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ORIGINAL PAPER

High-resolution mapping of the brown planthopper resistance gene *Bph6* in rice and characterizing its resistance in the 9311 and Nipponbare near isogenic backgrounds

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Abstract Brown planthopper (*Nilaparvata lugens* Stål, BPH) is one of the most destructive insect pests of rice. Exploring resistance genes from diverse germplasms and incorporating them into cultivated varieties are critical for controlling this insect. The rice variety Swarnalata was reported to carry a resistance gene (designated *Bph6*), which has not yet been assigned to a chromosome location and the resistance mechanism is still unknown. In this study, we identified and mapped this gene using the F_2 and backcrossing populations and characterized its resistance in *indica* 9311 and *japonica* Nipponbare using near isogenic lines (NILs). In analysis of 9311/Swarnalata F_2 population, the *Bph6* gene was located on the long arm of chromosome 4 between the SSR markers RM6997 and RM5742. The gene was further mapped precisely to a 25-kb region delimited between the STS markers Y19 and Y9; and the distance between these markers is 25-kb in Nipponbare genome. The *Bph6* explained 77.5% of the phenotypic variance of BPH resistance in F_2 population and 84.9% in BC_2F_2 population. Allele from Swarnalata significantly increased resistance to the BPH, resulted in a reduced damage score. In characterization of *Bph6*-mediated resistance, the BPH insects showed significant preference between NIL-9311 and 9311 in 3 h and between NIL-NIP and Nipponbare in 120 h after release. BPH growth and development were inhibited, and the insect's survival rates were lower on *Bph6*-NIL plants, compared with the parents 9311 and Nipponbare. The results indicate that the *Bph6* exerted prolonged antixenotic and antibiotic effects in *Bph6*-NIL plants, and NIL-9311 plants showed a quicker and stronger effect toward BPH than NIL-NIP plants.

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Introduction

The brown planthopper (*Nilaparvata lugens* Stål, BPH hereafter) is one of the most destructive insect pests to feed on rice (*Oryza sativa* L.). The BPH feeds on the rice phloem sap using its piercing-sucking mouthparts, which affects the growth of rice plants and results in "hopperburn" (Watanabe and Kitagawa 2000). The BPH also transmits viruses, such as the ragged stunt virus and the grassy stunt virus, and associated diseases (Khush and Brar 1991; Jena et al. 2006). In recent years, BPH infestations have intensified across Asia, causing heavy rice yield losses (Normile 2008). A reduction in BPH infestation of rice is needed to maintain and protect food production levels; controlling this pest is an important food safety issue.

Several approaches have been used to reduce BPH damage to rice. Conventional measures have included the application of chemical insecticides, but this is expensive, ineffective under some weather conditions and the chemicals can kill BPH predators, which may lead to BPH resistance and the resurgence of BPH populations (Tanaka et al. 2000). The use of resistant rice varieties is the most economical and efficient method for controlling the BPH (Alam and Cohen 1998; Renganayaki et al. 2002), therefore it is imperative to identify BPH-resistance genes from diverse sources and incorporate them into rice cultivars. To

date, 23 monogenically controlled BPH-resistance genes have been reported (Rahman et al. 2009). With the exception of *bph5*, *Bph6*, *bph7* and *Bph8*, all the other reported BPH-resistance genes have been previously mapped to chromosomes using molecular genetic approaches. An important advance in developing BPH resistance in rice was the cloning of the *Bph14* gene, which encodes a member of the CC-NB-LRR protein family (Du et al. 2009). *Bph14*-transgenic plants display an antibiosis toward the BPH. To effectively exploit BPH-resistance resources in rice to support sustained control of BPH pest, more BPH-resistance genes need to be identified and the associated resistance mechanisms need to be determined.

The Asian-cultivated rice *O. sativa* consists of two major groups, known by the subspecies names *indica* and *japonica*. In general, these subspecies vary in genome constitution, physiological characteristics and agronomic traits. It is noteworthy that the host genetic background is an important factor that influences the function of disease resistance genes. For example, the background of *japonica* rice facilitates the function of *Xa3* more than the background of *indica* rice (Cao et al. 2007). Furthermore, all of the BPH-resistance genes identified to date are derived from *indica* varieties or wild rice species. Therefore, two questions need to be addressed. Firstly, do the BPH-resistance genes have a resistant effect on the BPH in the *japonica* background? Secondly, is there any difference between the resistance level of the BPH-resistance genes in *japonica* and *indica* backgrounds? Although there were reports that BPH-resistance gene was incorporated into *japonica* varieties, and the plants showed resistance to the BPH in seedling bulk tests (Jena et al. 2006; Park et al. 2007), little is still known about the resistance level of the same resistance genes in different genetic backgrounds under more elaborate test conditions.

Swarnalata is an *indica* rice variety that was shown to be resistant to biotype 4 Bangladesh BPH insect populations (Kabir and Khush 1988). A dominant gene (designated *Bph6*) was found to control BPH resistance in this variety in a genetic segregation analysis, which was independent of the gene previously identified through allele tests. *Bph6* has not yet been assigned to a chromosome location. In the present study, we aimed to map the BPH-resistance gene in rice variety Swarnalata and precisely estimate the resistance it conferred in *indica* 9311 and *japonica* Nipponbare backgrounds using near isogenic lines. The results located the gene in a region flanked by the STS markers Y19 and Y9 on rice chromosome 4. Furthermore, we found that *Bph6*-mediated resistance had both antixenosis and antibiosis effects on the BPH, which were stronger in the 9311 genetic background than in the Nipponbare genetic background.

