

Development and characterization of *japonica* rice lines carrying the brown planthopper-resistance genes *BPH12* and *BPH6*

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Abstract The brown planthopper (*Nilaparvata lugens* Stål; BPH) has become a severe constraint on rice production. Identification and pyramiding BPH-resistance genes is an economical and effective solution to increase the resistance level of rice varieties. All the BPH-resistance genes identified to date have been from *indica* rice or wild species. The *BPH12* gene in the *indica* rice accession B14 is derived from the wild species *Oryza latifolia*. Using an F₂ population from a cross between the *indica* cultivar 93-11 and B14, we mapped the *BPH12* gene to a 1.9-cM region on chromosome 4, flanked by the markers RM16459 and RM1305. In this population, *BPH12* appeared to be partially dominant and explained 73.8% of the phenotypic variance in BPH resistance. A near-isogenic line (NIL) containing the *BPH12* locus in the background of the susceptible *japonica* variety Nipponbare was developed and crossed with a NIL carrying *BPH6* to generate a pyramid line (PYL) with both genes. BPH insects showed significant differences in non-preference in comparisons between the lines harboring resistance genes (NILs and PYL) and Nipponbare. BPH growth and development were

inhibited and survival rates were lower on the NIL-*BPH12* and NIL-*BPH6* plants compared to the recurrent parent Nipponbare. PYL-*BPH6* + *BPH12* exhibited 46.4, 26.8 and 72.1% reductions in population growth rates (PGR) compared to NIL-*BPH12*, NIL-*BPH6* and Nipponbare, respectively. Furthermore, insect survival rates were the lowest on the PYL-*BPH6* + *BPH12* plants. These results demonstrated that pyramiding different BPH-resistance genes resulted in stronger antixenotic and antibiotic effects on the BPH insects. This gene pyramiding strategy should be of great benefit for the breeding of BPH-resistant *japonica* rice varieties.

Introduction

The brown planthopper (*Nilaparvata lugens* Stål; BPH) is a typical piercing–sucking pest which feeds on rice phloem sap, affecting the growth of rice plants and resulting in “hopperburn” (Watanabe and Kitagawa 2000). Historically considered an occasional pest of rice in tropical Asia, BPH became a severe constraint on rice production following the introduction of high-yielding varieties in the 1960s (Way and Heong 1994). According to the *Statistics of China Agriculture Yearbook*, there were large outbreaks in 2005–2007 with over 25 million hectares of rice infested by rice planthopper (main BPH) populations in each of those years. Rice planthopper infestations have intensified across Asia and the rise in BPH outbreaks is considered to be one of the main reasons that the price of rice has increased fourfold since 2003 (Normile 2008).

Conventional methods of controlling BPH are primarily dependent on chemical insecticides, which are costly and environmentally unfriendly. The occurrence of resurgence, a phenomenon of pest population increase after application of

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insecticides, is also problematic (Heinrichs et al. 1982; Tanaka et al. 2000; Park et al. 2007). The most important factor in BPH management in rice-growing areas is minimizing insecticide use to promote integrated control, and the use of resistant rice varieties plays a significant role in this approach (Way and Heong 1994; Renganayaki et al. 2002). Resistant rice varieties can provide important “insurance” against BPH outbreaks caused by factors outside of farmers’ control, such as unusual weather patterns or insecticide over use in neighboring fields (Alam and Cohen 1998a).

To date, at least 23 major BPH-resistance genes from diverse *indica* varieties and wild rice species have been reported (Rahman et al. 2009; Jena and Kim 2010). Since the early 1970s, several BPH-resistance genes have been used extensively in rice-breeding programs. IR26, which contains the *Bph1* gene, was released in 1973 and initially provided control of BPH over a large area. However, the populations adapted to the *Bph1*-mediated resistance in a few years, and BPH outbreaks resumed (Cohen et al. 1997; Alam and Cohen 1998b). This scenario was repeated with varieties containing the *bph2* gene which were released in 1975 (Alam and Cohen 1998b). Improved rice cultivars carrying a single gene (e.g., *Bph1*, *bph2* or *bph4*) have lost their resistance against BPH in most rice-growing areas after cultivation for a few years (Jairin et al. 2007). In contrast, IR64, which contains the major gene *Bph1* and additional minor QTLs, was found to display moderate resistance to BPH and has retained its resistance in Central Luzon of the Philippines for more than 10 years (Cohen et al. 1997; Ram et al. 2010; Deen et al. 2010). These examples clearly demonstrate that BPH populations can rather easily overcome single gene-derived resistance, but that resistance from multiple genes or QTLs may address this problem. Hence, it is imperative to identify and characterize more resistance genes from diverse sources and incorporate them into rice cultivars, especially for *japonica* varieties which possibly lack or have only very weak BPH resistance (Chen et al. 2006; Jena et al. 2006).

It has long been proposed that polygenic resistance to disease or insect pests provides more durable and higher levels of resistance than single major genes and that pyramiding or combining these genes in a variety can be an effective way to achieve this goal (Heinrichs 1986; Cohen et al. 1997; Alam and Cohen 1998a; Palloix et al. 2009; Fujita et al. 2010). The pyramiding strategy has been examined in several plant-pathosystems and generally the pyramiding of two resistance genes/QTLs has been shown to result in an additive effect (Yoshimura et al. 1995; Huang et al. 1997; Singh et al. 2001; Zhang et al. 2006; Palloix et al. 2009; Tan et al. 2010). However, relatively few studies of pyramiding resistance genes in plant–pest interactions, especially involving rice and BPH, have been reported.

