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Full Length Research Paper

Genetic improvement of resistance to blast and bacterial blight of the elite maintainer line Rongfeng B in hybrid rice (*Oryza sativa* L.) by using marker-assisted selection

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Rice blast caused by the fungus *Magnaporthe grisea* and bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) are two major rice diseases in the world. An elite, early maturing maintainer line of hybrid rice, Rongfeng B hybrid rice is susceptible to both blast and BB. For improving its diseases resistance, BL122 and CBB23 were used as the donors of blast resistance genes *Pi1* and *Pi2* and BB resistance gene *Xa23*, respectively. These resistant genes were introgressed into Rongfeng B by using a marker-assisted backcross breeding programs, and two improved lines D521 and D524 with *Pi1, Pi2* and *Xa23* were developed. The results indicated that both improved lines showed high resistance to leaf and neck blast and BB. The resistance frequencies for the rice blast and the length of lesions resulting from BB ranged from 96.7 to 100% and 0.77 to 1.18 cm, respectively. The two improved lines showed the desired variation in the majority of evaluated agronomic traits, including the number of grains per panicle, the grains weight, plant height, and seed setting rate. A new cytoplasmic male sterile line, Rongfeng 3A, with *Pi1*, *Pi2,* and *Xa23*, was successfully developed through successive backcross breeding.

Key words: Gene pyramiding, marker-assisted backcross breeding, rice blast, bacterial blight.

INTRODUCTION

Plant diseases are one of the major limiting factors in rice production. Rice blast caused by the fungus *Magnaporthe grisea* and bacterial blight (BB) caused by l *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) are 2 major rice diseases which occur not only in Asia, but also in Australia, the United States, and in several rice growing countries of Latin America and Africa (Ou, 1985; Adhikari et al., 1995; Sere et al., 2007). In epidemic years of rice blastor BB, the losses in the rice yield were 10 to 90% (Sun et al., 1998). *M. griseais* is known for its tremendous genetic diversity, a diversity that has been observed in all planting areas (Chadha and Gopalakrishna, 2005; Chen et al., 2006). Due to the emergence of new pathogenic races and because the pathogenic isolates in a population, focus tend to shift from avirulent to virulent forms (and not vice versa) on a given host (Lau and Ellingboe, 1993), the resistance of elite rice varieties were often broken soon after their release. For BB, only *Xa4* and *Xa21* among more than 30 identified BB

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Abbreviations: BB, Bacterial blight; **CMS,** cytoplasmic male sterility; **MAS,** marker-assisted selection; **PCR,** polymerase chain reaction; **PH,** plant height; **HD,** heading date; **NET,** number of effective tillers; **NGP,** number of grains per panicle; **SR,** seed-setting rate; **TGW,** weight of 1000 grains; **SSR,** simple sequence repeat.

resistance genes are widely used in the development of hybrids and in the conventional cultivars of Indica-type rice in the main rice-growing regions in China (Zhang et al., 1998). The narrow genetic basis for resistance to BB in many Chinese rice cultivars has increased the likelihood of a breakdown in the resistance of rice cultivars with *Xa4* (Zhang et al., 1998). In addition, the BB resistance gene *Xa21* was recently broken down by new virulent strains in southern China and the Yangtze river valley (Zeng et al., 2002; Zhang, 2009).

The genetic improvement of resistance by using a marker-assisted backcross breeding program is the most economical and environment-friendly strategy for disease improvement in rice. Single-locus resistance can result in a short lifespan, often lasting for only 2 to 4 years (Babujee and Gnanamanickam, 2000). Pyramiding several resistance genes into a single rice cultivar is an effective way to prevent the resistance breakdown. Pyramided lines carrying the resistance gene combinations *Pi1* + *Piz-5* and *Pi1* + *Piz – 5* + *Pi-ta* broadened the resistance spectrum of each individual gene in both India and the Philippines (Hittalmani et al., 2000). Similarly, the widely cultivated aromatic rice hybrid Pusa RH10, carrying the introgressed BB resistance genes *Xa3* and *Xa21* in India, maintained the performance of agronomic traits and showed enhanced BB resistance (Basavaraj et al., 2009, 2010). Deployment of rice broad-spectrum resistance genes is a second widely adopted strategy to lessen the risk of the breakdown. For example, the blast resistance gene *Pi2* confers resistance to 455 blast isolates from different regions in the Philippines and most of the 792 isolates from 13 important rice-growing regions in China (Chen et al., 1996). Zhu et al. (2004) analyzed the resistance spectrum of different genes in near-isogenic lines and found that the resistance frequency of *Pi1* was 80.1%, as shown by artificial inoculation, with 146 isolates of rice blast from Guangdong Province, China. He et al. (2001) successfully introgressed *Pi1* and *Pi2* into CO39 to breed BL122, which showed a broader resistance spectrum than lines with only *Pi1* or *Pi2* alone. At the aspect of the improvement of BB resistance in rice, most of the reported BB resistance genes, besides *Xa4* and *Xa21*, have not been used in breeding programs. However, the *Xa4* gene in rice hybrids F_1 showed incomplete dominance, and the broad-spectrum resistance gene *Xa21* was only expressed at the late tillering stage and its resistance level was affected by the genetic background (Zhou et al., 2011). *Xa23*, a novel BB resistance gene from the wild rice species *Oryza rufipogon*, was found to be strongly resistant to all 20 strains of BB, which included 10 Philippine races, 7 Chinese races, and 3 Japanese races, throughout all rice growth stages and exhibited the broadest spectrum resistance of all identified BB resistance genes (Zhang et al., 2001, 2009). *Xa23* has been introgressed into the popular rice restorer line Minghui63, as well as into YR293 and Y1671. The improved lines showed strong resistance to BB (Zhou et al., 2011).