Materials and methods

Plant materials and mapping populations

The rice variety Swarnalata has been reported to contain the resistance gene *Bph6* and is resistant to Bangladesh BPH populations (Kabir and Khush 1988). In this study, two BPH-susceptible rice varieties, 9311 (*indica*) and Nipponbare (*japonica*), and Swarnalata were used as the parents for the crosses. An F_2 population, which was derived from a 9311/Swarnalata cross and consisted of 140 families, was used to identify and map the gene. Then markers flanking the *Bph6* locus were used to select three heterozygous plants from the BC_2F_1 population (designated 2-64, 2-72 and 2-79), from which a BC_2F_2 population comprising 216 families was generated and used to fine-map the gene. For high-resolution mapping we further screened 4,300 BC_3F_2 plants to obtain recombinants in the region of interest, derived from the positive BC_3F_1 plants containing the locus. All the rice seeds used in our experiments were germinated at 30°C, then transferred to soil in plastic boxes (58 × 38 × 9 cm) or plastic cups (0.4 L). Seedlings were grown in a greenhouse under natural light at 25–30°C.

BPH insects and evaluation of BPH resistance

The BPH insects used for infesting plants were collected from rice fields in Wuhan, China, and maintained on Taichung Native 1 (TN1, a susceptible *indica* variety) under natural conditions in a greenhouse at Wuhan University. For gene mapping, a seedling bulk test was performed on the F_3 and BC_2F_3 families as described by Huang et al. (2001). Sixty seeds were randomly sown in a plastic box in three 26-cm-long rows, with 2.5 cm between rows. Three lines of Swarnalata, 9311 and TN1 were randomly sown among the F_3 families as controls. At the third-leaf stage the seedlings were infested with BPH nymphs at a level of ten insects per seedling. When all of the TN1 seedlings had died (scored as 9), each seedling was given a score of 0, 1, 3, 5, 7, or 9 according to Huang et al. (2001); the lower scores indicate the higher resistance to the insect. The evaluation experiments were repeated three times. The resistance score of each F_2 individual was then inferred from the weighted average of the scores for the seedlings in the corresponding F_3 families.

DNA extraction, map construction and gene detection

Total DNA of all the individuals used in the study was extracted from fresh leaves using the CTAB method (see Murray and Thompson 1980), and sequences of interest were amplified using PCR protocols described by Yang

et al. (2002), with minor modifications. PCR products were separated on a 6% non-denaturing polyacrylamide gel and detected by silver staining. Genomic sequence and SSR markers were obtained from the GRAMENE (http://www.gramene.org/markers/index.html).

According to the phenotype of the F_3 families, two contrasting bulks were prepared, each containing DNA from ten extremely resistant or susceptible individuals from the F_2 population. Bulked segregant analysis (BSA) (Michelmore et al. 1991) was then used to screen SSR markers that were linked to BPH resistance. A local genetic linkage map of SSR markers from the BPH-resistance gene-containing region was constructed by JoinMap 3.0 (Van Ooijen and Voorrips 2001), and the effect of the BPH-resistance gene was analyzed by MapQTL 5.0 (Van Ooijen 2004).

Bph6-NIL development

The resistance gene identified in the Swarnalata rice was introgressed into 9311 and Nipponbare by successive backcrossing and molecular marker-aided selection. In this process, two flanking markers tightly linked to the locus were used to select the positive progenies for continuous backcrossing. At the same time, a total of 142–199 SSR markers, which were polymorphic between Swarnalata and 9311 or Swarnalata and Nipponbare, and evenly distributed on 12 chromosomes, were used for genetic background screening in the BC_4F_1 generation. Thus, the individuals that were positive for the target locus, and had nearly identical genetic constitutions to the recurrent parents 9311 or Nipponbare, were selected for selfing. Finally, two BC_4F_1 plants with the least background noise were selected to produce corresponding BC_4F_2 generations. Two BC_4F_2 individuals that were homozygous at the target region in the 9311 or Nipponbare genetic backgrounds were selfed to generate BC_4F_3 lines. The homozygous BC_4F_3 lines in the 9311 or Nipponbare genetic backgrounds were designated as near isogenic lines (NIL)-9311 and NIL-NIP, respectively, and used to perform the following experiments.

BPH host selection behavior

Two seedlings (14 days old) of both NIL-9311 and 9311 were planted in each of five plastic buckets (17 cm diameter, 15 cm height), with seedlings of the same genotype at opposite ends of roughly perpendicular diagonals (Supplemental Figure S1). Five buckets with NIL-NIP and Nipponbare seedlings were prepared in the same manner. To observe the host selection of the BPH, 60 second-instar nymphs were placed in each bucket and allowed to choose host plants (42 days old) on which to feed and reproduce over a 120-h period. The locations where the BPH had settled on each plant were observed 3, 6, 12, 24, 48, 96 and 120 h after release.

BPH development on rice plants

To measure BPH survival and growth on NIL-9311, NIL-NIP, wild-type 9311 and Nipponbare plants, seedlings were grown in individual 0.4-L plastic cups in a plastic box (68 × 41 × 18 cm) under natural conditions. One week before treatment with BPHs, the plants were cultured in a greenhouse at a constant temperature (26–28°C). To examine the BPH survival rate on plants (42 days old), each cup/plant was treated with 20 second-instar nymphs, and the survival of the BPHs in each cup/plant was recorded every day for 9 days.

BPH growth was measured after 4 days on NIL-9311, NIL-NIP, 9311 and Nipponbare plants using ten pre-weighed, second-instar nymphs. Fourteen replicates of 35-day-old seedlings were established for each genotype treatment. Four days after the treatment, the weight of the surviving BPHs was recorded. The population growth rate (PGR) of surviving nymphs was calculated according to Edwards (2001) and Klingler et al. (2005).

Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA and significant differences were identified by the LSD test at the 5% significance level.

