Development of molecular marker and introgression of Bph3 into elite rice cultivars by marker-assisted selection

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The brown planthopper (BPH) is a serious insect pest of rice and a substantial threat to rice production. Identification of new BPH resistance genes and their transfer into modern rice cultivars are effective breeding approaches to reduce the damage caused by BPH. In this study, we mapped a BPH resistance gene to a 50-kb genomic interval between two InDel markers 4M03980 and 4M04041 on the short arm of chromosome 4 in indica rice cultivar BP60, where the BPH resistance gene was mapped in Rathu Heenati by Liu et al. (2015) and named “Bph3”. This region contains two annotated genes Os04g0201900 and Os04g0202300, which encode lectin receptor kinases responsible for BPH resistance. We also developed a molecular marker “MM28T” for Bph3, and introgression Bph3 into susceptible rice restorer lines Guihui582 and Gui7571 by the marker-assisted selection (MAS) approach. The BPH resistance level is significantly enhanced in the Bph3-introgression lines, the resistance scores decrease from 8.2 to 3.6 for Guihui582 and decrease from 8.7 to around 3.8 for Gui7571. Therefore, developing molecular markers for the BPH resistance gene Bph3 and using them for molecular breeding will facilitate the creation of BPH-resistance rice cultivars to reduce damage caused by BPH.

Key Words: brown planthopper, fine mapping, resistance gene, molecular breeding.

Introduction

The brown planthopper (BPH, Nilaparvata lugens Stål) is one of the most destructive insect pests of rice plants in Asia. BPH infects rice by sucking phloem sap causing the plants to dry, and has been a threat to rice production in Asia for many years (Heong and Hardy 2009, Normile 2008). Serious damage by BPH infestation causes completely drying of rice crops, known as ‘hopperburn’ in the rice paddy. The usual approach of controlling BPH by spraying pesticides is costly, causes environmental pollution, and leads to the development of pesticide resistance in BPH (Cheng and Zhu 2006). The application of BPH resistance genes in rice breeding has generally been considered to be one of the most economical and environmentally sound strategies for BPH pest control (Khush 2001). Previous studies demonstrated that rice plants containing one or more endogenous BPH resistance genes can reduce BPH damage and increase yield. Therefore, development of BPH resistant rice cultivars with a molecular marker-assisted breeding approach is very important for controlling this insect in the field.

To date, more than 30 BPH resistance genes have been identified from wild species and cultivars of Oryza, and interestingly most of these genes are mainly located on chromosome 3, 4, 6, 12. Bph1, Bph13, Bph14 and Bph19 are located on chromosome 3 (Fujita et al. 2013, Hu et al. 2016). Nine genes (Bph3, Bph6, Bph12, Bph15-17, Bph20, Bph22, Bph27) are located on chromosome 4 (Fujita et al. 2013, Hu et al. 2016, Huang et al. 2013, Sun et al. 2005, Xiong et al. 2004, Yang et al. 2004). Bph4, Bph25 and bph29 are located on chromosome 6 (Kawaguchi et al. 2001, Myint et al. 2012, Wang et al. 2015), while Bph1, bph2, bph7, Bph9, Bph10, Bph18, bph19, Bph21 and Bph26 are located on chromosome 12 (Fujita et al. 2013, Hu et al. 2016). Some BPH resistance genes that are tightly linked in the same chromosomal regions might be allelic, but have either the same function or interaction with each other resulting in a gain of function for BPH resistance in rice.
plants. It is imperative to develop durable broad-spectrum BPH resistant varieties by identifying additional BPH resistance genes and novel alleles. The BPH resistance level of several cultivars and breeding lines was enhanced by pyramiding multiple BPH resistance genes in rice with the MAS approach (Liu et al. 2016).

