Pyramiding blast, bacterial blight and brown planthopper resistance genes in rice restorer lines

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Abstract

Rice blast, bacterial blight (BB) and brown planthopper (BPH) are the three main pests of rice. This study investigated pyramiding genes resistant to blast, BB and BPH to develop restorer lines. Ten new lines with blast, BB and/or BPH resistance genes were developed using marker-assisted selection (MAS) technique and agronomic trait selection (ATS) method. Only HR13 with resistance genes to blast, BB and BPH was obtained. In addition to blast and BB resistance, four lines (HR39, HR41, HR42, HR43) demonstrated moderate resistance to BPH, but MAS for BPH resistance genes were not conducted in developing these four lines. These data suggested that there were unknown elite BPH resistance genes in the Zhongzu 14 donor parent. A more effective defense was demonstrated in the lines with \( \text{Pi1} \) and \( \text{Pi2} \) genes although the weather in 2012 was favorable to disease incidence. Blast resistance of the lines with a single resistance gene, \( \text{Pita} \), was easily influenced by the weather. Overall, the information obtained through pyramiding multiple resistance genes on developing the restorer lines is helpful for rice resistance breeding.

Keywords: rice, blast, bacterial blight, brown planthopper, resistance, pyramid

1. Introduction

Rice (\( \text{Oryza sativa} \) L.) is a staple food crop in China that feeds more than 60% of the population, and it contributes nearly 40% of the total calorie intake (Cheng et al. 2007).

Compared with conventional varieties, hybrid rice can significantly increase rice yields and has made a large contribution to the self-sufficiency of the food supply in China. However, most of the hybrid rice varieties do not have resistance to specific biotic stresses (Khush and Jena 2009).

Rice blast, bacterial blight (BB) and brown planthopper (BPH) caused by \( \text{Magnaporthe grisea} \), \( \text{Xanthomonas oryzae pv. oryzae} \) (Xoo) and \( \text{Nilaparvata lugens} \) Stål, respectively, are the most destructive diseases and insects causing significant reduction in rice production throughout China and in other Asian rice-growing countries. Rice blast alone can cause annual yield losses of between 10 and 30% of the total harvest, and its occurrence was reported by the Ministry of Agriculture of China to be as high as 20% of the hybrid rice fields cultivated in 2006 (Jiang et al. 2012). BB disease, in its severe form, is known to cause yield losses ranging from 74 to 81% (Srinivasan and Gnanamanickam...
The damage caused by BPH feeding has the greatest effect on the growth and crop yield of the susceptible rice plant through the removal of assimilates and the reduction in photosynthetic rate of leaves, ultimately causing plant death in its severe form (Jirapong et al. 2007). Deployment of host plant resistance is considered to be the best option for managing the diseases and insects. Breeding rice varieties with multiple disease and insect resistance genes will broaden the resistance spectrum and increase the resistance durability for the varieties.

With the development of gene identification technologies, the marker-assisted selection (MAS) technique is typically used to improve disease and insect resistance. The scope of MAS breeding for targeted introgression of BB resistance genes (Huang et al. 1997; Chen et al. 2000; Chen et al. 2001; Sundaram et al. 2008, 2009), blast resistance genes (Amante-bordeos et al. 1992; Hittalmani et al. 2000) and BPH resistance genes (Sharma et al. 2004; Jena et al. 2006) has been successfully demonstrated. In addition, the introgression of two different diseases or insect resistances has been conducted (Jiang et al. 2004). However, to the best of our knowledge, there is no report on the simultaneous introgression of BB, blast and BPH resistance into the lines of hybrid rice.

