Genetic and breeding studies for resistance to brown planthopper (*Nilaparvata lugens* (Stål)) in rice (*Oryza sativa* L.)

(イネのトビイロウンカ抵抗性に関する遺伝育種学的研究)

Nguyen Dinh Cuong

2021

THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES
KAGOSHIMA UNIVERSITY

JAPAN

Genetic and breeding studies for resistance to brown planthopper (*Nilaparvata lugens* (Stål)) in rice (*Oryza sativa* L.)

(イネのトビイロウンカ抵抗性に関する遺伝育種学的研究)

A Dissertation Submitted in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

in

AGRICULTURE SCIENCE

Nguyen Dinh Cuong

2021

Dedication

To my mother and father, for bringing me into this world, and for all that you have done for me.

To my Beloved family, for always supporting, helping, and standing with me through good and difficult times.

Acknowledgments

I would like to thank the following people, without whom I would not have been able to complete this research, and without whom I would not have made it through my PhD degree.

First and foremost, I am extremely grateful to Associate Professor Daisuke Fujita of Saga University for his constant support and encouragement at the various stages of this PhD study. His enthusiasm and passion for plant breeding, genetics, and applied research have been a source of inspiration for this work. I would not have imagined having a better advisor and mentor for my PhD study.

My sincere thanks and gratitude also go to Professor Shao-Hui Zheng of Saga University and Professor Katsuyuki Ichitani of Kagoshima University for their invaluable suggestion and consistent encouragement.

My special thanks to Professor Hideshi Yasui of Kyushu University, Dr. Sachiyo Sanada-Morimura of Kyushu Okinawa Agricultural Research Center, Japan, and Dr. Masaya Matsumura of Central Region Agricultural Research Center, Japan, for the kind provision of experimental materials, guidance, and several support during this PhD study.

I thank the members of The United Graduate School of Agriculture Sciences, Kagoshima University for giving me this great opportunity to study under their program. I am also thankful to all the staff of Saga University for all the considerate guidance.

I also wish to thank Oba-san, Rasadul-san, Hirano-san, Zita-san, Nilsa-san, Yohei-san, Erina-san, Honda-san, Matsuoka-san, Ha-san, Nam-san, Kurihara-san, Baba-san, Day Shar-san, Kamal-san, Molla-san, and the other student members of the Tropical Crop

Improvement Laboratory for their physical assistance in the laboratory and greenhouse, and for their friendship.

I express my indebtedness to the Ministry of Agriculture and Rural Development, Vietnam, for providing me the scholarship to carry out this study.

I also would like to express my heartfelt gratitude to all the members of College of Food Industry (101B Le Huu Trac, Son Tra, Da Nang, Vietnam) for their support during this PhD course.

Finally, I am extremely grateful for my parents, my parent-in-law, all sisters, and brothers for all the unconditional support. Most importantly, I thank my beloved wife Nguyen Thi Van Hong and my little son Nguyen Dinh Phuc for their tremendous love and support throughout my studies.

Table of contents

Abbreviations

ADM : Adult mortality

ANOVA : Analysis of variance

AVE : Average

bp : base pair

BPH : Brown planthopper

CL : Culm length

DAS : Days after sowing

DNA : Deoxyribonucleic acid

DS : Damage score

DTH : Days to heading

FBRR : Fresh biomass reduction rate

IRRI : International Rice Research Institute

kbp : kilo base pair

LL : Flag leaf length

LW : Flag leaf width

MAS : Marker-assisted selection

Mbp : Mega base pair

mm^2 : square millimeter

MMTS : Modified mass tiller screening

MSST : Modified seedbox screening test

N : North

NIL : Near-isogenic lines

PCR : Polymerase chain reaction

PFWL : Percentage of plant fresh weight loss

PL : Panicle length

PN : Panicle number

PYL : Pyramided lines

QTL : Quantitative trait locus

S : South

SD : Standard deviation

SSR : Simple sequence repeat

T65 : Taichung 65

TBE : Tris boric acid EDTA

List of tables

List of figures

Abstract

Rice (*Oryza sativa* L.), the most important crop of the world population, is severely devastated by the brown planthopper (BPH, *Nilaparvata lugens* Stål.). Host plant resistance is generally regarded as the most effective and economical mean for controlling this insect. More than 46 genes for BPH resistance have been identified from rice cultivars and wild relatives. Understanding of genetic locations and resistance performances of genes are necessary to exploit resistance plants efficiently and sustainably in field condition.

In the first study, we developed seven near-isogenic lines (NILs) (*BPH2*-NIL, *BPH3*-NIL, *BPH17*-NIL, *BPH20*-NIL, *BPH21*-NIL, *BPH32*-NIL and *BPH17-ptb*-NIL) carrying a single BPH resistance gene and fifteen pyramided lines (PYLs) carrying two or three genes on the genetic background of the *japonica* rice variety, 'Taichung 65' (T65). The NILs and PYLs were assessed for resistance levels against two BPH populations collected in Japan in 1966 (Hadano-66) and 2013 (Koshi-2013). Many of the NILs and PYLs were resistant against the Hadano-66 population but were less effective against the Koshi-2013 population. Among PYLs, *BPH20+BPH32*-PYL and *BPH2+BPH3+BPH17*-PYL had relatively high BPH resistance against Koshi-2013.

In the second study, the locations of *BPH2*, *BPH17-ptb*, and *BPH32* were delimited using chromosome segment substitution lines derived from crosses between T65 and NILs for *BPH2* (*BPH2*-NIL), *BPH17-ptb* (*BPH17-ptb*-NIL), and *BPH32* (*BPH32*-NIL). The resistance mechanism of *BPH2*, *BPH17-ptb* and *BPH32* were determined by applying the tests for antibiosis, antixenosis and tolerance. *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.

The third research topic was conducted for identifying quantitative trait loci for BPH resistance from two *O. nivara* accessions (IRGC 89073 and IRGC 93005) and Sri Lanka rice variety, 'Rathu Heenati'. The segregation populations for these three-rice variety/accessions were developed. The frequency distributions of segregation populations showed continuous distribution, suggesting that IRGC 89073, IRGC 93005, and 'Rathu Heenati' contain multiple genes for BPH resistance.

These results will be useful in the monitoring of BPH virulence prior to exploiting resistant rice varieties, and in the improvement of BPH resistance of rice varieties in the context of regionally increasing levels of virulence.

Chapter 1

General introduction

1. Rice and insect pest threats

Rice (*Oryza sativa* L.) is one of the most important crops because it provides the calorie intake for more than two-thirds of the world population (Muthayya *et al*. 2014). At present, rice is cultivated on more than 165 million hectares across 114 countries in Asia, Latin America, Africa, Australia, Europe, and North America. The world rice production is approximately 745 million tons annually (Milovanovic *et al*. 2017). Among rice production areas, Asia contributes more than 90% for global rice production with approximately 640 million tons. Approximately 36 million tonnes are produced in the Americas and 29 million tonnes in Africa. China and India are the leading rice-producing countries in the world. The combined rice production in China and India is around 55% of the world (Milovanovic *et al*. 2017).

Rice provides the main income for many people, especially in South and Southeast Asian countries. There are approximately 144 million households whose income is mainly from rice producing. Many worlds' poor people rely on rice as producers or as consumers. In 2008, 94% of total rice area was in low- and lower-middle income countries. Rice accounts for 19% of total crop area harvested in these countries (Dawe and Timmer 2012). Rice plays an important role in economics of many countries as a foreign exchange earner. Rice exports are concentrated to in ten countries: India, Thailand, Pakistan, USA, Vietnam, Italia, Uruguay, Brazil, China, and Australia (Prasad *et al*. 2017). Together, they contribute to more than 80% of total rice transaction in the world.

Although rice yields are still growing in many regions, it has been detrimentally affected by several major constraints including pests, diseases, and weed. These biotic stress agents cause various types of diseases, infections, and damage to rice and ultimately affect the production. According to Oerke *et al.* (2006), the total yield loss of rice in a year is around 41% consists of 15% loss due to insects, 13% loss due to pathogens, and 13% loss due to weeds. The total amount of yield loss caused by biotic stress is equivalent to 400 billion US dollars.

Rice is an idea host for insects. It subjects to many insects at all growth stages from seedling to mature and on all body parts of rice plant. There are totally more than 800 insect pests of rice although majority of them causes very little damage. The number of major insects varies between different regions. In tropical Asia, there 20 insects that are importance and regular occurrence for rice (Horgan 2017). Based on the feeding types, the insects are classified to six types: root and stem feeders, stem borers, rice gall midges, leafhoppers and planthoppers, foliage feeders and panicle feeders. Nowadays, brown planthopper, whitebacked planthopper, green leafhopper, grasshoppers, rice leaf-folders, rice water weevil, and rice bug are the most important insects in many cultivation areas (Pathak 1994).

