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Fine mapping, candidate genes analysis, and characterization of a brown planthopper (*Nilaparvata lugens* Stål) resistance gene in the rice variety ARC5984

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Abstract The brown planthopper (*Nilaparvata lugens* Stål; BPH) is the most destructive rice pests worldwide. Identification and application of major resistance genes and cultivation of resistant varieties are the most efficient methods to control this insect. In this study, the rice variety, ARC5984, was detected to be highly resistant to BPH due to the presence of a major resistance gene, which has primarily been mapped in a 6-cM region on the long arm of chromosome 4. Fine mapping of the resistance gene was performed using the recombinants derived from BC_1F_2 individuals; the target gene was described to be a 46-kb region in the Nipponbare genome. Based on the information on the genomic sequences of the varieties Nipponbare, ZS97 and MH63, six, seven and 11 candidate genes were identified in the finally located region, respectively. The relative expression of the candidate genes *Os04g35190* and *Os04g35210* was significantly different between the resistant and susceptible plants after BPH infestation. The sequencing results indicated the coding domain sequence (CDS) of *Os04g35210* from ARC5984 to be identical to that of *Bph6*. But the CDS of *Os04g35190* was different between in ARC5984 and 9311. Moreover, the preliminary-near-isogenic lines (pre-NILs; the lines carrying resistance genes) with genetic background of the 9311 showed significant antixenosis to BPH, and thus, restricted its growth and development when compared with that in the susceptible line of 9311. Finally, a marker-assisted selection (MAS) technique was developed for the markers, RM5635 and BF9; its efficiency has been confirmed by applying it in screening of backcrossed individuals. The development in the fine mapping of the major gene will facilitate efforts of cloning this important resistance gene and to apply it in breeding of BPH-resistance rice varieties.

Keywords Brown planthopper · *Nilaparvata lugens* Stål · Genetic mapping · Preliminary-near-isogenic line · BPH resistance gene · Candidate gene analysis

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Introduction

The brown planthopper (*Nilaparvata lugens* Stål, BPH) is a monophagous rice (*Oryza sativa* L.) pest and seriously threatens rice production in the tropics and sub-tropics. It destroys crop by piercing the rice shoot or leaf and by sucking the phloem sap using its mouthparts, which negatively affects rice growth (Watanabe and Kitagawa 2000). It also transmits the ragged or grassy stunt virus and associated diseases (Khush and Brar 1991). Infestations of BPH have caused heavy rice yield losses and consequent economic losses across Asia (Normile 2008; Chen et al. 2006). According to the *Statistics of China Agriculture Yearbook*, there have been about 1–1.5 billion kg of rice production losses, which is equivalent to annual economic losses of multi-billions of Yuan (CNY). Therefore, it is not only important but urgent to control this rice pest, thereby protecting rice production.

Application of chemical insecticides is a conventional method that has been widely used in the rice-growing areas for reduction of the damage caused by BPH. However, the method is costly and non-ecofriendly in the long term. Furthermore, BPH has developed resistance against many insecticidal compounds, including isoprocarb, carbofuran, and even against synthetic pyrethroids (Lakshmi et al. 2010). Hence, safe and effective methods need to be explored and applied to add to the conventional ways of fighting this pest. The application of resistant rice varieties is considered to be an efficient way to minimize insecticide use and to promote integrated pest control (Way and Heong 1994; Renganayaki et al. 2002).

A total of 35 major BPH resistance genes from diverse rice species have been identified till date (Wang et al. 2018). Among the reported BPH resistance genes, 13 loci (*Bph1-9, Bph17*, *bph19*, *BPH31*, *Bph32*) were derived from diversified *indica* cultivars, while the other loci were identified in the wild rice species (Athwal et al. 1971; Lakshminarayana and Khush 1977; Khush et al. 1985; Kabir and Khush 1988; Nemoto et al. 1989; Sun et al. 2005; Chen et al. 2006; Huang et al. 2013; Jena et al. 2017). Great effort has been made to clone the BPH resistance genes in the past 10 year. In this regard, 12 genes (*Bph14*, *Bph18*, *Bph3*, *Bph26*, *Bph29*, *Bph1/7/9/10/21*, *Bph32*, and *Bph6*) have been cloned and primarily characterized (Du et al. 2009; Tamura et al. 2014; Liu et al. 2015; Wang et al. 2015; Ji et al. 2016; Zhao et al. 2016; Ren et al. 2016; Guo et al. 2018). However, the usage of major resistance genes in the molecular marker-assisted selection (MAS) of genes derived from different rice species is still a problem because it requires that the gene-linkage be overcome, which involves meticulous and laborious transfer of the target gene. Moreover, most of the identified resistance genes do not confer high resistance levels against the different BPH biotypes. Therefore, it is still imperative to identify newer and stronger resistance genes against BPH, especially in the diverse cultivars of rice, and transfer them to the elite BPH-susceptible cultivars to generate BPH-resistant rice varieties.