Generally, plants may employ three resistance mechanisms against insects with respect to physiological function. These mechanisms are: *antixenosis*, reduction in colonization or oviposition; *antibiosis*, reduction in insect survival, growth rate or reproduction after ingestion of host tissue; and *tolerance*, production of a crop of high quality and yield despite insect infestation (Kennedy et al. 1987; Alam and Cohen 1998a). Previous studies of IR64 have documented each of these mechanisms with regard to BPH–rice interactions (Cohen et al. 1997; Alam and Cohen 1998a, 1998b). In the case of *Bph14*, resistance appears to be due to antibiosis (Du et al. 2009). However, we recently fine mapped the *BPH6* (formerly *Bph6*) gene and found that it confers both antixenotic and antibiotic effects in *BPH6* near-isogenic line (NIL) plants (Qiu et al. 2010). Interestingly, *BPH6* conferred more rapid and stronger antixenotic and antibiotic effects in the NIL plants with 93-11 (*indica*) genetic background than those with Nipponbare (*japonica*) genetic background. The weaker resistance observed in Nipponbare suggested that pyramiding BPH-resistance genes might be particularly useful for improving resistance in *japonica* varieties.

The *BPH12*, formerly designated as *Bph12(t)*, gene was previously mapped to a 13.4-cM region on the short arm of rice chromosome 4 (Yang et al. 2002). Here we report the mapping of *BPH12* to a 1.9-cM region using an *indica/indica* F₂ population. We subsequently developed NILs harboring the *BPH12* locus in Nipponbare and constructed pyramid lines containing *BPH12* and *BPH6*. The objectives of this study were to: (1) map the *BPH12*-resistance gene with SSR markers to facilitate marker-assisted breeding, (2) characterize its resistance mechanisms when introgressed into a susceptible *japonica* rice variety, (3) estimate the pyramiding effects of *BPH12* and *BPH6* on the BPH-resistance level using NILs.

Materials and methods

Plant materials and mapping population

The rice line B14 has been reported to contain the BPH-resistance gene *BPH12* introgressed from *O. latifolia* and is resistant to BPH biotypes 1 and 2 (Yang et al. 1999; Yang et al. 2002). Two rice lines, 93-11 (*indica*) and Nipponbare (*japonica*), were used as the susceptible parents for the crosses. An F₂ population consisting of 126 families derived from a 93-11/B14 cross was used to identify and map the gene. Near-isogenic and pyramid lines in the Nipponbare background were produced from BC₄F₁ plants that were generated by successive backcrossing of the Nipponbare/B14 F₁ with Nipponbare (Supplementary Figure S1). During this process, the two nearest markers

flanking the *BPH12* locus were used to select heterozygous plants from each backcross populations for the further backcrossing.

BPH insects and evaluation of resistance

The BPH insects used for infesting plants were collected from rice fields in 2006 in Wuhan, China, and maintained on TN1 (a susceptible *indica* variety) plants under natural conditions in a greenhouse at Wuhan University. For gene mapping, a seedling bulk test was performed on the $F_{2:3}$ and $BC_4F_{2:3}$ families as described by Huang et al. (2001). Sixty seeds were randomly sown in a plastic box (58 × 38 × 9 cm) in three 26-cm-long rows, with 2.5 cm between rows. Three lines of B14, 93-11 and TN1 were randomly sown among the $F_{2:3}$ families as controls. Seedlings were grown in a greenhouse under natural light at 25–30°C. At the third-leaf stage the seedlings were infested with 2–3 instar 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 evaluation experiments were repeated thrice. The resistance score of each F_2 individual was then inferred from the weighted average of the scores for the seedlings in the corresponding $F_{2:3}$ families.

DNA extraction, map construction and QTL analysis

Total DNA was extracted from fresh leaves of individual plants by CTAB method (Murray and Thompson 1980). PCR was performed as described by Yang et al. (2002) with minor modifications. PCR products were separated on a 6% denaturing polyacrylamide gel and detected by silver staining. Genomic sequence and SSR markers were obtained from GRAMENE (<http://www.gramene.org/markers/index.html>).

For bulked segregant analysis (BSA), two contrasting DNA bulks were prepared based on the phenotype of the $F_{2:3}$ families. The bulks consisted of DNA from ten extremely resistant or susceptible individuals of the F_2 population and were screened with SSRs to identify linked to BPH resistance. A local genetic linkage map of SSR markers from the BPH-resistance gene-containing regions was constructed using JoinMap 3.0 (Van Ooijen and Voorrips 2001). QTL analysis of the BPH resistance was conducted with MapQTL 5.0 (Van Ooijen 2004).

