

Article

Molecular Mapping of a New Brown Planthopper Resistance Gene *Bph43* in Rice (*Oryza sativa* L.)

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Abstract: Brown planthopper (BPH) has become the most devastating insect pests of rice and a serious threat to rice production. To combat newly occurring virulent BPH populations, it is still urgent to explore more new broad-spectrum BPH resistance genes and integrate them into rice cultivars. In the present study, we explored the genetic basis of BPH resistance in IRGC 8678. We identified and mapped a new resistance gene *Bph43* to a region of ~380 kb on chromosome 11. Genes encoding nucleotide-binding domain leucine-rich repeat-containing (NBS-LRR)-type disease resistance proteins or Leucine Rich Repeat family proteins annotated in this region were predicted as the possible candidates for *Bph43*. Meanwhile, we developed near isogenic lines of *Bph43* (NIL-*Bph43*-9311) in an elite restorer line 9311 background using marker-assisted selection (MAS). The further characterization of NIL-*Bph43*-9311 demonstrated that *Bph43* confers strong antibiosis and antixenosis effects on BPH. Comparative transcriptome analysis revealed that genes related to the defense response and resistance gene-dependent signaling pathway were significantly and uniquely enriched in BPH-infested NIL-*Bph43*-9311. Our work demonstrated that *Bph43* can be deployed as a valuable donor in BPH resistance breeding programs.

Keywords: brown planthopper; gene mapping; near isogenic line; marker-assisted selection; RNA-Seq



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1. Introduction

Rice (*Oryza sativa* L.), being an important cereal crop in the Asian-Pacific region, accounts for a staple food resource for around four billion people worldwide [1]. Nevertheless, like other plants, the growth of rice is continuously threatened by pathogens and herbivore insects during its entire growth cycle and thus leads to an estimated annual loss of 10–30% of the total rice yield [2,3]. Among 20 kinds of known serious paddy pests, brown planthopper (BPH, *Nilaparvata lugens* Stål), is a migratory and monophagous one and becomes the most destructive pest of rice in recent years [4,5]. BPH has sharp, elongated mouthparts that penetrate plant cells and sucks the phloem sap from rice leaf sheath, during which process viral diseases are also transmitted [6]. Serious BPH feeding causes the complete dying of rice, leading to a phenomenon called “hopperburn” [7]. In China, the BPH-infested rice area was estimated at over 25 million hectares, resulting in a rice yield loss of 2.7 million tons between 2005 and 2008 [8,9]. In Asia, the economic loss caused by BPH could reach more than 300 million dollars per year [10].

In agricultural practice, BPH management is still depending heavily on chemical pesticides, which not only cause severe environment pollution and food safety concerns, but also induce increased BPH resistance to chemical pesticides [11]. Therefore, breeding and deploying of resistant cultivars have been the most economically effective and

environmental-friendly approach to BPH management [12]. Hence, the identification of BPH resistance genes and the dissection of the underlying resistance mechanism are critical for their successful use in rice breeding. To date, around 40 BPH resistance genes have been identified in the traditional cultivated rice varieties and wild rice species, most of which are usually present in clusters on chromosomes 3, 4, 6, and 12 [13]. Among them, *Bph14*, *Bph3/Bph15*, *Bph26/bph2*, *bph29*, *bph7/Bph9/Bph10/Bph21*, *Bph18/Bph1*, *Bph32*, *Bph6*, *Bph30/Bph40*, and *Bph37* have been isolated and characterized via a map-based cloning approach [14–25], which provide useful targets for marker-assisted selection (MAS) breeding.

However, the resistance of varieties carrying single BPH resistance gene is easy to be quickly broken down due to the rapid adaptation of BPH or evolution of new biotypes [26]. For example, IR26, the first resistant variety with single resistant gene *Bph1*, was released by the International Rice Research Institute (IRRI) in 1973. However, by 1975, IR26 and other *Bph1*-containing resistant varieties were incapable of reducing BPH damage due to the development of BPH biotype II [27]. In 1976, IRRI released IR36 and other resistant varieties harboring *bph2*. However, a few years later, these varieties were also adapted by the new BPH population [28,29]. Therefore, it is still urgent to explore more new broad-spectrum BPH resistance genes and integrate them into rice cultivars to combat the new virulent BPH populations and ultimately achieve durable and broad-spectrum resistance.

IRGC 8678, a Bangladesh indica rice cultivar, was reported to be resistant to BPH biotype 3 [30], but the underlying BPH resistance gene(s) have not been identified yet. In this study, we revealed that IRGC 8678 was highly resistant to the current BPH population of China, the more destructive Bangladesh type. We identified and mapped a new BPH-resistant gene, namely *Bph43*, in IRGC 8678. A highly resistant near-isogenic line (NIL-*Bph43*-9311) was developed by the introgression of *Bph43* into the elite restorer line 9311 through MAS. We also performed comparative transcriptome analysis and explored early defense responsive genes and pathways underlying *Bph43*. Our work identified a new BPH-resistant gene and demonstrated that *Bph43* can be deployed as a valuable donor in BPH resistance breeding programs.