Results

Genetic analysis of BPH resistance and gene identification

Swarnalata, which was previously reported to resist BPH populations in Bangladesh (biotype 4) (Kabir and Khush 1988), exhibited high resistance to BPH populations in China; its average resistance score was 2.9 in the seedling bulk test. The 9311 plants were highly susceptible to the BPH, with an average resistance score of 8.7. In the F_2 population, BPH-resistance scores showed a continuous range from 1.4 to 9.0, with an apparent valley between 7.0 and 7.9 in the distribution curve (Fig. 1a). Such a distribution indicates the involvement of a major gene in the segregation of BPH resistance in this population. On the basis of the genotype of RM6997 (a SSR marker near the *Bph6* locus, Fig. 2), the F_2 plants were classified into three classes: homozygous for the Swarnalata or 9311 alleles, and heterozygous (Fig. 1a). The average BPH-resistance scores of the F_2 homozygous plants for the Swarnalata allele at RM6997 tended to be lower than those of plants

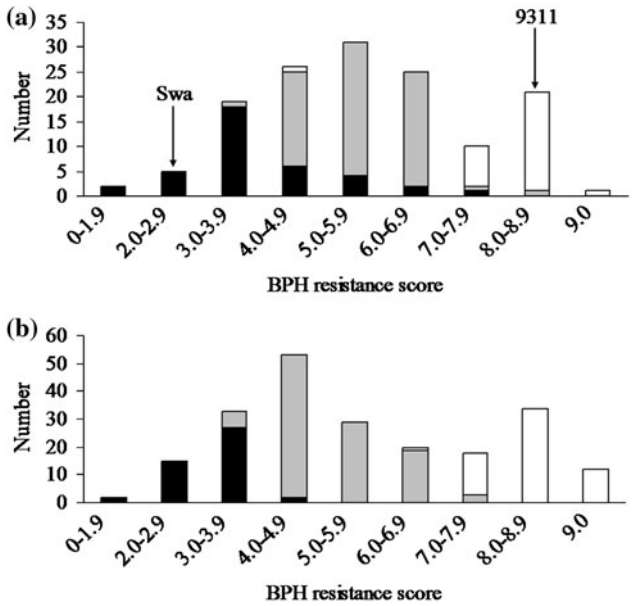

Fig. 1 Frequency distributions of the BPH-resistance score in **a** an F_2 population and **b** a BC_2F_2 population. Genotypes for the simple sequence repeat (SSR) marker RM6997 in the F_2 and RM17004 in the BC_2F_2 populations are shown. The *black*, *white* and *gray bars* denote the marker genotypes of Swarnalata homozygotes, 9311 homozygotes and heterozygotes, respectively. Rice (*Oryza sativa*) seedlings of 140 F_2 lines (3 leaf stage/11 days old) or 216 BC_2F_2 lines (3 leaf stage/11 days old) were treated with ten BPHs per plant for 9–10 days, respectively. The average resistance scores of the parents Swarnalata and 9311 was 2.9 and 8.7, respectively. *Lower scores* indicate higher resistance to the insect

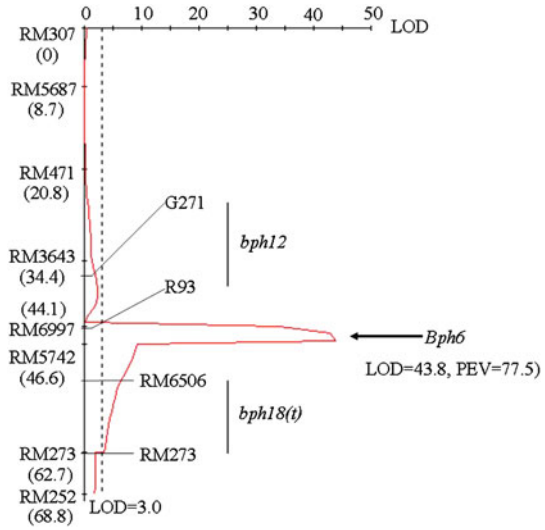

Fig. 2 Location of the *Bph6* gene on the linkage map of rice chromosome 4 constructed by SSR markers using an F_2 population derived from 9311/Swarnalata. Marker names are listed on the left hand side of the chromosome with the distances (in cM) shown in *brackets*. *PEV* percentage of total phenotypic variance explained by the locus. The positions of *bph12* and *bph18*(*t*) were integrated into the map based on the data from Hirabayashi et al. (1998) and Li et al. (2006)

that were heterozygous or homozygous for the 9311 allele. According to the scoring criterion in the seedling bulk test and previous studies (Huang et al. 2001; Yang et al. 2004), we considered the plants with scores within the ranges 0–4.0, 4.1–7.0 and 7.1–9.0 to have high resistance, moderate resistance and high susceptibility, respectively. Thus, based on the resistance score of the 140 F_2 plants, the segregation of the resistant to susceptible plants was in agreement with a 3:1 ratio (108:32; $\chi_c^2 = 0.24 < \chi_{0.05,1}^2 = 3.84$). This result indicates that there is one major gene controlling the segregation of BPH resistance in this F_2 population.

To map the gene, we performed a BSA analysis. A total of 536 SSR markers, distributed on 12 rice chromosomes, were used for the parental survey, and polymorphism between Swarnalata and 9311 was detected with 197 (36.8%) of the markers. Three markers, RM3643, RM6997 and RM5742, from a contiguous region on chromosome 4, were different between the resistant and susceptible bulks, indicating that the BPH-resistance gene is located in this region. Therefore, additional markers from chromosome 4 that were polymorphic between the parents were used to assay the 140 F_2 lines, and using the acquired data a local linkage map was constructed by JoinMap 3.0 (Fig. 2). The map covered 68 cM of chromosome 4, and the order of markers in the map was in agreement with previously published maps (McCouch et al. 2002; Yue et al. 2006). To detect the location of the gene, we analyzed the resistance scores and genotypes of the F_2 plants using MapQTL 5.0. Consequently, we detected one locus for BPH resistance with a LOD score of 43.8 in a 2.5-cM region between RM6997 and RM5742 on the long arm of chromosome 4 (Fig. 2); this gene was designated as *Bph6*. This locus explained 77.5% of the phenotypic variance of BPH resistance in the F_2 population.