NIL-*BPH12* and PYL-*BPH6* + *BPH12* development

BPH12 from the rice line B14 was introgressed into Nipponbare by successive backcrossing and molecular marker-assisted selection. The nearest flanking markers tightly

linked to the gene were used to select the positive backcross progenies for continuous backcrossing. At the same time, 188 SSR markers were used to screen the genetic background of the selected BC_4F_1 plant. Here, except for the markers of the target region, the number of markers with Nipponbare alleles divided by the total number of markers tested was used as a measure of the genetic identity of the BC_4F_1 plants. Consequently, individuals with the target locus and having nearly identical genetic constitutions to the recurrent parent Nipponbare were selected for self-pollination. Finally, a BC_4F_1 plant with the fewest B14 introgressions was selected to produce BC_4F_2 progeny. One BC_4F_2 individual that was homozygous at the target region of the *BPH12* was self-pollinated to generate BC_4F_3 lines. The homozygous BC_4F_3 lines were designated as NIL-*BPH12* and used in antibiosis and antixenosis tests (Supplementary Figure S1). Furthermore, the BC_4F_2 plants which were homozygous for Swarnalata and Nipponbare or heterozygous at the *BPH6* region in Nipponbare genetic background were designated as NIL2R, NIL2S and NIL2H, respectively; and the BC_4F_3 lines homozygous for the Swarnalata *BPH6* region in the Nipponbare genetic background were designated as NIL-*BPH6* (previously designated as NIL-NIP; Qiu et al. 2010), and also applied to antibiosis and antixenosis tests simultaneously.

The PYL population was developed by crossing two BC_4F_1 plants carrying *BPH6* and *BPH12*, respectively. The BC_4F_1 plant 4Y1100-2-5 carrying *BPH6* and having 93.9% identity to Nipponbare (Qiu et al. 2010) was used to pollinate a BC_4F_1 plant 4Y1249-53-2 containing the *BPH12* and having the fewest B14 introgressions (95.7% identity to Nipponbare; Supplementary Figure S2). The F_1 individuals were self-pollinated to produce F_2 populations from which the plants homozygous for both the Swarnalata and B14 alleles at the *BPH6* and *BPH12* regions were selected using the nearest flanking markers. Then these plants were self-pollinated to generate F_3 populations. The homozygous F_3 lines were designated as PYL-*BPH6* + *BPH12* and were used for the BPH performance tests (Supplementary Figure S1).

Host selection behavior

Two 14-day-old seedlings of the NIL-*BPH12* and Nipponbare or PYL-*BPH6* + *BPH12* and Nipponbare were transplanted in a plastic bucket (17 cm diameter, 15 cm height) with seedlings of the same genotype at opposite ends of roughly perpendicular diagonals. The bucket was then completely covered with fine, light-transmitting mesh. The experiment was conducted as described by Qiu et al. (2010) and a total of five buckets were used for each pair of genotypes. To observe the host selection of the BPH, 60 s-

instar nymphs were placed in each bucket and allowed to choose host plants (42-day old) on which to feed and reproduce over a 120-h period. The BPH insects that settled on each plant were counted at 1, 3, 6, 12, 24, 48, 96 and 120 h after release. NIL-*BPH6* and Nipponbare seedlings were tested at the same time and the data were published earlier (Qiu et al. 2010).

BPH development on rice plants

To measure the BPH survival and growth on the NIL-*BPH12*, NIL-*BPH6*, *PYL-BPH6* + *BPH12* 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 infested with 20 s-instar nymphs, and the surviving insect in each cup/plant was recorded every day for 9 days.

The BPH growth was measured after 4 days on the NIL-*BPH12*, *PYL-BPH6* + *BPH12* and Nipponbare plants using ten pre-weighed, second-instar nymphs. Nineteen replicates of 35-day-old seedlings were established for each genotype treatment of NIL-*BPH12*, *PYL-BPH6* + *BPH12* and Nipponbare. Four days after the treatment, the surviving nymphs on each plant were collected and the weight was recorded. The population growth rate (PGR) of surviving nymphs was calculated according to Edwards (2001) and Klingler et al. (2005). The BPH development on the NIL-*BPH6* plants was also tested simultaneously and the data were published earlier (Qiu et al. 2010).

Statistical analysis

The Chi-square test for goodness-of-fit was performed with MS-Excel; and the resistance data were analyzed using one-way ANOVA and comparing the LSD test at a 5% significance level.

Results

Genetic analysis and mapping of the *BPH12* gene

The rice line B14 has been reported to resist BPH biotypes 1 and 2 (Yang et al. 1999) and the resistance gene *BPH12* in B14 has been mapped to the short arm of rice chromosome 4 using a recombinant inbred line population (Yang et al. 2002). In this study, the B14 line exhibited resistance to the BPH insects with an average resistance score of 3.6 in the seedling bulk test. The 93-11 and Nipponbare plants were highly susceptible to the BPH with an average

resistance score of 8.7 and 8.9, respectively. In the 93-11/B14 F₂ population, the BPH-resistance scores showed a continuous range from 1.9 to 9.0, with an irregular distribution curve (Fig. 1). On the basis of the genotype of RM16459 (SSR marker nearest the *BPH12* locus, Fig. 2), the F₂ plants were classified into three classes: homozygous for the B14 or 93-11 alleles, and heterozygous (Fig. 1). The average resistance scores of the F₂ plants homozygous for the B14 allele at RM16459 tended to be lower than those plants that were heterozygous or homozygous for the 93-11 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 0–7.0 and 7.1–9.0 to have resistance and susceptibility, respectively. Thus, based on the resistance scores of the 126 F₂ plants, the segregation of the resistance to susceptible plants was in agreement with a 3:1 ratio (86:40; $\chi^2_c = 3.06 < \chi^2_{0.05,1} = 3.84$). Furthermore, the F₁ plants of the 93-11/B14 cross were resistant to BPH and had an average resistance score of 4.6 in the seedling bulk test (Fig. 1). These results indicate that a major gene controls the segregation of the BPH resistance in the F₂ population.