2. Brown planthopper

Brown planthopper (*Nilaparvata lugens* Stål.) is a causative agent to massive yield loss in many rice cultivation areas in Asia. China is the country most affected by BPH: around 1 to 2 million tons of rice loss annually, and over 25 million hectares were affected from 2005 to 2007. Rice in Thailand, Vietnam, Indonesia, as well as other South-eastern and Eastern Asian countries are severely damaged by BPH (Catindig *et al*. 2009). To

reduce the negative impact of BPH, it is essential to understand the biology and ecology of this insect that can help regulate its population.

BPH is a typical monophagous herbivore belonging to Delphacidae family. The development of BPH includes three stages: eggs, instar nymphs, and formation of mature dimorphic adults (fully-winged macropterous and truncate-winged brachypterous forms) of both sexes. The total life cycle of BPH is about 9 to 36.6 days depending on temperature and host cultivars (Pathak *et al*. 1994). Therefore, new generations of BPH monthly appear in natural environment. The oviparous female starts laying eggs from the day following bisexual mating. After embryonic development, the eggs of planthoppers hatch into first-instar nymphs. There are five nymphal stadia which are distinguished by the shape of the mesonotum and body size. It takes about 10 to 18 days from hatching of the first-instar nymph to adult stage and the adult planthoppers live for 18 to 20 days. However, these development period varies depending on environmental factors such as food sufficiency, density of population during development, temperature, light intensity, etc.

At adult stage, the male BPH is shorter than female in both types of macropterous (long wings) and brachypterous (short wings). Ratios of female macropterous and brachypterous in populations are changed depending on density of population, food availability and/or other environmental factors. Relative numbers of the brachypterous females increase under low-density population and favourable living conditions such as food redundance and suitable ambient temperature (Lynn *et al*. 2009). The brachypterous males do not appear at lowest density, but are more numerous at middle densities, and decrease again with higher density. The brachypterous females are generally larger and have longer legs and ovipositors compared with those of macropterous type. Nymphs and adults of brachypterous forms move by walking and hopping while adults of macropterous forms with long wing move by flying, walking, and hopping (Pathak *et al*. 1994). The

macropterous forms are adapted to migration of BPH and the relative numbers of macropterous of both sexes increase under high density and food insufficiency.

BPH resides in rice ecosystem and collects nutrients from rice plants by feeding plants. Both nymphs and adult BPH usually inhabit on the leaf-sheath of rice plants. BPH tends to gather in high humidity and low temperature places in the rice field. However, in case of high-density population, the adult BPHs swarm even on higher parts of a rice plant such as the flag leaves, the uppermost internodes of panicles, and the panicle axes. The BPH damages in a rice field are generally started with macropterous immigrants. In the next generation, when the environment factors are optimal, the brachypterous forms will be hatched from eggs. If the host plant is inadequate, insect develops the macropterous forms to facilitate migration to find new food sources (Lynn *et al*. 2009, Mochida and Okada 1979).

N. lugens is distributed in Asia, Australia, and the Pacific Islands. In Asia, it is inhabited in many rice cultivation areas in Bangladesh, Brunei, Myanmar, China, Hong Kong, India, Indonesia, Japan, Cambodia, Korea, Laos, Malaysia, Nepal, Pakistan, Philippines, Singapore, Sri Lanka, Taiwan, Thailand, and Vietnam. In Australia and the Pacific Islands, it is found on the Caroline Islands, Fiji, Mariana Islands, Papua New Guinea, and Solomon Islands (Reissig *et al.* 1986). In tropical areas, the BPH lives year-round on rice and rice ratoons. In temperate areas in Asia, BPHs are migrated by wind-assisted from China to Japan and Korea in spring and summer every year. BPHs spend three to four generations and afterward they move or die when rice is harvested and temperature decreases. BPH is unable to overcome the winter in Japan and Korea because of cold temperature (Dyck *et al*. 1979, Kuno 1979).

N. lugens is a phloem-feeding insect. The sucking of plant honeydew and removing of nutrients from plant sap reduce the net photosynthate resulting in reduction of plant

biomass and production. On susceptible varieties, high density of BPH population can appear in a single cropping season and generally causes the “hopperburn”: the severe and massive devastation of rice (Lynn *et al*. 2009, Mochida and Okada 1979, Pathak *et al*. 1994). On the other hand, the feeding and oviposition activities of BPH cause physical injuries of plants and consequently expose the plants to fungal and bacterial infection. The plant honeydew excreted by BPH accelerates the growth of sooty moulds. In addition to damage by direct feeding, BPH indirectly causes yield loss by being vectors transmitting viral diseases. Grassy stunt and ragged stunt are two dangerous viruses transmitted by BPH and are caused serious problems in rice field in South and Southeast Asia.

3. Host plant resistance for brown planthopper

As BPHs annually cause losses in crop production, scientists have devised several management ways to minimize these losses. Chemical application and varietal resistance to insects are the two prominent measures which have been widely applied. Farmers are commonly use insecticides to protect rice against the BPH because the insecticides have strong and immediate effects to BPH. However, the insecticides also kill the other insects including BPH’s natural predators which suppress BPH. This results in huge development of BPH when the BPH’s natural enemies are not available. Additionally, the resistance of BPH against insecticides has increased recently that urged farmers to raise insecticide doses to keep the BPH below the economic threshold. The overuse and applying insecticide permanently consequently destroy the ecological balance resulting in resurgence of stronger virulence of BPHs and other insect pests. Moreover, through the natural food chain, insecticides are bioaccumulated which can eventually become a risk to human. Therefore, development of natural alternatives, such as resistance variety, is important to secure rice production as well as human health (Horgan *et al*. 2016).

Resistance, a natural response of rice to BPH, have been found in many rice cultivars and wild rice accessions. According to Jackson *et al*. (1997) and Heinrichs *et al*. (1985), around 15.4% of rice cultivars and 48% of wild rice accessions had resistance against BPH "biotype 1". Utilization of resistance characteristic in combination with integrated pest management can contain BPH population at a low density that will not cause significant yield loss of rice. Therefore, the host plant resistance can reduce the impact of BPH on rice production as well as impact of insecticides on natural environment. Study for genetic basis of resistance cultivars/wild rice accessions facilitates application of host plant resistance in management of BPH damage (Du *et al*. 2020, Fujita *et al*. 2013, Horgan *et al*. 2018).

Studies of host plant resistance has been started by screening for BPH resistance of rice cultivars and wild rice accessions. Until recently, more than 40,000 rice varieties and wild rice accessions have been screened for BPH resistance (Pathak *et al*. 1979). *BPH1* (from *indica* variety 'Mudgo') and *BPH2* (from 'ASD7') are the first two BPH resistance genes that have been reported (Athwal *et al*. 1971). Consequently, the *BPH1* was utilized to develop many resistance varieties. In International Rice Research Institute (IRRI), approximately 30 varieties carrying *BPH1* were developed until 1979. These varieties were widely grown against the BPH-"biotype 1", especially in the Philippines and Indonesia. However, these varieties were immediately adapted by new BPH population-"biotype 2" from the Philippines, Indonesia, and Vietnam. For dealing with new "biotype" appearance, *BPH2* which had resistance against both "biotype 1" and "biotype 2" was subsequently used in rice breeding. *BPH2* was introduced into many varieties, such as 'IR36', 'IR38', 'IR40', 'IR42', 'IR1628-632-1', and 'IR1702-74-3' those have been commonly cultivated in Southeast Asian countries (Pathak *et al*. 1979). However, the varieties carrying *BPH2* were eventually damaged by the new virulence-"biotype 3", a laboratory strain produced in

the Philippines. *BPH2* was also overcome by populations appeared in North Sumatra, Indonesia. By large-scale screening of genetic resources for BPH resistance, many varieties have been found to resistance against the "biotype 3", such as 'Rathu Heenati' and 'PTB33'. These varieties carry *BPH3* which has been introduced to 'IR56' and 'IR60' varieties and widely cultivated in Indonesia and Philippines. Nevertheless, the varieties carrying *BPH3* had different reaction patterns against populations in South Asia (India and Bangladesh). This population was defined as "biotype 4" and is avirulent against *BPH4* (from 'Babawee') (Saxena and Barrion 1985, Jairin *et al*. 2007).