MAS is a highly efficient breeding approach for introducing genes to plants, as it can trace the target gene in plants rapidly and precisely (Gupta et al. 2010, Xiang et al. 2003). Previous studies have demonstrated that MAS is an effective method to select rice lines resistant to bacterial blight, blast, brown planthopper, etc (Cao et al. 2003, Conaway-Bormans et al. 2003, Jena et al. 2006, Hu et al. 2016, Liu et al. 2016, Mohan et al. 1997, Myint et al. 2012, Xu 2013). An increasing number of studies have demonstrated that introducing endogenous BPH resistance genes into rice plants can reduce BPH damage to rice. Bph18(t) was introduced into IR65482-7-216-1-2 and Junambyeo using the MAS approach, and BPH resistance level increased in the introgression line (Suh et al. 2011). BPH25(t) and BPH26(t) introgression lines were obtained from the BPH-susceptible japonica cultivar Taichung 65 using the MAS approach, and the introgression lines obtained BPH resistance to the BPH pest (Yara et al. 2010). The BPH resistance ability was improved in rice variety Huahui938 by pyramiding Bph14 and Bph15 into it using the MAS method (Wang et al. 2016). Qiu et al. (2012) used the MAS approach to pyramid Bph12 and Bph6 genes into indica and japonica rice cultivars for obtaining stronger antixenotic and antibiotic effects on the BPH insects.

In the present study, we identified an allele of Bph3 by the InDel mapping approach in the indica variety BP60. The gene was introduced into the BPH susceptible rice restorer line Guihui582 and Gui7571 by the MAS method to obtain the near-isogenic lines (NILs) for facilitating the breeding of BPH resistant rice varieties. Further, we used the near-isogenic line from Guihui582 to cross with sterile line TeA in breeding, and obtained a BPH-resistance hybrid rice cultivar TeYou373 for commercial cultivation in the south of China.

### Materials and Methods

#### Plant materials and BPH insects

**Indica** variety BP60, restorer lines Guihui582 (R582), Gui7571 and TeA were collected from Guangxi, China, and BP60 was used as BPH-resistant donor parent. The rice *japonica* line 02428 is a BPH susceptible rice cultivar used for crossing with BP60 to develop F1 and F2 mapping populations. Each F2 plant was self-pollinated to yield F2:3 lines, which were used to validate the BPH resistance level. Taichung Native 1 (TN1) with no resistance gene and Rathu Heenati (RH) with Bph3 (Liu et al. 2015, Zhang et al. 2015) were used as BPH susceptible and resistant controls, respectively.

The BPH colony used for the study was biotype 2, which was a predominant biotype in most of the rice-planting area in China (Chen et al. 2006). BPH insects were maintained on TN1 plants under natural conditions in a green-house at Plant Protection Research Institute, Guangxi Academy of Agricultural Sciences.

#### BPH bioassay for evaluation of resistance

Bioassays were conducted following the method described by Huang et al. (2013) with some modifications. Rice seeds were germinated in petri dishes, and about 20 well-sprouted seeds were sown in metal trays (52 × 37 cm). Three-leaf old rice seedlings grown in trays were infested with second- or third-instar nymphs of BPH, with 10–12 nymphs per seedling. The trays were then covered with a mesh cage after infestation. When all TN1 seedlings died, the plants of other lines were measured and a score of 0, 1, 3, 5, 7 or 9 was given to each seedling based on the published criteria (Huang et al. 2001). The bioassay experiments were repeated three times. The higher scores indicate lower resistance to the BPH insect. For evaluating resistance of adult plants, 80-day-old plants were transferred from paddy to a pool (2 × 1 m) in the green house. The distance between the plants was about 20 cm, and plants were covered with a mesh cage after about 1000 adult insects were placed on the 83-day-old plants. The bioassays experiments were repeated three times. The significance among varieties was analyzed using Tukey’s HSD (Honestly Significant Difference) test.

#### DNA preparation, genome sequencing and resistance gene mapping of BPH

Genomic DNA was extracted from fresh rice leaves using the CTAB method (Murray and Thompson 1980). The extracted genomic DNA was dissolved in TE buffer for further experiments. Genomic DNA of the BPH resistant indica cultivar BP60 was used for genome sequencing using the Illumina HiSeq × Ten sequencing system at Beijing Genomics Institute. PCR was performed as described by Sun et al. (2005). PCR-based InDel markers for mapping were developed on the basis of sequence differences between *Japonica* rice Nipponbare and *indica* cultivar 9311. 216 polymorphic InDel markers (Shen et al. 2004) were used to validate the individual progeny in the resistant group. PCR primers were designed using the software Primer Premier 5.0 (http://www.premierbiosoft.com).

Based on the BPH resistant phenotype of the F2:3 families, two contrasting groups were prepared, with each group containing genomic DNA from 10 resistant (R) or 10 susceptible (S) progeny from the F2 generation of 02428 × BP60. The bulked segregant analysis (BSA) (Michelmore et al. 1991) was used to select InDel markers linked to BPH resistance.

