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Research Paper

Substitution mapping and characterization of brown planthopper resistance genes from *indica* rice variety, 'PTB33' (*Oryza sativa* L.)

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Rice (*Oryza sativa* L.) yield is severely reduced by the brown planthopper (BPH), *Nilaparvata lugens* Stål, in Asian countries. Increasing resistance in rice against BPH can mitigate yield loss. Previous reports indicated the presence of three BPH resistance genes, *BPH2*, *BPH17-ptb*, and *BPH32*, in durable resistant *indica* rice cultivar 'PTB33'. However, several important questions remain unclear; the genetic locations of BPH resistance genes on rice chromosomes and how these genes confer resistance, especially with relationship to three major categories of resistance mechanisms; antibiosis, antixenosis or tolerance. In this study, locations of *BPH2*, *BPH17-ptb*, and *BPH32* were delimited using chromosome segment substitution lines derived from crosses between 'Taichung 65' and near-isogenic lines for *BPH2* (*BPH2*-NIL), *BPH17-ptb* (*BPH17-ptb*-NIL), and *BPH32* (*BPH32*-NIL). *BPH2* was delimited as approximately 247.5 kbp between RM28449 and ID-161-2 on chromosome 12. *BPH17-ptb* and *BPH32* were located between RM1305 and RM6156 on chromosome 4 and RM508 and RM19341 on chromosome 6, respectively. The antibiosis, antixenosis, and tolerance were estimated by several tests using *BPH2*-NIL, *BPH17-ptb*-NIL, and *BPH32*-NIL. *BPH2* and *BPH17-ptb* showed resistance to antibiosis and antixenosis, while *BPH17-ptb* and *BPH32* showed tolerance. These results contribute to the development of durable BPH resistance lines using three resistance genes from 'PTB33'.

Key Words: rice, brown planthopper, BPH resistance gene, 'PTB33', gene mapping.

Introduction

Rice (*Oryza sativa* L.), the primary food source for as much as one third of the world's population, frequently suffers from insect pests. The brown planthopper (BPH), *Nilaparvata lugens* Stål, is one of the most prevalent insect pests in rice cultivation areas in Asia and Australia (Bottrell and Schoenly 2012). BPH causes severe damage by feeding on the plant phloem sieve and transmitting serious viral diseases, such as rice grassy stunt virus, rice ragged stunt phytoreovirus, and rice wilted stunt virus (Fujita *et al.* 2013, Wei *et al.* 2018). The exponential increase in the population of BPH in rice fields can cause "hopper burn" and, consequently, huge yield losses. China is the country most severely affected by BPH, with approximately 8.7 million hectares of rice damaged in 2007; and approximately 0.57 million hectares of rice in Vietnam were devastated in 2007 (Catindig *et al.* 2009). Over 3 million hectares of rice were destroyed in Thailand between 2009 and 2011, and as much as 200,000 hectares of rice were damaged in Indonesia in 2011 (Horgan *et al.* 2015).

Since the late 1960s, the improvement of host plant resistance in rice has been one of the strategies to reduce BPH damage. To date, more than 40 loci for BPH resistance (designated as *BPH1* to *BPH40*) have been identified in rice (Akanksha *et al.* 2019, Balachiranjeevi *et al.* 2019, Du *et al.* 2020, Fujita *et al.* 2013, Li *et al.* 2019, Yang *et al.* 2019, Zhang *et al.* 2020). Among these, seven genes (*BPH6*, *BPH7*, *BPH15*, *BPH27*, *BPH28*(t), *BPH33*, and

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BPH36) have been mapped to specific chromosomal locations in large-scale populations (Hu *et al.* 2018, Huang *et al.* 2013, Li *et al.* 2019, Qiu *et al.* 2010, 2014, Wu *et al.* 2014, Yang *et al.* 2004). The other genes, *BPH20*, *BPH21*, *BPH25*, and *BPH31*, have been identified by linkage mapping or substitution mapping (Li *et al.* 2019, Prahalada *et al.* 2017, Rahman *et al.* 2009). Seven genes, *BPH9*, *BPH14*, *BPH17*, *BPH18*, *BPH26*, *BPH29*, and *BPH32*, have been cloned and characterized for BPH resistance (Du *et al.* 2009, Ji *et al.* 2016, Liu *et al.* 2015, Ren *et al.* 2016, Tamura *et al.* 2014, Wang *et al.* 2015, Zhao *et al.* 2016). Among the genes mapped to specific chromosomal locations, most of the BPH resistance genes were clustered on chromosome 12 (cluster A), chromosome 4S (cluster B), chromosome 6 (cluster C), and chromosome 4L (cluster D) (Fujita *et al.* 2013). Eight genes, *BPH1*, *BPH2*, *BPH7*, *BPH9*, *BPH10*, *BPH18*, *BPH21*, and *BPH26*, have been reported in cluster A. Seven resistance genes have been identified in cluster B: *BPH12*, *BPH15*, *BPH17*, *BPH17-ptb*, *BPH20*, *BPH22*(t), *BPH30* (Hou *et al.* 2011, Liu *et al.* 2015, Nguyen *et al.* 2019, Qiu *et al.* 2012, Rahman *et al.* 2009, Wang *et al.* 2018, Yang *et al.* 2004). Five genes, *BPH3*, *BPH4*, *BPH25*, *BPH29*, and *BPH32*, have been identified in cluster C (Jairin *et al.* 2007b, 2010, Lakshminarayana and Khush 1977, Myint *et al.* 2012, Ren *et al.* 2016, Sidhu and Khush 1978, Wang *et al.* 2015). Other genes, *BPH6*, *BPH18*(t) and *BPH27* (from *Oryza rufipogon* Griff.), *BPH27*(t) (from 'Balamawee'), and *BPH34* are located in cluster D (Guo *et al.* 2018, He *et al.* 2013, Huang *et al.* 2013, Kabis and Khush 1988, Kumar *et al.* 2018, Li *et al.* 2010). The identification and mapping of BPH resistance genes has facilitated the introduction of resistance genes using marker-assisted selection (MAS) and has helped to elucidate the resistance mechanisms.

There are generally three types of resistance mechanisms: antibiosis, antixenosis, and tolerance, with different roles contributing to plant resistance (Kogan and Ortman 1978, Painter 1951). In antibiosis, the plant hinders the normal development of the insect by producing compounds that are toxic to the insect or by inhibiting nutrient sucking. In antixenosis, the preference of the insect for the host plant results in less favorable settling or oviposition of the insect. In tolerance (the third type of resistance), the plant has some ability to compensate for the loss of nutrients or diminished yield due to the infestation (Kogan and Ortman 1978, Painter 1951). Among the three types of resistance mechanisms, antibiosis is commonly induced by many BPH resistance genes: *BPH1*, *BPH2*, *BPH3*, *BPH10*, *BPH17*, *BPH20*, *BPH21*, *BPH25*, *BPH26*, *BPH30*, and *BPH32* (Cohen *et al.* 1997, Jena *et al.* 2017, Nguyen *et al.* 2019, Ren *et al.* 2016, Wang *et al.* 2018). Other genes, *BPH6*, *BPH9*, *BPH12*, *BPH14*, *BPH15*, *BPH18*, *BPH27*, *BPH33*, and *BPH36*, express both antibiosis and antixenosis (Du *et al.* 2009, Guo *et al.* 2018, Hu *et al.* 2018, Huang *et al.* 2013, Ji *et al.* 2016, Li *et al.* 2011, 2019, Qiu *et al.* 2010, Zhao *et al.* 2016). *BPH7* and *BPH37* are related to tolerance (Qiu *et al.* 2014, Yang *et al.* 2019). Understanding the resistance mechanism(s) for each BPH resistance gene can be useful for enhancing and/or prolonging the resistance level through pyramiding with other genes (Du *et al.* 2020).