Until recently, more than 46 loci for BPH resistance have been identified on ten rice chromosomes (chromosomes 1, 2, 3, 4, 6, 8, 9, 10, 11, and 12). *BPH37* was identified on chromosome 1 from *indica* variety, 'IR64' (Yang *et al*. 2019). On chromosome 2, *BPH13*(t) was detected from *O. eichingeri* by Liu *et al*. (2001). Six loci, *BPH11*, *BPH13*, *BPH14*, *BPH19*(t), *BPH31*, and *qBPH3*, have been identified on chromosome 3 (Chen *et al*. 2006, Du *et al*. 2009, Hu *et al*. 2015 a, Prahalada *et al*. 2017, Renganayaki *et al*. 2002). More than 16 BPH resistance loci have been identified on chromosome 4. Most of these genes are located around two cluster regions for BPH resistance genes: cluster B and cluster D. The cluster B on chromosome 4S contains seven genes: *BPH12*, *BPH15*, *BPH17* (*BPH3*), *BPH20*, *BPH22*(t), *BPH30*, and *BPH33* (Hou *et al*. 2011, Hu *et al*. 2018, Liu *et al*. 2014, Qiu *et al*. 2012, Rahman *et al*. 2009, Wang *et al*. 2018, Yang *et al*. 2004). The cluster D on chromosome 4L carries nine genes: *BPH6*, *BPH18*(t), *BPH27*, *BPH27*(t), *BPH34*, *qBPH4*, *qBPH4.2*, *qBPH4.3*, and *qBPH4.4* (He *et al*. 2013, Hu *et al*. 2015 a, b, Huang *et al*. 2013, Kumar *et al*. 2018, Mohanty *et al*. 2017, Qiu *et al*. 2010, Sun *et al*. 2006). Chromosome 6S carries cluster C where six loci have been detected: *BPH3*, *BPH4*, *BPH25*, *BPH29*, *BPH32*, and *qBPH6*(t) (Jairin *et al*. 2007 a, b, Myint *et al*. 2009 a, 2012, Ren *et al*. 2016, Sidhu *et al*. 1978, Wang *et al*. 2015). A single BPH resistance, *BPH23*(t),

has been identified on chromosome 8 and *BPH*(t) was identified on chromosome 9 (Hou *et al*. 2011). *BPH30* was identified on chromosome 10 and *BPH28*(t) was mapped on chromosome 11 (Wu *et al*. 2014, Yang *et al*. 2012). Eight genes: *BPH1*, *BPH2*, *BPH7*, *BPH9*, *BPH10*, *BPH18*, *BPH21*, and *BPH26* were located at cluster A on chromosome 12L (Athwal *et al*. 1971, Cha *et al*. 2008, Ishii *et al*. 1994, Ji *et al*. 2016, Kabir *et al*. 1988, Nemoto *et al*. 1989, Rahman *et al*. 2009, Sun *et al*. 2006, Tamura *et al*. 2014).

Among those, eight genes, *BPH6*, *BPH9*, *BPH14*, *BPH17*, *BPH18*, *BPH26*, *BPH29*, and *BPH32*, have been cloned and characterized for BPH resistance. *BPH6* was isolated to a segment of 18.1-kbp on chromosome 4 from Bangladeshi rice landrace 'Swarnalata' (Guo *et al*. 2018). *BPH6* encodes a previously uncharacterized protein that localizes to exocysts and activates a coordinated cytokinin, salicylic acid, and jasmonic acid signaling pathway to broad resistance to BPH. *BPH9* is originated from *indica* variety 'Pokkali'. The fine mapping and cloning of *BPH9* revealed physical location of *BPH9*, which is 68-kbp from 22.7 to 22.9 Mbp on chromosome 12 (Zhao *et al*. 2016). *BPH9* encodes: a protein of 1,206 amino acids with a coiled-coil (CC) domain, two nucleotide-binding site (NBS) domains, and a leucine-rich repeat (LRR) domain (CC-NB-NB-LRR), which are expressed in the vascular bundle. *BPH14* from 'B5', an introgression from the wild rice species *O. officinalis*, is the first gene cloned through map-based cloning (Du *et al*. 2009). This gene was located to a 34 kbp region on chromosome 3L and contains two candidate genes, *Ra* and *Rb*, encoding for coiled-coil, nucleotide-binding, and leucine-rich repeat (CC-NB-LRR) protein. *BPH17* (*BPH3*) from 'Rathu Heenati' was delimited to a 79-kbp genomic segment on chromosome 4S and carries three candidate genes: *OsLecRK1*, *OsLecRK3*, and *OsLecRK3* encoding the plasma membrane-localized proteins and lectin reception kinases (Liu *et al*. 2014). *BPH18* is located on an approximately 27-kbp segment on chromosome 12 from 'IR65482-7-216-1-2', an introgression line from *Oryza australiensis*. *BPH18*

encodes the CC-NB-NB-LRR protein (Ji *et al*. 2016). *BPH26* from *indica* variety, 'ADR52', was detected in a 135-kbp region on chromosome 12 encoding CC-NB-NB-LRR protein (Tamura *et al*. 2014). *BPH29* is originated from 'RBPH54' variety, an *O. rufipogon* (Griff.) introgression line. *BPH29* was mapped to a 24-kbp region on the short arm of chromosome 6 (Wang *et al*. 2015). This gene encodes a B3 DNA-binding domain which induced to vascular tissue, the location of BPH attack. The other gene, *BPH32* from Indian variety, 'PTB33', was detected on chromosome 6. *BPH32*, which encodes a unique short consensus repeat domain protein, was cloned by bioinformatics and DNA sequencing approaches and (Ren *et al*. 2016).

4. Outlines of research

The main objective of this research is to develop breeding materials for BPH resistance genes that will eventually facilitate rice breeding and perform genetic analysis of resistance to brown planthopper in rice. Through the main object, three experiments were conducted. First experiment (Chapter 2) is entitled: "The development of the near-isogenic lines (NILs) and pyramided lines (PYLs) for BPH resistance genes on the genetic background of Taichung 65 (T65)". In this experiment, the NILs and PYLs for BPH resistance genes were developed and assessed for agronomic traits and BPH resistance level. Second experiment (Chapter 3): "Substitution mapping and characterization of BPH resistance genes from indica rice variety, 'PTB33' (*O. sativa*)", which describes the substitution mapping of three BPH resistance genes from 'PTB33' (*BPH2*, *BPH17-ptb* and *BPH32*). Additionally, the corresponded NILs for those BPH resistance genes were evaluated for BPH resistance by various methods for antibiosis, antixenosis and tolerance. Third experiment (Chapter 4) is entitled: "Genetic analysis for brown planthopper resistance gene in Sri Lanka variety 'Rathu Heenati' and two accessions of *O. nivara*",

which describes the development of segregation populations and the estimation of gene loci for BPH resistance from these three donors.

Chapter 2

Development and characterization of near-isogenic and pyramided lines carrying resistance genes to brown planthopper with the genetic background of *japonica* rice (*Oryza sativa* L.)

1. Introduction

Monogenic resistance is vulnerable to rapid adaptation by BPH populations. Research indicated that BPH populations included sufficient genetic variability to enable them to overcome specific resistance genes when selected on a resistant host over multiple generations (Horgan 2018, Ketipearachchi *et al*. 1998, Saxena and Barrion 1985). In the late 1970s, BPH populations adapted to varieties carrying the *BPH1 and/*or *BPH2* genes after these were widely deployed in rice varieties across Asia (Horgan 2018, Pathak *et al*. 1979, Saxena and Barrion 1985). A recent multi-nation study has indicated that BPH populations across much of Asia have adapted to feed on rice carrying the *BPH1, BPH2, BPH5*, *BPH7, BPH8, BPH9, BPH10* and *BPH18* genes (Horgan *et al*. 2015). Under laboratory condition, BPH populations adapted to resistant rice varieties carrying either *BPH1*, *BPH2, BPH3, BPH8, BPH9, BPH10* or *BPH32* when selected for between seven and 15 generations on the resistant hosts (Alam *et al*. 1998, Claridge *et al*. 1982, Ferrater *et al*. 2015, Ketipearachchi *et al*. 1998, Peñalver Cruz *et al*. 2011). Adaptation by BPH to resistance genes appears to have minimal ecological costs in modern rice production systems, such that BPH virulence remains stable for several decades (Horgan 2018, Horgan *et al*. 2017) Therefore, it is important to preserve the effects of resistance genes by preventing BPH adaptation.

To maintain the limited number of available BPH resistance genes, a strategy for deployment of resistance genes based on insect virulence is necessary (Horgan 2018).