To identify markers tightly linked to the BPH resistance, 484 SSR markers, distributed on 12 rice chromosomes, were used to survey B14 and 93-11. A total of 185 (38.2%) of the markers were polymorphic and were used for BSA. Only the markers RM335, RM518, RM8213 and RM261, which are all on chromosome 4, were found to differentiate between the resistant and susceptible bulks. Additional polymorphic markers from this chromosome were used to genotype the 126 F₂ plants and construct a local linkage map using JoinMap 3.0 (Fig. 2). The map covered 41 cM of chromosome 4, and the marker order was basically in

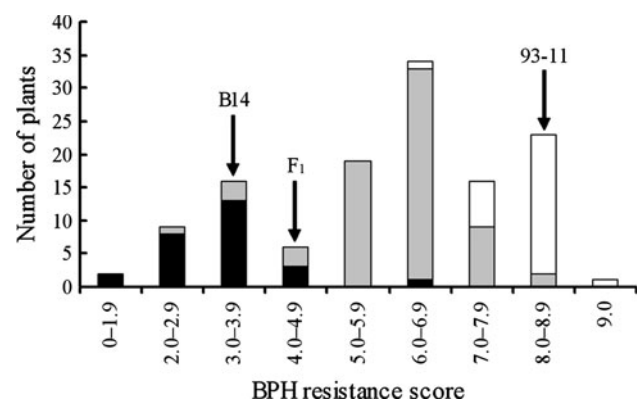


Fig. 1 Frequency distribution for the BPH-resistance scores of an F₂ population derived from the cross of 93-11/B14. The black, white and grey bars denote the RM16459 marker genotypes of B14 homozygous, 93-11 homozygous and heterozygous, respectively. Seedlings were treated with ten BPH per plant for 9–10 days. The average resistance scores of the parents B14, 93-11 and the F₁ plants were 3.6, 8.7 and 4.6, respectively. Lower scores indicate higher resistance

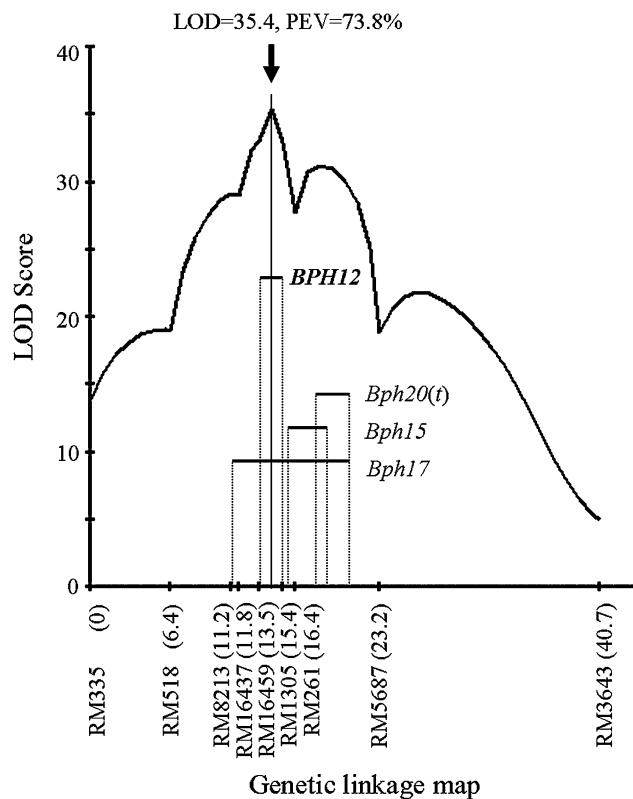


Fig. 2 Location of the *BPH12* gene on the linkage map of rice chromosome 4 constructed using an F_2 population derived from 93-11/B14. SSR markers are along the X-axis with distances (in cM) as shown and LOD scores are on the Y-axis. Vertical solid line indicates the location with the largest LOD score. PEV phenotypic variance explained by the locus. Positions of *Bph15*, *Bph17* and *Bph20(t)* are based on Huang et al. (2001), Sun et al. (2005) and Rahman et al. (2009), respectively

agreement with the previously published maps (Temnykh et al. 2000; McCouch et al. 2002). To detect the location of the resistance gene, we analyzed the resistance scores and genotypes of the F_2 plants by interval mapping using MapQTL 5.0. Consequently, one locus for BPH resistance was detected; and it had the largest LOD score of 35.4 in a 1.9-cM region between RM16459 and RM1305 on the short arm of chromosome 4 (Fig. 2), confirming the same location as reported previously (Yang et al. 2002). Variation at this locus explained 73.8% of the phenotypic variance of BPH resistance in the F_2 population. For the markers tightly linked to *BPH12*, alleles from the resistant parent B14 conferred increased resistance to BPH and the additive effects varied from 43.5 to 46.0; whereas the dominant effects of the marker loci varied from -1.3 to -4.1 (Table 1). In addition, the *BPH12* heterozygotes in F_2 or NIL populations exhibited a lower resistance to the BPH insects than *BPH12* homozygotes in the seedling bulk test (Fig. 1; Fig. 3). These results suggest that the resistance gene *BPH12* is partially dominant, but mainly exhibits an additive effect.