2. Materials and Methods

2.1. Plant Materials, Mapping Populations, and NIL-*Bph43*-9311 Construction

IRGC 8678, a Bangladesh indica rice cultivar, was previously found to be resistant to BPH biotype III [30]. We obtained IRGC 8678 from the IRRI and crossed it with a BPH-susceptible Chinese elite indica rice cultivar 9311. The resulted F₁ was self-pollinated to generate F₂ mapping population and the corresponding F₃ families for genetic analysis and gene mapping. At the same time, 9311/IRGC 8678 F₁ plant was backcrossed with 9311 to generate the BC₁F₁ population. The heterozygous BC₁F₁ plants showing the resistance to BPH were selected to generate BC₁F₂ populations for further mapping. The near isogenic lines of *Bph43* in 9311 background was developed by the successive backcrossing of the 9311/IRGC 8678 F₁ with 9311. During this process, the gene-linked markers 16–22 and 16–30 flanking the *Bph43* locus were used to select plants with heterozygous *Bph43* from each backcrossed populations for the next step of backcrossing. Finally, one BC₃F₂ individual carrying homozygous *Bph43* and with the least amount of genetic background of IRGC 8678 was selected and designated as NIL-*Bph43*-9311. The detailed procedure for the development of mapping populations and NIL-*Bph43*-9311 is illustrated in Supplementary Figure S1.

2.2. BPH Insects and Evaluation of BPH Resistance

The BPH insects used in this study were collected from rice fields and maintained on the susceptible cultivar Taichung Native 1 (TN1) in a greenhouse at Wuhan University as previously described [20].

For gene mapping, a seedling bulk test for BPH resistance evaluation was performed on the F₃ families as previously described [30,31]. Briefly, approximately 20 seeds per line were sown in a plastic box in 15 cm long rows, with 2.5 cm between rows. A total of

two rows of IRGC 8678, three rows of 9311, and three rows of TN1 were randomly sown among the F_3 families as controls. The seedlings were thinned to 15 plants per row on 7 days after sowing. At the third-leaf stage, the seedlings were infested with eight 2nd to 3rd instar BPH nymphs per seedling. The plastic box was completely enclosed in a fine mesh cage that allowed the light to shine through but prevented the escape of the BPH insects. When all of the seedlings of susceptible control TN1 died, the plants were examined and each seedling was given a resistance score of 0 (none damage, none of the leaves shrank and the plant was healthy), 1 (very slight damage or one leaf was yellowing), 3 (one to two leaves were yellowing, or one leaf shrank), 5 (one to two leaves shrank, or one leaf shriveled), 7 (three to four leaves shrank or two to four leaves shriveled, and the plant was still alive), or 9 (the plant died) based on the modified Standard Evaluation System for Rice [30,31]. A lower resistance score represents a higher BPH resistance level. The resistance score of each F_2 individual was then quantified with Microsoft Excel (ME) as the weighted average of the scores of its corresponding F_3 families. The experiments were conducted with at least three biologically independent replicates.

To verify the location of *Bph43*, a total of 320 BC_2F_2 seeds derived from heterozygous *Bph43* BC_2F_1 plant were sown in a plastic box, surrounded with susceptible 9311 and subjected to the BPH resistance assay. When all of the seedlings of 9311 died, each BC_2F_2 individual was examined and given a resistance score.

The performance of BPH insects on rice plants was evaluated as previously described [20]. In the two-host choice test, one 9311 and one NIL-*Bph43*-9311 plants were grown in the same plastic cup (10 cm in diameter, 15 cm in height). At the four-leaf stage, the cup was covered with a light-transmitting nylon mesh, and 20 2nd to 3rd-instar BPH nymphs were released in the cup. The numbers of BPH insects that settled on each plant were recorded at 3, 6, 24, 48, 72, 96, 120, 144, and 168 h after release. The experiments were repeated at least 20 times. The two-host choice test is an indicator of the BPH antixenotic factor.

For BPH weight gain and honeydew excretion assay, newly emerged female adults were weighed, individually enclosed in a pre-weighed Parafilm sachet and attached to the leaf sheath of the rice plant. After 2 days of insect infestation, both the sachets and BPH insects were weighed again. The weight difference of BPH insects was recorded as the weight gain of BPH, and the weight difference of the Parafilm sachet was recorded as honeydew excretion. At least 15 BPH insects were used for analysis.

2.3. DNA Extraction and Gene Mapping

Total genomic DNA was extracted from fresh rice leaves using modified CTAB protocol [32]. The extracted DNA was dissolved in a $1\times$ TE buffer. According to phenotype of F_2 mapping population, equal amounts of DNA from 11 extremely resistant F_2 plants were mixed to form a resistant bulk DNA pool. Similarly, equal amounts of DNA from 18 extremely susceptible F_2 plants were mixed to form a susceptible bulk DNA pool. The green super rice chip-based bulked segregant analysis method (BSA-chip) was used to map the BPH resistance gene. The DNA pools of the resistant bulk and the susceptible bulk as well as the DNAs of the two parents (9311 and IRGC 8678) were subjected to the green super rice chip GSR 40K array at the Greenfafa (Wuhan, China), according to the Infinium HD Assay Ultra Protocol (available online: <http://www.illumina.com/> (accessed on 17 September 2021)).

For the insertion-deletion (InDel) calling and development, the DNAs of the two parents (9311 and IRGC 8678) were deeply ($\sim 30\times$) sequenced on the platform of HiSeq X Ten. Low-quality reads were deleted or trimmed by a base-quality Q score with a Phred scale of <20 and a read size of <60 bp from raw data using Perl scripts. The cleaned data were mapped to the reference genome of Nipponbare retrieved from Rice Genome Annotation Project website (available online: http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/ (accessed on 20 October 2019)) using Burrows–Wheeler Aligner (BWA) software (version 0.5.8c) [33].