However, BPH virulence varies under different environments depending on the predominant rice cultivars, BPH migration paths and the period of population exposure to different resistance genes (Horgan 2018). Therefore, without pre-exposing resistance genes to BPH populations under controlled conditions, the potential effectiveness of the resistance genes for target regions would be difficult to predict. In previous studies, the virulence of BPH was characterized using resistant varieties (Ali *et al*. 2012, Horgan *et al*. 2015, Myint *et al*. 2009 a, Peñalver Cruz *et al*. 2011, Qiu *et al*. 2011). However, many BPH resistant varieties have multiple resistance genes, thus the effects of a single resistance gene cannot be assessed by using these resistant varieties. The effect of a single genes may be revealed in detail by using NILs carrying a single resistance gene on the genetic background of a susceptible variety. Recently, more than 16 NILs with BPH resistance genes (*BPH3*, *BPH4*, *BPH6*, *BPH9*, *BPH10*, *BPH12*, *BPH14*, *BPH15*, *BPH17*, *BPH18*, *BPH20*, *BPH21*, *BPH25*, *BPH26*, *BPH30* and *BPH32*) have been developed on different genetic backgrounds of several *indica* and *japonica* susceptible varieties and evaluated against different BPH populations from China, the Philippines and Japan (Jena *et al*. 2017, Liu *et al*. 2016, Qiu *et al*. 2012, Wang *et al*. 2018, Xiao *et al*. 2016 b, Yara *et al*. 2010).

Since the resistance of rice varieties carrying single genes is weaker and less durable to BPH (allowing rapid BPH adaptation), the pyramiding of two or more genes to enhance resistance levels has been widely proposed to reduce damage by BPH and avoid pest adaptation (Horgan *et al*. 2019). Combinations of multiple BPH resistance genes have been reported to increase levels of plant resistance to BPH. For example, a PYL with *BPH14* and *BPH15* had enhanced resistance against BPH from China compared to monogenic NILs with either *BPH14* or *BPH15* (Hu *et al*. 2012). Similarly, the pyramided lines *BPH6*+*BPH12* PYL and *BPH3*+*BPH27*-PYL exhibited greater resistance levels in

bulk seedling tests than monogenic lines with each of the genes present alone (Liu *et al*. 2016, Qiu *et al*. 2012), and *BPH17+BPH21* PYL had greater resistance against BPH in the Philippines than lines with either gene alone (Jena *et al*. 2017). Pyramiding the *BPH25* and *BPH26* gene into a single rice line was reported to have positive epistatic effects against BPH populations collected in Vietnam, the Philippines and Japan (Fujita *et al*. 2009, Myint *et al*. 2009 a). Therefore, the development of rice varieties carrying multiple BPH resistance genes might be an effective way to enhance BPH resistance.

In this study, seven NILs with BPH resistance genes (*BPH2, BPH3*, *BPH17, BPH20, BPH21, BPH32* and *BPH17-ptb*) and a *japonica* rice genetic background were developed to evaluate gene effects on BPH populations. Based on the developed NILs, 15 PYLs carrying two or three resistance genes were developed to enhance levels of resistance against BPH. Additionally, using the developed NILs and PYLs, the study compared resistance against two BPH populations collected in Japan: the first was collected in 1966 (before resistant varieties were widely released) and the second was collected in 2013 (recently migrated from China to Japan). Comparisons of the reactions by BPH from each population to the NILs and PYLs indicates the utility of resistance genes and their different combinations (some with epistatic effects) against modern BPH populations.

2. Materials and methods

2.1 Plant materials

To develop NILs with BPH resistance genes, a *japonica* rice variety, T65, that is susceptible to BPH, was used as a recurrent parent and three rice varieties resistant to BPH as donor parents. The donor lines were ‘IR71033-121-15’, ‘PTB33’ and ‘Rathu Heenati’.

‘IR71033-121-15’ contains two BPH resistance genes, *BPH20* and *BPH21*, from the wild rice species *O. minuta* (Acc. no. IRGC101141) (Rahman *et al*. 2009). ‘PTB33’ (Acc. no. IRGC19325) originated from India contains *BPH2* and *BPH32.* There has been no previous report of a BPH resistance gene on chromosome 4S of ‘PTB33’. However, amino acid sequences for the *BPH17* locus in ‘PTB33’ was identical to that of ‘Rathu Heenati’ (Liu *et al*. 2014). Thus, we assumed that ‘PTB33’ contains a gene for BPH resistance on chromosome 4S and tentatively named this as *BPH17-ptb*. ‘Rathu Heenati’ (Acc. no. IRGC 11730) originated from Sri Lanka carries *BPH3* and *BPH17* (Jairin *et al*. 2007 b, Sun *et al*. 2005). T65 was crossed with these donor parents and F_1 plants were backcrossed four times with T65 to generate BC_4F_1 plants (**Figure 2.1**). At each generation of backcrossing, plants carrying BPH resistance genes from the donor parents were selected by marker-assisted selection (MAS) using flanking simple sequence repeat (SSR) markers of the target BPH resistance genes (**Table 2.1**). The selected BC_4F_1 plants were self-pollinated to produce BC_4F_3, BC_4F_4 and BC_4F_5 plants with BPH resistance genes. Finally, seven NILs with either *BPH2, BPH3, BPH17, BPH20, BPH21, BPH32* or *BPH17-ptb* were developed. The NILs were used for surveying the genetic background and evaluating BPH resistance levels as well as agronomic traits. Two additional NILs, *BPH25*-NIL and *BPH26*-NIL were used in the development of the PYLs (Yara *et al*. 2010).

2.2 The development of PYLs with BPH resistance genes

All the PYLs for two or three BPH resistance genes were developed using the NILs descended from the BC_4F_1 generation, except *BPH20+BPH21*-PYL and *BPH32+BPH17-ptb*-PYL which were descended from the BC_3F_1 generation. The F_1 plants derived from crosses between NILs were self-pollinated to produce F_2 plants. From 96 F_2 plants, plants that were homozygous for two or three BPH resistance genes were selected by MAS.

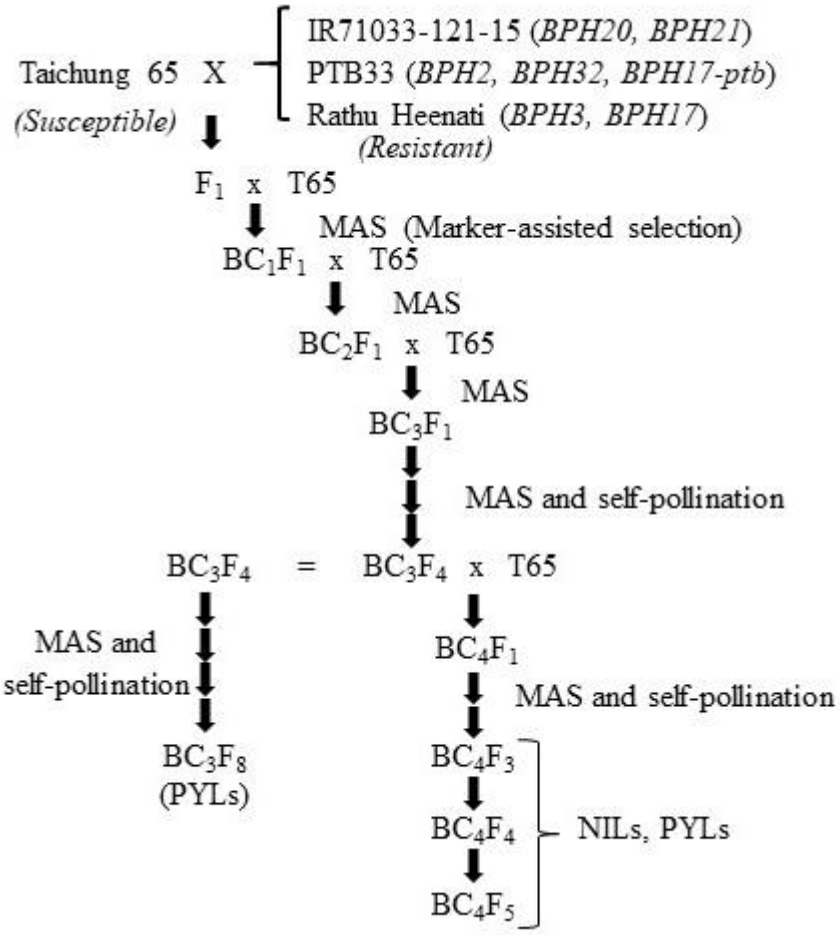


Figure 2.1. Breeding scheme for the development of near-isogenic lines and pyramided lines containing brown planthopper resistance genes from donor parents, 'IR71033-121-15', 'PTB33' and 'Rathu Heenati'.

Table 2.1. The SSR markers used for maker-assisted selection of nine genes for resistance to the brown planthopper.

