
MD12086-1089	*Xa23, Pi-9, Bph14,15*	0.48±0.13(HR)	0.32±0.07(HR)
MD12086-1351	*Xa23, Pi-9, Bph14,15*	0.49±0.13(HR)	0.37±0.07(HR)
R1005	-	10.39±3.16(MS)	2.28±1.15(R)
B5	*Bph14,15*	16.10±4.42(S)	9.88±8.82(MS)
75-1-127	*Pi-9*	11.48±3.79(MS)	21.35±2.87(HS)
CBB23	*Xa23*	0.35±0.20(HR)	1.43±0.89(R)
Rong feng A/MD12086-41	*Xa23, Pi-9, Bph14,15*	1.13±0.52（R）	0.43±0.08(HR)

	Genes	ZHE173	GD1358
Rong feng A/MD12086-293	*Xa23*, *Pi-9*, *Bph14,15*	0.45±0.26（ HR)	0.43±0.07(HR)
Rong feng A/MD12086-352	*Xa23*, *Pi-9*, *Bph14,15*	0.81±0.51(HR)	0.45±0.05(HR)
Rong feng A/MD12086-394	*Xa23*, *Pi-9*, *Bph14,15*	0.55±0.46(HR)	0.45±0.05(HR)
Rong feng A/MD12086-1089	*Xa23*, *Pi-9*, *Bph14,15*	0.56±0.31(HR)	0.89±0.47(HR)
Rong feng A/MD12086-1351	*Xa23*, *Pi-9*, *Bph14,15*	0.57±0.31(HR)	0.41±0.09(HR)
Rong feng A/R1005	-	13.25±3.79(S)	4.98±2.19(MR)
Rong feng A/B5	*Bph14,15*	17.80±5.22(S)	16.40±6.39(S)
Rong feng A/75-1-127	*Pi-9*	12.77±4.14(S)	16.10±2.77(S)
Rong feng A/CBB23	*Xa23*	2.82±1.30(R)	4.23±2.16(MR)
五优308（ CK1）	-	12.07±4.32(S)	17.15±2.76(S)
Q2A/MD12086-41	*Xa23*, *Pi-9*, *Bph14,15*	0.51±0.13(HR)	0.28±0.10(HR)
Q2A/MD12086-293	*Xa23*, *Pi-9*, *Bph14,15*	0.48±0.13(HR)	0.31±0.10(HR)
Q2A/MD12086-352	*Xa23*, *Pi-9*, *Bph14,15*	0.48±0.13(HR)	0.31±0.09(HR)
Q2A/MD12086-394	*Xa23*, *Pi-9*, *Bph14,15*	0.50±0.14(HR)	0.21±0.07(HR)
Q2A/MD12086-1089	*Xa23*, *Pi-9*, *Bph14,15*	0.51±0.14(HR)	0.24±0.09(HR)
Q2A/MD12086-1351	*Xa23*, *Pi-9*, *Bph14,15*	0.48±0.13(HR)	0.23±0.09(HR)
Q2A/R1005	-	7.88±2.48(MS)	2.59±0.95(R)
Q2A/B5	*Bph14,15*	13.00±4.10(S)	3.96±1.68(MR)
Q2A/75-1-127	*Pi-9*	9.89±2.62(MS)	5.30±1.67(MS)
Q2A/CBB23	*Xa23*	1.02±0.33(R)	0.99±1.33(HR)
Q优6号（ CK2）	-	12.00±3.75(MS)	2.44±0.73(R)
扬两优6号（ CK3）	-	2.88±1.33(R)	6.29±1.07(MS)
Hua1165S/MD12086-41	*Xa23*, *Pi-9*, *Bph14,15*	0.48±0.13(HR)	0.24±0.07(HR)
Hua1165S/MD12086-293	*Xa23*, *Pi-9*, *Bph14,15*	0.45±0.15(HR)	0.27±0.05(HR)
Hua1165S/MD12086-352	*Xa23*, *Pi-9*, *Bph14,15*	0.48±0.13(HR)	0.25±0.05(HR)
Hua1165S/MD12086-394	*Xa23*, *Pi-9*, *Bph14,15*	0.48±0.13(HR)	0.26±0.05(HR)
Hua1165S/R1005	*Bph14,15*	3.65±1.49(MR)	2.42±0.60(R)
Hua1165S/B5	*Bph14,15*	5.18±1.68(MS)	13.73±7.96(S)
Hua1165S/75-1-127	*Pi-9*, *Bph14,15*	5.83±2.70(MS)	17.75±2.62(S)
Hua1165S/CBB23	*Xa23*, *Bph14,15*	1.00±0.57(R)	1.64±0.63(R)

HR=high resistance, R=resistant, MS=middle susceptible, HS=high susceptible.