Several types of resistance mechanisms, such as antibiosis, antixenosis, or tolerance, are naturally employed by plants, when they are attacked by insect (Alam and Cohen 1998; Kennedy et al. 1987). Previous research has suggested that several major BPH resistance genes, including *Bph14*, *Bph6*, and *Bph9*, confer resistance via two different mechanisms (Du et al. 2009; Qiu et al. 2010; Zhao et al. 2016). Moreover, some major BPH resistance genes have been demonstrated to have one certain mechanism. For example, *BPH7* was reported to confer tolerance to BPH after it was mapped by a seedling bulk test (Qiu et al. 2012). Therefore, it is essential to study the mechanisms conferred by the resistance genes using preliminary-near-isogenic lines (pre-NIL). It is better to understand the different resistance mechanisms and to breed durable BPH-resistant varieties.

ARC5984 is an *indica* rice variety that has been reported to be resistant to BPH and gall midge insect populations (Kalode and Krishna 1979; Kumar et al. 1998). It was identified to be highly resistant to the BPH population collected from Nanning, Guangxi in our recent insect resistance experiments. Here, we aimed to finely map and analyze the candidate resistance genes in this rice variety and then explore it via MAS.

Materials and methods

Plant materials and mapping populations

ARC5984 is an *indica* rice variety that was shown to be resistant to BPH and gall midge insect populations (Kalode and Krishna 1979; Kumar et al. 1998). In the present insect resistance test, it was identified to be

highly resistant to the BPH population collected from Nanning, China. The 3-leaf seedlings of ARC5984 grew well after 12 days of infestation with BPH as compared with the susceptible line, 9311, whose average resistance score was 2.7 in the seedling bulk test (Supplementary Fig. 1). Then ARC5984 was crossed with 9311 to create an F_2 population which included 230 families. The mapping population was used to identify the resistance gene. The markers flanking the locus were applied to select heterozygous plants in the BC_1F_1 population (with 9311 genetic background). From among these, the BC_1F_2 lines were used to screen recombinants in the region-of-interest. The heterozygous BC_1F_1 plants were backcrossed twice with the susceptible 9311 line to obtain the BC_3F_1 generation. From this, several positive plants carrying the resistance gene were selected and allowed to self-pollinate twice to produce BC_3F_3 lines that were homozygous at the target region and were identified to be the same as the recurrent parent 9311 as per their agricultural traits. The homozygous BC_3F_3 lines were designated as pre-NILs. They were then used in antibiosis and antixenosis tests (Supplementary Fig. 2).

Rice seeds were germinated at 30 °C and then transferred to plastic boxes (58 × 38 × 9 cm) or plastic buckets (25 cm diameter, 15 cm height) with paddy field soil. Seedlings were grown with an average day/night temperature of 32 °C/26 °C, 75% relative humidity under natural light.

BPH insects and evaluation of BPH-resistance

The insects used in the present experiments were from rice fields in Nanning, China, and reared on Taichung Native 1 (TN1, a susceptible *indica* variety) with an average day/night temperature of 32 °C/26 °C, 75% relative humidity in a greenhouse at Guangxi University, China. The BPH resistance evaluation of F_3 families was performed as described previously (Qiu et al. 2010). The rice lines, ARC5984, 9311, and TN1, were taken as controls. Each plant was scored when all of the TN1 plants died. The experiments were conducted twice. The same method was performed with the BC_2F_2 or BC_3F_2 lines.

Genetic map construction and gene detection

Bulked segregant analysis (BSA) was used to create a local genetic linkage map and identify the resistance gene (Michelmore et al. 1991). Firstly, two DNA bulks contained ten extremely resistant or susceptible individuals were screened with molecular markers from 12 rice chromosomes. Then the resistance gene-containing region was analyzed with the phenotype and genotype of F_2 population by performed JoinMap 3.0 (Van Ooijen and Voorrips 2001) and MapQTL 5 (Van Ooijen 2004).

Marker-assisted selection

A CTAB method described previously by Murray and Thompson (1980) was used to isolate genomic DNA of fresh leaves. The sequence-of-interest was amplified in a 10 μL reaction system consisting of 1 μL (15–20 ng) of genomic DNA, 0.2 μM of the forward and reverse primers each, 5 μL of 2 X PCR mix, and 3.8 μL of ddH_2O. The PCR products were then examined by using the silver staining method in a 6% denaturing or non-denaturing polyacrylamide gel (PAGE) or 2.5% agar gel.

Host selection behavior of BPH

To observe the BPH's host selection behavior, four seedlings (14 days old) of pre-NIL and 9311 were transplanted in a bucket covered with a fine light-transmitting mesh. Then each 28-day-old plant was infested with 15 s-instar nymphs. The number of BPH settled on each plant was counted at 3, 6, 9, 24, 48, 72, 96, and 120 h after release. A total of seven buckets were used in the test.