Marker	Resistance gene tagged	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Chr.	Physical position	Predicted size (bp)*
RM1246[a]	*BPH2, BPH21*	GGCTCACCTCGTTCTCGATCC	CATAAATAAATAGGGCGCCACACC	12	19,156,149	195
RM28493[b]	*BPH2, BPH21*	ACCGTTAGATGACACAAGCAACG	GGTTAGCAAGACTGGAGGAGACG	12	23,279,853	259
RM508[c]	*BPH3, BPH32*	AGAAGCCGGTTCATAGTTCATGC	ACCCGTGAACCACAAAGAACG	6	441,752	158
RM588[c]	*BPH3, BPH32*	TCTTGCTGTGCTGTTAGTGTACG	GCAGGACATAAATACTAGGCATGG	6	1,612,412	97
RM8213[a]	*BPH17, BPH17-ptb*	TGTTGGGTGGGTAAAGTAGATGC	CCCAGTGATACAAAGATGAGTTGG	4	4,418,222	178
MS10[d]	*BPH17, BPH17-ptb, BPH20*	CAATACGAGAAGCCCCTCAC	CTGAAGGAACACGCGGTAGT	4	8,071,921	167
RM5900[a]	*BPH20*	TTCTACGTTTGACCGTCA	TCTAGGAGCGTTTGTAGGAG	4	13,773,131	248
S00310[e]	*BPH25*	CAACAAGATGGACGGCAAGG	TTGGAAGAAAAGGCAGGCAC	6	214,278	215
MSSR1[e]	*BPH25*	CTAGCTGCTCTGCTCTGCTG	CGGCAATCTCTCCGAATC	6	221,014	114
RM309[e]	*BPH26*	CACGCACCTTTCTGGCTTTCAGC	AGCAACCTCCGACGGGAGAAGG	12	21,521,910	177
RM28438[b]	*BPH26*	GTTCGTGAGCCACAACAAATCC	GTTAAATGCTCCACCAAACACACC	12	22,586,348	216
InD14[f]	*BPH26*	GGCCGAGTAGGATACTCTAGAAA	CTGCGAGAAAGGAGAGGTGG	12	22,864,811	387
RM28466[b]	*BPH26*	CCGACGAAGAAGACGAGGAGTACC	AGGCCGGAGAGCAATCATGTCG	12	22,975,528	93
RM28481[b]	*BPH26*	GTCAATTAACCATTGCCCATGC	TTCACGTGGGAACTACTCATGC	12	23,138,168	242
MSSR2[g]	*BPH26*	CATGTCGAAGAGGTTGCAGA	GGTTTCATCCAAGTCCACGA	12	25,033,993	265

* The PCR product size was estimated based on the Nipponbare genome sequence. Primer sequence information was obtained from: [a:] McCouch *et al.* 2002, [b:] International Rice Genome Sequencing Project (IRGSP). 2005, [c:] Temnykh *et al.* 2001, [d:] Yang *et al*. 2004, [e:] Temnykh *et al.* 2000, [f:] Zhao *et al.* 2016 and [g:] Myint *et al.* 2012. Chr.: chromosome.

Several plants from 96 F_3 plants with similar agronomic traits to T65 were selected as final PYLs. The following PYLs carrying two or three BPH resistance genes were evaluated for BPH resistance and agronomic traits: *BPH2+BPH17*-PYL, *BPH2+BPH25*-PYL, *BPH2+BPH32*-PYL, *BPH2+BPH17-ptb*-PYL, *BPH3+BPH17*-PYL, *BPH17+BPH21*-PYL, *BPH20+BPH21*-PYL, *BPH20+BPH32*-PYL, *BPH21+BPH25*-PYL, *BPH21+BPH17-ptb*-PYL, *BPH25+BPH17-ptb*-PYL, *BPH32+BPH17-ptb*-PYL, *BPH2+BPH3+BPH17*-PYL, *BPH2+BPH32+BPH17-ptb*-PYL, and *BPH20+BPH21+BPH32*-PYL (**Table 2.2**).

2.3 The MAS for BPH resistance genes

To conduct MAS, total DNA was extracted using the potassium acetate method (Dellaporta *et al*. 1983). An approximately 2 cm of leaf from two–week old seedling was collected and dried in the freeze drier for 48 h. After grinding in 1.5 mL tube, the sample was treated by 300 µL of DNA extraction buffer containing Tris-HCl (pH 8.0) 0.1M, EDTA (pH 8.0) 0.05M, NaCl 0.5M and SDS 1%. The sample was inoculated at 60ºC for 30 minutes and added by 60 µL of potassium acetate 5M before maintaining in ice for 30 minutes. After centrifuging, the supernatant was transferred to the other tube before adding 60 µL of Isopropanol. The sample was centrifuged, and the supernatant was removed. The sample was washed by adding 100 µL of ethanol 70%. The ethanol was discarded after centrifuging. After drying for one night at room temperature, DNA in tube was diluted by adding 100 µL of distilled water.

The genotypes of SSR markers on plants in each generation were determined by polymerase chain reaction (PCR) and electrophoresis. PCR amplification (8µL) contains 3 µL of 1X GoTaq® Green Master Mix (pH 8.5), 0.25 µM of primer and 4 µL of DNA. Each PCR amplification included one cycle at 96ºC for 5 min, 35 cycles at 96ºC for 30s, 55ºC

for 30s and 72ºC for 30s followed by one extension cycle at 25ºC for 1 min. PCR products were analyzed by electrophoresis at 200 V using 4% agarose gel with 0.5 μg/mL ethidium bromide in 0.5X TBE buffer for 1h and photographed under ultraviolet light. During MAS for resistance genes on chromosome 4S, the plants with *BPH17* and *BPH17-ptb*, were selected using two markers, RM8213 and MS10 (Sun *et al*. 2005), and the plants with *BPH20* were selected using MS10 and RM5900 (**Table 2.1**) (Rahman *et al*. 2009). The plants with *BPH3* and *BPH32* on the short arm of chromosome 6 were selected using two flanking markers, RM508 and RM588 (Jairin *et al*. 2007 b). The plants carrying *BPH2* and *BPH21* located on the long arm of chromosome 12 were screened using RM1246 and RM28493 (Rahman *et al*. 2009, Sun *et al*. 2006). The plants with *BPH25* were selected using S00310 and MSSR1, and the plants with *BPH26* were selected using RM309, RM28438, InD14, RM28466, RM28481 and MSSR2 (Yara *et al*. 2010, Zhao *et al*. 2016).

Table 2.2. Seven near-isogenic lines and 15 pyramided lines carrying brown planthopper resistance genes.

Entry	Gene (donor parent)						Generation
BPH2-NIL	*BPH2*	('PTB33')					BC_4F_3
BPH3-NIL	*BPH3*	('Rathu Heenati')					BC_4F_4
BPH17-NIL	*BPH17*	('Rathu Heenati')					BC_4F_4
BPH20-NIL	*BPH20*	('IR71033-121-15')					BC_4F_5
BPH21-NIL	*BPH21*	('IR71033-121-15')					BC_4F_5
BPH32-NIL	*BPH32*	('PTB33')					BC_4F_4
BPH17-ptb-NIL	*BPH17-ptb*	('PTB33')					BC_4F_3
BPH2+BPH17-PYL	*BPH2*	('PTB33')	*BPH17*	('Rathu Heenati')			BC_4F_3 equivalent
BPH2+BPH25-PYL	*BPH2*	('PTB33')	*BPH25*	('ADR52')			BC_4F_3 equivalent
BPH2+BPH32-PYL	*BPH2*	('PTB33')	*BPH32*	('PTB33')			BC_4F_3 equivalent
BPH2+BPH17-ptb-PYL	*BPH2*	('PTB33')	*BPH17-ptb*	('PTB33')			BC_4F_3 equivalent
BPH3+BPH17-PYL	*BPH3*	('Rathu Heenati')	*BPH17*	('Rathu Heenati')			BC_4F_4 equivalent
BPH17+BPH21-PYL	*BPH17*	('Rathu Heenati')	*BPH21*	('IR71033-121-15')			BC_4F_3 equivalent
BPH20+BPH21-PYL	*BPH20*	('IR71033-121-15')	*BPH21*	('IR71033-121-15')			BC_3F_8 equivalent
BPH20+BPH32-PYL	*BPH20*	('IR71033-121-15')	*BPH32*	('PTB33')			BC_4F_3 equivalent
BPH21+BPH25-PYL	*BPH21*	('IR71033-121-15')	*BPH25*	('ADR52')			BC_4F_3 equivalent
BPH21+BPH17-ptb-PYL	*BPH21*	('IR71033-121-15')	*BPH17-ptb*	('PTB33')			BC_4F_3 equivalent
BPH25+BPH17-ptb-PYL	*BPH25*	('ADR52')	*BPH17-ptb*	('PTB33')			BC_4F_3 equivalent
BPH32+BPH17-ptb-PYL	*BPH32*	('PTB33')	*BPH17-ptb*	('PTB33')			BC_3F_8 equivalent
BPH2+BPH3+BPH17-PYL	*BPH2*	('PTB33')	*BPH3*	('Rathu Heenati')	*BPH17*	('Rathu Heenati')	BC_4F_3 equivalent
BPH2+BPH32+BPH17-ptb-PYL	*BPH2*	('PTB33')	*BPH32*	('PTB33')	*BPH17-ptb*	('PTB33')	BC_4F_3 equivalent
BPH20+BPH21+BPH32-PYL	*BPH20*	('IR71033-121-15')	*BPH21*	('IR71033-121-15')	*BPH32*	('PTB33')	BC_4F_3 equivalent