Among the many BPH resistance genes, *BPH2*, *BPH17-ptb*, and *BPH32* primarily originate from the strong and broad-spectrum resistance cultivar 'PTB33' (Angeles *et al.* 1986, Horgan *et al.* 2015, Jairin *et al.* 2007b, Nguyen *et al.* 2019, Sidhu and Khush 1978). *BPH32* has been detected primarily on chromosome 6, between two markers RM19291 and RM8072 and has been cloned using bioinformatics methods (Jairin *et al.* 2007c, Ren *et al.* 2016). However, the locations of *BPH17-ptb* and *BPH2* are unclear. *BPH17-ptb* was detected on chromosome 4S based on the similarity in amino acid sequence for the location of *BPH17* between 'PTB33' and 'Rathu Heenati' (Sri Lanka rice variety). The region of *BPH17-ptb* (from 4.4 to 8.2 Mbp) was delimited as approximately 3.8 Mbp that possibly contains other factor(s) related to BPH resistance (Nguyen *et al.* 2019). *BPH2* is a recessive gene that has been detected primarily on 'ASD7' (Lakshminarayana and Khush 1977). Sidhu and Khush (1978) and Angeles *et al.* (1986), using conventional genetic analysis, reported that the BPH resistance of 'PTB33' is controlled by one dominant gene (*BPH3*) and one recessive gene (*BPH2*). Jairin *et al.* (2007a) failed to map *BPH2* from 'PTB33' because of the strong virulence of BPH populations from Thailand, causing *BPH2* plants to be overwhelmed by the pest. Accordingly, there is a knowledge gap in the resistance mechanisms of these genes. To date, these genes—*BPH2*, *BPH17-ptb*, and *BPH32*—have only been tested for antibiosis with regard to adult BPH mortality and/or anti-feeding activity but have yet to be tested for antixenosis or tolerance activity (Jena *et al.* 2017, Nguyen *et al.* 2019, Zhao *et al.* 2016). Therefore, it is crucial to detect the exact location of *BPH2* and *BPH17-ptb* as well as to characterize the resistance mechanism of these three genes.

Recently, in order to understand the genetic basis and resistance behavior of rice genes, three near-isogenic lines (NILs) for *BPH2* (*BPH2*-NIL), *BPH17-ptb* (*BPH17-ptb*-NIL), and *BPH32* (*BPH32*-NIL) have been developed on the genetic background of *japonica* cultivar 'Taichung 65' (T65) (Nguyen *et al.* 2019). In this study, the presence and detailed locations of *BPH2*, *BPH17-ptb*, and *BPH32* were verified through substitution mapping for target genes using chromosome segment substitution lines derived from the corresponding NILs. We then estimated the resistance mechanisms of the three genes using different methods of resistance evaluation. The detailed location of *BPH2* and characterization of the resistance mechanisms of *BPH2*, *BPH17-ptb*, and *BPH32* can accelerate the understanding of BPH resistance in 'PTB33' and facilitate MAS of these genes in rice breeding.

Materials and Methods

Development of populations for substitution mapping of BPH2, BPH17-ptb, and BPH32

The susceptible parent T65 was crossed with the BPH-resistant donor parent 'PTB33' (IRGC Acc. 19325) and F_1 plants were developed. The F_1 plants were continuously backcrossed with T65 and plants with *BPH2*, *BPH17-ptb*, or *BPH32* were selected by MAS at each generation. Through backcrossing and MAS, BC_4F_1 plants were developed and self-pollinated (**Fig. 1**). Ninety-six BC_4F_2 plants from each crossing were used to screen the recombinants with respect to *BPH2*, *BPH17-ptb*, and *BPH32*. Homozygous recombinant chromosome substitution lines were selected using simple sequence repeat (SSR) markers from the BC_4F_3 populations. BC_4F_4 plants carrying homozygous recombination events related to *BPH2*, *BPH17-ptb*, and *BPH32* were used for substitution mapping. Three NILs, *BPH2*-NIL (BC_4F_3), *BPH17-ptb*-NIL (BC_4F_3), and *BPH32*-NIL (BC_4F_4), were used to characterize the resistance mechanisms of the three genes, *BPH2*, *BPH17-ptb*, and *BPH32*, from 'PTB33', respectively (Nguyen *et al.* 2019).

DNA extraction and genotyping

Total DNA from BC_4F_2, BC_4F_3, and BC_4F_4 populations was extracted using the potassium acetate method (Dellaporta *et al.* 1983). The genotypes of plants were determined using PCR and agarose gel electrophoresis, as described in a previous study (Nguyen *et al.* 2019). Six SSR markers, RM277, RM1246, RM28493, RM1103, S12091B, and RM5479 on chromosome 12L, were used for genotyping BC_4F_2 and recombinant BC_4F_3 plants segregating at *BPH2* (**Table 1**). Three DNA markers, C61009, RM8213, and B40, on chromosome 4S were used for genotyping BC_4F_2 and recombinant BC_4F_3 plants segregating at *BPH17-ptb*. BC_4F_2 and recombinant BC_4F_3 plants for *BPH32* were genotyped using six DNA markers on chromosome 6S: RM6775, S00310, RM508, RM586, RM588, and RM19341. The BC_4F_4 homozygous recombinant lines for *BPH2* were genotyped with 13 additional DNA markers between RM1246 and RM28493 (RM28305, RM28346, RM28396, RM28404, RM28424, RM28433, RM28449, InD14, ID-28L4, ID-174, ID-161, ID-161-2, and RM3726). Similarly, additional 16 DNA markers between RM8213 and B40 (RM16460, RM3658, RM1305, RM16474, RM16479, RM16480, RM16482, RM3471, RHD3, WH2, RM16506, RM16508, RM16514, MS5, RM6156, and RM16531) were used for genotyping BC_4F_4 homozygous recombinant lines around the *BPH17-ptb* location. The BC_4F_4 lines for *BPH32* were genotyped with five additional DNA markers between RM508 and RM586 (RM19288, RM19291, RM19296, RM589, and RM19311).

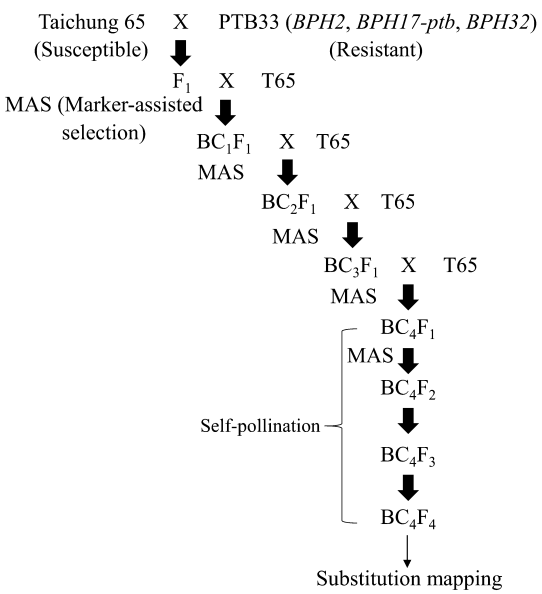

Fig. 1. The breeding scheme for development of substitution mapping populations for *BPH2*, *BPH17-ptb*, and *BPH32*.

