Breeding of R8012, a Rice Restorer Line Resistant to Blast and Bacterial Blight Through Marker-Assisted Selection

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Abstract: Genetic improvement is one of the most effective strategies to prevent rice from blast and bacterial blight (BB) diseases, the two most prevalent diseases jeopardizing rice production. Rice hybrids with durable resistance to blast and BB are needed for sustainable production of food. An incomplete diallele design resulted in 25 crosses between five blast and five BB resistant germplasm accessions. Only one pair of parents, DH146 × TM487, showed polymorphism for all the markers to identify one blast resistance gene P25 and three BB resistance genes, Xa21, xa13 and xa5, thus it was used in the marker-assisted selection (MAS). F2 individuals of DH146 × TM487 were genotyped using flanking markers of RM3330 and sequence tagged site (STS) marker SA7 for P25. The resistant F2 plants with P25 were used for pyramiding BB resistance genes Xa21, xa13 and xa5 identified by the markers pTA248, RM264 and RM153, respectively in subsequent generations. Finally, after selection for agronomic traits and restoration ability among 12 pyramided lines, we acquired an elite restorer line, R8012 including all four target genes (P25+Xa21+xa13+xa5). Hybrid Zhong 9A/R8012 derived from the selected line showed stronger resistance to blast and BB, and higher grain yield than the commercial checks uniformly in experimental plots, 2007 state-wide yield trial and 2008 nation-wide yield trial. This study provides a paradigmatic example to show that MAS is a practically feasible tool in effectively pyramiding multiple resistance genes. The resultant restoring line and its hybrid would play an important role in securing rice production in China.

Key words: rice restorer line; bacterial blight; blast; marker-assisted selection; gene pyramiding; breeding

Rice blast (caused by Magnaporthe grisea) and bacterial blight (BB, caused by Xanthomonas oryzae pv. Oryzae, Xoo) are two of the most destructive diseases leading to severe yield losses in rice production worldwide (Narayanan et al, 2004). The most effective approach to prevent the two diseases is the genetic improvement using resistant varieties (Khush et al, 1989). With few exceptions, the control of rice diseases depends on main-effect resistance genes in host varieties. So far, 73 blast resistance genes and 31 BB resistance genes have been identified (Ballini et al, 2008; Ruan et al, 2008) and some of them have been incorporated into modern rice varieties. However, because of large-scale monoculture of disease-resistant variety and the emergence of new pathogen physiological strains and biotypes, loss of resistance to diseases is a constant threat to rice production. Breeding cultivars with durable and multiple resistance is a new challenge for plant pathologists and breeders. Pyramiding multiple resistance genes into an existing variety proves to be an effective approach for achieving broad-spectrum and durable resistance in rice (Huang et al, 1997; Chen et al, 2009). However, the pyramiding is difficult in using conventional breeding methods due to the dominance and imperfectly penetrant effects of genes governing disease resistance. Conventional breeding selects resistant offspring from disease evaluation and the evaluation is difficult for multiple diseases and the same disease controlled by multiple genes. Furthermore, accuracy of the evaluation is largely affected by environments and technical operations. Fortunately, the availability of molecular markers closely linked with each of the resistance genes makes the identification of offspring with two or more genes possible, e.g. marker-assisted selection (MAS).

Numerous reports suggest that MAS is more effective and economical than traditional phenotypic selection in developing disease-resistant varieties (Sanchez et al, 2000; Chen et al, 2001; Singh et al, 2001; Cao et al, 2003; Narayanan et al, 2004). Through MAS as well as genetic transformation, indica varieties, ID50 and CO39 with enhanced BB and blast resistance, have been developed (Narayanan et al, 2002, 2004). Aiming to combine the important Basmati quality with resistance to BB, cultivar IRBB55 carrying BB
isolate *xa13* and *Xa21* was used to increase the BB resistance to Pusa Basmati-1 (Joseph et al., 2004).

We previously reported a novel blast resistance gene *Pi25* that confers resistance to both leaf and neck blast. It is derived from the variety Gumei 2 and located on chromosome 6. This gene has great potential in disease-resistance breeding programs (Wu et al., 2000; Zhuang et al., 2002). The objective of this study was to pyramid the *Pi25* gene for blast resistance and three genes *Xa21*, *xa5* and *xa13* for BB resistance through MAS, and to develop new restorer lines conferring resistance to both blast and BB. The bred lines can be used as valuable breeding resources for further development of new hybrid cultivars with high-yielding potential and broad-spectrum and dual-resistance to blast and BB in rice.