BPH development on rice plants

To quantify honeydew excretion and growth weight of BPHs, the seedlings were treated the same way as done for the host selection test. One pre-weighed BPH with a short wing (brachypterous) was released in a rectangular parafilm bag (3.5 cm length, 3 cm width) which was also pre-weighed and fastened on the rice shoot. Each BPH was collected and the weight was recorded after 2 days; the bag with honeydew excretion was also weighed. The honeydew excretion by the BPH and the growth weight were calculated according to the method of Qiu et al. (2010). Each plant was installed with two such parafilm bags; a total of ten plants for each genotype were tested.

To study insect survival and emergence on plants with different genotype, ten second-instar nymphs were released on each 24-day-old seedling covered with a light-transmitting plastic cup. Then the surviving insects were counted every day and the emerging insects were detected at the ninth day. Ten replicates were set up for each genotype.

Quantitative real-time PCR analysis

To detect the gene expression in pre-NIL and 9311, 14-day-old seedlings were treated with eight insects (third-instar) per plant at 0 (control), 12, 24, and 48 h after BPH release. Reverse transcription-PCR (RT-PCR) was conducted with total RNA according to the manufacturer's instructions of the PrimeScriptTM RT reagent kit (TaKaRa). Gene primers for qRT-PCR were designed according to the reference cDNA sequences of Nipponbare and 9311 (Table 1). The qRT-PCR reaction mixture had 2 μL of cDNA, 0.5 μL of 10 μM forward and reverse primers each, 10 μL of TransStart Top Green qPCR SuperMix (2×) (TRANS), and 7 μL of ddH_2O. The program was set as follows: 94 °C for 30 s, 40 cycles of 94 °C for 5 s, 56 °C for 15 s and 72 °C for 10 s, followed by a final step of 94 °C for 5 s, 56 °C for 15 s, and 72 °C for 10 s. Three biological replicates were analyzed for each sample. Relative gene expression was quantified using the $2^{-\Delta CT}$ method. *OsActin* (Accession no. AB047313) was used as a reference (internal control) for mRNA expression.

Statistical analyses

The Chi square test for goodness-of-fit was performed using MS-Excel. All other data were analyzed by one-way ANOVA and compared using the least significant difference (LSD) test at a 5% or 1% significance level.

Results

BPH resistance analysis and gene identification

Based on the insect resistance evaluation of the mapped population, the BPH resistance scores of F_2 lines showed a continuous range from 2.4 to 9.0, with an obvious valley between 7.0 and 7.9 in the distribution curve (Fig. 1). It suggested that there had major BPH resistance genes that control the phenotypic segregation in the mapped F_2 population.

A total of 1160 SSR and InDel markers from 12 rice chromosomes were used for the extremely phenotypic-based DNA bulk survey. Three markers,

Table 1 Primers for resistance gene mapping and quantitative real-time PCR analysis

Marker and gene ID	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Type
RM5635	TCGCGTCGTAGCTGAACACTGC	TAGCTTGCTCTCCCTCTGCTTGC	170	SSR
BF3	ACAGCGAGAGGATGAGAAGC	GATAGAATATGGCGGCGACCT	270	InDel
RM5757	GCTGATGCGGAACAAGGAGACC	GATCAGATCACCACCCGAATGAGC	180	SSR
BF9	ATGTTCATACCGCAGGGGTG	AGTCTGAACATTACCTCCCCCA	160	InDel
BF21	TCTTCCCGCTGACCTCGAACG	TGCGACCTCACCGTCTTCATGG	178	SSR
RM5742	GATCCTCAAACGGCCTCTGC	CCTTCAAAGTTTACTCACGCTCTGC	190	SSR
Os04g35190	AACTCTGGGCGCAAACAAC	GGAACAAACACAAATGGGGTC	189	qRT-PCR
Os04g35210	CAGCTTTGCATCACCACCTT	TGGAGAAACTGCCACTTCCT	120	qRT-PCR
Os04g35220	AATGGACATGCAACTGGAGA	TGCGATTTCTCTTTGCATTGC	91	qRT-PCR
Os04g35240	GTCGGTCGAGGAGATCATGA	GTTGTCCTCCTGCTCATCCT	109	qRT-PCR
OsActin	CAGCACATTCCAGCAGAT	GGCTTAGCATTCTTGGGT	120	qRT-PCR

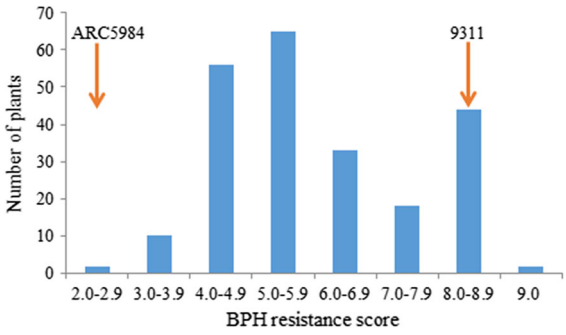

Fig. 1 Frequency distribution for the BPH resistance scores of an F_2 population derived from the crossing of 9311/ARC5984. Three-leaf seedlings were treated with eight BPHs per plant for 9–10 days. The average resistance scores of the parents ARC5984 and 9311, were 2.7 and 8.7, respectively. Lower scores indicate higher resistance