BPH population used for evaluating plant resistance

The BPH population collected in Kanagawa Prefecture, Japan, in 1966 (Hadano-66), was used in substitution mappings for evaluation of plant resistance and characterization of resistance mechanisms. Hadano-66 that has been maintained on *japonica* cultivar 'Reiho' at the Kyushu Okinawa Agricultural Research Center of the National Agriculture and Food Research Organization in Japan was provided. At Saga University, Hadano-66 reared on T65 seedlings under room conditions of 25°C and 16 h of light followed by 8 h of dark more than five generations.

Modified seedbox screening test (MSST) and modified mass tiller screening (MMTS)

The MSST was conducted to evaluate the resistance levels of BC_4F_4 homozygous recombinant lines for *BPH2*, *BPH17-ptb* and *BPH32* (Velusamy *et al.* 1986). Twenty seeds of each line were sown in a row in a plastic tray (23.0 × 30.0 × .5 cm) with 2.5 cm spacing between rows of seedlings. One row of 'PTB33' and three rows of T65 were sown as resistance and susceptible controls, respectively. Seven days after sowing (DAS), the plants were thinned to 15 seedlings per row and infested by second and third instar nymphs of Hadano-66 at a density of approximately 20 nymphs per plant. When T65 was dried by BPH sucking, the plants were scored following the evaluation system for rice from the International Rice Research Institute. The experiments were performed in triplicates.

MMTS described by Jairin *et al.* (2007b) was used for the evaluation of homozygous recombinant lines for *BPH32*. Seeds of each line, as well as 'PTB33' and T65, were separately sown in 3-L pots. At 60 DAS, the tillers with similar growth condition were separated and transplanted in a plastic box (50.0 × 30.0 × 10.0 cm). Ten days after transplanting, the plants were infested by the fourth and fifth instar BPH nymphs at a density of approximately

Table 1. Simple sequence repeat markers used for substitution mapping of *BPH2*, *BPH17-ptb*, and *BPH32*

Marker	Resistance gene tagged	Chromosome	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)	Physical location (bp)
RM277[a]	*BPH2*	12	CGGTCAAATCATCACCTGAC	CAAGGCTTGCAAGGGAAG	18,319,039
RM1246[b]	*BPH2*	12	GGCTCACCTCGTTCTCGATCC	CATAAATAAATAGGGCGCCACACC	19,156,149
RM28305[c]	*BPH2*	12	GTCATCTTCGCAAATGGTGATGG	GGTCGTCGTGGTGTTATTCTTGG	19,998,669
RM28346[c]	*BPH2*	12	GCCCAAAGTTAATATCGGTGTCTCC	AGCCTGCCTAGCACTCATAGACC	20,989,018
RM28396[c]	*BPH2*	12	CTGCTTGTTGTTGGGACTGGTTTCG	CTCGTACTGCAGCTGTGCATCTCG	21,765,229
RM28404[c]	*BPH2*	12	GTGGGAGTCGAGAGGCGATAAGG	AAAGGACGGCTCATAGGTGATGG	21,888,484
RM28424[c]	*BPH2*	12	TCCACACACTTCGCCAATAAACC	CCGCCACCACTCCTCTATCC	22,404,416
RM28433[c]	*BPH2*	12	AATAGCTGCATATACCCGGTTGG	TGTGTCTCTGATGATCCGTTTCG	22,600,596
RM28449[c]	*BPH2*	12	CACCCATTGATGTGAAACTCTGG	GGATTCATGATACAGTGTGCAACG	22,689,921
InD14[d]	*BPH2*	12	CCACTCTGAAAATCCCAAGC	ACCAGTTAAGTCACGCTCAAA	22,865,198
ID-28L4[e]	*BPH2*	12	GAAGGGAAATGGAAGCATGA	TACACCCGACAAGGAACACA	22,876,313
ID-174[e]	*BPH2*	12	TGCTCGTACGATGGAGTCAT	CGGGCTTCATTCATCGTTA	22,912,230
ID-161[e]	*BPH2*	12	CTGTCAAAATTGCGTTCGAT	CATTCCCCTGAATTTGAAACA	22,935,877
ID-161-2[e]	*BPH2*	12	ATCCTTTCGGACAGGGTGAT	GGACGGGATGATACCTCAGA	22,937,422
RM3726[c]	*BPH2*	12	TACACCCACCCACATACGTCAGC	GTCGTACTCCCGGATCTTCTTCC	23,275,244
RM28493[c]	*BPH2*	12	ACCGTTAGATGACACAAGCAACG	GGTTAGCAAGACTGGAGGAGACG	23,279,853
RM1103[b]	*BPH2*	12	GTCGGTGTGTACTCCGTGTTTGG	CATATGCAGTGGTCAGTGGAGTGG	23,606,775
S12091B[f]	*BPH2*	12	GGCTTTCTTCCTCACACTGC	CGAGGACGAGATGAGACGA	23,685,715
RM5479[b]	*BPH2*	12	CTCACCATAGCAATCTCCTGTGC	ACTTCGTTCACTTGCATCATGG	24,446,205
C61009[g]	*BPH17-ptb*	4	GGCCAGCAAGGTGTAGTAAG	ACAAACCCCAGCACCCTAAG	2,427,000
RM8213[b]	*BPH17-ptb*	4	TGTTGGGTGGGTAAAGTAGATGC	CCCAGTGATACAAAGATGAGTTGG	4,418,222
RM16460[c]	*BPH17-ptb*	4	ATTGCACCATTCAAACGGAACC	TTCCAAGCTGTCTTTCTGACATGACC	5,318,612
RM3658[b]	*BPH17-ptb*	4	GTAGCACTCCGCTGCTTCGTCTCC	AATCCCACCCGCCTCATCTCC	5,573,675
RM1305[b]	*BPH17-ptb*	4	GGTACTACAAAGAAACCTGCATCG	TCCTAGCTCAAATGTGCTATCTGG	5,624,467
RM16474[c]	*BPH17-ptb*	4	GGAGCCTGGATCTTTACCTCTCC	CGTGGCGTTCTCTGTCAAGG	5,752,955
RM16479[c]	*BPH17-ptb*	4	GGTCCGCATCATCATTATCACC	CTGCTTATCCTAGGGTGTGTTTGG	5,942,786
RM16480[c]	*BPH17-ptb*	4	GCCAAGATTGGTGCTTTCACTCTGG	GAGGGCCTGTGTGCATAAGATACGC	6,007,686
RM16482[c]	*BPH17-ptb*	4	TTCTGCAGGATTGATGGTGTGG	CCAGTTGATGTGCAGTTGTGTTGG	6,021,947
RM3471[b]	*BPH17-ptb*	4	AGAAACAGAGGGAGGGAGCAGAGG	GATCCCGACAGATGGTGACTTGC	6,279,483
RHD3[h]	*BPH17-ptb*	4	GGTAAGGTTGGGCGGTAG	AGTGAAGGGTGAGGGTGG	6,597,076
WH2[h]	*BPH17-ptb*	4	CCCACCACACCAGAGATAAA	ACACAACACCCGCATACAA	6,697,366
RM16506[c]	*BPH17-ptb*	4	GCAGTAGACCTCGTGCTGAATGC	CCACACCGCCGCAATATAAACC	6,926,963
RM16508[c]	*BPH17-ptb*	4	TTCATTGTCATCGCCTCATTGG	ACAGGTACAGCTGGGTAGAGAGAAGC	6,954,478
RM16514[c]	*BPH17-ptb*	4	GGCTACGTCAGGATGGAGAGG	GGATGTTACATGTCAGCTTGAGAGC	7,213,726
MS5[i]	*BPH17-ptb*	4	TTGTGGGTCCTCATCTCCTC	TGACAACTTGTGCAAGATCAAA	7,251,940
RM6156[b]	*BPH17-ptb*	4	CGTCCGCACGCAAGAAGAAGG	CCGTACGTGTGGCTTCAGATTGG	7,856,903
RM16531[c]	*BPH17-ptb*	4	CAGTGCAGGAACAAGATTCAGG	CATTGCAGTTGGGTTCTATTGG	7,935,067
B40[f]	*BPH17-ptb*	4	CAATACCGGATATCTTGACTCC	CGACCACGCTGCCTATATTC	8,214,283
RM6775[b]	*BPH32*	6	AATTGATGCAGGTTCAGCAAGC	GGAAATGTGGTTGAGAGTTGAGAGC	209,054.
S00310[f]	*BPH32*	6	CAACAAGATGGACGGCAAGG	TTGGAAGAAAAGGCAGGCAC	214,278
RM508[a]	*BPH32*	6	AGAAGCCGGTTCATAGTTCATGC	ACCCGTGAACCACAAAGAACG	441,752
RM19288[c]	*BPH32*	6	CGGAGCTGTTGCCGTTCTGC	CGATGTGCCATGTCAGGATGACC	1,173,479
RM19291[c]	*BPH32*	6	CACTTGCACGTGTCCTCTGTACG	GTGTTTCAGTTCACCTTGCATCG	1,215,950
RM19296[c]	*BPH32*	6	CTAGCTTGACGCCAAGGACACC	GCACAGACGCACACTGATCTCC	1,290,544
RM589[a]	*BPH32*	6	GTGGCTTAACCACATGAGAAACTACC	TCACATCATTAGGTGGCAATCG	1,380,931
RM19311[c]	*BPH32*	6	TGCGGTGCTGTTCACCTACTATCG	GCACTGAAGCTGGTGCAATCG	1,463,445
RM586[a]	*BPH32*	6	TGCCATCTCATAAACCCACTAACC	CTGAGATACGCCAACGAGATACC	1,476,905
RM588[a]	*BPH32*	6	TCTTGCTGTGCTGTTAGTGTACG	GCAGGACATAAATACTAGGCATGG	1,611,442
RM19341[c]	*BPH32*	6	GCTACAAATAGCCACCCACACC	CAACACAAGCAGAGAAGTGAAGC	1,764,661