InDels of 20 to 40 bp in length between IRGC 8678 and 9311 were extracted using SAMtools (version 0.1.12a) [34]. The upstream and downstream sequences of the candidate InDel loci on the reference genome Nipponbare were obtained and subjected to PCR primer design using Primer 3. The size of PCR products ranged from 160 to 250 bp, so that the polymorphism between IRGC 8678 and 9311 can be easily identified on a 1.5–2% agarose gel. Primers designed in this study are listed in Supplementary Table S1. PCR was performed as previously described [35], with minor modification for different InDel markers. PCR products were separated on a 1.5–2% agarose gel and stained with ethidium bromide.

2.4. RNA-Seq and Transcriptome Analysis

The seedlings of NIL-*Bph43*-9311 and 9311 plants were infested with BPH nymphs (8 s to third-instar nymphs per plant). At 3 h of BPH infestation, leaf sheaths of the rice plants infected by BPH were collected. The samples were referred to as NIL-3 for NIL-*Bph43*-9311 and 9311-3 for 9311, the number representing 3 h of BPH infestation. At the same time, leaf sheaths of NIL-*Bph43*-9311 and 9311 without BPH infestation were collected as an undamaged control and referred to as NIL-0 and 9311-0, respectively. Two to four biological replicates per treatment with 15 seedlings per replicate were used for RNA-seq analysis. Total RNA was isolated using an RNAPrep Pure Plant Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) following the manufacturer's instructions. Concentrations of RNA were checked and integrity was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cDNA library for each sample was constructed using a TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) and quantified on a 150 bp paired-end run by Agilent2100 and sequenced by illumina novaseq 6000 (Illumina, San Diego, CA, USA). Clean reads were obtained after the removal of adaptors, low-quality reads and reads with >5% unknown nucleotides with Trimmomatic software [36] and mapped on the rice genome of the cultivar Shuhui498 (available online: <http://www.mbkbase.org/R498/> (accessed on 29 October 2021)) using the Hisat2 (v2.2.1.0) [37]. Gene counts were obtained by HTseq, and gene expression was determined using the fragments per kilobase of the exon model per million mapped fragments (RPKM) method [38]. Differentially expressed genes (DEGs) were filtered by DESeq2 after significance. *p*-values were performed at absolute values of log₂FC of ≥ 1 ($p < 0.05$) [39]. The gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs were performed separately using R based on the hypergeometric distribution [40,41]. The summary of RNA-Seq data is presented in Supplementary Table S2. The Pearson's correlation coefficients for all tested samples are shown in Supplementary Figure S2.

2.5. Quantitative Real-Time PCR (qRT-PCR) Analysis

qRT-PCR was used to validate the DEGs analysis results. All of the rice samples used for qRT-PCR verification were prepared as described above. Total RNAs were isolated from rice plants using TRIzol reagent (TaKaRa) and then converted into first-strand cDNA using PrimeScript™ RT reagent kits accompanied with gDNA Eraser (TaKaRa, code no. RR047A) according to the manufacturer's instructions. Gene expression was analyzed by qRT-PCR using SYBR green supermixes and CFX96 Real-Time System (Bio-Rad). Primers are listed in Supplementary Table S1. The expression levels of genes were calculated with the $2^{-\Delta\Delta C_t}$ method using CFX Manager Software 2.1. Being stably expressed in rice various organs and developmental stages, *OsAct1* (AB047313) was used as the internal control for qRT-PCR [42]. Each experiment was performed in three biological replicates and three technical replicates.

2.6. Statistical Analysis

The *t*-test was used to examine the difference between two groups. One-way ANOVA was used to compare multiple samples. Statistical tests were conducted using the software GraphPad Prism 7.

3. Results

3.1. Genetic Analysis of BPH Resistance

IRGC 8678, which was previously reported to be resistant to BPH biotype III [30], exhibited high resistance to the current BPH population of China, the more destructive Bangladesh type [43]. The average resistance scores of IRGC 8678 were 1.15 in two independent tests (Figure 1A), suggesting that IRGC 8678 was highly resistant to BPH and might be a useful donor for BPH resistance breeding programs. We further investigated the performance of BPH fed on IRGC 8678 and 9311 plants. Compared to those on 9311 plants, BPH insects fed on IRGC 8678 showed a significantly lower weight gain, which was 0.1808 mg on average (Figure 1B), indicating a strong antibiosis effect on BPH.