RM5635, 4M19.06, and RM5742, from a contiguous region on chromosome 4 had polymorphic bands between the resistance and susceptibility DNA bulks. None polymorphic markers were found in other chromosomal regions. This result suggests that the major BPH resistance gene is located in this region. Therefore, together with RM5635, 4M19.06, RM5742, additional markers from the chromosomal region of interest of the parents were used to assay the genotype of 230 F_2 lines. Following this, a local linkage map was constructed by JoinMap 3.0 using the acquired genotypic data (Fig. 2). The map thus prepared covered 64-cM of chromosome 4, and the order of markers in this linkage map was in agreement with the reference genomes of Nipponbare and 9311. To detect the exact location of the resistance gene, we analyzed the resistance scores and genotypes of the F_2 lines by performing inter-mapping by MapQTL 5. As a result of this mapping, one locus for BPH resistance with an likelihood of odds (LOD) score of 59 was detected in a 6-cM region between markers RM5635 and RM5742 on the long arm of chromosome 4 (Fig. 2). This gene was tentatively designated as *qBph4*(*t*) according to the nominating rule. Variation at this locus explained 72% of the phenotypic variance in BPH resistance in the F_2 population. Some other most relevant markers investigated here: RM5635, RM5757, and RM5742, had large LOD scores of 36, 53, and 43; they explained 52%, 66%, and 58% of the phenotypic variance (Table 2).

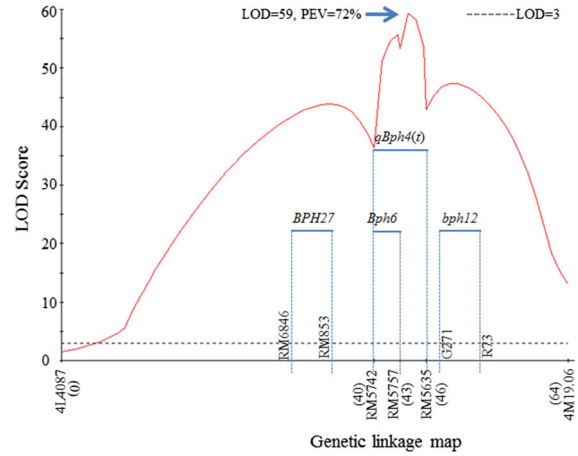

Fig. 2 Location of the *qBph4*(*t*) gene on the linkage map of rice chromosome 4 constructed using an F_2 population derived from 9311/ARC5984 crossing. Markers are along the X-axis with distances (in cM) shown and LOD scores on the Y-axis. The phenotypic variance (PEV) is explained by the locus. Positions of *BPH27*, *Bph6, and bph12* are based on reports of Huang et al. (2013), Qiu et al. (2010), and Hirabayashi et al. (1998), respectively

Fine mapping of the resistance gene

To finely map the resistance gene, we detected the genotype of 4200 BC_1F_2 seedlings to identify the recombinants of the markers, RM5742 and RM5635. A total of 56 recombinants were identified. In addition, we observed three polymorphic markers (BF3, BF9) in the target region by comparing with the genomic

Table 2 Genetic effect estimated on the progeny data by MapQTL 5

Position (cM)	Marker	LOD	PEV (%)[a]	A[b]	D
0	4L4087	2.1	3.6	0.37	− 0.13
40	RM5742	36.4	51.8	1.55	− 0.71
43	RM5757	53.2	65.6	1.76	− 0.71
46	RM5653	42.9	57.7	1.66	− 0.61
64	4M19.06	13.1	23.2	1.02	− 0.39

[a]The percentage of total phenotypic variance (PEV %) was explained by the locus

[b]A = (mu_A − mu_B)/2, D = mu_H − (mu_A + mu_B)/2; A, additive effect of the associated marker indicated from 9311; D, dominant effect of the associated marker indicated from 9311; mu_A : the estimated mean of the distribution of the quantitative trait associated with 'a' genotype; mu_B: idem for the 'b' genotype; mu_H: idem for the 'h' genotype (Van Ooijen 2004)

sequences of Nipponbare and 9311. Therefore, the markers RM5635, BF3, RM5757, BF9, and RM5742 were used to analyze the genotype of the screened recombinants (Table 3). Lines 1 and 2 that harbored the 9311 homozygous alleles at the BF3, RM5757, and BF9 loci were highly susceptible to the BPH and had average resistance scores of 8.4 and 8.5, respectively. Line 8 was highly resistance to BPH (a resistance score of 3.1), and carried the ARC5984 homozygous alleles at RM5757 and BF3 loci. Lines 3–7 had heterozygous alleles at the target loci and showed moderate resistance to BPH. It suggests that any lines with the parent allele of the resistance gene at the RM5757 loci showed resistance phenotype. But the lines with the 9311 alleles at the target region were highly susceptible to BPH infestation. Thus, the major resistance gene *qBph4*(*t*) was located in a 46-kb region and was bordered by markers, BF3 and BF9.