Table 4. Breed strains of brown plant hopper resistance.

Breeding lines	Date of survey				Resistance levels
	7/5	7/7	7/9	7/13	
MD12086-41	1	1	1	1	HR
MD12086-293	1	1	1	1	HR
MD12086-352	1	1	1	1	HR
MD12086-394	1	1	1	1	HR
MD12086-1089	1	1	1	1	HR
MD12086-1351	1	1	1	1	HR
TN1（ susceptible CK）	9	9	9	9	S
R1005（ recipient parent）	3	5	7	9	S
B5（ donor parent of *Bph14* and *Bph15*）	1	1	1	1	HR
75-1-127（ donor parent of *Pi9*）	9	9	9	9	S
CBB23（ donor parent of *Xa23*）	9	9	9	9	S

HR=high resistance, S=susceptible

Table 5. Yield and major agronomic traits.

Breeding Lines	HD（ d）	PH(cm)	P/P	PL(cm)	S/P	GP	GD/(10cm)	SSR (%)	1000GW (g)	Y/P(g)
MD12086-41	82	115.3	7.3	28.1	130.3	99.6	46.4	76.59	28.35	19.82
MD12086-293	82	107.3	11.7	26.5	122.6	95.9	46.2	78.74	25.49	23.36
MD12086-352	82	106.2	8.3	25.7	139.0	108.9	53.9	78.28	27.54	21.05
MD12086-394	82	111.7	6.0	26.4	133.7	102.5	50.8	77.09	27.47	18.89
MD12086-1089	82	113.3	8.7	29.6	99.5	75.0	35.0	74.93	27.86	25.75
MD12086-1351	80	112.7	6.7	27.4	131.4	88.6	47.7	67.13	27.52	20.21
R1005	84	110.0	7.7	23.8	124.1	94.6	52.1	76.26	28.43	23.53

HD: the days from seeding to heading, PH: plant height, P/P: panicles per plant, S/P: spikelet per panicle, G/P: filled grains per panicle, GD: grain density (grainspercm), SSR: seed set rate, 1000-GW: 1000 grain weight, Y/P: grain yield per pant

Table 6. Breed strains of rice quality performance.

Strain Name	BRR（ %）	RR（ %）	MRR（ %）	ChGR（ %）	Ch （%）	GL (mm)	A R	AC（ %）	GC (mm)	GTL
MD12086-41	75.7	64.74	47.91	opaque	opaque	6.3	3.1	11.79	91.3	2.0
MD12086-293	73.5	62.75	39.99	opaque	opaque	6.2	3.2	11.27	86.5	2.0
MD12086-352	76.2	64.42	52.82	opaque	opaque	6.4	3.2	11.23	87.3	2.0
MD12086-394	76.2	64.50	53.31	opaque	opaque	6.4	3.1	11.54	64.3	2.2
MD12086-1089	74.5	64.56	57.33	opaque	opaque	6.4	3.2	11.96	77.0	2.0
MD12086-1351	75.5	65.14	53.71	opaque	opaque	6.3	3.1	11.51	67.0	2.1
11005	73.7	62.94	41.62	opaque	opaque	6.6	3.1	11.87	80.0	2.4

BRR=Brown rice rate, RR= Rice rate, MRR= Milled rice rate, ChGR= Chalky grain rate,

CH= Chalkiness, GL= Grain length, AR= Aspect Ratio, AC= Amylose content, GC= Gel consistency, GTL= Gelatinization temperature.

Table 7. Production of hybrids, the period of growth and of great agronomic traits.