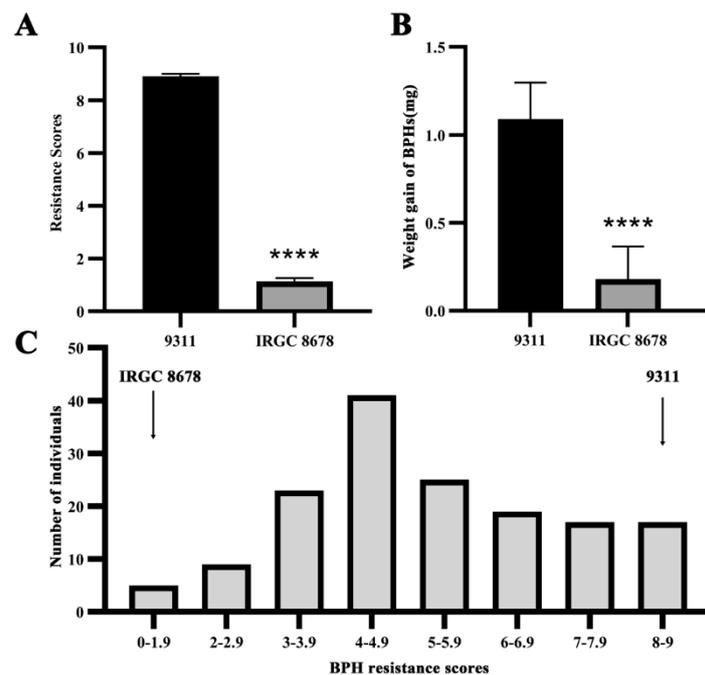


Figure 1. Genetic analysis of brown planthopper (BPH) resistance in IRGC 8678. (A) BPH resistance scores of 9311 and IRGC 8678; (B) weight gains of BPHs fed on 9311 and IRGC 8678 for 48 h. All data are means \pm the standard error of the mean (SEM). **** indicates a significant difference at $p < 0.0001$ by t -test; (C) frequency distributions of the BPH resistance scores in the F₂ population derived from 9311 \times IRGC 8678.

To explore the genetic basis of BPH resistance in IRGC 8678, we evaluated BPH resistance scores of the F₂ mapping population, which showed a continuous distribution with an apparent valley bottom between 7.0 and 7.9 (Figure 1C). According to a previous BPH resistance-scoring criterion [35], we categorized the plants with resistance scores below 6.99 as resistant plants, whereas those with resistance scores above 7.00 were considered as susceptible ones. The segregation of the resistant to susceptible plants was found to be 122:34, which was in agreement with a 3:1 ratio (122:34; $\chi^2_c = 0.504 < \chi^2_{0.05,1} = 3.84$), demonstrating that BPH resistance of IRGC 8678 was controlled by a major dominant gene.

3.2. Molecular Mapping of *Bph43*

To map the BPH resistance gene in IRGC 8678, we performed a bulked segregant analysis (BSA) analysis. According to the phenotype of F₂ population, 11 extremely resistant plants and 18 extremely susceptible plants were selected to prepare two contrasting bulks, respectively. The green super rice chip GSR 40K was then used to determine the region containing putative BPH resistance genes. It was revealed that there was only one contiguous region ranging from 8.0 to 18.0 Mb on chromosome 11 significantly differentiating between

the resistant and susceptible bulks (Figure 2A), suggesting that the BPH-resistant gene in IRGC 8678 is located in this region. We designed this gene as *Bph43*.

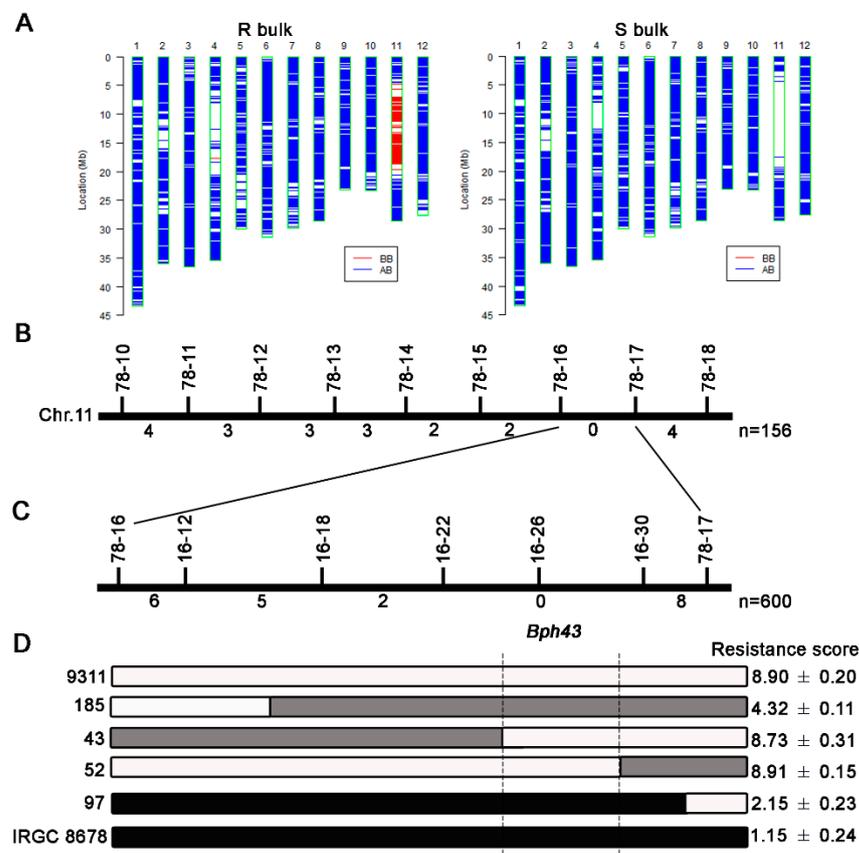


Figure 2. Molecular mapping of *Bph43*. (A) Mapping of *Bph43* on rice chromosome 11 using bulked segregant analysis (BSA)-based rice chip GSR 40K analysis. Red lines indicate the genetic background of IRGC 8678, while white ones indicate that of 9311. Blue lines indicate the heterozygous genomic fragments between IRGC 8678 and 9311. R, resistant; S, susceptible; (B) mapping of *Bph43* on the genomic region flanked by markers 78–16 and 78–17; (C) physical map of marker intervals 78–16 and 78–17. The numbers below the line in (A,B) indicate the numbers of recombinants between adjacent markers; (D) graphical genotypes and resistance phenotypes of the recombinants. The black, white, and grey bars denote the genotypes of IRGC 8678 homozygotes, 9311 homozygotes, and heterozygotes, respectively.

