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Research Paper

# 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 resistant 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. *Bph11*, *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

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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 $F_1$ and $F_2$ mapping populations. Each $F_2$ plant was self-pollinated to yield $F_{2:3}$ 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 $F_{2:3}$ families, two contrasting groups were prepared, with each group containing genomic DNA from 10 resistant (R) or 10 susceptible (S) progeny from the $F_2$ 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 $BC_2F_1$ generation containing the target locus were used for selfing seven times to obtain $BC_2F_8$ homozygous generation. The $BC_2F_8$ 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 $F_1$ 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**).

$F_3$ progeny of 158 $F_2$ individuals derived from the $F_2$ mapping population of 02428 × BP60 were screened for BPH reaction on the basis of resistant scores. Segregation of $F_2$ plants for BPH resistance showed a range from complete susceptibility (42 plants) to segregating (78 plants) to resistance (38 plants). The segregation of $F_2$ showed a 1:2:1 segregation ratio; the resistant to susceptible plants corresponded to a 3:1 ratio (116:42; $x_c^2 = 3.43 < x_{0.05}^2 = 3.84$). This result indicated that a major dominant gene contributes to BPH resistance in this $F_2$ populations.

**Table 1.** The resistance score of the two parents and $F_1$ varieties

| Cultivar[a] | Number of seedlings tested | Resistance score (0–9) | Significance[b] |
|---|---|---|---|
| Taichung Native 1 (TN1) | 66 | 8.4 | A |
| BP60 | 56 | 3.8 | C |
| 02428 | 52 | 8.2 | A |
| BP60/02428 $F_1$ | 63 | 5.3 | B |
| RH | 68 | 3.2 | C |

[a] TN1 and RH were used as susceptible and resistant controls, respectively.
[b] Tukey's HSD test, $p < 0.05$.

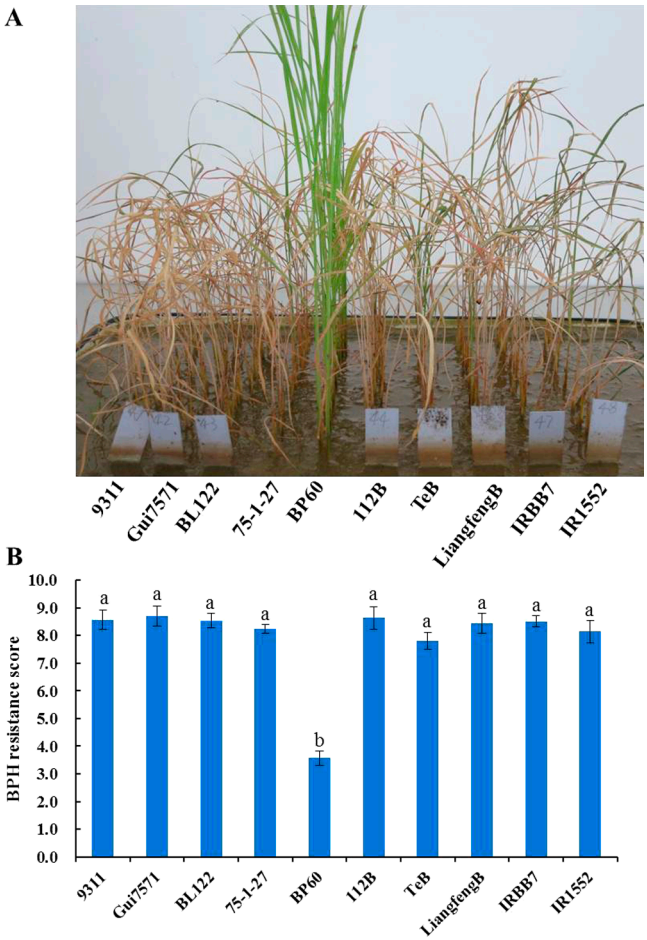


**Fig. 1.** The evaluation result of BPH resistance of BP60 and other nine BPH susceptible lines ten days after infestation (A). The data are presented as mean ± SD (three biological repeats), and letters indicate a significant difference at $p < 0.05$ by Tukey's HSD test (B).