Currently, the production of hybrid rice is primarily based on the three-line hybrid system, which involves a cytoplasm male sterile (CMS) line, a corresponding isonuclear maintainer line and a genetically diverse restorer line. In addition, the sterile line is maintained by being crossed with its maintainer line, and hybrid seed is produced by crossing the sterile line with the restorer line (Cheng et al. 2012). Generally, restorer lines are much easier to be improved through breeding techniques than sterile lines because no sterility is considered. Shuhui 162 and Zhongzu 14 are two restorer lines in hybrid rice. Shuhui 162 is resistant to sterility is considered. Shuhui 162 and Zhongzu 14 are normally introgressed into the new lines (Table 1). The Shuhui 162 restorer line contains the Pita gene. The Zhongzu 14 restorer line contains Pi1, Pi2 and xa5 genes, and it is resistant to BB, blast and BPH. The BPH-resistance gene donor RH contains the Bph3 gene. CBB23 and HN88 contain the Xa23 gene. HN88 originated from CBB23 and is a new restorer line with high productive-tiller-rate and thousand-grain weight.

Two crosses, namely Shuhui 162/CBB23//HN88///RH (cross 1) and Zhongzu 14/CBB23 (cross 2), were conducted. After obtaining compound F1 or F2, self-pollination was continuously performed for several generations to make the resistance genes homozygous using the MAS technique and to stop other agronomic traits segregation through the agronomic trait selection (ATS) method and pedigree selection. Herein, the ATS method involves selecting agronomic traits of the progenies similar to the restorer parents by artificially judging for the background selection. Crosses between Xieqingzao A and the new lines were further conducted to evaluate their restoring fertility for CMS lines.

2.2. MAS technique

Six markers were used to select corresponding genes in the breeding of each generation (Table 2). DNA samples were extracted from fresh leaves using a simple one-step method (Ji et al. 2014). Leaves with a length of approximately 3 mm were immersed in buffer A containing 100 mmol L\(^{-1}\) Tris-HCl (pH 9.5), 1 mol L\(^{-1}\) KCl and 10 mmol L\(^{-1}\) EDTA (EDTANa\(_2\)·2H\(_2\)O). The samples were crushed using a multi-sample tissue lyser (Jingxin Technology Co. Ltd., Shanghai, China), and the supernatants were collected by centrifugation at 4000 r min\(^{-1}\) for 5 min for DNA amplification.

Polymerase chain reaction (PCR) was performed in a 15-µL reaction volume containing 0.8 µL of supernatant, 2× PCR buffer (including Tris-HCl, KCl and MgCl\(_2\)), 2 mmol L\(^{-1}\) dNTPs, 0.9 µmol L\(^{-1}\) primer pairs, and 0.3 U KOD FX polymerase (Toyobo Co. Ltd., Shanghai, China). The reaction mixture was initially denatured at 94°C for 2 min followed by 30 cycles of PCR amplification with the following parameters: 10 s of denaturation at 98°C, 30 s of primer annealing at 50°C (53°C for marker C189), and 1 min of primer extension at 68°C. Finally, the reaction mixture was maintained at 68°C for 7 min before completion. The amplified product was electrophoretically resolved on a 2% agarose gel using GelRad staining for C189 and YL155/YL187, and it was also resolved on an 8% denaturing polyacrylamide gel using silver staining for RM122, RM224, (Indel) Pi2-4 and RM589.

2.3. Disease and BPH resistance evaluation

After several successive segregating generations, new lines pyramiding multiple resistance genes were sown on June
5th, and transplanted on June 26th, in the field in 2012 and 2013 at the China National Rice Research Institute, Fuyang, China. Resistance to BB and leaf blast was evaluated by artificial inoculation on August 5th in the field in the 2 yr. Isolates of the two diseases prevalent at the area were provided by Mr. Tao Rongxiang of Zhejiang Academy of Agricultural Sciences. The lines planted in the field were inoculated with BB disease isolates using the leaf-clipping method. Nine leaves of three plants were inoculated with BB pathogens, and lesion length (LL) was recorded for each leaf 25 d after inoculation. The heartleaf-injecting method was used during the middle of the tillering stage to evaluate the level of blast resistance. Five heartleaves for each line were inoculated with blast pathogens, and the LL of blast infection was recorded 2 wk after inoculation. The susceptible controls to blast and BB were Zhongzheyou 1 and Jingang 30, respectively.