Primer sequence information was obtained from: [a] Temnykh *et al.* (2001), [b] McCouch *et al.* (2002), [c] International Rice Genome Sequencing Project (IRGSP 2005), [d] Zhao *et al.* (2016), [e] Tamura *et al.* (2014), [f] Rahman *et al.* (2009), [g] Shirasawa *et al.* (2004), [h] Liu *et al.* (2015), and [i] Yang *et al.* (2004) . The physical positions of primers for each marker were obtained from The Rice Annotation Project Database (Sakai *et al.* 2013).

20 nymphs per tiller. Ten days after infestation (DAI), the damage scores (DSs) of the substitution lines and parents were determined.

Antibiosis on feeding rates

The feeding rates of BPH on the NILs were determined following the methods described by Heinrichs *et al.* (1985) with minor modifications. Seeds of the NILs and parents were individually sown in 220-mL plastic cups with five replications. A plastic chamber with ventilators was placed at the base of the plant to maintain the insects. A filter paper treated with 0.1% bromocresol green in ethanol was placed inside the chamber to absorb plant honeydew excreted by the insects. The yellow-orange filter papers turned blue when honeydew was absorbed. Before infestation, the insects (Hadano-66) were starved for 2 h in a plastic box with paper towel saturated with distilled water to maintain sufficient moisture. Each plant was infested with two adult female BPHs with the small abdomen. At 24 h after infestation, the filter papers were collected, and the area of honeydew was measured using ImageJ software (ver. 1.53a; National Institutes of Health, Maryland, USA, https://rsb.info.nih.gov/ij).

Antixenosis test

Two plants from each NIL (*BPH2*-NIL, *BPH17-ptb*-NIL, and *BPH32*-NIL) and two plants from T65 were sown in 420-mL plastic cups with three replications. At 30 DAS, the plants in each cup were covered with plastic tubes with ventilators. Each cup was infested with twenty second-instar BPH nymphs. The number of insects that settled on the NILs and T65 was recorded at 1, 2, 3, and 4 DAI. The antixenosis level was calculated as the percentage of insects settled on each plant per total insects on the NIL and T65 of each cup at 1, 2, 3, and 4 DAI.

Tolerance test

The tolerance test was conducted following the methods described by Heinrichs *et al.* (1985). Two plants from each of *BPH2*-NIL, *BPH17-ptb*-NIL, *BPH32*-NIL, and parents were sown in 1-L plastic cups with three replications. At 30 DAS, the plants in the cups were separately covered with mesh and infested by three adult female BPHs. The other three identical cups for each entry were maintained without infestation as controls. During the first week of infestation, dead insects were replaced by new ones. The insects could feed and lay eggs to increase the population for one generation. One month after infestation, the plants were cut at the soil surface, and the fresh weight was measured. The percentage of plant fresh weight loss (PFWL) was used as an inverse measure of tolerance; i.e., plants showing the smaller PFWL have higher tolerance. PFWL is calculated as:

$$\text{PFWL}(\%) = \frac{\text{Fresh weight of control plants} - \text{Fresh weight of infested plants}}{\text{Fresh weight of control plants}} \times 100\%$$

Statistical analysis

The mean values of the damage score of the homozygous recombinant chromosome substitution lines, antibiosis, and tolerance level of NILs were compared using one-way ANOVA. Dunnett's test was conducted for multiple comparisons of the damage scores of the homozygous recombinant chromosome substitution lines compared with that of T65. Tukey Kramer's test was applied for multiple comparisons of resistance levels in antibiosis and tolerance tests, using the R software version 3.5.3.

Results

Substitution mapping of the BPH2, BPH17-ptb, and BPH32

To identify BPH resistance genes from PTB33, the BC_4F_4 progenies were used for substitution mapping. Among 96 BC_4F_2 plants segregating at *BPH2*, 14 plants carrying recombinant events that occurred between the two markers, RM277 and RM5479, on chromosome 12 were selected. Ten BC_4F_4 lines carrying different sizes of 'PTB33' substituted chromosomal segments were developed (**Fig. 2**). Four lines were homozygous for 'PTB33' at all marker loci from RM277 to different positions of DNA markers: RM28404 for line 17-4, RM28424 for line 19-3, ID-161 for line 10-1, and ID-161-2 for line 17-1. The other three lines were homozygous for 'PTB33' introgression from RM5479 to: InD14 for line 15-2, RM28449 for line 24-1, and RM28424 for line 9-3. Line 20-2 was homozygous for T65 from RM277 to RM28493, and for 'PTB33' from RM1103 to RM5479. Line 1-1 and line 23-3, homozygous for T65 and 'PTB33', respectively, between RM277 and RM5479, were used as control lines. These lines were used for evaluation of BPH resistance against Hadano-66 using MSST. As a result, T65 (DS = 6.3) and 'PTB33' (DS = 1.8) were determined to be susceptible and resistant, respectively. The DS of 'PTB33' was significantly different from that of T65. Among the 10 BC_4F_4 lines, four lines (1-1, 20-2, 17-4, and 19-3) homozygous for T65 at all marker loci between RM28449 and ID-161-2 were susceptible to BPH. The DS values of the four lines (greater than 5.4) were not significantly different from that of T65. In contrast, four lines (17-1, 24-1, 9-3, and 23-3) that were homozygous for 'PTB33' at all marker loci between RM28449 and ID-161-2 were resistant to BPH. The DS values of these four lines (less than 3.6) were significantly different from that of T65. Two lines, 10-1 and 15-2, that had common 'PTB33' homozygous segments flanked by two markers, RM28449 and ID-161-2, were also resistant to BPH. Therefore, *BPH2* was delimited between