Verification and fine mapping of the gene

To further confirm that the gene was located on the long arm of chromosome 4 and identify its exact location, another mapping population of BC_2F_2 was developed, which contained 216 plants. The frequency distribution of BPH-resistance scores in this population showed continuous variation from 1.2 to 9.0 based on the progeny test (Fig. 1b). At the same time, more SSR markers were developed between RM5742 and RM6997 from the public database released by GRAMENE, and four polymorphic markers were obtained. These markers were surveyed to assay the genotype of the BC_2F_2 plants and the markers were ordered on the linkage map by JoinMap 3.0 (Fig. 3). Analysis of the BPH-resistance scores and genotypes of the BC_2F_2 showed that the SSR markers RM119, RM17004 and RM17008 were tightly linked to the BPH-resistance

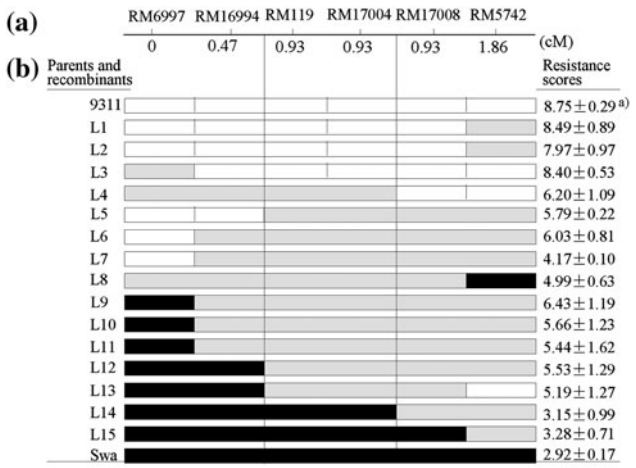

Fig. 3 The local genetic linkage map of the *Bph6* gene molecular marker genotypes and phenotypes of the recombinants used for fine mapping. **a** The local genetic linkage map. The *numbers below* the linkage map show the genetic distances (in cM). **b** Molecular marker genotypes and phenotypes of the recombinants. The *black*, *white* and *gray bars* denote the marker genotypes of Swarnalata homozygotes, 9311 homozygotes and heterozygotes, respectively. L1, L2, L3, L5, L7, L8 and L13 were the individuals from the BC_2F_2 population. L4, L6, L9, L10, L11, L12 and L14 were the individuals from the F_2 population. *9311* and *Swa* denote the parents. Superscript "a" BPH-resistance score (mean ± SD, $n = 6$ replicates)

locus; and all these markers had a LOD score of 88.6 in the BC_2F_2 population detected by MapQTL5.0. This locus accounted for a large proportion (84.9%) of the total phenotypic variation in the BC_2F_2 population (Table 1). We classified the BC_2F_2 plants into 9311 or Swarnalata homozygotes and heterozygotes based on the genotype of RM17004 (Fig. 1b). At all marker loci, alleles from the resistant parent Swarnalata conferred significantly increased resistance to the BPH and the additive effect was estimated as 2.5. The dominant effects of the loci were −0.6, and the heterozygotes showed moderate resistance to the BPH. These results indicate that the *Bph6* resistance gene is partially dominant (Table 1).

Table 1 QTL scanning results using 216 BPH-resistance scores and SSR marker genotypes of BC_2F_2 (9311/Swarnalata//9311///9311) by MapQTL 5

Locus	Position (cM)	LOD	PEV (%)	*A*	*D*
RM6997	0	79.0	81	2.48	−0.57
RM16994	0.47	87.5	84	2.52	−0.65
RM119	0.93	88.6	85	2.55	−0.64
RM17004	0.93	88.6	85	2.55	−0.64
RM17008	0.93	88.6	85	2.55	−0.64
RM5742	1.86	79.7	82	2.51	−0.59

The genetic effect estimated on the progeny data by MapQTL5.0. Additive effect was equal to the half of the trait value difference between two homozygotes, dominant effect equal to the trait value difference between heterozygote and the middle value of two homozygotes

PEV Percentage of total phenotypic variance explained by the locus, *A* additive effect on the Swarnalata allele, *D* dominant effect on the Swarnalata allele

Within the F_2 and BC_2F_2 populations, there were 15 recombinants between SSR markers RM6997 and RM5742. The markers RM119 and RM17004 co-segregated with *Bph6*, and there were three recombinant events between RM16994 and *Bph6*, and two between RM17008 and *Bph6* (based on the genotypes and phenotypes of the recombinants). Among the recombinants, line L14, which was homozygous for Swarnalata alleles at RM119 and RM17004, showed a high level of resistance with an average resistance score of 3.15, while lines L4, L5, L12 and L13, which were heterozygous at both loci, had moderate resistance to the BPH and scored 6.20, 5.79, 5.53 and 5.19, respectively. Thus, based on the analysis of the genotypes and resistance scores of the recombinants, *Bph6* was localized between RM16994 and RM17008. The physical distance between these markers is ca. 154 or 300 kb, according to the Nipponbare and 9311 genome sequences, respectively.

High-resolution mapping of *Bph6*

To map *Bph6* at high resolution, we further screened the genotypes of 4,300 BC_3F_2 seedlings to obtain recombinants between SSR markers RM16994 and RM17008. Forty-one recombinants were identified. In addition, we obtained four informative SSR markers for Swarnalata and 9311 between RM16994 and RM17008, based on a comparison of the genomic sequences of Nipponbare and 9311. In addition to RM16994, RM119, RM17004 and RM17008, another 4 markers were surveyed to analyze the genotypes of the 41 recombinants (Fig. 4, Supplemental Figure S2). Lines N1, N2 and N3, which harbored the 9311 homozygous alleles at Y19 and Y9, had resistance scores of 8.85, 8.66, and 8.82, respectively; while line N5, which carried the Swarnalata homozygous alleles at both loci, had a resistance score of 2.99. Line N4, which had heterozygous alleles at these loci had moderate resistance to the BPH in the seedling bulk tests. These results suggest that any lines with the Swarnalata alleles at Y19 and Y9 were resistant to the BPH. In contrast, those lines with the 9311 alleles were highly susceptible to the BPH. Results for the other recombinants confirmed these findings (Supplemental Figure S2). Among these recombinants, lines N6 and N7, which showed moderate resistance to the BPH, had a recombination event between Y19 and Y9. Therefore, *Bph6* was finally located in a region bordered by the STS markers Y19 and Y9; the corresponding region is 25-kb on BAC clone OSJNBa0084A10 of Nipponbare.