**MATERIALS AND METHODS**

**Rice materials and markers for resistance genes**

Recombinant inbred line (RIL), '3-11', derived from Zhenshan 97B/Milyang 46, was crossed to three BB resistant varieties, IRBB58, IRBB59 and IRBB60, respectively. Five pyramided lines of BB genes with superior agronomic traits were selected from the three F6 populations consisting of 203 lines as BB donors: TM443 (*Xa21*+*xa5*), TM475 (*Xa21*+*xa13*+*xa5*), TM487 (*Xa21*+*xa13*+*xa5*), TM490 (*Xa21*+*xa13*+*xa5*) and TM510 (*Xa21*+*xa13*+*xa5*+*Xa4*). Five *Pi25* homozygous lines with restorer genes and superior agronomic traits named DH146, DH166, DH190, DH326 and DH388 derived from Gumei 2/Zhong 156 were used as the donors of *Pi25* (Zhou et al., 2008).

Twenty-five crosses designed by incompletely diallel cross were made between the above-mentioned two sets of selected lines for identification of proper cross for MAS. Based on our previous results for mapping *Pi25* (Wu et al., 2000, 2004), simple sequence repeat (SSR) marker RM3330 and sequence tagged site (STS) marker SA7 on each side of *Pi25*, respectively, were used for selection of *Pi25* gene (Zhou et al., 2008). Tightly linked STS marker pTA248 and SSR markers RM264 and RM153 that have been reported for BB resistance genes, *Xa21*, *xa13* and *xa5* (Ronald et al., 1992; Matthew et al., 2003; Basharat et al., 2006), respectively, were used for selection of these genes in each generation of offspring (Table 1).

The prerequisite for MAS is to obtain DNA markers polymorphic between the parents. The above mentioned markers for blast resistance gene *Pi25*, and BB resistance genes, *Xa21*, *xa13* and *xa5*, were polymorphic only between DH146 and TM487 (Fig. 1). Thus, only the cross of DH146 × TM487 was used for the following MAS. Progenies of the other 24 crosses were used as breeding materials and advanced to higher generations on basis of field performance. *F1* seeds of the cross between DH146 and TM487 were harvested and extended to *F2* generation in Hainan Province, China. The *F2* plants were inoculated with a selected *M. grisea* isolate 05-20-1 to screen plants carrying blast resistance gene *Pi25*, which is verified by genotyping DNA markers flanking *Pi25* on both sides.

**Marker-assisted selection for resistance genes**

Mini-scale DNA isolation for PCR analysis of the parents and progenies in each segregating generation for selection of the resistance genes was carried out from 25-day old seedlings according to the procedures described by Zheng et al (1995). The sequences for all

![Fig. 1. Reaction of pyramiding parents inoculated with *M. grisea* isolate 05-20-1 for neck blast resistance.](image)

Parent TM487 was susceptible while parent DH146 was resistant to the isolate 05-20-1.
the resistance markers were from the Rice Genome Research Program (RGP) in Japan and further synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, China. The primers for resistance gene analogue (RGA) were amplified based on the methods described by Zhou et al (2008). The PCR products were detected on either 1.5% agarose gel or 6% denaturing polyacrylamide gels using silver staining.

**Bacterial blight evaluation**

Those selected lines from molecular marker analysis were inoculated with five most predominant Xoo isolates, 05-10, 05-20, 94-11, Zhe 173 and C23, which popularly occur in the South China. By leaf-clipping method (Zhang et al, 2000), five leaves of a plant were clip-inoculated with bacterial suspensions of $10^6$ cfu/mL at the booting stage. Three plants of each line were inoculated with each of the five isolate-genotypes. Lesion areas of each inoculated leaf were measured 15 d after inoculation. Reaction of rice plant to bacterial blight was determined by the ratio ($P$) of the lesion area to entire leaf area, a standard grading scale of 1–9, as resistance (R) with scores 1–2 and $P \leq 5\%$; moderately resistance (MR) with scores 3–4 and $5\% < P \leq 20\%$; moderately susceptible (MS) with scores 5–6 and $20\% < P \leq 50\%$; susceptible (S) with scores 7–8 and $50\% < P \leq 75\%$; and highly susceptible with score 9 and $P \geq 75\%$.