Evaluation of *BPH12* and *BPH6* NILs and PYL

In the BC_4F_2 progenies of the selected BC_4F_1 plants, plants homozygous for Nipponbare *BPH12* region (NIL-*BPH12* S) were highly susceptible to BPH with an average resistance score of 8.5 (Nipponbare, score 8.9) in the seedling bulk tests (Fig. 3). The plants heterozygous for the B14 *BPH12* region (NIL-*BPH12* H) showed moderate resistance to BPH compared to Nipponbare (average score of 6.5, $F = 28.4$, $P < 0.001$). The NIL-*BPH12* R plants that were homozygous for the B14 *BPH12* region and B14 plants were both resistant to BPH (average scores of 4.4 and 3.6, respectively). At the same time, the lines homozygous for the Swarnalata (NIL2R) or Nipponbare (NIL2S) *BPH6* region were scored 3.3 and 8.6, respectively (Qiu et al. 2010). The PYL-*BPH6* + *BPH12* plants showed high resistance to BPH (average score of 2.9). One-way ANOVA analysis showed that there was a significant difference between PYL-*BPH6* + *BPH12* and NIL-*BPH12* R ($F = 12.7$, $P = 0.006$), and between PYL-*BPH6* + *BPH12* and NIL2R ($F = 4.5$, $P = 0.047$). These findings indicate that pyramiding the *BPH6* and *BPH12* genes in a Nipponbare genetic background could significantly improve the BPH resistance of this *japonica* rice variety.

Antixenotic effect of *BPH12* and *BPH6* toward BPH insects

To test the antixenosis in the BPH resistance conferred by *BPH6* and *BPH12*, the NIL-*BPH12*, NIL-*BPH6* and PYL-*BPH6* + *BPH12* lines were used to examine the host preference of the BPHs. In the BPH host choice test, most of the BPHs jumped onto the rice and fastened themselves to the shoots at 3 h after release (Fig. 4). The average number of settled BPHs on the NIL-*BPH12*, NIL-*BPH6*, PYL-*BPH6* + *BPH12* and Nipponbare plants increased over the 12-h observation period. One-way ANOVA analysis showed that the BPH insects had non-preference for the NIL-*BPH12* and Nipponbare, NIL-*BPH6* and Nipponbare or PYL-*BPH6* + *BPH12* and Nipponbare after 12 h (Supplementary Table S1). The average number of settled BPHs remained relatively constant on the NIL-*BPH12* or NIL-*BPH6* plants from the observation period of 24–120 h, whereas the BPHs on Nipponbare plants increased over this observation period and showed a significant difference compared with the NIL-*BPH12* or NIL-*BPH6* plants at 120 h (Fig. 4a, b; Supplementary Table S1). As for the PYL-*BPH6* + *BPH12* and Nipponbare plants, the average number of settled BPHs on the susceptible plants varied slightly from 24 to 120 h, while the BPHs on the resistant plants decreased greatly over this observation period and showed a significant difference compared to Nipponbare (Fig. 4c; Supplementary Table S1). From these results, we can infer that antixenotic

Table 1 Genetic effect of *BPH12* identified by tightly linked markers in F₂ (93-11/B14) population by MapQTL 5.0

Locus	Position (cM)	LOD	PEV (%)	A	D
RM8213	11.2	29.1	65.5	43.5	-4.1
RM16437	11.8	29.0	65.3	43.7	-2.3
RM16459	13.5	33.1	70.1	45.6	-2.9
RM1305	15.4	33.0	70.0	46.0	-2.9
RM261	16.4	27.8	63.7	44.1	-1.3

The genetic effect estimated on the progeny data by MapQTL 5.0. Additive effect was equal to the half of the trait value difference between two homozygotes; and dominant effect was 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 of the B14 allele, D dominant effect of the B14 allele

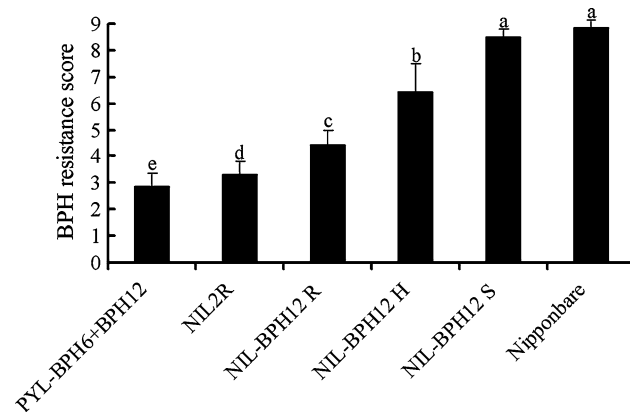


Fig. 3 BPH-resistance phenotype in NILs, PYLs and parents as measured using the seedling bulk test. Bars represent means of 5–8 replicates. Error bars represent the SD. Means labeled with the same letter are not significantly different at a level of $P = 0.05$

factors were present in the NIL-*BPH12* and NIL-*BPH6* plants, and this antixenotic effect was probably quicker and stronger in the PYL-*BPH6* + *BPH12* plants compared with the single introgression lines NIL-*BPH12* or NIL-*BPH6*.