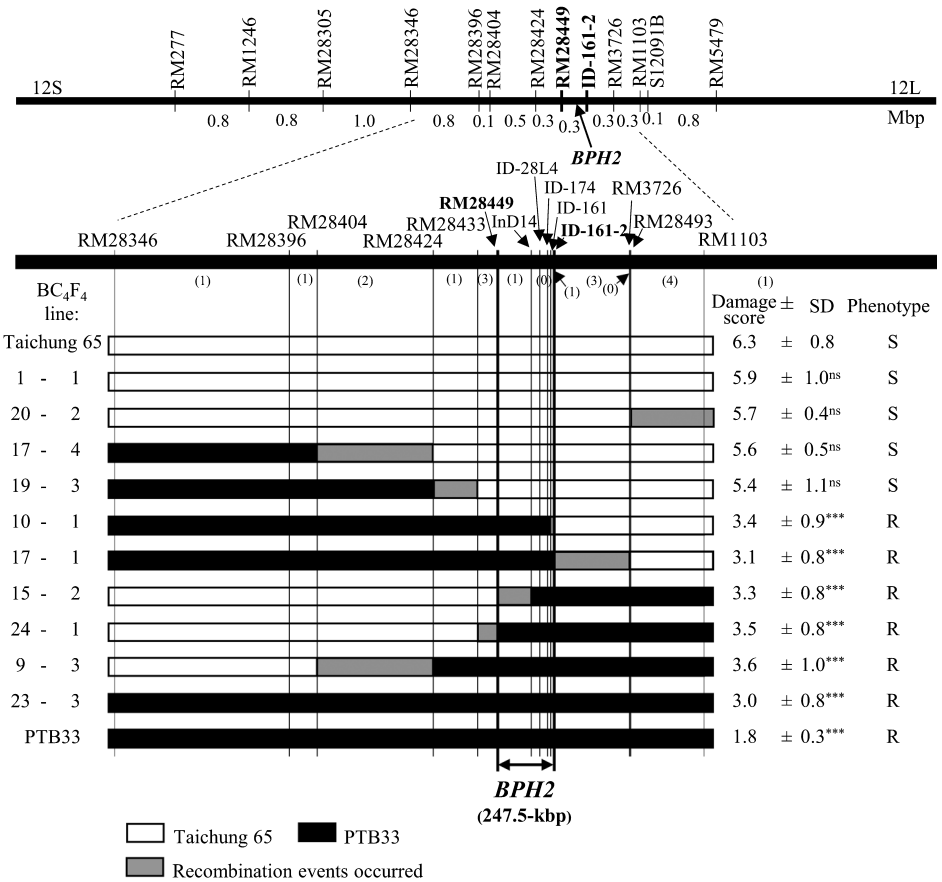

Fig. 2. The substitution map of *BPH2* on chromosome 12. Upper line indicates the physical position of DNA markers around location of *BPH2* on chromosome 12L. Vertical bars indicate the positions of DNA markers and the numbers below the upper bar indicate physical distance between markers. The numbers in parentheses indicate the number of recombinants. White rectangles are 'Taichung 65' homozygous; black rectangles are 'PTB33' homozygous; grey rectangles are the position where recombinant events occurred. The asterisk and ns are significantly different from damage score of 'Taichung 65' and no significance ($P < 0.001$, Dunnett's multiple comparison tests against 'Taichung 65'), respectively. SD: standard deviation; S: susceptible; R: resistant.

RM28449 and ID-161-2 on chromosome 12, with an interval of approximately 247.5 kbp based on the 'Nipponbare' genome sequence.

For the population segregating at *BPH17-ptb*, 11 of 96 BC_4F_2 plants carrying recombinant events that occurred between C61009 and B40 on chromosome 4 were selected. Four lines homozygous for 'PTB33' segments encompassing overlapping regions of *BPH17-ptb* were developed from the 11 selected recombinant plants (**Fig. 3**). Among these, line 46-5 was homozygous for 'PTB33' at all marker loci from C61009 to RM16460; line 47-1 was homozygous for 'PTB33' at all marker loci from C61009 to MS5; and line 44-5 was homozygous for 'PTB33' segments from RM16479 to RM16531. Line 44-4 as control was homozygous for 'PTB33' between C61009 and B40, while line 43-6 was homozygous for T65 in this region. The selected BC_4F_4 lines were evaluated for BPH resistance using MSST against Hadano-66. Lines 43-6 and 46-5, which have in common a T65 homozygous segment flanked by two markers, RM1305 and RM6156, were susceptible. The DS values of lines 43-6 (6.8) and 46-5 (6.2) were not significantly different from that of T65 (7.8). Three lines, 47-1, 44-5, and 44-4, which have common 'PTB33' segments flanked by RM1305 and RM6156 showed resistance to BPH. The DS of the three lines (<3.0) was significantly lower than that of T65. The results suggest that *BPH17-ptb* is located between two markers, RM1305 and RM6156, on chromosome 4, with a physical distance of approximately 2.23 Mbp based on the 'Nipponbare' genome sequence.

From 96 BC_4F_2 plants for *BPH32*, ten plants with recombination events between RM6775 and RM19341 were selected. Using the additional markers between RM6775 and RM19341, six lines with different homozygous substitutional chromosomal segments from 'PTB33' were developed from the selected recombinant plants (**Fig. 4**). Line 41-2 was homozygous for 'PTB33' at S00310 and T65 between RM508 and RM19341. Line 34-1 was homozygous for 'PTB33' between RM6775 and RM508. Line 35-1 was homozygous for 'PTB33' between RM6775 and RM588 and line 32-2 was homozygous for 'PTB33' between RM19288 and RM19341. Line 37-1 was homozygous for 'PTB33' between RM508 and RM19341 and