#### Bph3-NILs development and breeding application

The procedure of introducing Bph3 into Guihui582 and Gui7571 was described in Supplemental Fig. 1. The BPH
resistance gene identified in the rice cultivar BP60 was introduced into rice restorer line Guihui582 (R582) by successive backcrossing for two generations and MAS. Two flanking markers, which are tightly linked to the locus, were used to screen the positive progeny during the backcrossing process. As a result, the individuals of the BC2F1 generation containing the target locus were used for selfing seven times to obtain BC2F8 homozygous generation. The BC2F8 generation named R373, and its agronomic trait is stable in the field. R373 was used for crossing with the sterile line TeA for obtaining the BPH resistant hybrid rice cultivar TeYou373. The hybrid rice cultivar TeYou582, which without containing Bph3 as a negative control in BPH bioassay. The same procedure were used to obtain NIL lines for Gui7571, and a molecular marker “MM28T” was used for validation (Supplemental Table 1). Four lines have similar phenotype to Gui7571, which contain Bph3 gene, were obtained and named Gui-22, Gui-23, Gui-24, Gui-25, respectively.

Results

BPH resistance evaluation and fine mapping of the allele of Bph3

In the BPH bioassay experiment, the resistance scores of BP60 and 02428 were 3.8 and 8.2, respectively. The F1 plants from the cross between BP60 and 02428 showed more resistance to BPH than 02428 line with significant difference (Table 1). Nine other rice varieties (9311, Gui7571, 75-1-27, BL122, 112B, TeB, LiangfengB, IRBB7 and IR1552) were also used in the BPH bioassay experiment, and their resistance scores ranged from 7.8 to 8.7 (Fig. 1B). The phenotyping results also showed that most of the BP60 plants still had normal growth after 10 days of infestation by BPH, while the nine other varieties were completely dead (Fig. 1A).

F3 progeny of 158 F2 individuals derived from the F2 mapping population of 02428 × BP60 were screened for BPH reaction on the basis of resistant scores. Segregation of F2 plants for BPH resistance showed a range from complete susceptibility (42 plants) to segregating (78 plants) to resistance (38 plants). The segregation of F2 showed a 1:2:1 segregation ratio; the resistant to susceptible plants corresponded to a 3:1 ratio (116:42; $x^2 = 3.43 < x^2_{0.05} = 3.84$). This result indicated that a major dominant gene contributes to BPH resistance in this F2 populations.

To screen molecular markers that are linked with the BPH resistance gene, DNA mixture pools from 10 homozygous susceptible and 10 homozygous resistant lines were used in the survey. The bulked segregation analysis (BSA) method was used to efficiently identify the InDel markers linked with the BPH resistance gene. A total of 216 InDel primer pairs distributed along 12 chromosomes of rice were tested for parental polymorphism survey. Among these selected markers, two markers (RI05751 and RI05781) have polymorphic difference between both parents, the susceptible and resistant groups (Supplemental Fig. 1). The two markers are located in a contiguous region on the short arm of chromosome 4 (Fig. 2), and were considered as candidate markers for the resistance gene.

In order to fine map the BPH resistance gene, four InDel markers within the RI05751–RI05781 interval were used for polymorphism screening between BP60 and 02428 in 116 F2 plants. The gene was mapped to the 3078-kb region between markers 4M03176 and 4M04763. To narrow down

| Table 1. The resistance score of the two parents and F1 varieties |
|---------------------------------------------------------------|
| Cultivar          | Number of seedlings tested | Resistance score (0–9) | Significance |
| Taichung Native 1 (TN1) | 66                        | 8.4                     | A           |
| BP60             | 56                        | 3.8                     | C           |
| 02428            | 52                        | 8.2                     | A           |
| BP60/02428 F1    | 63                        | 5.3                     | B           |
| RH               | 68                        | 3.2                     | C           |

*TN1 and RH were used as susceptible and resistant controls, respectively.

*Tukey’s HSD test, $p < 0.05$. 

Fig. 1. The evaluation result of BPH resistance of BP60 and other nine BPH susceptible lines ten days after infestation (A). The data are presented as mean ± SD (three biological repeats), and letters indicate a significant difference at $p < 0.05$ by Tukey’s HSD test (B).
the region, an additional 5,431 F₂ plants were used for PCR-based InDel marker validation, the gene was mapped to a 50-kb interval between markers 4M03980 and 4M04041 (Fig. 2).