To detect the exact location of *Bph43* on chromosome 11, we developed 13 polymorphic InDel markers on chromosome 11 and used them to determine the genotypes of 156 F₂ plants with evaluated BPH resistance scores, including those 11 extremely resistant plants and 18 extremely susceptible plants used for BSA-chip analysis. Consequently, *Bph43* was primarily mapped to the 16.2–17.6 Mb region on chromosome 11 flanked by markers 78–16 and 78–17 (Figure 2B). A further analysis of 600 BC₁F₂ plants using the flanking markers 78–16 and 78–17 identified 21 recombinants. These recombinants were genotyped with polymorphic InDel markers within the mapping interval (Figure 2C). According to the genotypes and BPH resistance phenotypes of the recombinants, *Bph43* was located between InDel markers 16–22 and 16–30 (Figure 2D), within intervals of 350 and 380 kb according to R498 and Nipponbare reference genomes, respectively. Putative genes were predicted in the corresponding candidate regions of R498 and Nipponbare reference genomes by using a rice genome annotation project database [44]. A gene cluster encoding putative nucleotide-binding domain leucine-rich repeat-containing (NBS-LRR) proteins and LRR family proteins was identified in *Bph43* mapping regions of both R498 and Nipponbare

reference genomes (Supplementary Table S3). Interesting, one of them was found to be specifically induced in NIL-*Bph43*-9311 upon BPH infestation as revealed by RNA-Seq (Supplementary Table S3). As most BPH resistance genes isolated to date encode NBS-LRR proteins, we considered these putative genes encoding NBS-LRR and LRR family proteins as the candidates for *Bph43*.

3.3. Verification of *Bph43* in BC₂F₂ Populations

To confirm the location of *Bph43*, BC₂F₁ plants that were heterozygous between InDel markers 16–22 and 16–30 were selected to produce BC₂F₂ generations. BPH resistance evaluation revealed 236 and 84 seedlings were resistant and susceptible to BPH, respectively, and the segregation of the resistant to susceptible plants was in agreement with a 3:1 ratio (236:84, $\chi^2_c = 0.267 < \chi^2_{0.05,1} = 3.84$). We further genotyped these surviving resistant 236 BC₂F₂ plants with InDel markers 16–22 and 16–30. All of them contained either homozygous or heterozygous alleles from the resistant parent IRGC 8678, verifying the existence of *Bph43* on the region between markers 16–22 and 16–30.

3.4. Development and Characterization of NIL-*Bph43*-9311

9311, an elite restorer parent for hybrids in China, is highly susceptible to BPH. To improve its BPH resistance, 9311 was used as the recurrent parent to backcross with IRGC 8678 (Supplementary Figure S1). In the process, the flanking markers 16–22 and 16–30 tightly linked to *Bph43* locus were used to select the positive progenies for continuous backcrossing. Simultaneously, background selections using rice chip GSR 40K were conducted. One BC₃F₁ plant containing a heterozygous *Bph43* region and the least amount of genetic background of IRGC 8678 was selected to produce BC₃F₂ populations. Finally, one line homozygous for *Bph43* locus and morphologically similar to 9311 was selected as NIL-*Bph43*-9311 (Supplementary Figure S3). Thus, *Bph43* has been successfully introgressed into 9311 through MAS.

We further characterized BPH resistance of NIL-*Bph43*-9311. NIL-*Bph43*-9311 plants that were homozygous or heterozygous for *Bph43* exhibited high resistance to BPH, and no significant difference was detected between *Bph43*-heterozygous and -homozygous plants in terms of resistance scores (Figure 3A,B). The results suggested that *Bph43* is valuable in hybrid rice breeding. BPH insects fed on NIL-*Bph43*-9311 plants showed a significantly lower honeydew excretion and weight gain than those of 9311 (Figure 3C,D), indicating that *Bph43* conferred strong antibiosis effects on BPH. In two-host choice tests, many more BPH insects preferred to settle on 9311 compared with on NIL-*Bph43*-9311 plants (Figure 3E), suggesting that *Bph43* also had a stronger antixenotic effect on BPH.