In this study, we used the elite rice cytoplasmic male sterility (CMS) maintainer line Rongfeng B, which has a short growth period, good combining ability and high out-crossing rate. Several hybrid rice combinations developed using this line, have been released for commercial production. One of these hybrids, Ganxin203, has been identified as a super-hybrid rice combination by the Ministry of Agriculture, P. R. China, in 2009. However, the poor resistance to blast and BB in Rongfeng B and the related Rongfeng A the cytoplasmic male sterile line has limited their wide application in hybrid rice breeding. Therefore, the improvement of BB and blast resistance in Rongfeng B is necessary for its broader use. This study reports the successful improvement of resistance to rice blast and BB in Rongfeng B by pyramiding the *Pi1*, *Pi2,* and *Xa23* resistance genes in this line, using marker-assisted selection (MAS).

MATERIALS AND METHODS

Plant materials and the gene-pyramiding procedure

The plant materials included the recurrent parent Rongfeng B, an elite, early maturing CMS maintainer line, and the donors BL122, carrying the blast-resistant genes *Pi1* from West Africa cultivar LAC23 and *Pi2* from Columbia variety 5173 and CBB23, with the BB-resistant gene *Xa23* from the wild rice species *Oryza rufipogon*. Pyramiding the resistance genes into the rice hybrid parental lines Rongfeng B (the maintainer line) and Rongfeng A (the CMS line) was performed by using a marker-assisted backcross breeding program. Rongfeng B was crossed with BL122 and CBB23 in the early season of 2004. Respective backcross programs were then performed continuously to the BC_4F_1 generation. The plants with *Pi1* and *Pi2* or with *Xa23* were identified by using MAS in every backcrossing generation. BC_4F_1 plants carrying *Pi1* and *Pi2* genes were crossed with the BC_4F_1 plants, with *Xa23* in the late season of 2006. In the early season of 2007, the F_1 generation was grown and self-crossed. The subsequent F_2 to F_4 generations were continuously grown and self-crossed, and the elite individual plants with *Pi1*, *Pi2*, and *Xa23* were selected by using both conventional breeding methods and MAS. The F_4 plants with the 3 resistant genes were selected, crossed, and backcrossed to the original CMS line Rongfeng A to BC_4F_1 (Figure 1).

DNA markers and primer sequences

Pi1, *Pi2*, and *Xa23* were detected by using the 3 microsatellite markers MRG4766, AP22, and RM206, which were linked to these 3 resistance genes at a genetic distance of 1.3, 1.2, and 1.9 cM, respectively (Chen et al., 2005; Wu et al., 2002; Wang et al., 2005). The accuracy of selection for *Pi1*, *Pi2*, and *Xa23* using MRG4766, AP22, and RM206 was 98 to 100, 97, and 91.3%, respectively (Chen et al., 2005; Wang et al., 2005). The DNA marker and primer sequences are listed in Table 1.

Polymerase chain reaction (PCR) analysis

The genomic DNA of the recurrent parent, donors, and improved lines was isolated from 5 cm of fresh seedling leaf material using