**Candidate genes in the target region**

In order to obtain the DNA sequence of the BPH resistance gene, we re-sequenced the genome of the BP60 variety and compared it with the Nipponbare genome sequence data. There are four predicted ORFs in the 50-kb interval, Os04g0201500 encoding an amino acid/polyamine transporter II family protein, Os04g0201800 encoding a hypothetical protein, Os04g0201900 and Os04g0202300 encoding a putative lectin receptor kinases (Fig. 2). The two alleles of Os04g0201900 and Os04g0202300 responsible for BPH resistance were reported by Liu et al. (2015). Based on the DNA sequence of these two genes, we designed PCR primers (Supplemental Table 2) and PCR-amplified the two genes. We then sequenced the amplified genes to validate their sequences. The sequencing data showed that the ORF of each gene shares 98% sequence identity with Os04g0201900 and Os04g0202300 in Nipponbare (Supplemental Fig. 3), and shares 100% sequence identity with Bph3 in RH (Liu et al. 2015).

**Gene marker design and PCR validation for marker-assisted selection of Bph3**

Based on DNA sequence differences between Bph3, the BPH resistance rice cultivar BP60 and nine other BPH susceptible *indica* rice cultivars (9311, Gui7571, 75-1-27, BL122, 112B, TeB, LiangfengB, IRBB7 and IR1552) from nucleotide alignments, three PCR primers were designed in this gene to differentiate Bph3 between BPH resistance plants and BPH susceptible plants (Fig. 3). One nucleotide mutation (A to C) on the IR intermediate reverse primer, and other 22 nucleotides are specific to Bph3 of BP60 cultivar, but the 3’ end has mismatches with the nine other rice cultivars. Therefore, the IR primer is more specific to BP60 than the others, and the predicted result was that two PCR fragments would be produced from BP60 with three primers in PCR reaction, while only one PCR fragment would be amplified from other nine rice cultivars.

In a PCR validation experiment to test the molecular marker, rice cultivars (9311, Gui7571, 75-1-27, BL122, 112B, TeB, LiangfengB, IRBB7 and IR1552) from nucleotide alignments, three PCR primers were designed in this gene to differentiate Bph3 between BPH resistance plants and BPH susceptible plants (Fig. 3). One nucleotide mutation (A to C) on the IR intermediate reverse primer, and other 22 nucleotides are specific to Bph3 of BP60 cultivar, but the 3’ end has mismatches with the nine other rice cultivars. Therefore, the IR primer is more specific to BP60 than the others, and the predicted result was that two PCR fragments would be produced from BP60 with three primers in PCR reaction, while only one PCR fragment would be amplified from other nine rice cultivars.

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BS

112B, TeB, LiangfengB, IRBB7 and IR1552) were used. The results showed two PCR fragments (330 bp and 809 bp) were amplified from the BPH resistant rice cultivars BP60, while only one PCR fragment (809 bp) was amplified from the BPH susceptible rice cultivar 9311, Gui7571, 75-1-27, BL122, 112B, TeB, LiangfengB, IRBB7 and IR1552 (Fig. 4). This result suggested that the molecular marker can be used for screening BPH resistance plants, containing the 

Bph3 gene.

Bph3 enhances BPH resistance in the introgression lines

In order to validate whether Bph3-introgession line of Guihui582 with MAS selection can enhance BPH resistance, the 3-4 leaf-old seedlings were evaluated for BPH resistance under greenhouse conditions. There was no visible damage on the Bph3 introgression line R373 and its hybrid line TeYou373 after 10 days of BPH infestation, while the control lines R582, TeYou582 and TN1 were completely dead (Fig. 5A). According to the BPH resistance score analysis, the BPH resistance level of the Bph3 introgression line was higher than that of the R582 line, and there was a significant difference between these two lines (Fig. 5C). For hybrid lines, TeYou373, which contains Bph3, was also significantly more resistant to BPH than TeYou582 (Fig. 5C). The BPH resistance evaluation was also performed for adult plants of hybrid varieties YeTou582 and TeYou373 under greenhouse conditions. The results showed that the Bph3 containing the hybrid variety TeYou373 also displayed higher resistance to BPH than the control hybrid variety TeYou582 (Fig. 5B). Therefore, introducing Bph3 into rice plants can enhance BPH resistance significantly.