Candidate genes in the target region

Six candidate genes were harbored in the 46-kb region of the Nipponbare genome, based on crosschecking with the database of Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/) (Table 4). As per their functional category, the candidate genes included F-box domain containing protein (*LOC_Os04g35190*), leucine-rich repeat protein family (*LOC_Os04g35210*), calcium/calmodulin-dependent protein kinases (CAMK) (*LOC_Os04g35240*), and three expressed proteins (*LOC_Os04g35200*, *LOC_Os04g35220*, and *LOC_Os04g35230*). According to information on the same region in the genomes of ZS97 and MH63 lines, the physical distances were 47 kb and 75 kb, respectively, and 7 and 11 candidate genes were harbored in their corresponding regions, respectively (http://rice.hzau.edu.cn/rice/) (Table 4). Based on the functional category of ZS97, three candidate genes–*ZS04g0517700* (co-chaperone GrpE protein), *ZS04g0517900* (acetyltransferase), and *ZS04g0518300* (scarecrow-like protein)–were different from Nipponbare. As for the genome of MH63, seven candidate genes were different from those in Nipponbare. Among them, one candidate gene, *MH04g0430300*, was predicted to be a putative disease resistance gene. After that, the candidate gene (*LOC_Os04g35190*, *ZS04g0517800*, *MH04g0429800*) was predicted to be a defense-related F-box protein gene in the genomes of ZS97 and MH63.

Expression and sequence analysis of the candidate genes

As the two candidate genes *Os04g35190* and *Os04g35210* were most possibly associated with

Table 3 Marker genotypes and phenotypes of the recombinants used for gene fine mapping

Parents and recombinants	RM5635 (20.88 Mb)[1]	BF3 (21.38 Mb)	RM5757 (21.42 Mb)	BF9 (21.43 Mb)	RM5742 (21.56 Mb)	Resistance score
9311	A[2]	A	A	A	A	8.8 ± 0.2a[3]
1	A	A	A	A	H	8.5 ± 0.7a
2	H	A	A	A	A	8.4 ± 0.5a
3	H	H	H	A	A	6.1 ± 0.8b
4	A	A	H	H	H	5.6 ± 0.3b
5	A	H	H	H	H	6.2 ± 0.8b
6	B	H	H	H	H	5.7 ± 1.1b
7	B	B	H	H	H	5.3 ± 0.9bc
8	B	B	B	H	H	3.1 ± 0.9d
ARC5984	B	B	B	B	B	2.7 ± 0.2d

[1]Number in bracket was physical distance of the associated marker referring to Nipponbare genome

[2]A, B, and H bars denote the marker genotypes of 9311 homozygotes, ARC5984 homozygotes, and heterozygotes, respectively. Also, 1–8 are the lines from BC_1F_2, while 9311 and ARC5984 denote the parents. The BPH resistance score is given as mean ± SD (n= 6 replicates)

[3]Means with the same letter within a column are not significantly different ($P > 0.05$)

Table 4 Candidate genes in the target region based on the genomes of Nipponbare, ZS97, and MH63

Nipponbare ID	Gene length	ZS97ID	Gene length	MH63 ID	Gene length	Candidate function
–	–	ZS04g0517700	2597	–	–	Co-chaperone GrpE protein
Os04g35190	4243	ZS04g0517800	5511	MH04g0429800	4253	Defense-related F-box protein 1
–	–	ZS04g0517900	1278	MH04g0429900	2531	Acetyltransferase
Os04g35200	2287	–	–	–	–	Expressed protein
–	–	–	–	MH04g0430000	2211	Putative AC transposase
Os04g35210	5156	ZS04g0518000	12846	MH04g0430100	3564	Leucine Rich Repeat family protein
Os04g35220	2999	ZS04g0518100	2711	MH04g0430200	2699	Expressed protein
–	–	–	–	MH04g0430300	6307	Putative disease resistance protein
–	–	–	–	MH04g0430400	2570	Expressed protein
–	–	–	–	MH04g0430500	1253	Hypothetical protein
–	–	–	–	MH04g0430700	6728	Transposon Ty3-I Gag-Pol polyprotein
Os04g35230	3362	–	–	–	–	Expressed protein
Os04g35240	5461	ZS04g0518200	5514	MH04g0430800	5756	Serine/threonine-protein kinase SAPK7
–	–	ZS04g0518300	2235	MH04g0430900	2233	Scarecrow-like protein

resistance and were detected in the genomes of Nipponbare, ZS97, and MH63 (Table 4). Moreover, the Nipponbare genome was usually a suitable reference for homologous analysis. Therefore, we analyzed all the six candidate genes' response to BPH infestation by real-time PCR (Fig. 5). Subsequently, the expression of *Os04g35190* gene was significantly higher in the resistant plants after 48 h of BPH release in comparison with that in the susceptible plant ($F = 8.07$, $P = 0.03$). Also, the same phenomenon was indicated after 12 h and 48 h of BPH infestation, when it was checked in the gene expression of *Os04g35210* ($F = 12.9$, $P = 0.02$ for 12 h; $F = 14.1$, $P = 0.019$ for 48 h). The other two genes (*Os04g35220* and *Os04g35240*) showed no significant difference in their relative expressions between the resistant and susceptible plants during the treatment period. However, the expression products of *Os04g35200* and *Os04g35230* genes were not detected in the test.