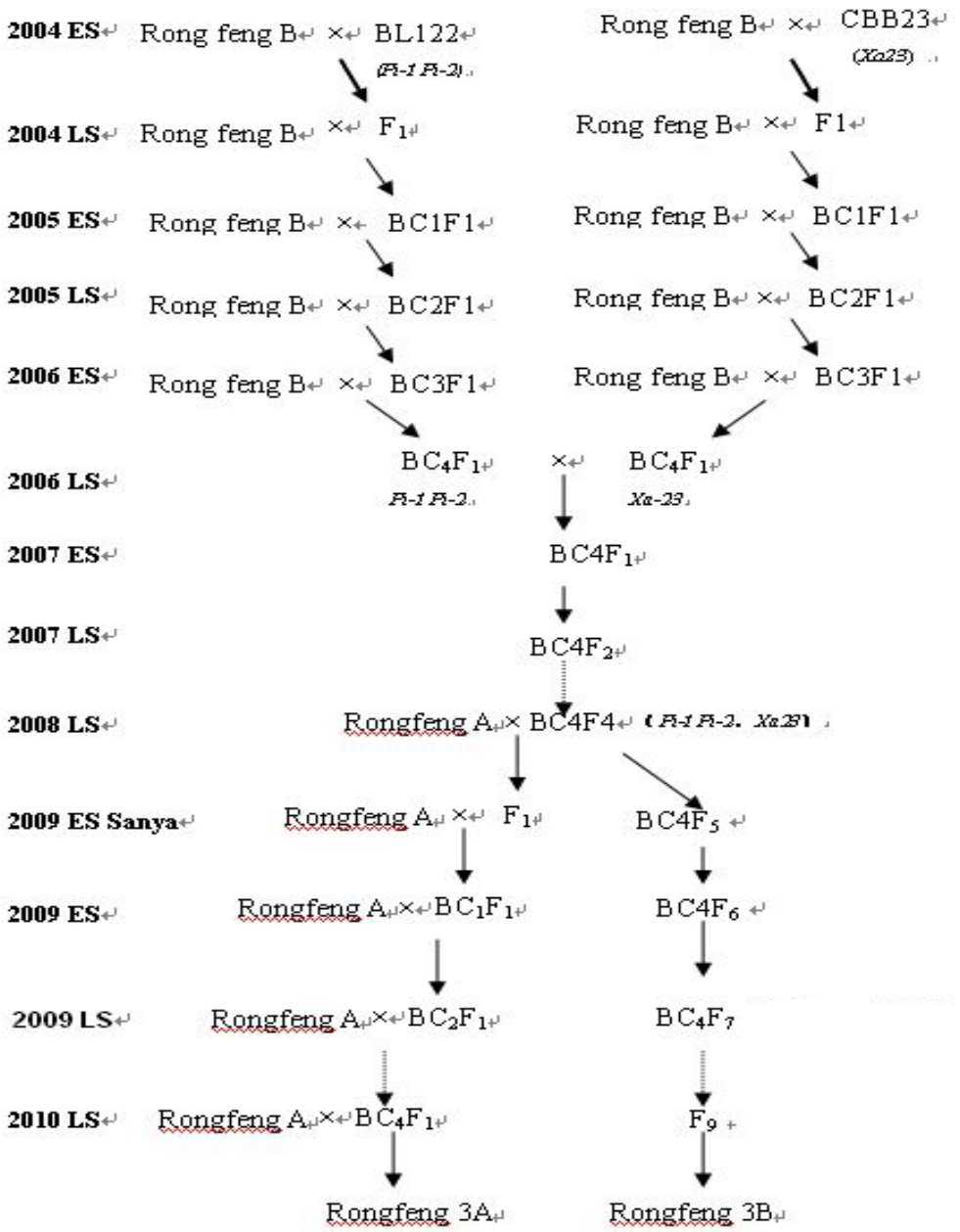

Figure 1. Procedure of pyramiding blast and bacterial blight resistance genes into parental lines rongfeng A/B.

Table 1. Tightly linked markers used of *Pi-1*, *Pi-2* and *Xa23* genes and their primer sequences.

Marker	Primer sequence	Clone	Length (bp)	Motif
MRG4766	F: ATTGCTGCAAAGTGGGAGAC R: AAGTGGAGGCAGTTCACCAC	AC136064	104	$(AGA)_9$
AP22	F: GTGCATGAGTCCAGCTCAAA R: GTGTACTCCCATGGCTGCTC	AP003522	143	$(GCC)_9$
RM206	F: CCCATGCGTTTAACTATTCT	AF344027	147	$(CT)_{21}$

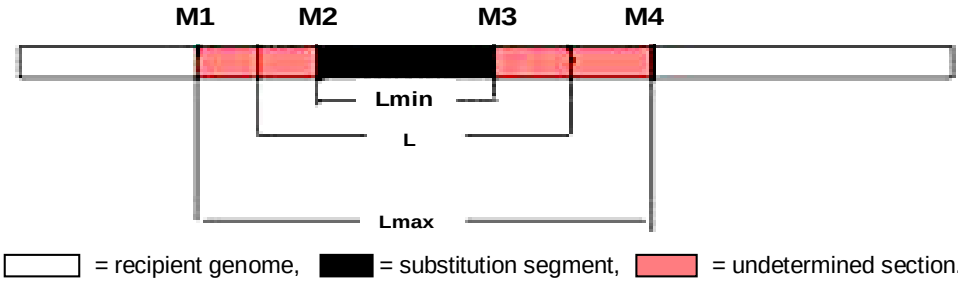

Figure 2. Diagrammatic representation of segment length estimation. Notes: M2, M3 polymorphic substituted markers, M1, M4 polymorphic non-substituted markers. Lmin represents the minimum segment length; Lmax represents the maximum segment length; L represents the estimated segment.