**Blast evaluation**

The parents and their progenies that contained the pyramided resistance genes resulted from MAS were evaluated for blast resistance using a highly pathogenic isolate ‘05-20-1’, a predominant isolate in Zhejiang Province, China. The evaluation replicated twice was conducted in the rice blast nursery at Zhejiang Academy of Agricultural Science, China. 30–40 seeds of each line were sown to a plastic tray for inoculation with rice blast isolate 05-20-1 after three weeks. The plants in each tray were sprayed with 50 mL of inoculum ($5 \times 10^5$ cfu/mL), maintained at 25 °C in a dew chamber for 24 h, transferred to a mist chamber at 25 °C and rated for disease reaction one week later. Seven days after the first rating, those survived plants were re-evaluated for resistance to blast isolate 05-20-1. Those healthily resistant plants were transplanted to breeding nursery in China National Rice Research Institute (CNRRI). Disease scoring was expressed as a percentage of the diseased leaf area (DLA) over the total area. The DLA was estimated visually following the Standard Evaluation System (SES) for rice (International Rice Research Institute, 1996), as a standard, lesion types 0–3 were scored as resistant and lesion types 4 and 5 as susceptible. An in vitro inoculation technique (Chai and Jin, 1995) was used to evaluate the neck blast resistance. Panicles were collected from main culms of five different plants of each line when they just emerged. Necks of 5–6 cm long were cut from panicles and put onto the filter paper in petri dishes. The filter paper was pre-soaked in benzimidazole solution. Both ends of the neck were covered with absorbent cotton which was also pre-soaked in benzimidazole solution. The necks were smeared with aqueous conidia suspension containing 2% carboxymethyl cellulose. These petri dishes were covered and placed under light at 28 °C. The disease reaction was scored 10 d after inoculation.

**RESULTS**

**Phenotypic screening of blast resistant plants**

The neck blast survey confirmed the expected blast reaction of the susceptible parent TM487 and resistant parent DH146 to isolate 05-20-1. Phenotypic analysis on 883 F2 plants in the cross of DH146 and TM487 was conducted to confirm the presence of Pi25 for selection of resistant plants (Fig. 2). There were 121 susceptible and 762 resistant plants identified in the first round blast evaluation. The number of resistant plants was reduced to 688 by the second round evaluation. Eventually, further selection on agronomic traits among the 688 resistant plants resulted in 186 plants for further analysis and pyramiding effort with bacterial blight in the subsequent generations. All the other F2 plants were eliminated from the subsequent analysis and breeding effort.
Pyramiding BB with blast resistance genes through MAS

F3 families derived from the 186 resistant F2 plants were genotyped using the flanking markers tightly linked to Pi25, SA7 and RM3330. The STS marker SA7 identified 84 plants homozygous and 31 heterozygous for resistance gene Pi25. The SSR marker RM3330 identified 48 plants homozygous and 97 plants heterozygous for Pi25, which left 41 plants without allele Pi25 and exhibited a goodness of fit for the expected Pi25 gene segregation ratio, 1:2:1 in F2 population. The 48 homozygous plants for Pi25 were used to further screen for BB resistance genes Xa21, xa13 and xa5 by the linked markers pTA248, RM264 and RM153, respectively. The genotyping screening showed 36 lines with Xa21, 12 lines with xa13, and 3 lines with xa5. Then, these homozygous resistant plants with two resistance genes, Pi25 for blast and one for BB, were further selected by the linked marker for the third and fourth BB resistance genes. Final results of the selected lines with various combinations of the resistance genes were presented in Table 2. We obtained 4 lines with the resistance gene combination of Pi25/Xa21/xa13/xa5, 5 lines with Pi25/Xa21/xa13, 4 lines with Pi25/Xa21/xa5, and 23 lines with Pi25/Xa21.

Reaction to BB and blast pathogens

Twelve selected F3 lines derived from F2 population that had different combinations of resistance genes with superior agronomic traits were evaluated by inoculation with five BB isolates and one blast isolate 05-20-1. Both of their parents plus a resistant check IRBB60 were also included in the evaluation. Results of the evaluation for the selected lines were displayed in Table 3 and corresponding analysis of genotypes were listed in Fig. 3. Ten F3 lines showed resistance to BB from moderate resistance (MR) to resistance (R) for all the isolates with two exceptions, L3 susceptible to the BB isolates 05-10 and C23, and L6 susceptible to the isolates 4-11 and C23. Resistance donor parent TM487 was resistant to all the BB isolates. All the 12 lines were resistant to both leaf and neck blast pathogens.