A modified seedbox screening technique (MSST) was used to evaluate the BPH resistance. Seedlings of the lines at the same growth stage were planted for BPH infestation in a greenhouse. At the 2nd-leaf stage, the seedlings were infested with the 2nd to 3rd instar BPH nymphs at a density of 10 insects per seedling. When 70% of the seedlings of the TN1-susceptible control were dead, the percent mortality of the lines was determined. The BPH resistance of the lines was evaluated with scores of 0, 1, 3, 5, 7 or 9 according to the criteria adapted from the International Rice Research Institute (IRRI 1988).

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The BB, blast and BPH resistance evaluations were replicated in three plots.

### 2.4. Weather data collection

The rice lines grew to the heading stage in mid-August and matured in late September on the same farm for 2012 and 2013. A small weather station (Watchdog 2475, SPEC-TRUM Technologies, Inc.) was used to collect meteorological data, including temperature, rainfall and humidity, during the growth period from June 5 to September 30 in the two years. The data were collected by the station every half hour each day and were averaged for analysis.

### 3. Results

#### 3.1. Pyramiding of different resistance genes into new lines

The two crosses were conducted with the MAS technique and the ATS method. The status of these plants carrying heterozygous or homozygous resistance genes are shown in Figs. 1 and 2. After obtaining plants with all homozygous resistance genes, pedigree selection was used to breed elite lines. In cross 1, the MAS technique was used to identify 8 compound F1-1 plants, 10 compound F 1-2 plants, 7 F2 plants, and 102 F3 plants from compound F1-1, compound F1-2, F2 and F3 generations, respectively. The ATS method was further used to select 66, 50 and 35 lines to generate next generations from F4, F5 and F6 populations, respectively (Fig. 1). One line from 15 F7 lines (PitaPitaXa23Xa23Bph3Bph3) and three lines from 20 F8 lines (PitaPitaXa23X-
were named HR13 and HR15, HR22, and HR34, respectively. In cross 2, the MAS technique was used to identify 5 F2 and 24 F3 plants from F2 and F3 generations. The ATS method was further used to select 20, 18 and 15 lines to generate next generations from F4, F5 and F6 populations, respectively (Fig. 2). Six lines from 15 F1 lines were designated as HR39, HR41, HR42, HR43, HR45, and HR47.

Ten new lines containing BB, blast and/or BPH resistance genes were obtained (Table 3). The aim of cross 1 was to pyramid Pita, Xa23 and Bph3 genes together with the multiple crosses and MAS techniques. However, only one line, HR13, containing the three resistance genes was obtained. Another three lines were pyramided with BB and blast resistance genes. The aim of cross 2 was to introgress the Xa23 gene into Zhongzu 14. Six lines pyramiding the Xa23 gene with xa5, Pi1 and Pi2 genes were achieved. The 10 newly obtained lines further restored the fertility of Xieqingzao A to a normal level in the F1 generation.

3.2. Diseases and BPH resistances of the new lines obtained in two years

After the artificial inoculation of blast, BB and BPH in 2012 and 2013, the diseases and BPH resistance levels of the new lines were evaluated (Table 3). All of the lines showed high resistance to blast and BB. There was a small change in blast resistance between the two years as the blast resistance in 2012 was lower than that in 2013. The lines obtained from cross 2 showed a higher resistance to blast than the lines from cross 1 in 2012 because most of the lines with the Pita gene (from cross 1) had only moderate resistance (3-level) to blast and the lines pyramiding Pi1 and Pi2 (from cross 2) maintained high resistance (0- to 1-level). A more effective defense against blast was demonstrated in the new lines containing Pi1 and Pi2 genes.

Only four lines showed BPH resistance. HR13, the line containing the Bph3 gene, showed moderate resistance (3-to 5-level in both years) to BPH. The four lines obtained from cross 2 demonstrated moderate resistance (3-level in both years) to BPH although they had no Bph3 gene. These results suggested that Zhongzu 14 might be the donor of BPH resistance demonstrated by the four lines.