Combination name	*HD（ d）*	*PH (cm)*	*P/P*	*PL (cm)*	*S/P*	*FG/P*	*GD/ (10cm)*	*SSR (%)*	*1000G -W(g)*	*Y/P (g)*
Rong feng A/MD12086-41	83	117.8	8.7	26.3	178.5	157.1	67.9	88.00	27.50	44.54
Rong feng A/MD12086-293	81	116.4	10.5	26.0	167.2	144.0	64.3	86.15	26.52	42.42
Rong feng A/MD12086-352	84	117.8	9.7	26.0	173.9	144.1	66.8	82.83	27.05	36.71
Rong feng A/MD12086-394	83	118.8	9.8	25.7	170.9	142.1	66.5	83.30	27.23	41.06
Rong feng A/MD12086-1089	85	121.4	10.5	25.8	171.4	142.9	66.3	83.30	27.40	44.02
Rong feng A/MD12086-1351	82	116.6	11.8	25.7	161.8	137.9	62.6	84.71	27.50	41.98
Rong feng A/R1005	83	115.5	9.5	25.0	176.7	144.4	70.8	81.61	28.10	38.11
Rong feng A/75-1-127	80	111.9	10.2	25.8	167.2	143.9	64.9	86.07	24.59	40.15
五优308（ CK1）	79	110.5	9.2	24.4	230.5	196.6	94.5	85.22	21.39	43.66
Q2A/MD12086-41	85	129.7	9.3	28.9	192.6	160.7	66.6	83.49	26.59	42.82
Q2A/MD12086-293	87	124.9	8.7	27.9	177.9	145.8	63.6	81.88	25.81	36.43
Q2A/MD12086-352	86	124.6	7.2	27.3	170.5	136.2	62.4	79.79	26.03	41.72
Q2A/MD12086-394	86	128.8	8.7	29.1	189.0	157.1	64.8	83.15	26.66	36.79
Q2A/MD12086-1089	86	126.7	10.0	28.2	194.7	155.0	68.9	79.54	26.22	41.17
Q2A/MD12086-1351	85	126.5	8.8	28.7	199.0	159.9	69.2	80.39	26.19	37.09
Q2A/R1005	86	127.3	8.5	27.8	217.9	175.3	78.2	80.40	26.26	38.42
Q优6号（ CK2）	86	125.5	7.5	27.7	204.9	163.8	73.9	79.97	26.59	39.51

Combination name	HD（d）	PH (cm)	P/P	PL (cm)	S/P	FG/P	GD/ (10cm)	SSR (%)	1000G -W(g)	Y/P (g)
扬两优6号（CK3）	90	130.9	8.8	27.1	178.0	142.9	65.6	79.98	27.24	34.04
Hua1165S/MD12086-41	81	120.5	11.3	28.0	148.8	123.1	53.2	82.76	27.33	39.83
Hua 1165S/MD12086-293	81	118.4	9.7	28.7	158.6	132.5	55.2	83.33	26.42	36.84
Hua 1165S/MD12086-352	81	118.9	11.8	25.5	143.2	110.1	60.6	77.20	26.77	37.15
Hua 1165S/MD12086-1089	82	120.3	10.7	27.7	146.9	120.2	52.9	81.96	26.40	38.49
Hua 1165S/MD12086-1351	81	117.0	10.7	27.1	151.0	121.9	55.8	80.31	26.73	37.11
Hua 1165S/R1005	83	118.1	9.8	28.0	160.0	132.2	57.2	82.68	28.59	41.22
Hua 1165S/B5	82	115.0	14.7	27.3	136.1	104.4	49.9	76.85	25.71	36.82
Hua 1165S/75-1-127	80	111.7	13.2	27.2	139.4	110.5	51.1	79.07	26.10	34.96
Hua 1165S/CBB23	81	112.3	12.8	26.3	146.9	123.5	55.8	84.38	26.12	34.87
Rong feng A/MD12086-394	83	118.8	9.8	25.7	170.9	142.1	66.5	83.30	27.23	41.06
Rong feng A/MD12086-1351	82	116.6	11.8	25.7	161.8	137.9	62.6	84.71	27.50	41.98
Rong feng A/R1005	83	115.5	9.5	25.0	176.7	144.4	70.8	81.61	28.10	38.11

Table 8. Hybrid combinations of rice quality performance.

Combination name	BRR（%）	BRR（%）	MRR（%）	ChGR（%）	Ch（%）	GL (mm)	A R	AC（%）	GC (mm)	GTL
Rong feng A/MD12086-41	79.0	65.10	43.70	87.0	51.4	6.1	2.9	20.51	45.3	2.7
Rong fengA/MD12086-293	77.9	65.35	44.04	77.0	36.1	6.1	2.9	20.94	51.5	2.8
Rong fengA/MD12086-352	78.1	68.27	64.36	47.0	20.9	6.2	3	12.77	77.5	2.7
Rong fengA/MD12086-394	85.0	65.06	33.72	93.0	46.9	6	2.8	19.86	65.0	3.2
Rong fengA/MD12086-1089	79.4	66.05	53.37	76.0	38.2	6.2	2.9	19.95	52.0	2.3
Rong fengA/MD12086-1351	78.7	67.76	43.15	76.5	33.3	6.4	2.9	20.50	51.5	2.9
Q2A/MD12086-41	78.6	69.08	65.89	96.5	65.1	6.2	2.9	14.72	77.0	3.2
Q2A/MD12086-352	78.1	68.27	64.36	47.0	20.9	6.2	3	12.77	77.5	2.7
Q2A/MD12086-1089	78.5	66.62	56.75	opaque	opaque	6.3	3	13.40	74.5	3.2
Q2A/R1005	78.2	67.40	57.23	69.0	14.9	6.3	3	13.40	76.0	3.1
Q优6号（CK2）	78.6	67.57	61.25	40.0	18.7	6.2	3	13.93	76.5	3.0
Hua1165S/MD12086-41	79.3	68.69	59.63	opaque	opaque	6.5	3.1	13.48	71.0	2.9
Hua1165S/MD12086-1089	76.3	65.52	56.84	opaque	opaque	6.4	3.1	11.50	64.5	2.8
Hua1165S/R1005	77.6	68.49	63.93	14.0	2.8	6.6	3.2	13.07	71.3	2.9