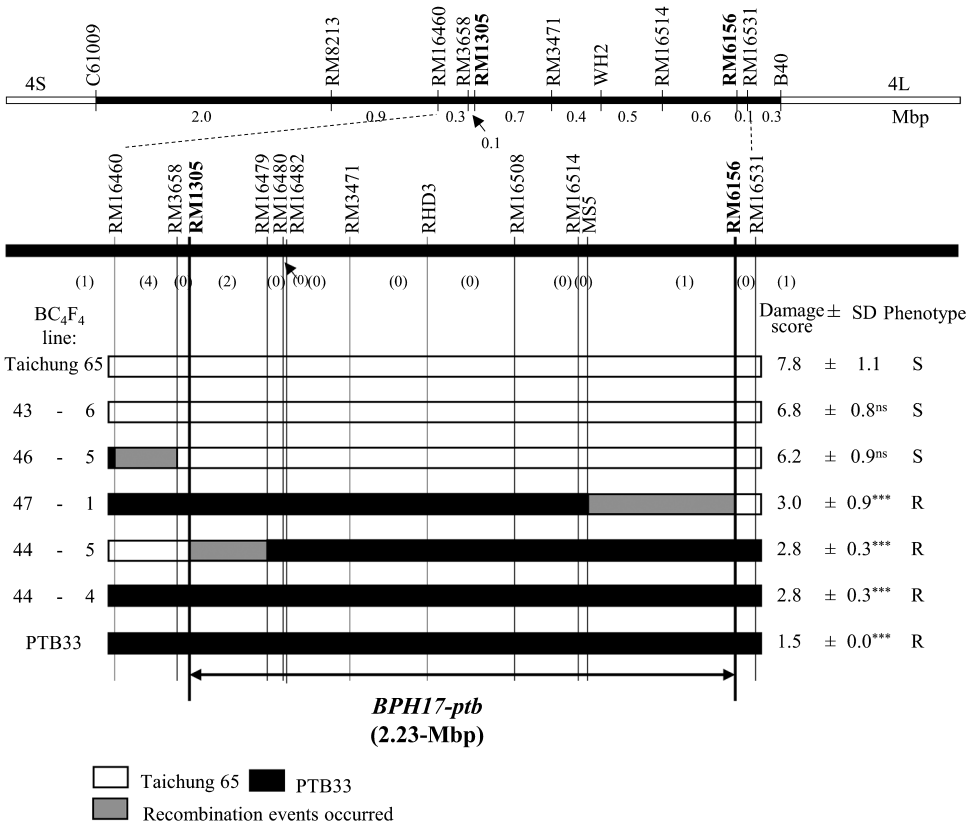

Fig. 3. The substitution map of *BPH17-ptb* on chromosome 4. Upper line indicates the physical position of DNA markers around location of *BPH17-ptb* on chromosome 4S. Vertical bars indicate the position of DNA markers and the numbers below the upper line indicate physical distance between markers. The numbers in parentheses indicate the number of recombinants. White rectangles are 'Taichung 65' homozygous; black rectangles are 'PTB33' homozygous; grey rectangles are the position where recombinant events occurred. The asterisk and ns are significantly different from damage of 'Taichung 65' and no significance ($P < 0.001$, Dunnett's multiple comparison tests against 'Taichung 65'), respectively. SD: standard deviation; S: susceptible; R: resistant.

line 40-1 was homozygous for T65 between RM6775 and RM588. As controls, line 36-1 was homozygous for T65 between RM6775 and RM19341, while line 35-3 was homozygous for 'PTB33' in this region. All the selected lines were evaluated for BPH resistance against Hadano-66 using the plants at the seedling stage. However, there was no difference in resistance level between the homozygous lines. Therefore, the BPH resistance of lines was repeated using plants at tillering stage. As a result, the DS values of parents and homozygous lines were classified into two groups: susceptible (DS greater than 5.0) and resistant (DS less than 5.0). T65 and four lines, 36-1, 40-1, 41-2, and 34-1, which have a common T65 homozygous segment flanked by RM508 and RM19341 were susceptible (the DS higher than 5.0). 'PTB33' and four lines, 35-1, 32-2, 37-1, and 35-3, had a common 'PTB33' homozygous segment flanked by RM508 and RM19341 were resistant (DS lower than 3.8). Based on these results, *BPH32* was located between two markers, RM508 and RM19341, on chromosome 6 with a physical distance of approximately 1.32 Mbp based on the 'Nipponbare' genome sequence.

Comparison of resistant levels among BPH resistance genes

To understand resistance mechanism (such as antibiosis, antixenosis, and tolerance) of each BPH resistance gene, the NILs for BPH resistance genes were evaluated by feeding rate on honeydew area, percentage of settling insect, and PFWL. For antibiosis by honeydew test, the area of honeydew excreted was 70.34 mm^2 for T65 and 8.23 mm^2 for 'PTB33' (**Fig. 5A**). The area of honeydew excreted by insects between T65 and 'PTB33' was significantly different ($P < 0.05$, Tukey-Kramer's test). Among the NILs, *BPH2*-NIL had the lowest amount of honey dew (12.85 mm^2) and highest level of antibiosis. The *BPH17-ptb*-NIL had a smaller area (25.13 mm^2) than *BPH32*-NIL and higher level of antibiosis than *BPH32*-NIL. The area of honeydew on *BPH32*-NIL was largest among the NILs and antibiosis level on *BPH32*-NIL was similar to that of T65.

The degree of antixenosis of the three genes was compared based on the number of insects that settled on pairs of each NIL and T65 after BPH infestation (**Fig. 5B–5D**). The number of insects on *BPH2*-NIL was always lower than that on T65 from 1 to 4 DAI. The percentage of settling insects on *BPH2*-NIL was 11.2 % at 1 DAI, 32.0 % at

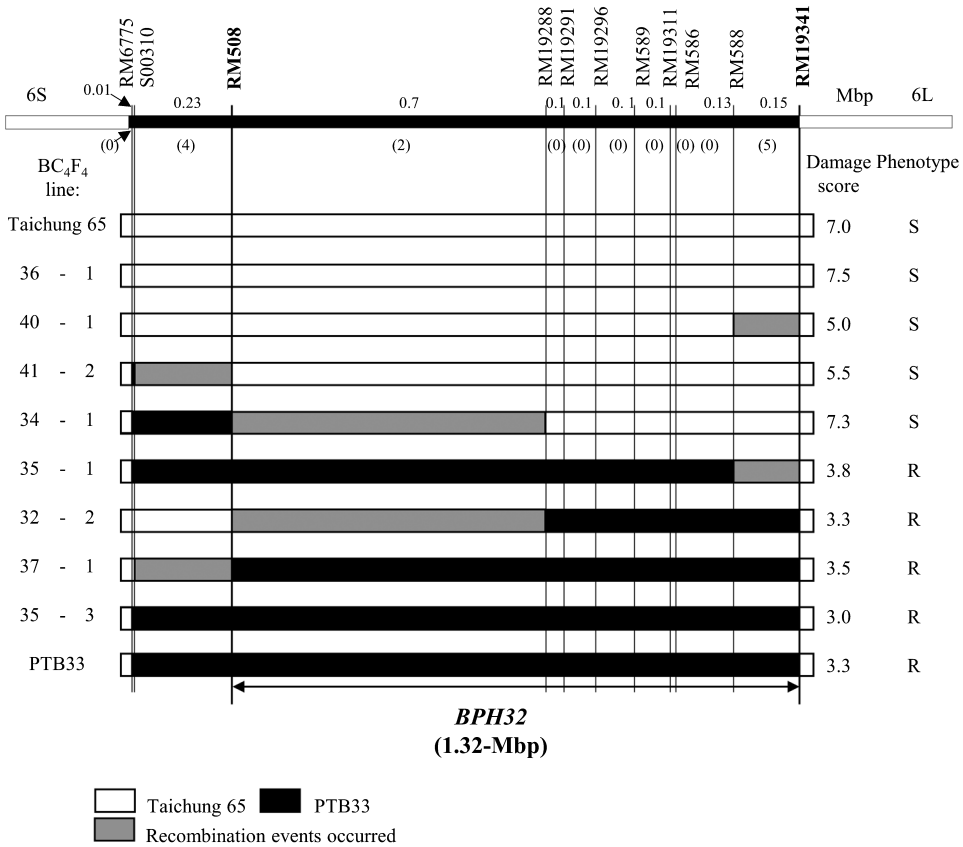

Fig. 4. The substitution map of *BPH32* on chromosome 6. Vertical bars indicate the position of DNA markers. The numbers above the top bar indicate physical distance between the markers and the below ones indicate the number of recombinants. White rectangles are 'Taichung 65' homozygous; black rectangles are 'PTB33' homozygous; grey rectangles are the position where recombinant events occurred. S: susceptible; R: resistant.