the alkali treatment method developed by Sang et al. (2003). The PCR reaction was performed in a 25 μl reaction mixture containing 2 μl of template DNA (50 ng), 2.5 μl of 10× PCR buffer with $MgCl_2$, 2 μl of 2.5 mM dNTPs, 2 μl of 1.25 mM forward and reverse primers, and 1 U *Taq* DNA polymerase. The PCR reaction was initiated by denaturation at 94°C for 5 min, followed by 30 cycles of PCR amplification (denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 30 s) and finally incubation at 72°C for 10 min. The PCR products for MRG4766, AP22, and RM206 were mixed with 10× loading dye (bromophenol blue) and resolved through electrophoresis on an 8% polyacrylamide gel. The electrophoresis was conducted for about 1.5 h (180 V) with a 1× TBE buffer in an electrophoresis set (Model-DYY III28D, Beijing Liu Yi Instrument Factory, Beijing). The PCR banding patterns were recorded after silver staining.

Recovery ratio of the genetic background

The recovery ratio of the genetic background was calculated on the basis of molecular-marker analysis by using the formula used in the study by Hospital et al. (1992):

$$G(g) = [L + X(g)] / (2L) = 1/2 + (1/2)\,(X(g) / L)$$

Where, G(*g*) is the recovery ratio of the genetic background at *g*th generation, X(*g*) is the number of polymorphic markers showing the band type of the recipient or recurrent parent at the *g*th generation, L is the total number of markers used in the analysis, and *g* is the backcross generation. The theoretical recovery ratio of the genetic background was calculated by using the following formula:

$$E\,[G(g)] = 1 - (1/2)^{g+1}$$

Where, *g*, is the backcross generation.

Evaluation of the length of residual donor segments in the improved lines

The lengths of residual chromosomal donor segments were evaluated in the improved lines following the study of Hospital (2002) and Xi et al. (2006). When 2 polymorphic markers (M2 and M3) showed the donor type, the segment between them was considered to be a residual segment from the donor, and the length of the segment was considered to be the minimum length (Lmin). When the flanking polymorphic markers (M1 and M4) of M2 and M3 showed the recipient type, the length of the segment between M1 and M4 was considered to be the maximum length (Lmax) of the residual donor segment. The minimum length plus one-half the length of the segments from M1 to M2 and from M3 to M4 was considered to be the length of residual donor segments in the improved lines (Figure 2). The marker position was determined based on the physical map of Nipponbare by Basic Local Alignment Search Tool (BLAST) analysis (http://www.gramene.org/Multi/blastview), and then the value of the segment length between two markers was calculated according to 1 cM of genetic distance, being equal to about 250 kb of physical distance in rice (Wu and Tanksley, 1993).

Evaluation of resistance to rice blast and BB

At the 3-leaf stage, the seedlings of the improved lines and the recurrent parent Rongfeng B were sprayed with highly virulent blast isolates collected from the infected rice plants in an epidemic region of Guangdong province. The disease reaction was scored on a scale ranging from 0 (resistant) to 9 (susceptible), 8 days after inoculation (Ou, 1985). In addition, these materials were grown in the blast nursery in Lvtian, Guangzhou, to evaluate other grades of resistance to leaf and neck blast. BB resistance was evaluated by artificially inoculating plants in the improved lines, Rongfeng B, and donor *Xa23* lines, at the maximum tillering stage, with isolate IV of *Xoo* (provided by the Institute of Plant Protection Reserarch Institute,, Guangdong Academy of Agricultural Sciences), following the standard procedure described by Fang (1990). The lesion lengths were evaluated 20 days after inoculation, with the mean lesion lengths recorded for 6 leaves.

Measurement of agronomic traits

The agronomic traits measured included plant height (PH), heading date (HD), number of effective tillers (NET), number of grains per panicle (NGP), seed-setting rate (SR), and the weight of 1000 grains (TGW). PH was measured from the soil surface to the tip of the panicle. HD was recorded when 50% of the plants headed. NET, NGP, and SR were measured on the basis of 5 individual plants randomly selected in each plot at maturity.