3.5. Comparative Transcriptome Analysis of NIL-*Bph43*-9311 and 9311 Underlying BPH Infestation

To further understand the molecular mechanism underlying BPH resistance conferred by *Bph43*, RNA-Seq analysis was performed using the stems of NIL-*Bph43*-9311 and 9311 at the early stage (3 h) of BPH infestation (Supplementary Table S2 and Supplementary Figure S2). One hundred and ninety constitutive DEGs between NIL-*Bph43*-9311 and 9311 without BPH infestation were identified (Supplementary Table S4) and significantly enriched in the defense response, defense response signaling pathway (resistance gene-dependent), plant-type hypersensitive response, and so on (Supplementary Table S5 and Supplementary Figure S4A). At 3 h after BPH infestation, a higher number of DEGs (449 vs. 281 DEGs) were identified in NIL-*Bph43*-9311 plants than in 9311, with 208 down- and 241 upregulated, respectively (Supplementary Table S4). These DEGs were mainly enriched in the regulation of the jasmonic acid mediated signaling pathway, monoterpene biosynthetic process, terpenoid biosynthetic process, regulation of defense response, and so on (Supplementary Table S5 and Supplementary Figure S4C). Upregulated DEGs in NIL-*Bph43*-9311 and 9311 upon BPH infestation were further analyzed. In total, there were 47 upregulated DEGs (G2) shared by NIL-*Bph43*-9311 and 9311, 194 upregulated DEGs (G1) unique to

NIL-Bph43-9311, and 183 upregulated DEGs (G3) unique to 9311 (Figure 4A and Supplementary Table S6). We then focused on analyzing DEGs in G1, as these DEGs are putatively involved in the molecular mechanism underlying *Bph43*-mediated resistance. GO enrichment analysis revealed that G1 was significantly enriched in the root development, defense response and defense response signaling pathway (resistance-gene-dependent), hydrogen peroxide catabolic process, plant-type hypersensitive response, and so on (Figure 4B and Supplementary Table S6).

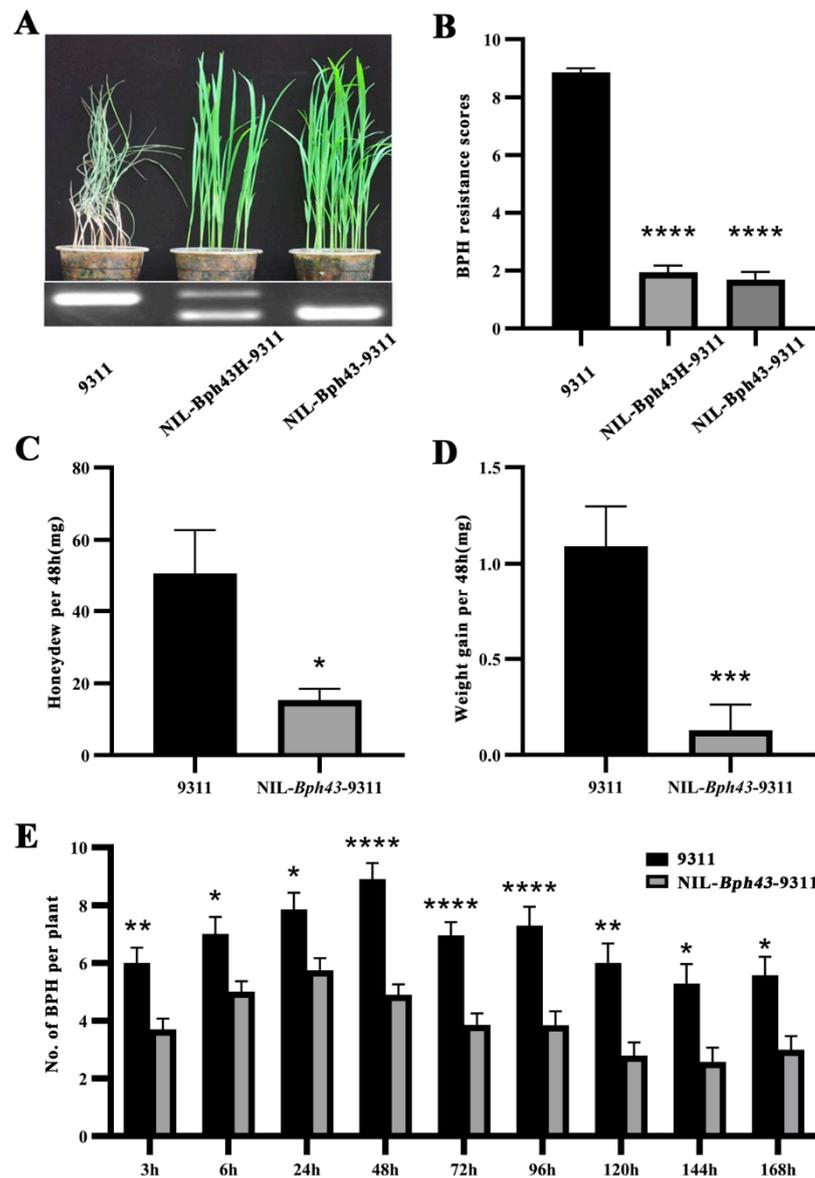


Figure 3. Characterization of BPH resistance of *NIL-Bph43-9311*: (A) seedling resistance tests of heterozygous and homozygous *NIL-Bph43-9311* plants. The PCR band patterns amplified with insertion-deletion (InDel) markers 16–26 showed that *NIL-Bph43-9311* was homozygous for *Bph43* and heterozygous for *NIL-Bph43H-9311* and 9311 contained no *Bph43*; (B) BPH-resistance scores of 9311, *NIL-Bph43H-9311*, and *NIL-Bph43-9311*; (C) honeydew excretion; and (D) weight gains of BPH feeding on *NIL-Bph43-9311* and 9311 for 2 days; (E) numbers of BPH insects settled on *NIL-Bph43-9311* and 9311 in the two-host choice test. Data represent means \pm SEM of 20 replicates. The asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; Student's *t*-test).