2.4 The genetic background survey of the NILs

In the genetic background survey of the NILs, the bulked DNA from five plants was used. A total of 384 SSR markers distributed on 12 rice chromosomes were used during polymorphism tests with T65 and the donor parents (McCouch *et al*. 2002). Among the 384 SSR markers, 254 SSR markers with polymorphism between 'IR71033-121-15' and T65 were utilized to identify substituted chromosomal segments from 'IR71033-121-15' on *BPH20*-NIL and *BPH21*-NIL. Additionally, 244 of 384 SSR markers with polymorphism between 'PTB33' and T65 were used to detect substituted chromosomal segments from 'PTB33' on *BPH2*-NIL, *BPH32*-NIL and *BPH17*-*ptb*-NIL. To identify substituted chromosomal segments from 'Rathu Heenati' on *BPH3*-NIL and *BPH17*-NIL, 204 of 384 SSR markers with polymorphism between 'Rathu Heenati' and T65 were used. The whole genome compositions of the developed NILs were graphically displayed following the concept of the graphical genotype proposed by Young and Tanksley (1989) using GGT software version 2.0 (Young *et al*. 1989).

2.5 The BPH populations and characterization of BPH resistance

Two BPH populations from Japan (Hadano-66 and Koshi-2013) were used to evaluate the NILs and PYLs for their resistance. Hadano-66 was collected from Hadano city, Kanagawa Prefecture, Japan in 1966 (Myint *et al*. 2009 a), and Koshi-2013 was collected from Koshi city, Kumamoto Prefecture, Japan in 2013. Both BPH strains were maintained on the susceptible *japonica* rice variety, 'Reiho', under 25^{o}C and 16h/8h of light/dark at Kyushu Okinawa Agricultural Research Center of the National Agriculture and Food Research Organization in Japan.

To evaluate resistance, an adaptation of the modified seedbox screening test (MSST) was applied at 25^{o}C using the Hadano-66 strain (Horgan *et al*. 2015, Naeemullah *et al*. 2009). To conduct the test, about 30 seeds of each of the NILs, PYLs and parent lines were sown to single rows in a plastic tray (23.0 x 30.0 x 2.5 cm) with 2.5 cm between successive rows of seedlings. Two sets of trays: one tray infested by BPH and the other without infestation (the control tray) were used to measure BPH effects on plant biomass. One row of 'Rathu Heenati' was added as a resistant control, while three rows of T65 were sown at the center and the two edges as a susceptible control. At seven days after sowing (DAS), the plants in the trays were thinned to 20 plants per row. One tray was infested by second and third instar nymphs at a density of around 20 BPHs per plant. The experiment was replicated three times. When all the plants of T65 were completely desiccated due to BPH feeding, the damage scores (DSs) of all lines was graded following the standard evaluation system for rice of the BPH (*Standard evaluation system (SES) for rice*. 2014). The plants from each row in the two trays were cut above the soil surface and weighed. The fresh biomass reduction rate (FBRR) was calculated using the following formula:

$$\text{Fresh biomass reduction rate (FBRR)(\%)} = \left[1 - \frac{\text{Infested plant weight (g)}}{\text{Non-infested plant weight (g)}}\right] \text{x } 100$$

Antibiosis tests were conducted at 25^{o}C following the method described by Myint *et al*. (2009 a). Five plants of each NIL, PYL and parent line were individually sown in 200 mL plastic cups. At four weeks after sowing, the plants were trimmed to 15 cm height and covered with a plastic cage with insect screen windows for ventilation. Each cage was infested with five thin-abdomen brachypterous female BPHs. At five days after infestation, the adult mortality (ADM) was recorded.

2.6 Characterization of NILs and PYLs for agronomic traits

The NILs and PYLs were grown in a paddy field at Saga University (Saga, Japan) in 2018 and characterized for agronomic traits compared with T65. Seedlings were transplanted at 28 DAS as one plant per hill, with 20 cm between hills and 25 cm between rows. Each entry was planted as at least three rows (12 plants per a row). Six agronomic traits: days to heading (DTH), culm length (CL), panicle length (PL), leaf length (LL), leaf width (LW) and panicle number (PN) were measured for five plants in the same row. DTH was the days from sowing until 50% of panicles flowered. CL was measured from the soil surface to the panicle neck. PL is the length from tip to panicle neck of the longest panicle. The flag leaf width and length were measured from the largest and longest flag leaf of each sampled plant. Panicle number is the number of reproductive panicles of each plant at maturity.

2.7 Statistical analysis

Mean values of BPH resistance on the NILs and PYLs (DS, FBRR and ADM) and agronomic traits were compared using one-way ANOVA. Dunnett's test and Tukey Kramer’s test were conducted for multiple comparisons of BPH resistance and agronomic traits, respectively, using R software version 3.5.2.

3. Results

3.1. Development of seven NILs for BPH resistance

Seven NILs with BPH resistance genes on the genetic background of T65 were developed through MAS and backcrossing (**Table 2.2**, **Figure 2.1**). The substituted

chromosomal segments were detected by polymorphic SSR markers that were equally distributed across the whole genome (**Table 2.3, Figure 2.2**). The genetic background of *BPH2*-NIL was analyzed using 203 polymorphic SSR markers (**Figure 2.2A**). The ratio of substituted segments from 'PTB33' on *BPH2*-NIL was about 11.9% (total 44.0 Mbp). One substituted segment with approximately 21.8 Mbp encompassing *BPH2* was detected between RM247 and RM5479 on chromosome 12. The other three segments were detected between RM5426 and RM248 on chromosome 7 with a size of about 4.0 Mbp, between RM5688 and RM444 on chromosome 9 with a size of approximately 7.6 Mbp and between RM7492 and RM216 on chromosome 10 with the size of about 10.6 Mbp.

The genetic background of *BPH3*-NIL was confirmed using 195 polymorphic SSR markers, and the ratio of substituted segment from 'Rathu Heenati' was about 2.0% (total 7.5 Mbp) (**Figure 2.2B**). One segment with approximately 1.7 Mbp including *BPH3* was detected between MSSR1 and RM1369 on the short arm of chromosome 6. The other substituted segments were detected between RM1359 and RM1155 on chromosome 4 with a size of approximately 2.3 Mbp and between RM1345 and RM3155 on chromosome 8 with a size of about 3.5 Mbp.

The genetic background of *BPH17*-NIL was surveyed using 173 polymorphic SSR markers. The ratio of substituted segments was about 4.1% (total 15.2 Mbp) containing one segment located between RM8213 and B40 on chromosome 4, including the *BPH17* region (**Figure 2.2C**). The genetic background of *BPH17-ptb*-NIL was analyzed using 229 polymorphic SSR markers, and the ratio of substituted segments from 'PTB33' on *BPH17-ptb*-NIL was about 5.1% (total 19.0 Mbp). One substituted segment with approximately 9.5 Mbp encompassing *BPH17-ptb* was detected between C61009 and B40 on chromosome 4. Two other substituted segments were detected at RM3126 on chromosome 3 with a size of

about 1.4 Mbp and between RM7048 and RM6971 on chromosome 9 with a size of approximately 8.1 Mbp (**Figure 2.2G**).

The genetic background of *BPH20*-NIL was confirmed using 237 polymorphic SSR markers and the ratio of substituted segments of 'IR71033-121-15' was about 7.6% (total 28.1 Mbp). One segment with approximately 16.9 Mb containing *BPH20* was detected between RM335 and RM5900 on chromosome 4 (**Figure 2.2D**). Two other substituted segments were detected between RM224 and RM5926 on chromosome 11 (approximately 4.6 Mbp) and between RM7315 and RM3103 on chromosome 12 (approximately 6.6 Mbp).

The genetic background of *BPH21*-NIL was surveyed using 229 polymorphic SSR markers and the ratio of substituted segments from 'IR71033-121-15' was about 9.5% (total 35.3 Mbp). One segment with a size of approximately 23.5 Mbp including *BPH21* was detected between RM1880 and RM28493 on chromosome 12 (**Figure 2.2E**). Three other segments were detected between RM6841 and RM3348 on chromosome 5 with a size of approximately 4.8 Mbp, around RM1328 on chromosome 9 with a size of about 2.3 Mbp, and between RM224 and RM5926 on chromosome 11 with a size of approximately 4.7 Mbp.