3.3. The influence of weather on diseases

The meteorological data collected in 2012 and 2013 were

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**Fig. 1** Scheme of cross 1 (Shuhui 162/CBB23//HN88///RH (Rathu Heenathi)) showing the use of the marker-assisted selection (MAS) technique and agronomic trait selection (ATS) method to develop new restorer lines containing the Pita, Xa23 and/or Bph3 genes.
The humidity and rainfall were different between the two years. In 2012, the total rainfall from June to September was 184.00 mm, and the total rainfall in 2013 was only 88.10 mm. Therefore, the rainfall for this period in 2012 was 2-fold more than the rainfall in 2013. Furthermore, during the week following August 5th (Fig. 3), which was the day of the artificial inoculation of blast and BB, the humidity in 2012 was almost

![Fig. 2](image-url) Scheme of cross 2 (Zhongzu 14/CBB23) showing the use of the MAS technique and ATS method to develop new restorer lines containing the Pi1, Pi2, Xa23, and xA5 genes.

Table 3  Pyramiding disease and insect resistance genes and evaluation of resistance by artificial inoculation for the two years in the new lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Origin</th>
<th>Pyramiding resistance genes by MAS1)</th>
<th>Resistance results by artificial inoculation2)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Blast resistance gene</td>
<td>BB resistance gene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pita Pi1 Pi2 Xa23 xA5 Bph3</td>
<td>2013</td>
</tr>
<tr>
<td>HR13</td>
<td>Shuhui 162/CBB23/HN88///RH</td>
<td>+ – – + – +</td>
<td>0 1</td>
</tr>
<tr>
<td>HR15</td>
<td>+ – – + – +</td>
<td>1 3 0 0 9 9</td>
<td>HR22</td>
</tr>
<tr>
<td>HR34</td>
<td>+ – – + – +</td>
<td>0 3 0 0 9 9</td>
<td>HR39</td>
</tr>
<tr>
<td>HR41</td>
<td>– + + + + –</td>
<td>0 1 0 0 3 3</td>
<td>HR42</td>
</tr>
<tr>
<td>HR43</td>
<td>– + + + + –</td>
<td>0 1 0 0 3 3</td>
<td>HR44</td>
</tr>
<tr>
<td>HR45</td>
<td>– + + + + –</td>
<td>0 1 0 0 3 3</td>
<td>HR47</td>
</tr>
<tr>
<td>RH</td>
<td>– – – 7 +</td>
<td>7 7 7 1 1</td>
<td>CBB23</td>
</tr>
<tr>
<td>Shuhui 162</td>
<td>+ – – – –</td>
<td>0 0 5 5 9 9</td>
<td>Zhongzu 14</td>
</tr>
</tbody>
</table>

1) MAS, marker-assisted selection; BB, bacterial blight; BPH, brown planthopper. + means that positive band is shown using the marker; – means negative band is shown using the marker.

2) In BB resistance level column, lesion length (LL)<1 cm means high resistant level (0-level) and 1.1 cm<LL<3 cm means resistant level (1-level). For blast resistance level, 0-level (high resistant level) means no lesion was found; 1-level (resistant level) means that the size of the lesion was that of a needle head; 3-level (moderately resistant level) means that the lesion diameter was approximately 1–2 cm (Tao et al. 2006).

Table 3 Pyramiding disease and insect resistance genes and evaluation of resistance by artificial inoculation for the two years in the new lines.
2-fold more than that in 2013, and the rainfall was almost zero for the same week in 2013. Thus, the weather the week after inoculation in 2012 was favorable to disease incidence.

By combining the disease resistance with the weather difference in the two years (Tables 3 and 4 and Fig. 3), we showed that weather did not influence BB resistance (near 0-level in both years). However, blast resistance differences (varying from 0- to 1-level or 0- to 3-level) in the two years were observed, which suggested that the weather might have some influence on the blast resistance of the lines. The influence of weather on BPH resistance was not analyzed because the BPH resistance evaluation was conducted in a greenhouse under controlled conditions.