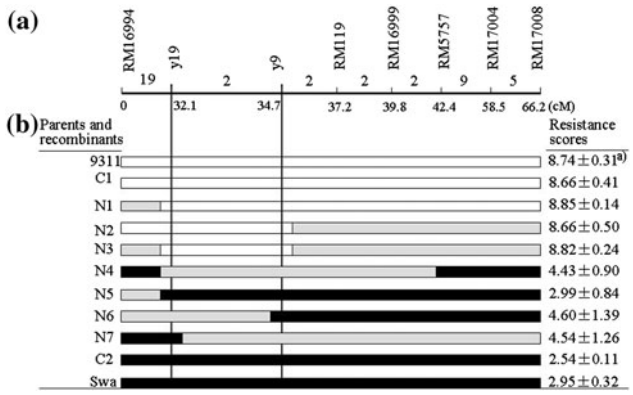

Fig. 4 High-resolution genetic map of the *Bph6* gene and the molecular marker genotypes and phenotypes of part recombinants. **a** High-resolution genetic linkage map. The *numbers above* the linkage map indicate the number of recombinants between markers; the *numbers below* the linkage map show the genetic distances (in cM). **b** Molecular marker genotypes and phenotypes of the recombinants. The *black*, *white* and *gray bars* denote the marker genotypes of Swarnalata homozygotes, 9311 homozygotes and heterozygotes, respectively. N1–N7, recombinants from the BC_3F_2 population; C1, control 1, homozygous for *Bph6* in the target region; C2, control 2, homozygous for 9311 in the target region; 9311, the susceptible parent; Swa, the resistant parent Swarnalata. Superscript "a" BPH-resistance score (mean ± SD, $n = 7$ replicates)

Bph6-NIL development and resistance evaluation

In the process of developing advanced backcross lines carrying the resistance locus, we used two flanking markers tightly linked to the locus to select the positive progenies for continuous backcrossing. At the same time, we also conducted background selections using SSR markers that were distributed on 12 chromosomes (Table 2, Supplemental Table S1). In the BC_4F_1 populations eight plants that were heterozygous at the target *Bph6* region were selected; these plants showed 94.56–97.37% genetic identity to 9311 and 90.85–93.85% genetic identity to Nipponbare (Table 2, Supplemental Table S1). The best individuals with the least amount of genetic background noise for 4Q1102-10-1 (2.63% in 9311 background) and 4Y1100-2-5 (6.15% in Nipponbare background) were used to produce BC_4F_2 and BC_4F_3 populations.

In the BC_4F_2 progenies of the selected BC_4F_1 plants, lines homozygous for 9311 and Nipponbare at the *Bph6* region, and both 9311 and Nipponbare were highly susceptible to the BPH, with scores of 8.35, 8.63, 8.52 and 8.75, respectively in the seedling bulk tests (Fig. 5). Lines heterozygous for the Swarnalata allele at the *Bph6* region showed moderate resistance to the BPH compared to 9311 and Nipponbare ($F = 199.9$, $P < 0.001$ for heterozygotes compared to 9311; $F = 52.5$, $P < 0.001$ for heterozygotes compared to Nipponbare). Plants that were homozygous for Swarnalata at the *Bph6* region in a Nipponbare genetic background and the resistant parent Swarnalata showed high resistance to the BPH (scores of 3.25 and 2.93, respectively), compared to 9311 ($F = 416.1$, $P < 0.001$ for homozygotes with Nipponbare background compared to 9311; $F = 335.1$, $P < 0.001$ for Swarnalata compared to 9311). Among all the tested lines, those homozygous for Swarnalata at the *Bph6* region in a 9311 genetic background showed the highest resistance and only the lowest leaf yellowing in the resistance tests ($F = 47.6$, $P < 0.001$ for homozygotes with 9311 background compared to Swarnalata; $F = 103.2$, $P < 0.001$ for homozygotes with 9311 background compared to homozygotes with Nipponbare background). These findings indicate that the BPH-resistance gene *Bph6* functioned in both *indica* and *japonica* backgrounds, but that the resistance was higher in the 9311 background than in the Nipponbare background.

Antixenotic effect of *Bph6*-NILs toward BPH insects

NIL-9311 and NIL-NIP, viz the homozygous BC_4F_3 lines, were used to characterize the resistance conferred by *Bph6*. In the BPH host choice test, the BPHs quickly jumped onto the rice, and most of them fastened themselves to the shoots. The average number of settled BPHs remained relatively constant on the 9311 plants over the 120-h observation period, whereas the BPH on NIL-9311 decreased significantly over the study period (Fig. 6a). One-way ANOVA analysis showed that there was a significant difference in BPH preference for NIL-9311 and 9311 after release ($F = 6.8$, $P = 0.03$ at 3 h; $F = 9.4$, $P = 0.02$ at 6 h; $F = 19.7$, $P < 0.002$ at 12, 24, 48, 96 and 120 h). As shown in Fig. 6b, the average number of settled BPHs remained relatively constant on both NIL-NIP and Nipponbare up to 48 h after their release, suggesting that there was no immediate effect of an antixenotic factor in NIL-NIP. After 96 and 120 h, the number of BPHs on Nipponbare remained stable, whereas the number of BPHs that had settled on NIL-NIP declined; these numbers were significantly different at 120 h ($F = 14.1$, $P = 0.006$). These findings indicate that antixenotic factors were present in the *Bph6*-NILs, and that NIL-9311 had a much quicker and stronger antixenotic effect on the BPH than NIL-NIP.