RESULTS

Polymorphisms of the tightly linked markers in the recipient and donor lines

The tightly linked simple sequence repeat (SSR) markers

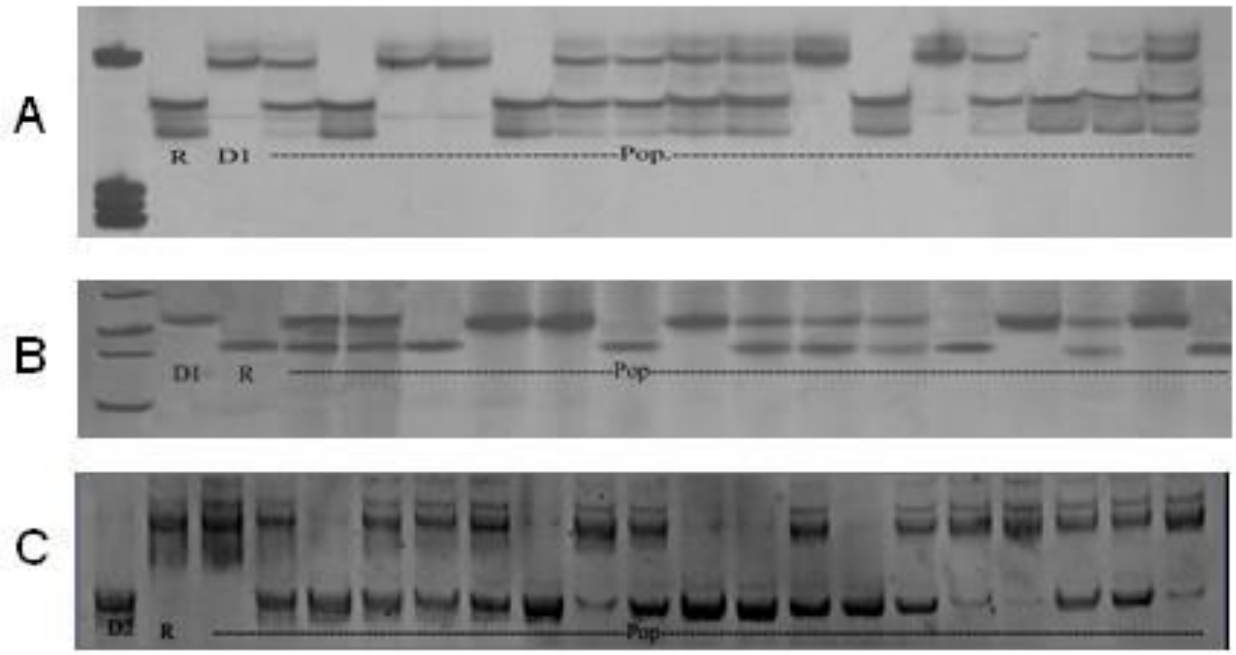

Figure 3. The band types of the PCR products amplified with the specific markers in F_2 populations. A: Screening *Pi1* gene with the specific marker MRG4766; B: screening *Pi2* gene with the specific marker AP22; C: screening *Xa23* gene with the specific marker RM206; R: the recurrent parent Rongfeng B; D_1: the donor BL122; D_2: the donor CBB23.

MRG4766, AP22, and RM206 were used for the blast resistance genes *Pi1* and *Pi2* and the BB resistance gene *Xa23*, respectively, in the PCR analysis of the recipient parent Rongfeng B and the donors BL122 and CBB23. The PCR banding patterns revealed that these markers were polymorphic in the recurrent parent, the donors, and their F_1 progeny (Figure 3).

Evaluation of rice blast resistance in the improved lines

The blast resistance of the 2 improved lines D521 and D524 (BC_4F_7; both carried *Pi1*, *Pi2*, and *Xa23*), Rongfeng B, and BL122, was evaluated with 30 isolates of *M. grisea* belonging to the B13, B15, C13, C15, and F01 races. D521 displayed resistance to 29 of the 30 blast isolates used; the single exception to resistance was isolate 09-0167a. D521 showed a resistance frequency of 96.7%. D524 showed resistance to all the isolates used, with a resistance frequency of up to 100%. However, 13 of the 30 isolates were able to infect Rongfeng B, which showed a resistance frequency of only 56.7%. BL122 showed resistance to all 30 isolates used and had a resistance frequency of 100% (Table 2). These results indicated that the resistance of the 2 improved lines was much stronger than that of Rongfeng B, and the introgression of *Pi1* and *Pi2* remarkably enhanced the level of resistance to B13. The 2 improved lines and Rongfeng B were grown in Lvtian, a natural disease nursery, and surrounded by the susceptible variety Guanglu'ai 4 (the induced line). The improved lines showed high resistance to leaf and neck blast, resulting in an infection score of 0 with both lines. In contrast, Rongfeng B was highly susceptible to leaf and neck blast (infection score, 7).

Evaluation of BB resistance in the improved lines

The BB resistance of the improved lines and Rongfeng B was evaluated by using isolate IV of *Xoo* (Figure 4). The mean lesion length was 31.5 cm in Rongfeng B, whereas the mean lesion lengths in D521 and D524 were 1 and 1.18 cm (Table 3), respectively, indicating that the introgression of *Xa23* significantly enhanced the resistance of the 2 improved lines to BB.

The performance of agronomic traits in the improved lines

An analysis of variance for several agronomic traits is presented in Table 3. Significant variances were found for NGP, SR, PH, TGW, and HD among the 2 improved lines and Rongfeng B. The NGP, SR, PH, TGW, and HD of Rongfeng B were 99.06, 82.41%, 86.83 cm, 24.45 g, and 56 days, respectively. The means for D521 and D524 were 150.16 and 132.56 (GNP), 86.25 and 89.22% (SR), 92.83 and 94.67 cm (PH), 23.77 and 25.10 g (TGW), and

Table 2. The resistance to 30 isolates of blast pathogen for the improved lines.