Table 2. Genotype of F2 plants identified by the flanking markers for blast resistance gene Pi25(t) and bacterial blight resistance genes Xa21, xa13 and xa5.

<table>
<thead>
<tr>
<th>No. of plants</th>
<th>P25(t)</th>
<th>Xa21</th>
<th>xa13</th>
<th>xa5</th>
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<tbody>
<tr>
<td>SA7</td>
<td>RM3330</td>
<td>pTA248</td>
<td>RM264</td>
<td>RM153</td>
</tr>
<tr>
<td>4</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>5</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>2</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>11</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

++, +− and −− represent homozygote, heterozygote and negative genotypes of the flanking markers, respectively.

Table 3. Reaction of the 12 selected pyramided F3 lines after inoculation with bacterial blight and blast pathogens.

<table>
<thead>
<tr>
<th>Rice line</th>
<th>Resistance genes genotyped by flanking markers</th>
<th>BB reaction 05-10</th>
<th>BB reaction 05-20</th>
<th>BB reaction 94-11</th>
<th>BB reaction Zhe173</th>
<th>BB reaction C23</th>
<th>Blast reaction LR</th>
<th>Blast reaction NR</th>
</tr>
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<tbody>
<tr>
<td>L1</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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</tr>
<tr>
<td>L2</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>MR</td>
<td>MR</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>L3</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>L4</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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</tr>
<tr>
<td>L5</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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</tr>
<tr>
<td>L6</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>S</td>
<td>MR</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>L7</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>MR</td>
<td>R</td>
<td>R</td>
<td>MR</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>L8</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>MR</td>
<td>R</td>
<td>R</td>
<td>R</td>
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</tr>
<tr>
<td>L9</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>L10</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>MR</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>L11</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>L12</td>
<td>++ ++ ++ ++ ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>TM487</td>
<td>-- -- -- -- -- -- --</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>IRBB60</td>
<td>-- -- -- -- ++ ++ ++</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>DH146</td>
<td>++ ++ ++ -- -- --</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

++, +− and −− represent homozygote, heterozygote and negative genotypes of the flanking markers, respectively.

LR, Leaf blast resistance; NR, Neck blast resistance; R, Resistance; S, Susceptible; MR, Moderate resistance.

Fig. 3. Resistance reaction of pyramided lines inoculated with bacterial blight strains.

1, A leaf of a resistant plant TM487; 2, A leaf of a susceptible plant DH146; 3, A leaf of a susceptible plant L3; 4, A leaf of a moderately resistant plant L4.
inoculated by the isolate 05-20-1 at the seedling and booting stages, respectively. The resistance was similar to the blast resistance donor DH146 (Table 3). In summary, we pyramided different BB and blast resistance genes into 10 F$_3$ lines successfully using MAS, and obtained 10 lines dually resistant to BB and blast.

**Breeding dual disease-resistance restorer lines**

Six lines with superior agronomic traits and strong dual resistance were selected from the aforementioned 10 F$_3$ lines, and advanced to F$_4$ generation till 2005 through continuous selfing. In each selfing population, DNA markers RM3330 and SA7 linked to *Pi*25, pTA248 linked to *Xa*21, RM264 linked to *xa*13 and RM153 linked to *xa*5 were used for MAS, respectively. Two lines numbered H1 and H2 which both carried *Pi*25, *Xa*21, *xa*13 and *xa*5 were selected for agronomic traits. Test-crossing H1 and H2 with the cytoplasmic male sterile (CMS) line Zhong 9A and Neixiang 5A were made, respectively and their hybrids were field-tested at the CNRRI experimental station in Hangzhou of 2005.

In experimental plots, Zhong 9A/H1 showed dual resistance to BB and blast, superior to Shanyou 63, therefore we named the restorer line H1 as R8012. Its hybrid ‘Zhong 9A/R8012’ had more spikelets per panicle and greater seed setting rate than Shanyou 63, indicating better restoration ability and higher yield potential than Shanyou 63. In 2006 national trial over South China, Zhong 9A/R8012 exhibited not only greater resistance to BB and blast, but also a 3.74% higher grain yield than the control hybrid IIYou 838. Because of these results, R8012 is an elite restorer line with multiple resistance genes into breeding lines, and its hybrid Zhong 9A/R8012, a newly developed three-line hybrid resistant to major diseases, is of great potential for large scale commercial production in rice.