BPH performance was reduced on *Bph6*-NILs plants

To determine whether *Bph6*-NIL plants affect BPH growth and development, we compared the BPH PGR on NIL-9311, NIL-NIP, 9311 and Nipponbare (Fig. 7a). By the fourth day after treatment the resistant trait caused a 2.4-fold reduction in PGR of the BPH in NIL-9311, compared to 9311 or Nipponbare ($F = 44.5$, $P < 0.001$ for NIL-9311 and 9311; $F = 46.7$, $P < 0.001$ for NIL-9311 and Nipponbare), and 0.4- and 0.5-fold reductions in NIL-NIP,

Table 2 Number of SSR markers used to analyze the introgression ratio of the resistant donor parent variety Swarnalata in the BC_4F_1 positive plants

Chromosome	4Q1102-10-1			4Y1100-2-5		
	No. of markers tested	No. of markers allelic to Swarnalata	SSR markers	No. of markers tested	No. of markers allelic to Swarnalata	SSR markers
1	15	3	RM220, RM243, RM594	18	4	RM220, RM522, RM315, RM165
2	16	0		15	1	RM555
3	15	1	RM168	18	0	
4	11	4	RM6997, RM16994, RM5742, RM252	14	4	RM185, RM6997, RM16994, RM5742
5	15	0		19	2	RM548, RM194
6	23	0		17	2	RM1356, RM176
7	15	0		20	0	
8	19	0		16	2	RM515, RM477
9	17	0		17	0	
10	17	0		14	1	RM8207
11	20	1	RM2110	15	0	
12	11	0		16	0	
Total and ratio[a] (%)	194	9	2.63%	199	16	6.15%

[a] The markers of the target region on chromosome 4 were excluded when calculating the ratio

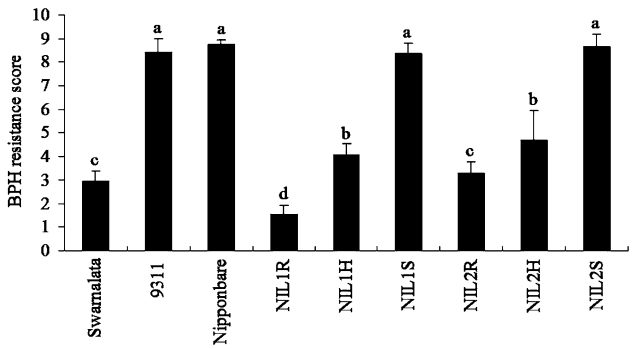

Fig. 5 BPH-resistance phenotype in NILs and parents as measured using the seedling bulk test. *Each bar* represents the mean of 5–15 replicates. *Error bars* represent the SD. NIL1R, NIL1S and NIL1H denote the BC_4F_2 plants that were homozygous for Swarnalata and 9311 or heterozygous at the *Bph6* region in 9311 genetic background; NIL2R, NIL2S and NIL2H denote the BC_4F_2 plants that were homozygous for Swarnalata and Nipponbare or heterozygous at the *Bph6* region in Nipponbare genetic background. Swarnalata, 9311 and Nipponbare were the parents. Means labeled with the *same letter* are not significantly different at the level of $P = 0.05$

compared to 9311 and Nipponbare, respectively ($F = 10.9$, $P = 0.003$ for NIL-NIP and 9311; $F = 12.3$, $P = 0.002$ for NIL-NIP and Nipponbare), indicating that the BPH growth and development were inhibited on the *Bph6*-NIL plants. In addition, the PGR of the BPHs on NIL-9311 was significantly less than that for NIL-NIP ($F = 14.8$, $P < 0.001$). The BPH survival rate was lower on *Bph6*-NIL plants. As shown in Fig. 7b, the average number of surviving BPHs on NIL-9311 and 9311 plants remained relatively constant for the first 2 days after the BPH treatment ($F = 2.7$, $P = 0.12$ at 1 day; $F = 2.1$, $P = 0.17$ at 2 days). However, from the third day, the average number of surviving BPHs on 9311 still remained constant, but the number of BPHs on NIL-9311 plants decreased quickly, and there were significant differences in numbers at later time points ($F = 7.4$, $P = 0.02$ at 3 days; $F = 9.1$, $P = 0.009$ at 4 days; $F = 20.0$, $P < 0.001$ at 5, 6, 7, 8, 9 days). In contrast to the BPH survival rates on NIL-9311 and 9311 plants, the average number of surviving BPHs on NIL-NIP and Nipponbare remained constant up to the seventh day after the BPH treatment, but differed significantly during the last 2 days ($F = 4.7$, $P = 0.046$ at 8 days; $F = 5.5$, $P = 0.033$ at 9 days; Fig. 7c). These results suggest that the BPH probably has an immediate effect on an antibiotic factor in NIL-9311, whereas the effect was weak in NIL-NIP.

Discussion

The BPH is a major biotic stress of rice production across the world. At present, 23 BPH-resistance genes have been reported. It is noteworthy that nine resistance genes (designated *Bph1–Bph9*) were identified in *indica* cultivars using a classical genetic segregation approach in the 1970s and 1980s, of which *Bph1*, *bph2*, *Bph3*, *bph4* and *Bph9* were further identified using molecular genetic approaches.