We sequenced the corresponding genomic regions containing the CDS of the candidate genes (Supplementary File 1). As a result, the nucleotide acid sequence 'TG' in *Os04g35190* of ARC5984 was changed to 'CA' in 9311. The CDS of *Os04g35200* was identical in the resistant and susceptible varieties. Furthermore, the CDS of *Os04g35210* in ARC5984 had 100% identity comparing with the gene *Bph6* cloned in Swarnalata, which caused four amino acid changes. But a lot of SNP changes were identified in the 5′-UTR and 3′-UTR of *Os04g35210* gene compared to *Bph6*. After that, there are also several different SNPs in the genes *Os04g35230* and *Os04g35240* between the ARC5984 and 9311 lines. The CDS of *Os04g35220* failed to be amplified.

Characterization of the pre-NILs

The pre-NIL plants were used to detect the antixenotic effect in the BPH resistance conferred by *qBph4*(*t*). The number of BPHs that settled on the resistant plants was significantly more than that on the susceptible plants over a 6 h observation period in the BPH host preference test. The difference in this number was larger during the observation period of 9–120 h (Fig. 3a). These observations suggest that the resistant plants showed strong antixenosis to the BPH insects.

To determine whether the resistant plants affect BPH growth and development, growth weight addition and honeydew excretion in BPH on the resistant and susceptible plants were quantified and compared. Two days after treatment, the average increase in BPH

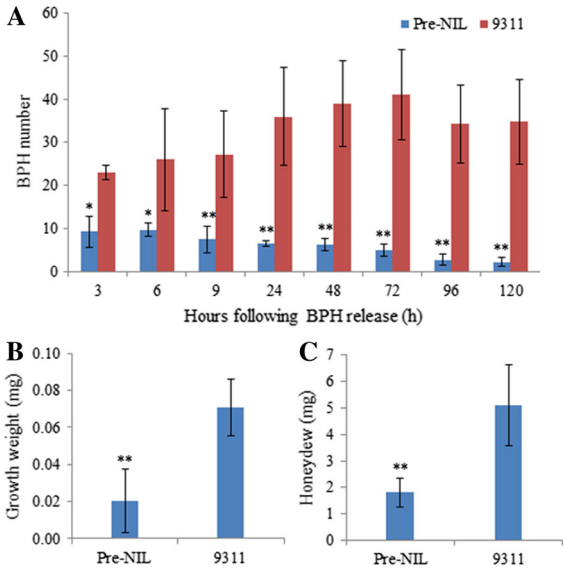

Fig. 3 BPH host choice and injection response on the different genotype plants. A: Results of BPH host choice test on pre-NIL and 9311. *Bars* represent means of six replicates. *Error bars* represent the SD. B: BPH growth weight addition in the pre-NIL and 9311. C: BPH honeydew excretion on pre-NIL and 9311. *Bars* represent means of 20 replicates. *Error bars* represent the SD. * and ** represent significant difference at $P = 0.05$ and 0.01, respectively

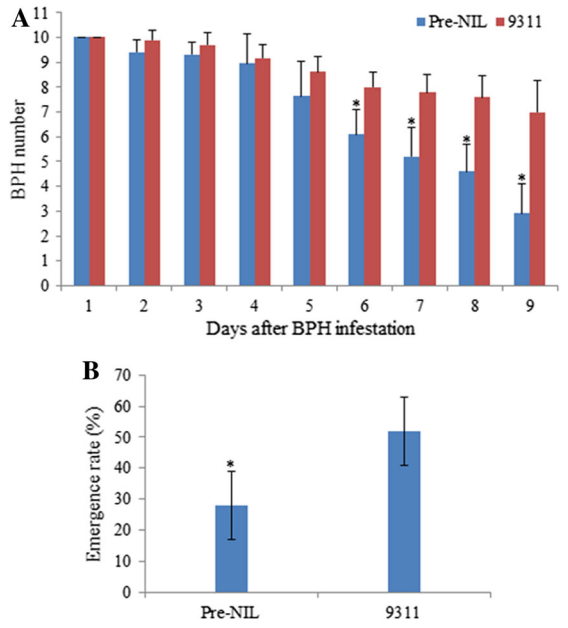

Fig. 4 Growth and development of BPH on plants of different genotype. A: The BPH survival rate on pre-NIL and 9311. *Bars* represent means of 10 replicates. *Error bars* represent the SD; B: The BPH emergence rate on pre-NIL and 9311. *Bars* represent means of 10 replicates. *Error bars* represent the SD. * and ** mean significant difference at $P = 0.05$ and 0.01, respectively