2 DAI, 22.8 % at 3 DAI, and 24.2 % at 4 DAI. The percentage of settling insects on *BPH32*-NIL at 1 DAI (44.8%), 2 DAI (52.6%), 3 DAI (51.1%), and 4 DAI (61.8%) was similar to the corresponding percentage on T65. For *BPH17-ptb*-NIL, the percentage of settling BPH was lower than that of T65 during the experiment. The percentage of settling insects on *BPH17-ptb*-NIL was 22.4% at 1 DAI, 35.5% at 2 DAI, 36.1% at 3 DAI and 39.8% at 4 DAI. Among the three NILs, *BPH2*-NIL had the highest antixenosis level. The antixenosis level of *BPH17-ptb*-NIL was higher than that of *BPH32*-NIL that was no antixenosis.

The tolerance of the NILs was measured as the percentage of PFWL due to BPH (**Fig. 5E**). Among the tested plants, T65 had the highest PFWL (52.2%), which was significantly different from that of 'PTB33' (1.2%). *BPH17-ptb*-NIL (19.3% PFWL) showed the lowest PFWL among the NILs and thus the highest tolerance index. The PFWL of *BPH32*-NIL (37.1%) was higher than that of *BPH17-ptb*-NIL, but lower than that of *BPH2*-NIL (with 51.9% PFWL). Therefore, tolerance index of *BPH32*-NIL was lower than that of *BPH17-ptb*-NIL and *BPH2*-NIL showed the lowest tolerance index among the NILs.

Discussion

Recently, many BPH resistance genes have been overcome by several specific BPH populations in tested; however, those of genes have effect against other BPH populations with lower virulence. The BPH resistance genes with no effective against BPH with strong virulence are also useful for pyramiding with other BPH resistance genes to enhance the resistance level. For example, *BPH25* was susceptible to BPH populations from Vietnam but showed strong resistance against those from China, Taiwan, and Mindanao Island in the Philippines (all collected in 2006) (Fujita *et al.* 2009). Additionally, pyramiding line carrying *BPH25* and *BPH26* showed resistance against Isahaya-99 BPH population (collected at Nagasaki, Japan in 1999), even if the lines with a single BPH resistance gene (*BPH25* or *BPH26*) were susceptible. Therefore, the understanding of genetic basis and resistance mechanism of low or non-effective resistance genes is still importance to enhance BPH resistance level in rice breeding.

The characterization of low or non-effective BPH resistance genes are required a low virulent BPH population. Among the BPH colonies maintained in the laboratory in

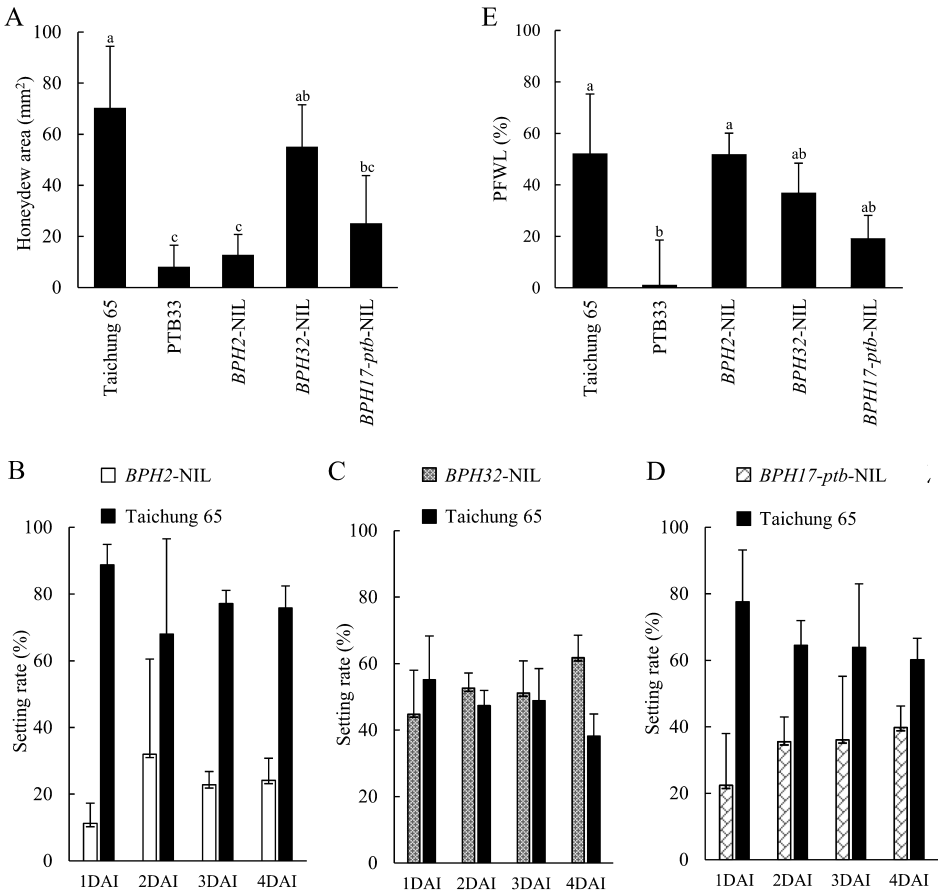

Fig. 5. Antibiosis, antixenosis, and tolerance level of *BPH2*-NIL, *BPH17-ptb*-NIL, and *BPH32*-NIL against Hadano-66. (A) Honeydew area excreted by insect feeding. (B) The percentages of insects settling on *BPH2*-NIL, (C) *BPH32*-NIL and (D) *BPH17-ptb*-NIL at 1–4 days after infestation (DAI). (E) Percentage of fresh weight loss on NILs by insect attacking. The different letters above the bars indicate the significant difference according to Tukey-Kramer's test at $P < 0.05$. PFWL: percentage of fresh weight loss.

Japan, Hadano-66 was collected before the first BPH-resistant variety, 'IR26', with the *BPH1* from 'Mudgo' was released. The Hadano-66 has lower BPH virulence compared with other BPH populations collected in Japan: Chikugo-89 (collected at Fukuoka in 1989), Isahaya-99, Japan-KG-06 (collected at Kagoshima in 2006), Nishigoshi-05 and Koshi-2013 (collected in Kumamoto in 2005 and 2013) (Myint *et al.* 2009b, 2012, Nguyen *et al.* 2019). The resistance levels of varieties carrying *BPH1*, *BPH2*, *BPH4*, and *BPH8* against Hadano-66 were higher than those of Chikugo-89, Isahaya-99, and Nishigoshi-05 (Myint *et al.* 2009b). The effectiveness of *BPH25* and *BPH26* against Hadano-66 was stronger than those of Isahaya-99 and Nishigoshi-05 (Myint *et al.* 2009a). Additionally, the resistance levels of *BPH2*, *BPH3*, *BPH17*, *BPH17-ptb*, *BPH20*, *BPH21*, *BPH26*, and *BPH32* against Hadano-66 were higher than that of Koshi-2013. Therefore, a low virulence BPH population, such as Hadano-66, can facilitate the mapping and characterization of a single BPH resistance with less effective against the current BPH populations having strong virulence.