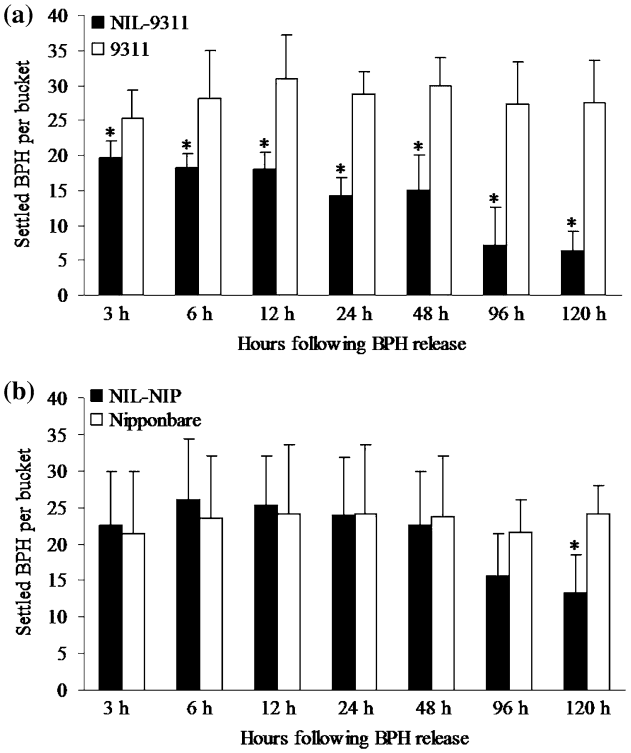

Fig. 6 BPH settlement in a choice test conducted in a greenhouse. **a** NIL-9311 (the BC_4F_3 plants were homozygous for Swarnalata at the *Bph6* region in the 9311 genetic background) and 9311; **b** NIL-NIP (the BC_4F_3 plants were homozygous for Swarnalata at the *Bph6* region in the Nipponbare genetic background) and Nipponbare. *Black bars* NIL-9311 or NIL-NIP; *white bars* 9311 or Nipponbare. *Bars* represent means of five replicates. *Error bars* represent SDs. Means labeled with *asterisks* are significantly different ($P < 0.05$)

Bph1 and *bph2* were identified in rice varieties Mudgo and Karsamba Red ASD7, respectively, through genetic segregation analysis (Athwal et al. 1971). These genes were eventually mapped on the long arm of chromosome 12 through restriction fragment-length polymorphism (RFLP) and amplified fragment-length polymorphism (AFLP) analysis (Hirabayashi and Ogawa 1995; Murai et al. 2001). *Bph3* and *bph4* were identified in rice varieties Rathu Heenati and Babawee, respectively, through later genetic segregation analysis, and were mapped to the short arm of chromosome 6 through SSR or RFLP analysis (Lakshminarayana and Khush 1977; Kawaguchi et al. 2001; Jairin et al. 2006). Molecular genetic approaches have not been previously applied to *bph5*, *Bph6*, *bph7* or *Bph8*. In this study, *Bph6* was identified on the long arm of chromosome 4 from an F_2 population by SSR markers and its position was further verified using BC_2F_2 and BC_3F_2 populations. This gene was finally located to a region flanked by the STS markers Y19 and Y9 through high-resolution mapping. According to the primer sequences information, the physical distance between Y19 and Y9 is estimated to be 25-kb in Nipponbare genome (http://www.gramene.org/Oryza_sativa/Location). To date, 20 BPH-resistance genes have been mapped on rice chromosomes. Interestingly, these BPH-resistance genes appear to cluster on rice chromosomes 3, 4, 6 and 12. In summary, seven BPH-resistance genes (*Bph1*, *bph2*, *Bph9*, *Bph10*, *Bph18*(*t*), *bph19*(*t*) and *Bph21*(*t*)) cluster in one location that spans an estimated 5.5-Mb region on chromosome 12 based on the sequence of Nipponbare between markers G2140 and RM5479. Another seven genes cluster at two regions on chromosome 4: one that covers about 4.9-Mb between markers C946 and RM5953 and contains *Bph12*(*t*), *Bph15*, *Bph17* and *Bph20*(*t*); and another that includes *bph12*, *bph18*(*t*) and *Bph6*, spanning 4.1-Mb between markers G271 and RM273 (Hirabayashi et al. 1998; Li et al. 2006). Four genes (*bph11*, *Bph13*(*t*), *Bph14* and *bph19*) have been mapped to chromosome 3. *Bph3* and *bph4* are located on chromosome 6. It is well known that rice disease resistance genes also cluster in the same chromosome regions (Ramalingam et al. 2003). Several hypotheses have been proposed to explain the relationship between these clustered resistance loci including: it is possible that they are different but tightly linked loci; or that they represent different alleles at the same locus; or they are the same allele, but show differential reactions to different BPH biotypes (Wang et al. 1994; Monna et al. 2002; Ramalingam et al. 2003; Chen et al. 2006). There are another two recessive genes (*bph12*, *bph18*(*t*)) beside *Bph6* on the long arm of rice chromosome 4. The *bph12* has been mapped to a region bordered by two RFLP markers G271 and R93; and the *bph18*(*t*) is located between the SSR markers RM6506 and RM273 (Fig. 2). From an analysis of the located marker information and genome sequence of the reference cv. Nipponbare, we infer that *Bph6* is probably located between *bph12* and *bph18*(*t*). One way to test this hypothesis is to construct a high-resolution map and then isolate these genes (Inukai et al. 1996).

Generally, plants may employ different mechanisms of resistance viz: antixenosis and antibiosis for reducing insect damage. The mechanism of resistance to BPH still remains largely unknown in rice. In one of the few published studies the resistant cultivar IR64, which contains the major gene *Bph1* and other additional minor QTLs, was found to display slight to moderate levels of antibiosis, antixenosis and tolerance to the BPH (Cohen et al. 1997). Du et al. (2009) studied the mechanism of resistance conferred by the resistance gene *Bph14*, and found that it confers antibiosis, reducing the feeding, growth rate and longevity of the BPH, but no significant difference was observed in the numbers of insects that settled on *Bph14*-transgenic plants and wild-type plants 48 h after infestation. In the study presented here, *Bph6* exhibited both antixenosis and antibiosis effects toward the BPH. The gene deterred the BPH from settling on NIL-9311 plants within 3 h