**DISCUSSION**

Research for disease resistance genes has always been a topic of interest for breeders (Tabien et al, 2002; Cao et al, 2003). Pyramiding major resistance genes to increase resistance spectrum in commercial cultivars proves to be effective and useful (Xu et al, 1996; Huang et al, 1997; Hittalmani et al, 2000; Chen et al, 2001; Singh et al, 2001; Zhan and Cheng, 2001; Narayanan et al, 2002, 2004; Joseph et al, 2004; Chen et al, 2008). Marker-assisted selection for genes and disease evaluation for genetic verification can be conducted in laboratory conditions during seedling stage at first. The following selection for complex traits such as combining ability and grain quality, can be carried out in the fields during heading and maturity stage. The combination of laboratory and field and of early and late stages makes MAS breeding more efficient than conventional breeding. Once the disease resistance genes are selected by MAS, rice breeding can be focused on combining and restoring the agronomic traits. The two phases of selection in breeding program overcome the defects of conventional hybridization breeding in which restorer genes and resistance genes usually have low recombinant frequency (Cao et al, 2003). The low frequency requires great effort to identify the individuals combining both the resistance and restoring ability in breeding program.

Nowadays, MAS shows an increasing use in rice breeding. For example, Deng et al (2005) pyramided three resistance genes *Xa*21, *Xa*23 and *Xa*4 together by means of multiple crossing and MAS. In terms of breeding for blast resistance, three blast resistance genes *Pi*-d(t), *Pi*-b and *Pi*-t*a* from breeding lines Digu, BL-1 and Pi-4, respectively, were pyramided into an elite maintainer line Gang 46B (Chen et al, 2004). Chen et al (2008) used rice lines C101LAC and V101A51 as the donors of the resistance genes to rice blast, and three resistance genes (*Pi*-1, *Pi*-2 and *Pi*-33) were introgressed into an elite variety Jin 23B. The pyramided lines with multiple resistance genes exhibited higher level of resistance to the selected isolates than those carrying only one resistance gene in the above research, implying that pyramiding multiple resistance genes into breeding lines can broaden resistance spectrum.

In this study, we used the tightly linked SSR marker RM3330 and STS marker SA7 to identify the blast resistance gene *Pi*25, and used the tightly linked STS marker pTA248, SSR markers RM264 and RM153 in identifying BB resistance genes *Xa*21, *xa*13 and *xa*5, respectively. The elite restorer line R8012 carrying multiple genes (*Pi*25/*Xa*21/*xa*13/*xa*5) were new-bred through MAS, in which all the resistance genes can confer resistance to BB and blast, and we also succeeded in development of one hybrid rice.
combination Zhong 9A/R8012, which performed dual resistance to BB and blast, good grain quality and high yield potential in the national rice trial of South China in 2009. Incorporating different BB and blast resistance genes into restorer line can broaden its resistance to BB and blast, which indicates that MAS technology is very valuable and useful in pyramiding different resistance genes for rice breeding.

The blast resistance gene Pi25 was identified by Wu et al. (2000). It is located at chromosome 6 and shows a wide-spectrum of leaf and neck blast resistance. In the present study, the selected plants carrying Pi25 gene all performed high resistance to neck and leaf blast, and the plants with two or three pyramided BB resistance genes all performed resistance to five BB isolates, proving that pyramiding different BB and blast resistance genes together can increase the resistance spectrum, and it is a feasible and efficient way to breed restorer lines with multiple and durable resistance.

The goal of modern agriculture is ‘high and stable yield, good quality and low-pollution’, therefore, it is necessary to develop rice varieties combining super high-yielding, good-quality and multiple-resistance. With increasing number of cloned resistance genes and mapping of useful genes for rice genetic transformation, multiple gene pyramiding is a wise and efficient strategy in rice resistance breeding. Currently, as safety evaluation of transgenic crops remains dispute, pyramiding multiple genes by means of MAS represents the predominant and effective method in multiple-resistance rice breeding. It is expected that more rice varieties pyramided different resistance genes can be developed and released to production.

ACKNOWLEDGEMENTS

We thank ZHENG Kangle, WU Jianli, FAN Yeyang and CHEN Jie for guidance and generous help in carrying out the research work. This work was financially supported in part by the National Natural Science Foundation of China (Grant No. 30623006, No. 31101209), the National High Technology Research and Development Program of China (Grant No. 2006AA10Z1E8) and the Program of Introducing International Advanced Agricultural Science and Technologies (948 Program) of Ministry of Agriculture of China (Grant No. 2006-G51).

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