In this study, using MSST by Hadano-66, *BPH2* from 'PTB33' was successfully mapped to a 247.5-kbp between two markers, RM28449 and ID-161-2, on the long arm of chromosome 12. The physical location of *BPH2* is approximately 22.69 to 22.94 Mbp, which differs from that of *BPH7* (19.95–20.87 Mbp) based on the 'Nipponbare' genome sequence (Qiu *et al.* 2014). This result confirms that *BPH2* is a different gene or allelic type of *BPH7*, as mentioned by Zhao *et al.* (2016). The location of *BPH2* partly overlaps that of *BPH1* (22.8–22.93 Mbp), *BPH9* (22.85–22.91 Mbp), *BPH10* (19.66–23.42 Mbp), and *BPH18* (22.87–22.90 Mbp) (Cha *et al.* 2008, Ishii *et al.* 1994, Ji *et al.* 2016, Zhao *et al.* 2016). The delimited location of *BPH2* completely covers that of *BPH26* (22.77–22.91 Mbp) on chromosome 12 (Tamura *et al.* 2014). Tamura *et al.* (2014) reported that the amino acid sequences and resistance levels of *BPH2* from 'ASD7' are identical to that of *BPH26* from 'ADR52'. In future studies, to confirm whether *BPH2* from 'PTB33' is identical to *BPH26*, a comparison of the amino acid sequence of *BPH2* from 'PTB33' would be necessary.

BPH17-ptb was mapped between two markers, RM1305 and RM6156, at approximately 5.63 to 7.86 Mbp on

chromosome 4S, based on the 'Nipponbare' genome sequence. This result confirms the presence of a BPH resistance gene on chromosome 4S of 'PTB33' in previous research (Nguyen *et al.* 2019). The location of *BPH17-ptb* partially overlapped with those of *BPH12* (5.21–5.66 Mbp), *BPH15* (6.90–6.95 Mbp), *BPH17* (6.94–6.97 Mbp), and *BPH22*(t) (4.14–6.58 Mbp). The delimited region of *BPH17-ptb* is 2.23 Mbp that possibly contains multiple BPH resistance genes. To confirm whether the other BPH resistance genes are located in this region of *BPH17-ptb*, fine mapping using a large population might be necessary in future studies. Another gene, *BPH32*, was detected between two markers, RM508 and RM19341, which locates from 0.44 Mbp to 1.76 Mbp on chromosome 6. The delimited region encompassed the location of *BPH32* (1.24 to 1.41 Mbp) reported by Jairin *et al.* (2007b) and Ren *et al.* (2016). This result confirms the presence of *BPH32* on the *BPH32*-NIL plants developed in a previous study (Nguyen *et al.* 2019).

BPH32 have been evaluated for BPH resistance in several studies and the effects for *BPH32* were fluctuated by plant growth stage, kinds of BPH population, and genetic background of plant materials (Jairin *et al.* 2007b, Jena *et al.* 2017, Nguyen *et al.* 2019, Ren *et al.* 2016). In this study, to evaluate BPH resistance on chromosomal substitution lines for *BPH32*, the plants at one week after sowing were used for MSST. However, there was no difference in the resistance levels between the substitution lines. In Jairin *et al.* (2007b), *BPH32* was evaluated using the plant at the tillering stage and the Thai BPH population. Therefore, in this study, we also used plants at the tillering stage for the evaluation of BPH resistance in chromosomal substitution lines for *BPH32*. On the other hand, in a study of NIL carrying *BPH32*, it was reported that *BPH32* is resistant to four BPH populations from the Philippines and one from China at three leaf stages (Jena *et al.* 2017, Ren *et al.* 2016). However, we found that the gene has low effect of resistance against Hadano-66 although the amino acid sequence of *BPH32* was identical to that of *BPH32* in Ren *et al.* (2016) (unpublished data). The different resistance levels might be related to the different BPH populations or other genetic factors around the *BPH32* region. Additionally, *BPH32* from 'PTB33' was introduced to the 'IR24' genetic background, indicating resistance to BPH by Jena *et al.* (2017), whereas the resistance level on *BPH32*-NIL with T65 genetic background showed a low resistance level in our study. In several studies, the gene behavior was demonstrated to fluctuate depending on the genetic background (Marcel *et al.* 2008, Palloix *et al.* 2009, Sun *et al.* 2006). This difference in resistance levels might be related to the different genetic backgrounds. The characterization of genes against various BPH populations and genetic backgrounds will be necessary to understand the behavior of BPH resistance genes.

The host plant resistance is a complex caused by different gene behaviors against different virulence factors of BPH, genetic backgrounds, and gene interactions in plants. Understanding the resistance mechanism is essential for the development of an appropriate breeding strategy (Qiu *et al.* 2014). In the present study, although *BPH2*, *BPH17-ptb*, and *BPH32* are derived from the same donor variety 'PTB33', their resistance effects were relatively different. *BPH2*-NIL showed the highest levels of both antibiosis and antixenosis, but the lowest level of tolerance among the three NILs. Therefore, antibiosis and antixenosis may be the major mechanisms of this gene. For the genes on the long arm of chromosome 12, *BPH9*, and *BPH18* conferred both antibiosis and antixenosis that were similar to that of *BPH2*. The other genes, *BPH1*, *BPH10*, *BPH21*, and *BPH26*, demonstrated antibiosis, but they have not been characterized for antixenosis or tolerance. Therefore, three of the four allelic types on chromosome 12L (*BPH1/BPH10/BPH18/BPH21*, *BPH9*, and *BPH2/BPH26*) might confer antibiosis (and antixenosis), whereas tolerance is the major component of the other type-*BPH7* (Qiu *et al.* 2014). *BPH17-ptb* showed resistance in the form of antibiosis, antixenosis, and tolerance. However, the antibiosis and antixenosis effects of *BPH17-ptb* were lower than that of *BPH2*. *BPH17-ptb* showed the highest level of tolerance among the three NILs. The density of BPH populations on *BPH17-ptb* in the tolerance test was lower than that of T65, which suggests that the high tolerance level of *BPH17-ptb* might be a result of antibiosis (unpublished data). In future studies, attention should be given to BPH populations with strong virulence to evaluate the tolerance of *BPH17-ptb* (excluding the effects of antibiosis and antixenosis). *BPH32* showed moderate tolerance, whereas the levels of antibiosis and antixenosis were almost similar to that of T65. The low resistance (antibiosis) level of *BPH32* as evident in the BPH feeding rate is consistent with the antibiosis effect on adult BPH mortality in a previous study (Nguyen *et al.* 2019).

Characterization of the resistance mechanisms of each gene from 'PTB33' might facilitate the understanding of BPH resistance of 'PTB33'. NILs carrying a single gene (*BPH2*, *BPH17-ptb*, and *BPH32*) have been overwhelmed by BPH populations. However, the donor parent 'PTB33' shows prolonged resistance against BPH for at least several decades (Nguyen *et al.* 2019, Saxena and Barrion 1985, Sidhu and Khush 1978). Although there are several minor QTLs for BPH resistance that have not been identified on 'PTB33', three genes, *BPH2*, *BPH17-ptb*, and *BPH32*, might be the essential genes for BPH resistance on 'PTB33' based on the similarity in resistance levels between 'PTB33' and pyramiding of these three genes (Nguyen *et al.* 2019). The differences in resistance mechanisms among these genes from 'PTB33' might be the key factor for the durability of this variety against BPH. Therefore, the characterization of the resistance of pyramided lines with different resistance mechanisms is crucial for understanding the effect of different mechanisms to strengthen resistance level.

Author Contribution Statement

DF, HY, and CDN designed the study. HY, MM, and SZ provided support for conducting the research and writing the manuscript. SS-M and MM provided insects for conducting the research. CDN and DF performed the research, developed the plant materials, and wrote the paper.

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