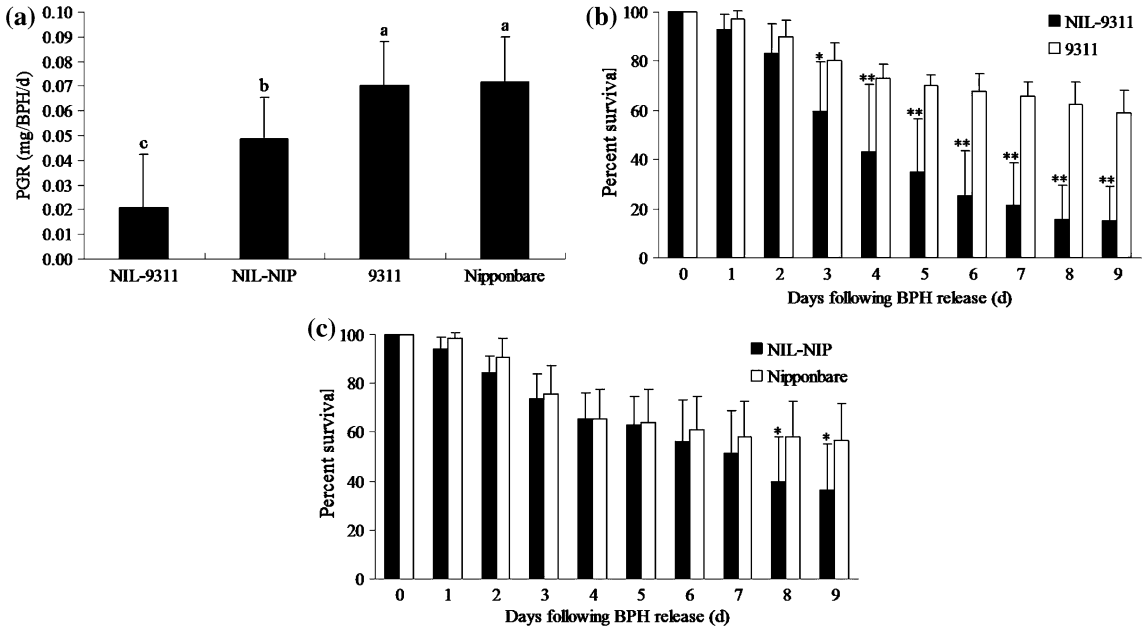

Fig. 7 Effects of plant genotype on the BPH population growth rate (mg/BPH/day) and BPH survival. **a** NIL-9311 (the BC_4F_3 plants were homozygous for Swarnalata at the *Bph6* region in the 9311 genetic background), NIL-NIP (the BC_4F_3 plants were homozygous for Swarnalata at the *Bph6* region in the Nipponbare genetic background), 9311 and Nipponbare (the susceptible parents). Population growth rate was measured as described by Edwards (2001). **b** NIL-9311 and 9311; **c** NIL-NIP and Nipponbare. *Bars* represent means of 14 replicates for **a**, 17 replicates for **b** and 16 replicates for **c**. *Error bars* represent SDs. Means labeled with the *same letter* are not significantly different at the level of $P = 0.05$ for **a**. Means labeled with *asterisks* are significantly different ($P < 0.05$) for **b** and **c**

and on NIL-NIP plants within 120 h of release. The survival rates and PGR of settled BPHs on the *Bph6*-NILs and recurrent parents also differed (these plants also differed in the bulked seedling test). The decreased survival of BPHs on *Bph6*-NILs, compared to 9311 or Nipponbare, may have been due to the accumulation of a small, but significant, deleterious effect of resistant plants on BPH biology, which may have been induced locally by the presence of the BPH. If the BPH resistance is based on the phloem properties of *Bph6*-NILs, the causal factor may be produced within infested tissue. One possible mechanism for local and phloem-specific resistance is physical blockage of sap uptake through rapid polymerization and deposition of macromolecules such as phloem proteins (Will et al. 2007) and callose (Hao et al. 2008; Du et al. 2009). Another possible mechanism is the biosynthesis of resistance factors in the vicinity of BPH feeding sites. For example, sieve elements in opium poppy (*Papaver somniferum*) can produce and accumulate alkaloid allelochemicals in their parietal cytoplasm (Bird et al. 2003). Considering the host choice and BPH performance on plants, the seedling bulk test results can be explained by a combination of antixenosis (fewer BPHs settling on plants) and antibiosis (reduced survival and growth rates). The survival results and PGR evaluation of BPH performance on *Bph6*-NIL plants showed that BPH reproduction was possible on this resistant genotype. This contrasts with *Meu1*-mediated resistance against *Macrosiphum euphorbiae* in tomato, which caused 100% mortality within 10 days in a study by Kaloshian et al. (1997), but supports previous *Bph14*-mediated resistance findings (see Du et al. 2009). To control the damage caused by the BPH, it is important to maintain low and stable populations of the insect in rice fields (Cohen et al. 1997). Since *Bph6*-mediated resistance in rice permits a low level of reproduction, it should impose a relatively moderate selection pressure on the BPH and thereby retain durability in the field.

Among the reported BPH-resistance genes, eleven loci (*Bph1*, *bph2*, *Bph3*, *bph4*, *bph5*, *Bph6*, *bph7*, *Bph8*, *Bph9*, *Bph17* and *bph19*) have been identified in diversified *indica* cultivars, while the other loci have been identified in wild 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). None of these BPH-resistance genes is originally identified in a *japonica* cultivar. Previous studies have shown that the genetic background can considerably affect the resistance level or durability of a resistance gene/QTL of plant to the pathogens (Marcel et al. 2008; Palloix et al. 2009). In this study, we compared the resistance level of the *Bph6* gene in susceptible 9311 (*indica*) and Nipponbare (*japonica*). The experimental data demonstrated that *Bph6* conferred a higher resistance when it was introgressed into an *indica* susceptible genetic background, than when it was

introgressed into a *japonica* susceptible genetic background. In the BPH host choice test, NIL-9311 had a quicker and stronger antixenotic effect on the BPH insects, compared with NIL-NIP (3 vs. 120 h, Fig. 6). The PGR of BPHs on NIL-9311 was significantly lower than that of BPHs on NIL-NIP (Fig. 7a). The average number of surviving BPHs on NIL-9311 decreased quickly compared with 9311, whereas the average number of surviving BPHs on NIL-NIP and Nipponbare remained constant up to the seventh day after release. These results suggest that the gene probably has an immediate antixenotic effect and a stronger antibiosis in NIL-9311, whereas the effect is weaker and slower in NIL-NIP. As a result, the resistance of NIL-9311 plants was stronger than NIL-NIP in the seedling bulk test. We have also observed that several BPH-resistance genes did not show resistance to BPH when introgressed into *japonica* background (data not shown). Further experiments are needed to elucidate the interaction between BPH-resistance gene and genetic background.

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