The genetic background of *BPH32*-NIL was confirmed using 233 polymorphic SSR markers. The ratio of substituted segments of 'PTB33' on *BPH32*-NIL was about 3.0% (total 11.2 Mbp). One segment with approximately 2.4 Mbp containing *BPH32* was detected between RM6775 and RM190 on chromosome 6. Three other segments from the donor parent were detected between RM5755 and RM3280 on chromosome 3 with a size of approximately 6.5 Mbp, between RM1306 and RM248 on chromosome 7 with a size of approximately 1.9 Mbp and between RM5349 and RM5961 on chromosome 11 with a size of approximately 0.4 Mbp (**Figure 2.2F**).

Table 2.3. Background survey analysis of seven near-isogenic lines using SSR polymorphic markers.

NIL	Donor	No. of SSR markers			Genome ratio (%)		Total physical distance of donor segment (Mbp)
		T65	Donor	Total	T65	Donor	
BPH2-NIL	'PTB33'	183	20	203	88.1	11.9	44.0
BPH3-NIL	'Rathu Heenati'	181	14	195	98.0	2.0	7.5
BPH17-NIL	'Rathu Heenati'	170	3	173	95.9	4.1	15.2
BPH20-NIL	'IR71033-121-15'	224	13	237	92.4	7.6	28.1
BPH21-NIL	'IR71033-121-15'	210	19	229	90.5	9.5	35.3
BPH32-NIL	'PTB33'	220	13	233	97.0	3.0	11.2
BPH17-ptb-NIL	'PTB33'	219	10	229	94.9	5.1	19.0

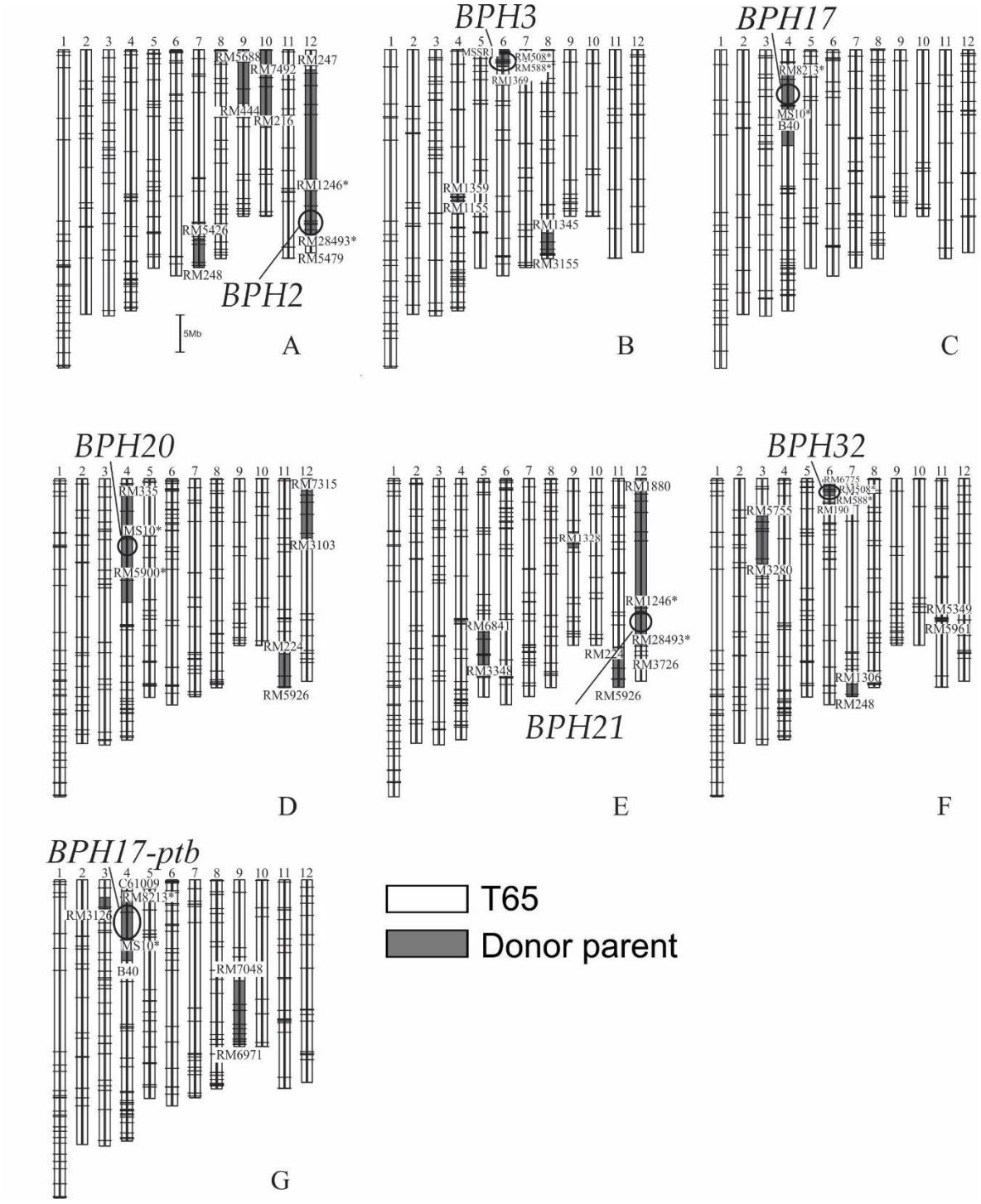


Figure 2.2. Graphical genotypes of *BPH2*-NIL (A), *BPH3*-NIL (B), *BPH17*-NIL (C), *BPH20*-NIL (D), *BPH21*-NIL (E), *BPH32*-NIL (F), *BPH17-ptb*-NIL (G). The 12 bars indicate 12 chromosomes of rice. Horizontal lines across the chromosomes show the positions of polymorphic SSR markers. Circles indicate the approximate positions of brown planthopper resistant genes.

3.2. Development of 15 PYLs carrying two or three BPH resistance genes

Twelve PYLs carrying two BPH resistance genes (*BPH2+BPH17*-PYL, *BPH2+BPH25*-PYL, *BPH2+BPH32*-PYL, *BPH2+BPH17-ptb*-PYL, *BPH3+BPH17*-PYL, *BPH17+BPH21*-PYL, *BPH20+BPH21*-PYL, *BPH20+BPH32*-PYL, *BPH21+BPH25*-PYL, *BPH21+BPH17-ptb*-PYL, *BPH25+BPH17-ptb*-PYL and *BPH32+BPH17-ptb*-PYL) and three PYLs containing three BPH resistance genes (*BPH2+BPH3+BPH17*-PYL, *BPH2+BPH32+BPH17-ptb*-PYL, *BPH20+BPH21+BPH32*-PYL) were developed using NILs and PYLs with BPH resistance gene(s) (**Table 2.2**). The PYLs were confirmed for resistance genes through foreground selection using flanking SSR markers tightly linked to each resistance gene. Most of PYLs were selected from the BC_4F_3 equivalent generation, except *BPH3+BPH17*-PYL from the BC_4F_4 equivalent generation, *BPH20+BPH21*-PYL from the BC_3F_8 generation and *BPH32+BPH17-ptb*-PYL from the BC_3F_8 generation.

3.3. Comparison of resistance levels against Hadano-66 by MSST

T65 was highly damaged (damage score [DS] = 8.2) by the Hadano-66 population (**Figure 2.3A**). The DS of the donor parents was significantly lower (0.7 for 'IR71033-121-15', 0.7 for 'PTB33' and 0.2 for 'Rathu Heenati') than that of T65. The donor parents also had higher levels of resistance compared with their NILs and PYLs. Among the NILs, *BPH2*-NIL (DS: 3.0) and *BPH17*-NIL (3.2) showed the highest resistance levels. The other NILs, *BPH3*-NIL (6.0), *BPH20*-NIL (6.0), *BPH21*-NIL (6.5), *BPH25*-NIL (6.7), *BPH26*-NIL (4.8), *BPH32*-NIL (6.7) and *BPH17-ptb*-NIL (5.7), had lower DS than T65 but were not significantly different from the T65. The range of DS among the 15 PYLs was from 2.3 to 6.0. Among PYLs, the DS of 10 PYLs: *BPH2+BPH17*-PYL (2.7), *BPH2+BPH25*-PYL (2.5), *BPH2+BPH32*-PYL (3.0), *BPH2+BPH17-ptb*-PYL (3.0), *BPH17+BPH21*-PYL

(2.3), *BPH20+BPH21*-PYL (2.3), *BPH21+BPH25*-PYL (3.3), *BPH21+BPH17-ptb*-PYL (2.7), *BPH2+BPH3+BPH17*-PYL (3.0) and *BPH20+BPH21+BPH32*-PYL (2.3) was equal to or less than 3.3, while the DS of five PYLs: *BPH3+BPH17*-PYL (5.0), *BPH20+BPH32*-PYL (5.3), *BPH25+BPH17-ptb*-PYL (6.0), *BPH32+BPH17-ptb* (5.0) and *BPH2+BPH32+BPH17-ptb*-PYL (4.3) was more than 4.3. Although the DSs between NILs and PYLs were not significantly different, the resistance levels of the PYLs tended to be higher than that of the NILs.