BPH performance on NIL and PYL plants

To determine whether the NIL and PYL plants affect the BPH growth and development, we compared the BPH PGR on the NIL-*BPH12*, NIL-*BPH6*, PYL-*BPH6* + *BPH12* and Nipponbare plants. As shown in Fig. 5a, by the fourth day after treatment a 17.6 or 35.7% reduction in PGR of the BPHs was observed on the NIL-*BPH12* or NIL-*BPH6* plants compared to Nipponbare, reflecting a significant difference between them ($F = 4.7$, $P = 0.036$ for NIL-*BPH12* and Nipponbare; $F = 12.3$, $P = 0.002$ for NIL-*BPH6* and Nipponbare). Similarly, 46.4, 26.8 and 72.1% reductions in PGR were observed on the PYL-*BPH6* + *BPH12* plants in comparison to the NIL-*BPH12*, NIL-*BPH6* and Nipponbare plants, respectively ($F = 12.4$, $P = 0.001$ for PYL-*BPH6* + *BPH12* and NIL-*BPH12*;

$F = 4.37$, $P = 0.03$ for PYL-*BPH6* + *BPH12* and NIL-*BPH6*; $F = 29.7$, $P = 0.001$ for PYL-*BPH6* + *BPH12* and Nipponbare). These results indicate that the BPH growth and development were inhibited on the NIL-*BPH12* plants and especially on the pyramiding plants PYL-*BPH6* + *BPH12*.

To test whether antibiosis is a component of the BPH resistance conferred by the *BPH12* and to compare NIL-*BPH12*, NIL-*BPH6* and PYL-*BPH6* + *BPH12* plants, we measured the BPH survival rates on the NIL-*BPH12*, NIL-*BPH6*, PYL-*BPH6* + *BPH12* and Nipponbare plants every day for 9 days. As shown in Fig. 5b, the average number of surviving BPHs on the NIL-*BPH12*, NIL-*BPH6*, PYL-*BPH6* + *BPH12* and Nipponbare plants remained relatively constant for the first 2 days after the BPH infestation. However, by the third and fourth days, the average number of surviving BPHs on the PYL-*BPH6* + *BPH12* plants decreased and showed a significant difference in number compared with Nipponbare ($F = 7.4$, $P = 0.01$ at 3 days; $F = 9.9$, $P = 0.004$ at 4 days); but there was no significant differences between the PYL-*BPH6* + *BPH12* and NIL-*BPH12* ($F = 0.9$, $P = 0.34$ at 3 days; $F = 2.8$, $P = 0.11$ at 4 days) or PYL-*BPH6* + *BPH12* and NIL-*BPH6* ($F = 1.7$, $P = 0.18$ at 3 days; $F = 3.6$, $P = 0.09$ at 4 days). From the fifth day, the average number of the BPHs on the PYL-*BPH6* + *BPH12* plants decreased quickly, and they were significantly different in numbers compared with the NIL-*BPH12*, NIL-*BPH6* or Nipponbare plants at later time points (Fig. 5b). In addition, the average numbers of the surviving BPHs on the NIL-*BPH12* plants were also reduced along with the days of the BPH infestation, and showed a significant difference at the ninth day, compared to Nipponbare ($F = 6.2$, $P = 0.02$ at 9 days). The same significant difference was observed at the eighth and ninth days between the NIL-*BPH6* and Nipponbare plants ($F = 4.7$, $P = 0.046$ at 8 days; $F = 5.5$, $P = 0.033$ at 9 days; Fig. 5b). These findings suggest that the BPH insects probably had an effect on the antibiotic factors in the NIL-*BPH12* plants and that this effect was weaker than that in the PYL-*BPH6* + *BPH12* plants.