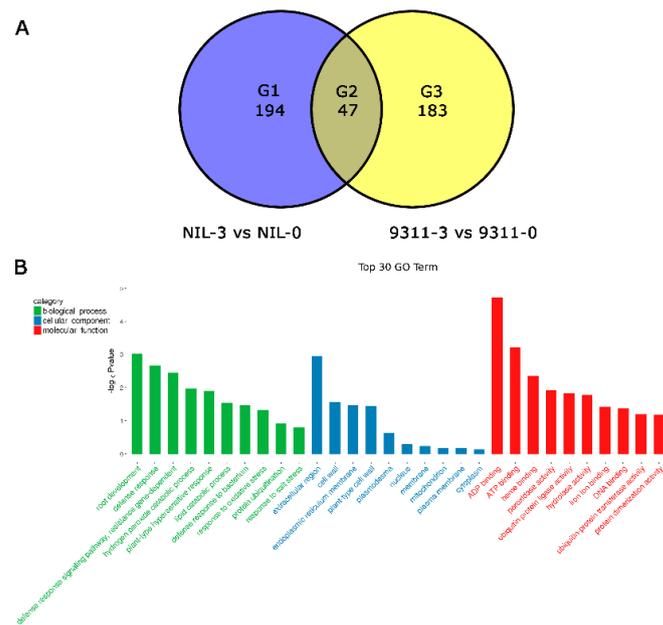


Figure 4. Gene ontology (GO) annotation of upregulated differentially expressed genes (DEGs): (A) Venn analysis of upregulated DEGs between NIL-*Bph43*-9311 and 9311; (B) top 30 GO terms of specific upregulated DEGs in NIL-*Bph43*-9311. 9311-0 and 9311-3 refer to 9311 sampled at 0 and 3 h of BPH infestation, respectively, and NIL-0 and NIL-3 refer to NIL-*Bph43*-9311 plants sampled at 0 and 3 h of BPH infestation, respectively.

To validate the RNA-Seq results, three DEGs of G2 upregulated in both NIL-*Bph43*-9311 and 9311 and five DEGs of G1 upregulated unique to NIL-*Bph43*-9311 were randomly chosen for the validation of the transcriptomic data using qRT-PCR. The expression profiles of eight genes in qRT-PCR were consistent with the RNA-Seq (Figure 5), confirming the accuracy and reproducibility of the RNA-Seq in the present study.

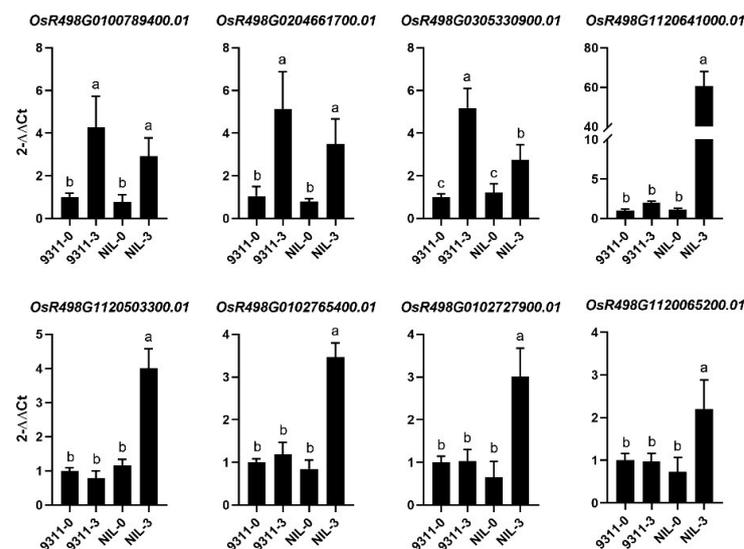


Figure 5. Quantitative real-time PCR (qRT-PCR) validation of the selected DEGs in 9311 and NIL-*Bph43*-9311. *OsAct1* was used as an internal control. The relative expression levels of each gene were measured using the 2^{-ΔΔCT} method. Data represent the means (three biologically independent experiments for gene expression) ± SEM. Different letters above the bars indicate significant differences ($p < 0.05$).

4. Discussion

BPH has become the most destructive pest of rice, resulting in a huge annual rice yield loss [4,5]. Breeding and deploying of resistant cultivars is the most effective, economical, and environment-friendly solution for BPH control [12]. So far, around 40 BPH resistance genes have been identified, and 17 of them have been isolated via a map-based cloning approach [13–25]. Most BPH resistance genes are clustered on specific chromosomes. For example, *Bph1*, *bph2*, *bph7*, *Bph9*, *Bph10*, *Bph18*, *Bph21*, and *Bph26* are clustered on chromosome 12L [20,45–49]. Twelve genes are clustered in three regions on chromosome 4 (*Bph30* and *Bph33* in a 0.91–0.97 Mb region; *Bph3/17*, *Bph12*, *Bph15*, *Bph20(t)*, and *bph22(t)* in a 4.1–8.9 Mb region; and *Bph6*, *bph18(t)*, *Bph27*, *Bph27(t)*, and *Bph34* in a 19.1–25.0 Mb), whereas *Bph3*, *bph4*, *bph25*, *bph29*, and *Bph32* are located in a 0.2–1.7 Mb region on chromosome 6 [16,19,50–52]. In the present study, *Bph43* was mapped to a region of ~380 kb on chromosome 11 flanked by InDel markers 16–22 and 16–30. Previously, *Bph28(t)* in rice variety DV85 was physically defined to an interval of 64.8 kb on chromosome 11 [53]. A protein transport Sec24-like gene (LOC_Os11g29200), a tetratricopeptide-like helical domain-containing protein gene (LOC_Os11g29230), a PHD zinc finger (LOC_Os11g29240), and three hypothetical protein genes are predicted in the interval [53]. *Bph28(t)* was located in the region of 16.92–16.99 Mb on chromosome 11 according to Nipponbare genome sequence. The position of *Bph43* was outside this interval. Thus, *Bph43* should be different from *Bph28(t)*. It is reasonable to conclude that *Bph43* is a novel major gene for BPH resistance.