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 the parental polymorphism survey. Among these selected markers, two markers (RI05751 and RI05781) have polymorphic difference between both parents, the susceptible and resistance groups (**Supplemental Fig. 2**). 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 $F_2$ plants. The gene was mapped to the 3078-kb region between markers 4M03176 and 4M04763. To narrow down

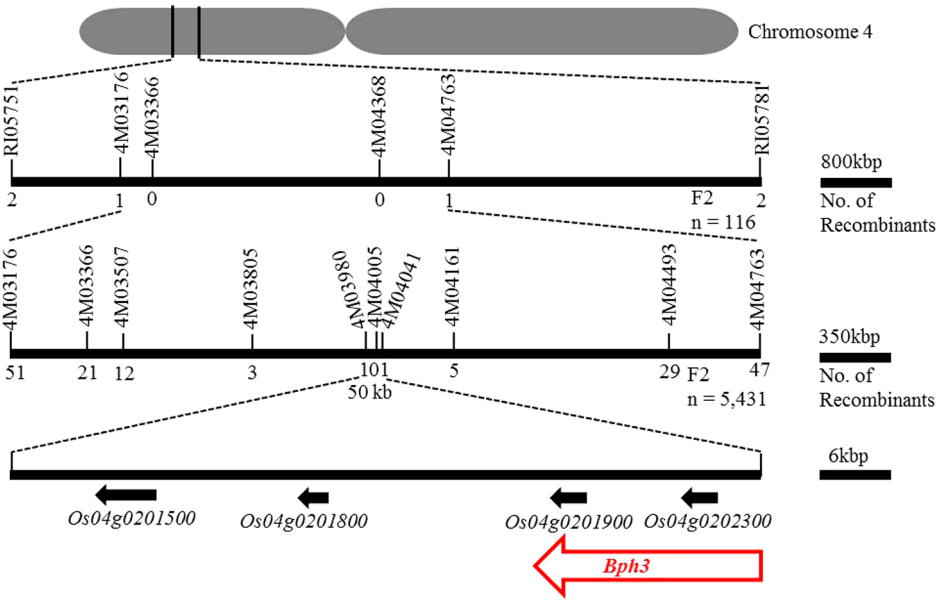


**Fig. 2.** Fine mapping of *Bph3* allele to a 50-kb genomic region. Four predicted ORFs are in the mapped interval. Numbers under the linkage map indicate the number of recombinants detected between the DNA markers closely linked to *Bph3*. Gene models annotated in The Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/) are shown as black arrows, and the actual *Bph3* gene is shown with a red arrow.

the region, an additional 5,431 $F_2$ 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,

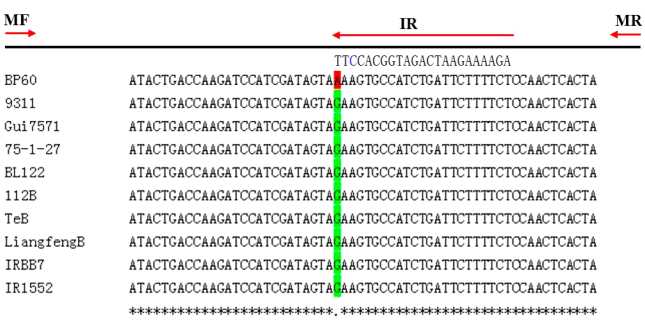


**Fig. 3.** Molecular marker design based on alignment of the *Bph3* sequence from different rice cultivars. According to *Bph3* sequence difference between BP60 and other rice cultivars, an additional intermediate reverse primer (IR) was designed to amplify a 330 bp PCR product only from BP60, and a 809 bp PCR product from all rice cultivars. One nucleotide mutation (blue color) on the IR primer to get one nucleotide mismatch with BP60 and two nucleotides mismatch with other rice cultivars. DNA sequence alignment was performed on website: https://www.ebi.ac.uk/Tools/msa/mafft/.