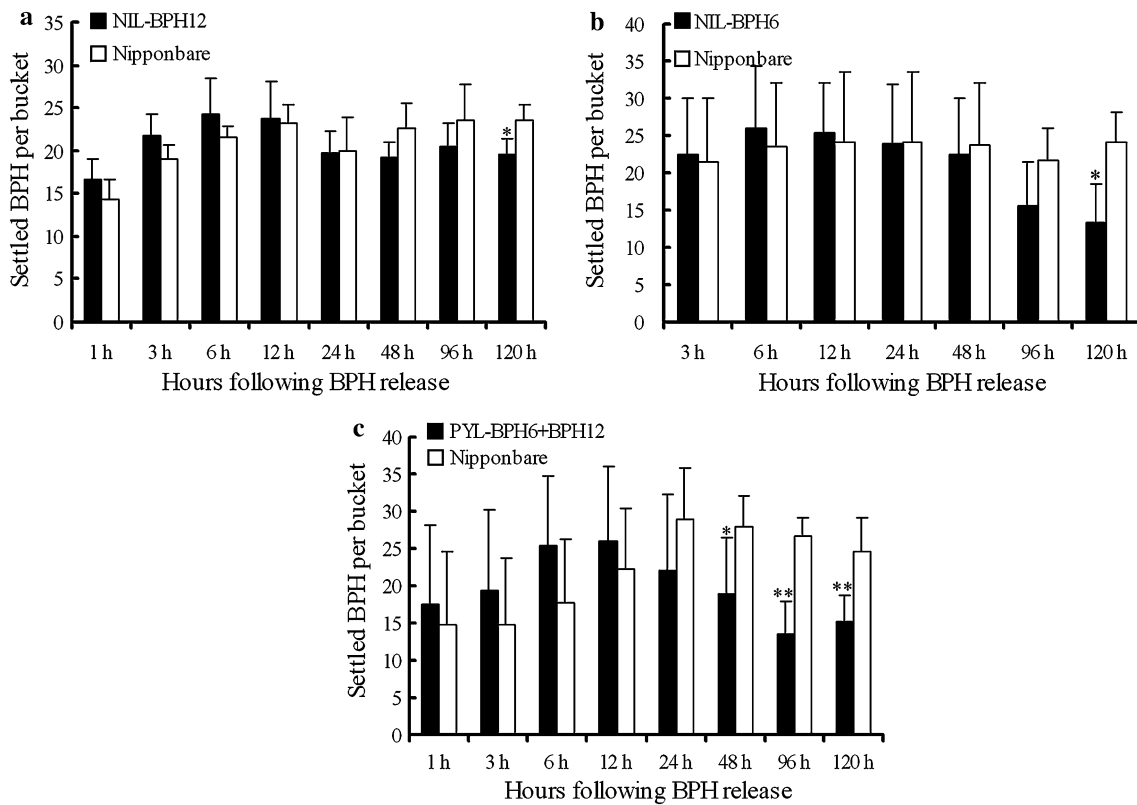


Fig. 4 Results of BPH host choice test. **a** NIL-*BPH12* and Nipponbare; **b** NIL-*BPH6* and Nipponbare; **c** PYL-*BPH6* + *BPH12* and Nipponbare. Bars represent means of five replicates. Error bars

represent the SD. Means labeled with asterisks are significantly different ($P < 0.05$). NIL-*BPH6* data were previously reported in Qiu et al. (2010)

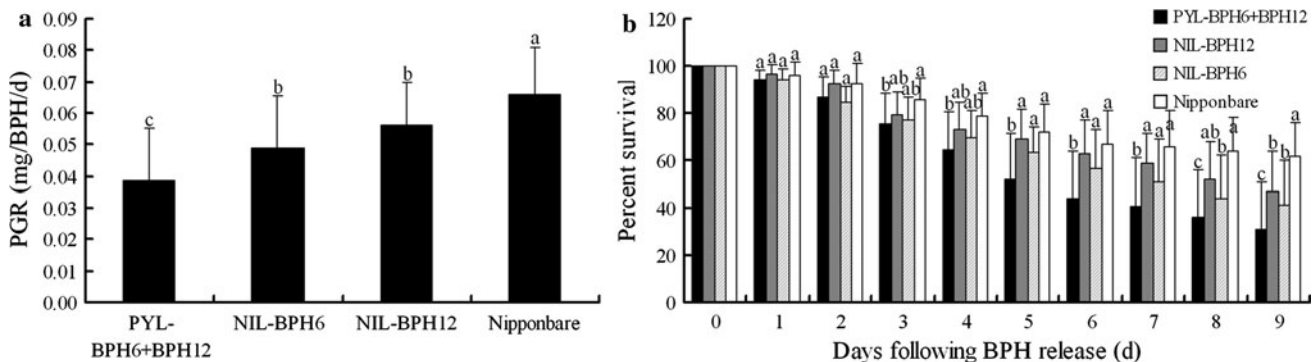


Fig. 5 Effects of plant genotype on the BPH population growth rate (mg/BPH/day, PGR) and BPH survival. **a** PGR of the NIL-*BPH12*, NIL-*BPH6*, PYL-*BPH6* + *BPH12* and Nipponbare. PGR was measured as described by Edwards (2001). **b** BPH survival rates on NIL-*BPH12*, NIL-*BPH6*, PYL-*BPH6* + *BPH12* and Nipponbare. Bars

represent means of 19 replicates for **a**, and 16 replicates for **b**. Error bars represent the SD. Means labeled with the same letter are not significantly different at a level of $P = 0.05$. NIL-*BPH6* data were previously reported in Qiu et al. (2010)

Discussion

The BPH-resistance gene *BPH12*, derived from the wild rice species *O. latifolia* (Yang et al. 1999), was previously mapped to a 13.4-cM region the short arm of rice chromosome 4 flanked by RFLP marker C946 and SSR marker RM261 (Yang et al. 2002). In this study, the location of *BPH12* was further refined to a region 1.0-cM south of

marker RM16459 and 0.9-cM north of marker RM1305. The physical distance between the nearest markers is approximately 400–430 kb, according to the 93-11 and Nipponbare reference genomes. It has been noted that many BPH-resistance genes appear to be clustered on rice chromosomes (Jena and Kim 2010; Qiu et al. 2010; Yara et al. 2010) and several other genes including *Bph15*, *Bph17* (derived from rice cultivar Rathu Heenati) and