It has been proposed that a large number of BPH resistant varieties come from South Asia [27,30,54]. In this study, IRGC 8678, which is originated from Bangladesh and reported to be resistant to BPH biotype III [30], also showed high resistance to the current BPH population of China, the more destructive Bangladesh type (Figure 1A,B). These results implied that *Bph43* in IRGC 8678 could be a potential durable and broad-spectrum resistance gene. Generally, rice may employ three resistance mechanisms against BPH, including antixenosis, antibiosis, and tolerance [55]. We have demonstrated that *Bph43* in IRGC 8678 confers strong antibiosis and antixenosis effects on BPH. Meanwhile, *Bph43* is a dominant gene, as plants homozygous or heterozygous for *Bph43* both exhibited high resistance to BPH at a similar level (Figure 3A,B). These characteristics make *Bph43* a valuable candidate for hybrid rice breeding and production.

Most BPH resistance genes isolated to date encode nucleotide-binding site and leucine-rich repeat receptors (NLRs) proteins [13–25]. For example, *Bph14* and *Bph9* and its alleles encode classical NLR proteins [17,20]. *Bph6* and *Bph30* encode novel atypical LRR proteins [23,24]. We identified a gene cluster encoding putative NLR and LRR proteins in the *Bph43* mapping region. One of them was specifically induced in NIL-*Bph43*-9311 (Supplementary Table S3). We considered these NBS-LRR and LRR family proteins encoding genes as the candidates for *Bph43*. It has been found that *Bph3* in Rathu Heenati (RH) comprises a cluster of three genes encoding plasma membrane-localized lectin receptor kinases (OsLecRK1–OsLecRK3) which function together to confer broad-spectrum and durable insect resistance in rice [21]. *Bph43* may function in a similar way. Whether these genes work alone or function together requires further investigation.

Transcriptome analysis is an effective way to explore rice–BPH interactions [43,56]. In this study, we also used RNA-Seq to explore the molecular mechanism underlying *Bph43*-mediated resistance at the early stage of BPH feeding. Genes related to defense pathways were enriched in un-infested NIL-*Bph43*-9311 plants, suggesting that the plants might be in a primed state. After BPH infestation, NIL-*Bph43*-9311 initiated more intensive and prompt defense responses. Comparative analysis revealed that there were 194 upregulated DEGs unique to NIL-*Bph43*-9311 upon BPH infestation, significantly enriched in the root development, defense response and defense response signaling pathway (resistance gene-dependent), hydrogen peroxide catabolic process, and plant-type hypersensitive response. These genes are putatively involved in BPH resistance mechanism mediated by *Bph43*.

5. Conclusions

Due to the rapid adaptation of BPH or evolution of new biotypes, it is still urgent to explore and utilize more new broad-spectrum BPH resistance genes. In the current study, we explored the genetic basis of BPH resistance of rice cultivar IRGC 8678. We identified and mapped a new resistance gene *Bph43* to a region of ~380 kb on chromosome 11. *Bph43* was successfully introgressed into elite restorer line 9311 through MAS. The resulted near isogenic lines NIL-*Bph43*-9311 had strong antibiosis and antixenosis effects on BPH. Comparative transcriptome analysis revealed genes related to the defense response and resistance gene-dependent signaling pathway were significantly and uniquely enriched in *Bph43* plants and might be the underlying molecular mechanism for BPH resistance. Our work provides a valuable BPH resistance gene for rice breeding programs.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12040808/s1>, Table S1: Primers used in this study. Table S2: Summary of RNA-Seq data. Table S3: Candidate genes predicted in the reference genomes and their expression analysis by RNA-Seq. Table S4: Differentially expressed genes (DEGs) in response to BPH feeding between NIL-*Bph43*-9311 and 9311. Table S5: Gene ontology (GO) annotations of DEGs in response to BPH feeding between NIL-*Bph43*-9311 and 9311. Table S6: Go annotations of upregulated DEGs in response to BPH feeding between NIL-*Bph43*-9311 and 9311. Figure S1: Strategy used to develop NIL-*Bph43*-9311. Figure S2: FPKM box diagram of all tested samples and heatmap of Pearson's correlation coefficients for all tested samples. Figure S3: Genetic background assay of NIL-*Bph43*-9311 with rice chip GSR 40K array. Figure S4: Enriched GO annotations of DEGs in response to BPH feeding between NIL-*Bph43*-9311 and 9311.

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