4. Discussion
Diseases and insects are major biotic stresses that cause significant yield losses globally. With the development of a comprehensive molecular genetic map of rice, at least 83 major resistance genes for blast, 38 resistance genes for BB and 27 resistance genes for BPH have been identified (China National Rice Data Center, http://www.ricedata.cn/gene). Gene pyramiding using molecular techniques for conventional breeding is now a common technology, especially in rice breeding for disease and insect resistance. Pyramiding of multiple resistance genes into a single genetic background leading to the simultaneous expression of more than one gene in a variety is a strategy to prevent or delay the breakdown of resistance as the probability of simultaneous pathogen mutations for virulence to defeat two or more effective genes is much lower than for a single gene (Mundt 1990). In our study, pyramiding genes for resistance to different diseases and BPH as well as pyramiding different genes resistant to one disease were performed.

Because resistance genes from restorer lines in a three-line hybrid rice display heterozygous genotypes, a completely dominant resistance gene with a broad resistance spectrum is needed (Ji et al. 2014). The xa5 gene, which is naturally found only within the Aus subpopulation of rice (Garris et al. 2003), provides recessive resistance to several Xoo races from the Philippines. Conversely, the Xa23 gene has a broader resistance spectrum to different BB races, displays a high resistance level during all growth stages and is highly heritable (Zhang et al. 1998; Zhang et al. 2001). Zhou et al. (2011) determined that there is no genetic background effect on the expression of the Xa23 gene, suggesting that Xa23 is of great value in a hybrid rice breeding program with BB resistance. Hence, the 10 lines in this study were introgressed with the Xa23 gene donated by CBB23, and high BB resistance was demonstrated in the lines (Table 3). The ability of the new lines to restore fertility in CMS lines was further confirmed.

The blast resistance gene, Pi1, was originally identified in the cultivar LAC23 (Mackill and Bonman 1992), an upland cultivar from Liberia, and it has a broad resistance spectrum. Only 10.35% of strains of the 792 Chinese isolates collected in central and southern China could infect the near-isogenic line (NIL) C101 LAC, which contains the Pi1 gene and the susceptible cultivar CO39 background (Chen et al. 2001). The Pi2 gene was first introgressed from a highly resistant indica cultivar, 5173, into the susceptible cultivar,
CO39 (Mackill and Bonman 1992). Extensive field tests in several countries have indicated that Pi2 is one of the rice blast resistance genes with a broad resistance spectrum (Chen et al. 1996). *Pita* is a single copy resistance gene in which the resistance specificity is determined by a single amino acid (Wang et al. 2010). The Pi-ta resistance allele was introduced from the Asian “Tetep” landrace variety, which is resistant to all common races of the blast fungus (Jia et al. 2004). With the introgression of the genes, the blast resistance of the new lines was demonstrated in our study. The effect of pyramiding *Pi1* and *Pi2* was similar to that of the *Pita* gene in 2013. However, in 2012, the lines with *Pi1* and *Pi2* showed higher blast resistance than the lines with the *Pita* gene. The weather of the 1st wk after inoculation in 2012 had higher humidity and rainfall (Fig. 3), which was favorable to disease incidence. Certain cultivars show durable resistance because they “... remain resistant ... even though they are extensively cultivated in environments favorable to disease” (Johnson 1981). Hence, a more effective defense was demonstrated in the lines with *Pi1* and *Pi2* genes.

Deployment of resistant varieties carrying various resistance genes has been successful for BPH control. *Bph1, bph2, Bph3, and bph4* (Sharma et al. 2004; Sun et al. 2006; Jirapong et al. 2010; Peñafalver et al. 2011) have been used extensively. Rice cultivars carrying *Bph3* have shown a higher degree and a broader spectrum of resistance against BPH (Jirapong et al. 2007). Nevertheless, the new line, HR13, containing the *Bph3* gene introgressed from Rathu Heenathi (RH), showed a moderate resistance to BPH. There might have been a certain genetic background effect on the *Bph3* gene because only moderate resistance to BPH was demonstrated compared to that of the donor RH. In contrast, the four lines (HR39, HR41, HR42, HR43) with *Bph3* on the *gene* because only moderate resistance to BPH was demonstrated compared to that of the donor RH. Further evaluation and gene mapping of the BPH resistance genes of Zhongzu 14, a more effective defense was demonstrated in the lines with *Pi1* and *Pi2* genes.

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