growth weight, when present on the resistant plants, was only 28.8% of that on the susceptible plants (Fig. 3b). Also, a 64.5% reduction in the BPH honeydew excretion on the resistant plants was observed as compared with that on the susceptible plants (Fig. 3c). Moreover, the BPH survival on the plants with different genotypes was measured every day for 9 days; and the emergence rates were detected at the ninth day. As a result, the average number of surviving insects on the pre-NIL and 9311 plants decreased with the increasing duration of insect occurrence on the plants (Fig. 4a). However, the number of insects present was found significant less on the resistant plants comparing with that on the susceptible plants after 6 days of insect release ($F = 6.7$, $P = 0.04$ at 6 days). Afterwards, more emerging BPHs were observed on the susceptible plants at the ninth day after infestation (Fig. 4b, $F = 12$, $P = 0.01$). These results suggest that the pre-NIL plants could seriously restrict insect growth and have a significant antibiotic effect on BPH.

Marker-assisted selection technique

Five tightly linked markers have been identified in the present study (Table 1). The PCR product of markers RM5757 and BF3 only were observed to be polymorphic between the parents on a 6% denaturing PAGE; while the other three markers can be shown polymorphic on the 6% non-denaturing PAGE (Supplementary Fig. 3A). Moreover, we detected that the SSR marker, RM5635, and the InDel marker, BF9, exhibited obvious polymorphism between the parents on the 2.5% Agar gel. They were then used to check the genotype of the backcrossed individuals (Supplementary Fig. 3B, C). Based on the results of the seedling bulk test, positive individuals with the parent band for resistance amplified using RM5635 or BF9 markers exhibited resistance to BPH (Supplementary Fig. 3). Therefore, the major resistance gene can be a simple and efficient application in the MAS for rice breeding by using the markers, RM5635 and BF9.

Discussion

BPH resistance gene mapping

The BPH is a major biotic stress for the rice crops grown all across the rice-cultivating regions of the world. To date, a total of 32 major BPH resistance genes have been identified in *indica* varieties and six wild rice species (Fujita et al. 2013; Ali and Chowdhury 2014; Wu et al. 2014). Analysis of gene location revealed the BPH resistance genes to cluster on rice chromosomes. For instance, chromosome 4 had one region that included *bph12*, *BPH27*, and *Bph6*, indicated by markers G271 and RM16853 (Fig. 2). The most probable reason is that they represent different alleles at the same locus. *BPH9* and other seven major BPH resistance genes were identified to show multiple allelism at the same locus (Zhao et al. 2016). Also, it is a possibility that they were present on different but tightly-linked loci (Monna et al. 2002; Chen et al. 2006). Based on the reported BPH resistance genes, three resistance genes *bph12*, *BPH27*, and *Bph6* were found to be located closed to the resistance gene detected in ARC5984 (Fig. 2). Although the rice variety ARC5984 was never reported to carry BPH resistance gene before, together with an analysis of the located marker information, gene expression and sequence, we have inferred here that the BPH resistance gene identified in the present study is an allelic gene to *Bph6*.

Possible nature of the gene

In this study, the resistance gene was detected to be present on the chromosome 4 based on analysis of an F_2 population with SSR and InDel markers. Furthermore, it was further finely mapped to a 46-kb region using the recombinants derived from BC_1F_2 individuals. Among the 12 cloned BPH resistance genes, *BPH1/7/9/10/21*, *Bph14*, *Bph18*, and *BPH26* were proved to be members of the nucleotide-binding and leucine-rich repeat (NB-LRR) disease resistance gene family. This means that they provide resistance to BPH via a mechanism that is fundamentally similar to the defense mechanisms against pathogens (Du et al. 2009; Tamura et al. 2014; Zhao et al. 2016; Ji et al. 2016). Another BPH resistance gene, *Bph6*, was reported to be a novel type of gene and encodes an exocyst-localized protein (Guo et al. 2018). The present located chromosome region contains six candidate genes in the Nipponbare genome; also, there were seven and 11 genes in the ZS97 and MH63 genomes, respectively. Gene expression analysis suggested that the *Os04g35210* gene (leucine-rich repeat family of proteins) significantly responded to BPH infestation in the pre-NILs (Fig. 5). After that, the CDS from ARC5984 was also identified to be identical to *Bph6*. Therefore, it is possible that the *Bph6* gene was also presented in the resistance variety, ARC5984. However, the *Os04g35190* gene was predicted to be a defense-related F-box protein gene; it was also observed to have an obvious up-regulative expression in the resistant plants after insect release. The sequencing results showed that sequence difference was present in the CDS of the resistant and susceptible plants. Moreover, one more candidate gene, *MH04g0430300*, was predicted to be a putative disease resistance gene in the genome of MH63. It should be checked in the rice variety ARC5984. And then the associated candidate genes should be cloned and transferred to the susceptible variety to confirm their trait of BPH resistance in future studies.