Bph20(t) have also been mapped to the short arm of chromosome 4. The *Bph15*, *Bph17* and *Bph20(t)* genes have been mapped to a region flanked by the markers C820 and S11182 (Huang et al. 2001), MS10 and RM5953 (Rahman et al. 2009) and RM8213 and RM5952 (Sun et al. 2005), respectively. Based on these reports and the rice reference genome sequences, *BPH12* is probably located to the north of the *Bph15* and *Bph20(t)* (Fig. 2), but additional genetic analyses (e.g., high-resolution mapping, allelism tests, gene cloning) are needed to clarify the relationship of *BPH12* and other BPH-resistance genes in the region.

One of the objectives of this research was to characterize the mechanism(s) of the BPH resistance conferred by *BPH12*. According to the tests of the BPH host preference and performance on the NIL-*BPH12* and Nipponbare plants, *BPH12*-mediated resistance involves both antixenosis and antibiosis in Nipponbare genetic background. Previously, we found that the *BPH6* gene in 93-11 or Nipponbare genetic background also confers antixenosis and antibiosis to the BPH insects (Qiu et al. 2010). Both the *BPH6* and *BPH12* genes deterred the BPHs from settling on the NIL plants within 120 h of release. The decreased survival rates of the BPHs on NIL-*BPH12* compared to Nipponbare were significantly different at the ninth day after the BPH treatment. Similarly, a significant difference between NIL-*BPH6* and Nipponbare occurred at the eighth and ninth days; however, based on the seedling bulk test, the BPH-resistance level conferred by the *BPH6* was generally higher than that of the *BPH12*, consistent with the average resistance scores of the donor parents (Swarnalata and B14 scored 2.9 and 3.6, respectively). As for the *japonica* NILs of the *BPH6* and *BPH12*, the average resistance scores of the BC₄F₂ lines for Swarnalata allele at the *BPH6* region were 3.3 (homozygous) and 4.7 (heterozygous); whereas lines with the B14 allele at the *BPH12* region were 4.4 and 6.5, respectively. It is probable that other factors such as BPH biotypes or tolerance affect the BPH-resistance levels (Alam and Cohen 1998a; Myint et al. 2009). Overall, the *BPH12* in Nipponbare genetic background had resistance to the BPHs and exhibited both antixenotic and antibiotic effects toward the BPHs.

Another objective of the present research was to pyramid two functional BPH resistance genes originating from wild rice species and *indica* cultivar into a susceptible *japonica* background and to compare their resistance levels. Most studies showed an additive effect of pyramiding two genes of plant resistance to pathogen or insect. Barloy et al. (2007) indicated that a higher level of resistance against cereal cyst nematodes was gained when the *CreX* and *CreY* genes were pyramided in wheat. More recently, Fujita et al. (2010) demonstrated a gene pyramiding effect that significantly increased resistance when two green rice leafhopper-resistance genes were combined in the rice

variety Taichung 65. However, when pyramiding the BPH-resistance genes *Bph1* and *Bph2* into a *japonica* cultivar, Sharma et al. (2004) found that while the pyramided line exhibited a higher level of resistance than the *Bph2*-single introgression line, its resistance level was only equivalent to that of the *Bph1*-single introgression line. In the present study, the PYL-*BPH6* + *BPH12* resulted in a higher resistance level than that of the NIL-*BPH12* or NIL-*BPH6* lines and showed an additive effect of the BPH resistance genes. In the seedling bulk test, the PYL-*BPH6* + *BPH12* lines had an average resistance score of 2.9 and showed a higher resistance to the BPHs than the resistant parent B14 and the near-isogenic lines NIL-*BPH12* R and NIL2R (the BC₄F₂ lines with homozygous for Swarnalata at the *BPH6* region in Nipponbare genetic background, Qiu et al. 2010), whose average scores were 3.6, 4.4 and 3.3, respectively. The survival rates of the BPH on the PYL-*BPH6* + *BPH12* plants decreased quickly and showed significant difference compared to Nipponbare, NIL-*BPH12* and NIL-*BPH6* plants at the third, fifth and fifth days after the BPH treatment, respectively; the survival rates of BPH on NIL-*BPH12* and NIL-*BPH6* were significantly different from Nipponbare at the ninth and eighth days, respectively. Thus, pyramiding these two BPH-resistance genes in a *japonica* rice cultivar Nipponbare did increase the resistance level.

More important than economic considerations, this strategy is possibly valuable because of the epidemiological and evolutionary considerations. Besides raising the resistance level, pyramiding major resistance genes might contribute to the durability of plant resistance to disease or insect (Cohen et al. 1997; Alam and Cohen 1998a; Chen et al. 2003; Tan et al. 2010). This has proven to be true in the studies of the disease resistance because the pathogen would need double or multiple mutations to overcome the resistance (Palloix et al. 2009; Zhou et al. 2009; Tan et al. 2010). However, it is still unknown whether the lines pyramided with two or more BPH-resistance genes can improve the durability of the rice resistance to the BPH.

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