BRR=Brown rice rate, RR= Rice rate, MRR= Milled rice rate, ChGR= Chalky grain rate, CH= Chalkiness, GL= Grain length, AR= Aspect Ratio, AC= Amylose content, GC= Gel Consistency, GTL= Gelatinization temperature level.

Discussion

In this study, the markers assisted pyramiding of *Xa23, Pi-9, Bph14 and Bph15* resistances genes into R1005 and its hybrids has facilitated the development of resistance against pathogens and pests in rice. Pyramiding of several resistance genes is an important and effective method that can protect rice plants from diseases and the environment of toxic effects arising from usage of pesticides. We introgressed four dominant resistant genes *Xa23, Pi-9, Bph14* and *Bph15* into an elite restorer line. Most studies reported an additive effect and epistasis by pyramiding two genes or more genes controlling plant resistance to pathogen or insects. Pyramiding two dominant bacterial blight resistance genes, *Xa7* and *Xa21*, into Minghui 63 and its hybrids showed a significant additive effect of two genes (Zhu *et al.,* 2004 Jie *et al.,* 2012). The identification of BACs carrying the most closely linked markers is a crucial step towards the cloning of a gene (Haiyuan *et al.,*

2004). However, pyramiding two BPH resistance genes, *Bph1* and *Bph2* revealed that the resistance level of the pyramided line was only equivalent to that of the *Bph1* single introgression line, which showed enhanced resistance compared with the *Bph2* single introgression. In this study, the pyramiding of *Xa23, Pi-9, Bph14* and *Bph15* into R1005 and its hybrids, showed higher resistance than the *Xa23, Pi-9, Bph14* or *Bph15* single introgression line. *Ba7* resistance gene was identified on the long arm of chromosome 6 where two dominant genes (*Xa7* and *Xa27*) were reported. *Xa7* was originally identified in rice cultivar 'DV85' (Sidhu *et al.,* 1978, Yang *et al*., 2004, J *et., al* 2006; Q, *et al.,* 1998). A tightly linked marker of Xa7, RG1091 was mapped to position 107.5 CentiMorgans (Cm) on the rice genome research program (RGP) map (T, 1996). Molecular markers linked to the target genes can be used in MAS programs which is particularly advantageous for improvement of resistance to diseases and insects. Therefore, it is necessary to identify and introduce several BB, BL, BPH resistance genes into rice breeding program and identify tightly linked molecular markers for MAS/MABC. MAS have distinct advantages in pyramiding of multiple genes. In the present research, the DNA markers were co-dominant, therefore, homozygous pyramid lines were readily selected from BC_2F_1 generation. The DNA markers can be identified by MAS hybridization or by using PCR. Gene pyramiding with marker technology can be integrated into plant breeding programs. Genetic background and growth stage of a restorer line play important roles in determining gene action. Therefore, it is useful to pyramid the restorer lines with several different resistant genes in a hybrid rice breeding program and develop a cropping area where pathogens and pests caused serious damage to rice farmers in the world (Hsieh *et al.,* 1988; I *et al*., 2012). We performed MAS by pyramiding four resistance genes, *Xa23, Pi-9, Bph14* and *Bph15* into R1005, and we evaluated the effect of the pyramided genes in conferring resistance to BB, BL and BPH in hybrid rice. Therefore, it is advisable to use these four pyramided resistance genes for rice improvement programs. Today, with the pyramiding of genes, new opportunities are opening up for breeders. The identification of molecular markers linked to genes that control resistance to diseases and insects can aid in selection studies (Yong-Li, *et al*., 2009; Basavaraj *et al.,* 2010; Chongyun *et al.,* 2012). The resistant line created as a result of pyramiding can be further evaluated to ascertain its stability through successive generations.

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