Characterization and potential exploitation of BPH resistance genes for rice improvement

Among the known physiological resistance mechanisms against insect attack, the most widely accepted mechanisms are antixenosis (non-preference), antibiosis, and tolerance (Painter 1951). We previously found that the BPH resistance genes (e.g. *Bph6*, *Bph12, BPH27*) also confer the properties of antixenosis and antibiosis against BPH (Qiu et al. 2010, 2012; Huang et al. 2013). According to the tests of BPH host preference and growth in the pre-NIL and 9311 plants, the resistance gene mapped in the present study involved both antixenosis and antibiosis in plants with the 9311 genetic background. The number of BPHs that settled on the pre-NIL plants was significantly less than that on the 9311 plants during the observation period of 3–120 h (Fig. 3a). Moreover, the resistant plants obviously restricted the growth and development of BPH based on the detection of insect growth weight and honeydew excretion.

As the MAS technique can speed up the process of recovering recurrent parental genetic background, and thus, shorten the breeding period, it has been frequently used to transfer important genes/QTLs into

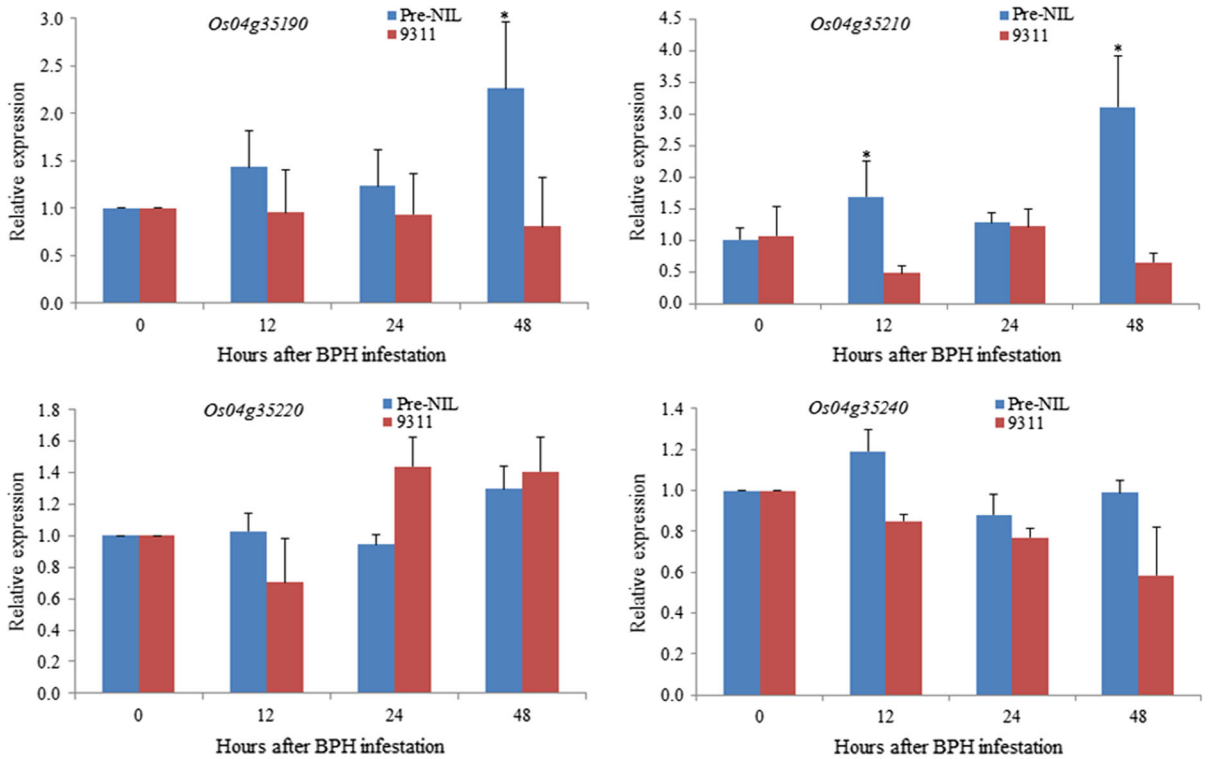

Fig. 5 Quantitative real-time PCR analysis of the candidate genes in pre-NIL and 9311. *Means significant difference at $P = 0.05$

various high-quality varieties (Jena and Mackill 2008; Lewis and Kernodle 2009; Jairin et al. 2009). For example, the InDel marker B14 and STS marker B15 were identified to be tightly linked with *Bph14* and *Bph15*, respectively, and were used to perform MAS and develop new BPH-resistant rice lines (Xu 2013; Hu et al. 2016; Jena et al. 2017) successfully created 25 NILs carrying different BPH resistance genes according to the reported linkage markers. Here, we identified several tightly linked molecular markers to the genes-of-interest with distinctly amplified bands between the two parents. The technique also proved to be efficient in the detection of the positively resistant individuals (Supplementary Fig. 3). The MAS technique developed for the detected gene here will be beneficial for the BPH-resistant rice breeding.

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