Race	Isolate	Rongfeng B (CK)	BL122 (donor)	Improved lines	
				D521	D524
B13	04-0099a	S	R	R	R
B13	93-0286a	S	R	R	R
B13	95-0059a	R	R	R	R
B13	08-0679a	S	R	R	R
B13	W08-59a	S	R	R	R
B13	08-0265a	R	R	R	R
B13	09-0037a	S	R	R	R
B13	08-0923a	R	R	R	R
B15	00-0193a	S	R	R	R
B15	08-0937a	S	R	R	R
B15	98-0288a	S	R	R	R
B15	00-0173a	R	R	R	R
B15	09-0203a	S	R	R	R
B15	06-0141a	S	R	R	R
B15	09-0004-3	R	R	R	R
B15	09-0238-1	S	R	R	R
B15	09-0644a	R	R	R	R
B15	W08-28a	R	R	R	R
B31	08-0507a	R	R	R	R
C13	04-0206a	R	R	R	R
C13	09-0526a	R	R	R	R
C13	08-T29	R	R	R	R
C13	09-0281a	S	R	R	R
C13	09-0094a	R	R	R	R
C13	09-0174a	R	R	R	R
C13	09-0167a	R	R	S	R
C13	09-0111a	R	R	R	R
C13	09-2004a	R	R	R	R
C15	09-0235a	S	R	R	R
F01	09-0091a	R	R	R	R
Resistance frequency (%)		56.7	100	96.7	100

Figure 4. Resistant reactions of improved lines D521 and D524, and control line Rongfeng B to *Xanthomonas oryzae pv. Oryzae (Xoo)*.

Table 3. Agronomic traits, BB reaction and recurrent parent genome (RPG) recovery ratio of improved lines.

Line	NET	NGP	SR (%)	TGW	PH	HD	BB lesion length (cm)	RPG (%)
Rongfeng B (CK)	9.58	99.06	82.41	24.45	86.83	56	31.5	
D521	8.56	150.16	86.25	23.77	92.83	64	1	96.18
D524	8.78	132.56	89.22	25.10	94.67	65	1.18	95.56

Notes: PH: Plant height, HD: heading date, NET: numbers of effective tillers, NGP: numbers of grains per panicle, SR: seed setting rate, TGW: weight of 1000 grains, RPGR: recurrent parent genome recovery.

Table 4. Polymorphic markers among the improved lines and recurrent parent.

Chromosome	Polymorphic markers between D521 and Rongfeng B	Polymorphic markers between D524 and Rongfeng B
2	RM71	RM71
4	RM471, RM307, PSM101	RM307, PSM101
6	RM564, RM253, AP22	RM564, RM253, AP22
7	RM351	RM351
11	RM21,PSM366, MRG4766, RM206, PSM346	PSM366,RM21.MRG4766, RM206, PSM346

64 and 65 days (HD), respectively. Although the improved lines and Rongfeng B were intensively evaluated for significant differences in HD, these lines showed photoperiod insensitivity. As for NET, Rongfeng B had a slightly higher value than the 2 improved lines, but the difference was not significant. In general, both the improved lines had Rongfeng B-like characteristics, and most of the agronomic traits were improved.

Recovery ratio of the genetic background in the improved lines

For identifying polymorphic markers for the backcross selection, 346 SSR markers evenly spanning all 12 rice chromosomes, were used to identify polymorphisms between Rongfeng B and the donors BL122 and CBB23. Among these, 94 (26.26%) and 100 (27.93%) markers were found to be polymorphic between Rongfeng B and BL122 and between Rongfeng B and CBB23, respectively. A total of 131 markers (36.31%) showed polymorphisms among Rongfeng B and the donors BL122 and CBB23. These 131 polymorphic markers were used to test the recovery ratio of the genetic background for the improved lines. The results suggested that 13 and 12 markers were polymorphic between D521 and Rongfeng B, and between D524 and Rongfeng B, respectively (Table 4). After 4 backcrosses, the recovery ratios of the recurrent-parental genome at non-target loci for D521 and D524 were 96.18 and 96.56%, respectively (Table 3). The majority of the residual segments from the donor genome were distributed on Chromosome 4, 6 and 11. One residual donor segment was observed on Chromosome 2 and 7. Perfect recovery of the recurrent parent's chromosomes was observed on Chromosome 1, 3, 5, 8, 9, 10 and 12. The physical distance between RM564 and AP22, which was tightly linked with *Pi2* on Chromosome 6, was approximately 500 Kb (~2 cM). RM21 on Chromosome 11 was found to be closely linked with RM206 and *Xa23.*

The data of minimum length, maximum length and estimated length are listed in Table 5. The estimated length of the residual segments RM457--RM21-PSM366-RM5997 on Chromosome 11 and RM539--AP22-RM564--RM193 on Chromosome 6 was up to 6.55 and 21.62 cM, respectively. The estimated length of the residual segment RM70--RM351--RM505 on Chromosome 7 and RM1341--RM206--RM2859 on Chromosome 6 was 7.2 and 5.4 cM, respectively. Whereas, the estimated length of the other residual segments was less than 5.0 cM. RM185--RM471---PSM321 on Chromosome 4 was the shortest segment at 1.73 cM. These large residual segments may carry genes affecting the agronomic traits of the improved lines. The distribution of the segments which introgressed into Rongfeng B from the donors is displayed in Figure 4.