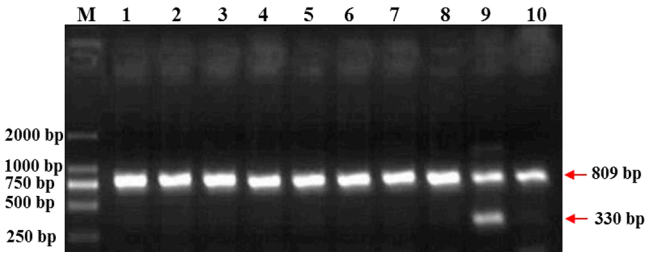


**Fig. 4.** Validation of the *Bph3* molecular marker using different rice cultivars. M: DNA marker 2000, 1: 9311, 2: Gui7571, 3: 75-1-27, 4: BL122, 5: 112B, 6: TeB, 7: LiangfengB, 8: IRBB7, 9: BP60, 10: IR1552. Using three primers in the PCR reactions, two different PCR products (809 bp and 330 bp) were obtained when the rice cultivars contained the *Bph3* gene. As BP60 contains the *Bph3* gene, both the 809 bp and 330 bp fragments were amplified in the PCR reaction, while the nine other cultivars only had the 809 bp PCR fragment after PCR amplification.

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*-introgression line of Guihui582 with MAS selection can enhance BPH resistance, the 3- to 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*-introgression lines of Gui7571 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

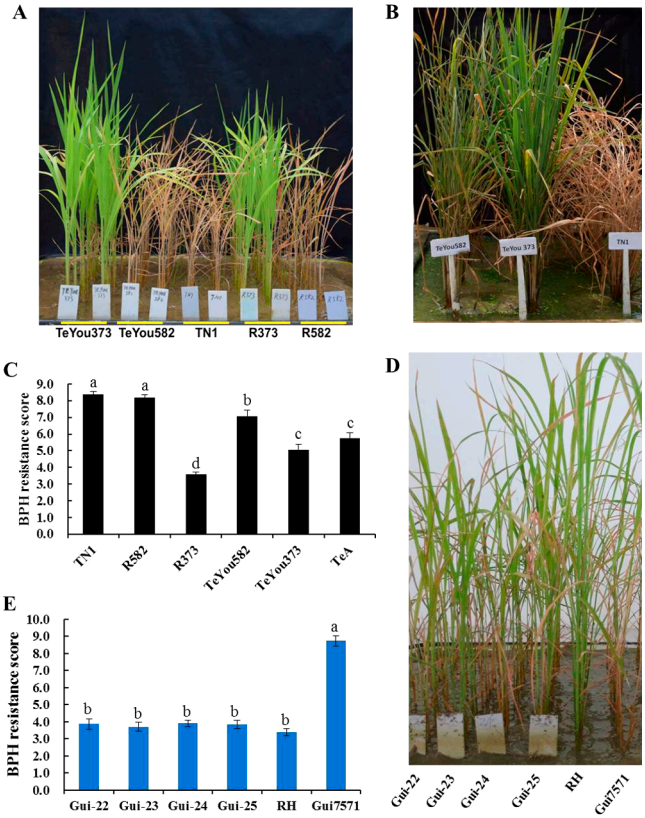


**Fig. 5.** BPH-resistance phenotype in *Bph3*-containing introgression lines and hybrid lines. (A and D) Seedling stage plants after 10 days of infestation by BPH; (B) 83-day-old plants after 18 days of infestation by BPH; (C and E) The data are presented as means ± SD (three biological repeats), and letters indicate a significant difference at $p < 0.05$ by Tukey's HSD test.

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 Gui7571 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

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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 maker 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.

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