Bph3-introgession lines of Gu7571 with MAS selection also displayed resistance against BPH and have similar resistance levels with the resistance control RH (Fig. 5D). Almost all plants grew normally after 10 days of infestation by BPH insects, and their resistance scores ranged from 3.2 to 3.8, which has no significant difference (Fig. 5E) based on Tukey’s HSD test. Bph3 conferring resistance to BPH in the introgression lines can enhance resistance levels of rice plants. Taken together, these results indicate that introducing Bph3 into rice cultivars can protect them from damage caused by BPH in the field.

Discussion

The main finding of the present study was the mapping of an allele of Bph3 by InDel marker analysis to a 50-kb segment of genomic DNA, which contains two functional genes for BPH resistance in indica cultivar BP60, and the introduced of the gene into rice varieties Guihui582 and Gu7571 by the MAS approach. The location and sequence of both functional genes were also reported by Liu et al. (2015), and both are members of Bph3. The Bph3 locus has been reported to map on rice chromosome 4, 6, 7 and 10 (Ikeda and Kaneda 1981, Jairin et al. 2007, Sun et al. 2005, Yang et al. 2002), and was even cloned from rice chromosome 4 in rice cultivar RH (Liu et al. 2015). Our study has also demonstrated that BP60 showed BPH resistance as high as RH in the evaluation assay (Table 1).

Conventional rice breeding for BPH resistance is time-consuming and highly dependent on environmental conditions. The development of novel methods for BPH resistance, such as marker-assisted breeding, can significantly reduce the time and resources required for resistance introgression.
conditions in the field, while MAS is a robust approach for tracking one or more target genes of resistance phenotype or selecting agronomic traits during breeding program. In rice, transferring BPH resistance genes into breeding lines to improve its capability to endure damage caused by BPH has been well reported (Hu et al. 2015, Liu et al. 2015). In the present study, we successfully introduced the BPH resistance gene Bph3 into indica rice restorer variety R582 and Gui7571 with the MAS approach to obtain the BPH resistance lines. The Bph3-introgression line R373 was used for molecular breeding to generate the BPH resistant hybrid variety TeYou373 for commercial planting in the south of China. Plant growth and thousand seed weight were unaffected with Bph3 introgression to rice varieties R582 and Gui7571 (data not showed). Therefore, the BPH-resistance gene Bph3 is useful in molecular breeding with the MAS approach for enhancing rice BPH resistant level in the paddy.

It is difficult to design marker primers to differentiate the gene in different rice cultivars on the basis of gene SNPs, as mismatches of only one or two nucleotides with the DNA template cannot guarantee there is no PCR product in reaction. In this study, according to the Bph3 sequence from BP60, there is only one nucleotide difference with the nine other cultivars in the same position. Therefore, we designed a reverse primer with a 3’ end of ‘T’ to only match the genomic DNA of BP60, and a mismatched nucleotide (the third nucleotide on the 3’ end) was introduced in the primer at the same time. Thus, the two-nucleotide mismatch with the genomic DNA of nine BPH susceptible cultivars, should not amplify the fragment; while having only one nucleotide mismatch with BPH resistance cultivar BP60 can result in amplification of the fragment. To check the quality of the genomic DNA template for PCR, a reverse primer, which has 479 bp distance to the SNP, were used in PCR reaction at the same time to amplify a longer DNA fragment. Two different size PCR products can be separated in agarose gel, and Bph3 genotype can be showed on the gel. So the molecular marker can detect the gene very accurately by PCR validation for screening Bph3 introgression plants.

To solve the problem of outbreaks of new biotypes BPH in the field, the identification of additional BPH resistance genes and pyramiding more resistance genes are required for widening the genetic base. The pyramiding of two or more BPH resistance genes in rice is an advantageous strategy against BPH, as the insects are unlikely to overcome multiple resistance genes simultaneously under natural conditions. In this case, transferring the BPH resistance genes into rice using MAS should be very useful for developing cultivars carrying multiple resistance genes. Therefore, developing molecular marker for the BPH resistance dominant gene Bph3 and using it in molecular breeding should greatly assist in the development of rice varieties that display durable resistance to BPH.

This work was supported by the National Natural Science Foundation of China (grant no. 31460344), Guangxi Science and Technology Base and Special Talents (grant no. GuiKe AD17129064) and Science-Technology Development Funding of Guangxi Academy of Agricultural Science (grant no. 2015JZ18). We thank Dr. Caren Chang (University of Maryland) for proofreading the manuscript.

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