DISCUSSION

The acceleration in the use of broad-spectrum resistance genes has been an effective strategy in alleviating the risk of a breakdown in resistance. Monitoring markers closely linked with resistance genes by MAS can be used to overcome the limitations of conventional breeding methods, and to increase the efficiency and probability of selecting the target traits during backcrossing (Jena and Mackill, 2008). Rongfeng B is an elite, early maturity

Table 5. Length of the target and residual segments from the donor.

Line	Residual segment	Chromosome	Minimum length (cm)	Maximum length (cm)	Estimated length (cm)
D521	RM145--RM71--RM438	2	0	7.23	3.62
	PSM111--PSM101--PSM103	4	0	3.5	2.6
	PSM357--RM307--PSM358	4	0	5	2.5
	RM185--RM471---PSM321	4	0	3.45	1.73
	RM276--RM253--RM314	6	0	9.5	4.75
	RM539--AP22—RM564--RM193	6	3.57	39.66	21.62
	RM70--RM351--RM505	7	0	14.4	7.2
	RM457--RM21—PSM366—RM5997	11	2.7	10.4	6.55
	RM4069-- MRG4766--RM2136	11	0	8.1	4.1
	RM1341--RM206--RM2859	11	0	10.8	5.4
	RM2859--PSM346--RM7277	11	0	7.6	3.8
D524	RM145--RM71--RM438	2	0	7.23	3.62
	PSM111--PSM101--PSM103	4	0	3.5	2.6
	PSM357--RM307--PSM358	4	0	5	2.5
	RM276--RM253--RM314	6	0	9.5	4.75
	RM539--AP22—RM564--RM193	6	3.57	39.66	21.62
	RM70--RM351--RM505	7	0	14.4	7.2
	RM457--RM21—PSM366—RM5997	11	2.7	10.4	6.55
	RM4069-- MRG4766--RM2136	11	0	8.1	4.1
	RM1341--RM206--RM2859	11	0	10.8	5.4
	RM2859--PSM346--RM7277	11	0	7.6	3.8

CMS maintainer line, with good breeding qualities; however, its poor resistance to rice blast and BB has been an important constraint in its wide use in hybrid rice-breeding programs. The primary objective of this study was to improve the resistance to rice blast and BB, to enhance the utilization of Rongfeng B. By combining MAS with phenotype-based visual selection for agronomic traits, the blast resistance genes *Pi1* and *Pi2* and the BB resistance gene *Xa23* were successfully introgressed into Rongfeng B, and a new maintainer line Rongfeng 3B (D524) and its relative cytoplasmic male sterile line Rongfeng 3A, which carry *Pi1*,*Pi2* and *Xa23*, were developed by backcrossing D524 to the original Rongfeng A.

Performance of blast resistance for the improved lines D521 and D524

MAS for the transfer of resistance genes have been successfully used in several studies (Chen et al., 2000; Basavaraj et al., 2009; Wongsaprom et al., 2010). To breed durable resistant rice varieties, pyramiding varous R genes with different resistance spectra into a single rice cultivar, to reduce the selection pressure on a single blast isolate is an effective way (Skamnioti and Gurr, 2009). Hittalmani et al. (2000) pyramided three major genes (Pi1, Piz-5 and Pita), and developed some pyramid lines with two and three-gene combinations; all of them were found to be resistant to the compatible isolates tested for all the three genes. He et al. (2001) found that the pyramided line BL122, which carried *Pi1* and *Pi2,* showed broad-spectrum and durable resistance to *M. grisea*. Yang et al. (2008) analyzed the race specificity of major rice blast resistance genes to *M. grisea* isolates collected from Guangdong, China, and found that the pyramiding of the resistance genes *Pi1* and *Pi2* could be effective in obtaining broad-spectrum and durable blast resistance in rice breeding in Guangdong. Therefore, we selected the genes *Pi1* and *Pi2* to improve blast resistance in Rongfeng B. Compared with the blast resistance of the original Rongfeng B, the improved lines D521 and D524 carrying *Pi1* and *Pi2* showed significantly enhanced blast resistance. It is further verified that *Pi1* and *Pi2* are two effective genes in breeding rice varieties with high-level resistance against blast. Since *Pi1* gene was originated from the West Africa cultivar LAC23, and *Pi2* was derived from Columbia variety 5173, we believe that the improved parental line with *Pi1* and *Pi2* could be effectively used to develop rice varieties of blast resistance to cope with this devastating disease of rice, not only in Asia but also in Africa and Latin America.

In this study, we found that the improved line D521 was

infected by the isolate 09-0167a (Table 2), while the recipient Rongfeng B and the improved line D524, as well as the donor BL122 were not infected by it. it is possible that a given small segment harboring minor-effect QTL conferring resistance to the isolate 09-0167a in Rongfeng B was exchanged, or the resistance to the isolate 09-0167a was a result of the interaction between two or among more QTLs conferring blast resistance, since the improved line D521 has 11 residual segments of the donor Bl122 to be left on Chromosome 2, 4, 6, 7 and 11, respectively, and the genetic background of D521 was different from D524.

Performance of BB resistance for the improved lines D521 and D524

Prevalence of BB disease in Asia, Africa and Latin America is endangering the popularization of the popular hybrids as a result of their susceptibility. CBB23 with *Xa23* gene is an effective donor against all races collected from China, Philippines and Japan (Zhang, 2005). By combining MAS with artificial inoculation, it was possible to incorporate BB resistance genes *Xa23* into the genetic backgrounds of Rongfeng B. *Xa23* has been introduced into several conventional cultivars and restorer lines (Luo et al., 2005; Li et al., 2006; Qin et al., 2007; Zhou et al., 2009, 2011). The resistance level of F_1 hybrid carrying *Xa23* gene in different genetic backgrounds was evaluated, and it was found that there was no genetic background effect on the expression of this gene (Zhou et al., 2011), which suggest that *Xa23* is of great value in breeding rice hybrids with BB resistance. Wang et al. (2005) found that the accuracy of RM206 MAS for *Xa23* was 81 to 95%. In this study, RM206 was used to select *Xa23*, and the resultant pyramided lines carrying *Pi1, Pi2*, and *Xa23* did not only express high resistance with broad spectrum during the entire growth stages, but also exhibited good agronomic traits. These improved lines could serve as immediate sources of BB resistance for hybrid rice breeding and the direct use of their relative sterile lines in hybrid rice seed production. Since the breeding of the sterile line is of long period and large amount of work, the development of BB resistant maintainer and male sterile lines will be playing a crucial role in controlling BB in hybrid rice -growing countries.

The residual donor segments and their effects on the traits

In the study, some residual donor segments were found in the improved lines. In these residual segments, the majority of residual segments were found at the centromeric regions or the telomeric and subtelomeric regions. Although, 346 SSR markers were used to analyze the recovery ratio, less than 30 SSR markers on each chromosome was still insufficient to finely evaluate the length of the residual segments. In addition, 1% recombinant rate around centromeric regions was equivalent to 1000 to 2500 kb physical distance (Copenhaver et al., 1999). The above factors could be the cause that the estimated length of the target segments of AP22 was up to 21.62 cM. The centromeric regions are highly conserved in function and are hetero chromatic (Henikoff et al., 2001; Sullivan et al., 2001); the residual segments in the centromeric regions should not result in significant variation in most traits. Mizuno et al. (2006) found that the rice chromosome ends were heterogeneous in both sequence and structure, and that the length of the telomere repeats was variable. This group suggested that this type of variation might be the result of genetic or epigenetic differences among the subtelomeric sequences. If so, this would indicate that the variation in the telomeric regions does not influence the variation for some traits. Since the genomic segments and the manner of the interaction contributing towards heterosis are not characterized, it is important to recover the original genome as far as possible, to maintain the level of heterosis of the original parent. Several hybrid rice combinations derived by the relative male sterile lines of two improved lines have been arranged in the regional yield trials of Guangdong Province, and showed good general agronomic traits.

In addition to blast and BB resistance, the improved lines showed desirable variation for several agronomic traits, including NGP, TGW, PH, SR, and TGW. However, the significant delay in HD of the improved lines would increase the risk of the use of this variety, especially in hybrid rice, for some ecological regions. The variance in HD was found to be caused by the introgression of *Pi2* (data not shown). Fujino and Sekiguchi (2008) reported that the QTL *qDTH6-2* was located 30.5 cM from *Hd1* on Chromosome 6 and plays an important role in controlling the HD among cultivars with extremely low photo-sensitivity. The polymorphic marker RM253 was found to be located at the region of *qDTH6-2,* as revealed by an analysis of genome information in rice (http:// www.gramene.org). However, the appropriate delay of heading date is not necessarily a bad thing, and could help more in the popularization and application for the maintainer and its related male sterile line with short heading date. Although the improved lines carried several residual segments from donors, the improved lines showed the desirable agronomic traits and significantly stronger blast and BB resistance, compared with Rongfeng B. The corresponding male sterile lines of the improved lines showed good breeding quality (data not shown) and have been used in hybrid rice seed production. Additionally, several new combinations of newly developed hybrid rice will be involved in regional yield trials. The improved maintainer lines of Rongfeng3B are considered to be good donors of resistant genes in further breeding for resistance.

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