Additionally, fresh biomass reduction rate (FBRR) of the NILs and PYLs was calculated as an indicator of resistance (**Figure 2.3B**). T65 had the highest FBRR (89.0%) and was significantly different from the donor parents: 'IR71033-121-15' (35.7%), 'PTB33' (39.2%) and 'Rathu Heenati' (20.4%). Among the NILs, *BPH17*-NIL (58.7%) had the lowest FBRR and was significant different from T65. The other NILs, *BPH2*-NIL (68.6%), *BPH3*-NIL (82.4%), *BPH20*-NIL (77.3%), *BPH21*-NIL (84.3%), *BPH25*-NIL (85.3%), *BPH26*-NIL (73.8%), *BPH32*-NIL (86.7%), and *BPH17-ptb*-NIL (77.6%) had lower FBRRs than T65, however, the differences were not significant. The FBRR of four PYLs: *BPH2+BPH32*-PYL (59.1%), *BPH2+BPH17-ptb*-PYL (56.7%), *BPH21+BPH17-ptb*-PYL (50.1%), and *BPH2+BPH3+BPH17*-PYL (57.6%) was less than 60%. The FBRR of five PYLs: *BPH2+BPH17*-PYL (64.5%), *BPH2+BPH25*-PYL (68.4%), *BPH20+BPH21*-PYL (62.3%), *BPH2+BPH32+BPH17-ptb*-PYL (64.0%) and *BPH20+BPH21+BPH32*-PYL (63.2%) ranged from 60% to 70%, and the FBRR of six PYLs: *BPH3+BPH17*-PYL (79.3%), *BPH17+BPH21*-PYL (70.9%), *BPH20+BPH32*-PYL (71.6%), *BPH21+BPH25*-PYL (70.3%), *BPH25+BPH17-ptb*-PYL (78.9%), and *BPH32+BPH17-ptb*-PYL (74.4%) ranged from 70% to 80%. Additionally, DS and FBRR were positively correlated (Pearson's C = 0.89, $P < 0.001$).

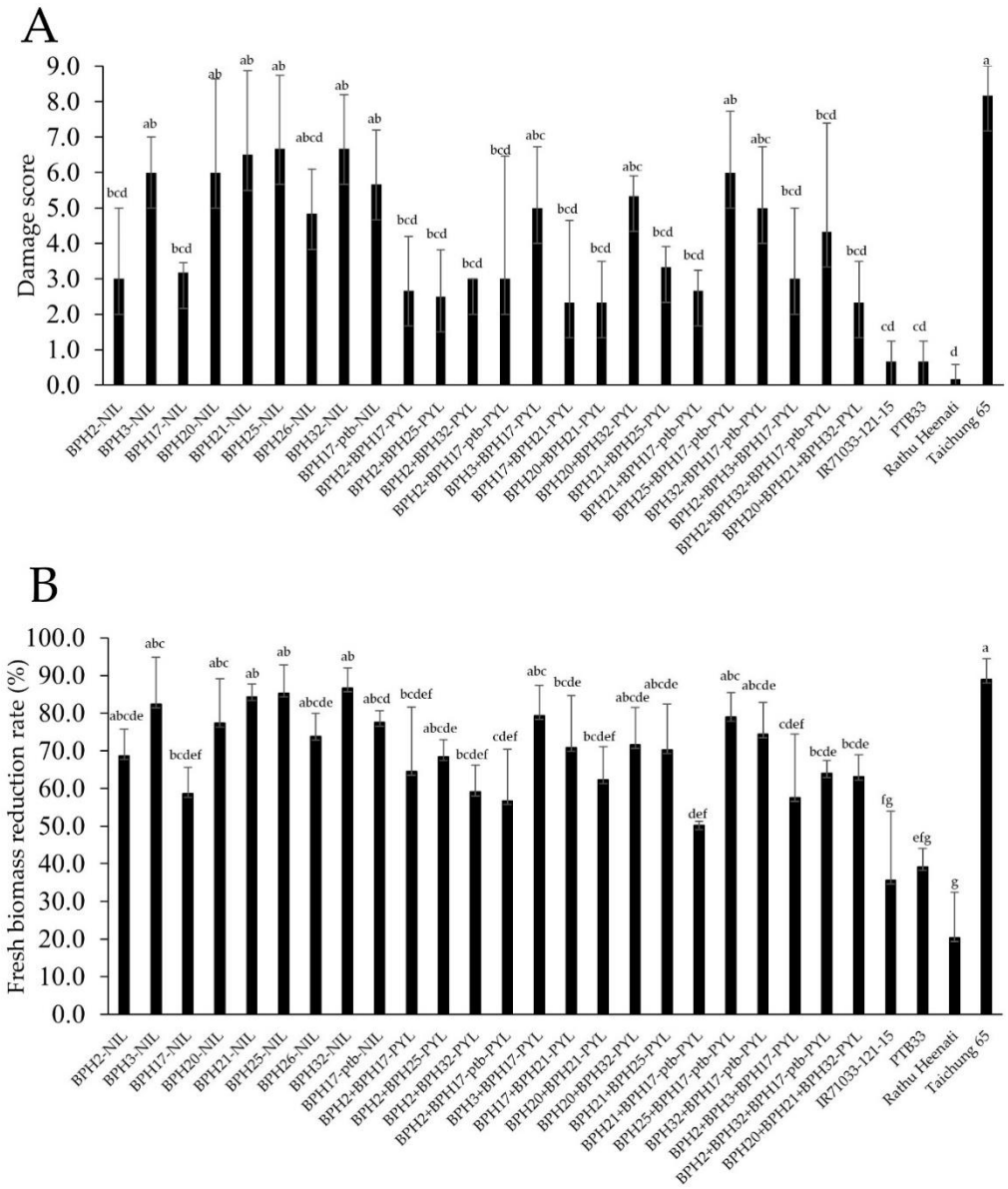


Figure 2.3. Damage score (A) and fresh biomass reduction rate (B) of near-isogenic lines and pyramided lines infested by the Hadano-1966 BPH population using the modified seedbox screening test at the seedling stage. The lower damage scores and fresh biomass reduction rates indicate higher resistance levels.

3.4. Comparison of adult mortality (ADM) of the Hadano-66 population on the NILs and PYLs

The ADM of the donor parents 'IR71033-121-15', 'PTB33' and 'Rathu Heenati' (100%), was significantly higher than that of T65 (17.6%) (**Table 2.4**). Among the NILs, *BPH2*-NIL and *BPH17*-NIL had the highest ADM, 68.9% and 59.0%, respectively. The ADM on other NILs was not significantly different from that on T65. The PYLs carrying the *BPH2: BPH2*+*BPH17*-PYL (75.0%), *BPH2*+*BPH25*-PYL (87.5%), *BPH2*+*BPH32*-PYL (84.0%), and *BPH2*+*BPH17-ptb*-PYL (84.0%) showed the highest ADMs among the PYLs with two genes and higher than any of the corresponding NILs. The ADMs of seven PYLs: *BPH3*+*BPH17*-PYL, *BPH17*+*BPH21*-PYL, *BPH20*+*BPH32*-PYL, *BPH21*+*BPH25*-PYL, *BPH21*+*BPH17-ptb*-PYL, *BPH25*+*BPH17-ptb*-PYL and *BPH32*+*BPH17-ptb*-PYL, ranged from 50.0% to 68.0%, while the ADM of *BPH20*+*BPH21*-PYL was 33.3%. The ADMs of PYLs for three genes: *BPH2*+*BPH3*+*BPH17*-PYL (96.0%), *BPH2*+*BPH32*+*BPH17-ptb*-PYL (95.8%), and *BPH20*+*BPH21*+*BPH32*-PYL (92.0%) were higher than those of the corresponding NILs and PYLs for two genes and were similar to the ADMs of the donor parents (100%). Furthermore, the ADMs showed a negative correlation with DS (Pearson's C = -0.79, $P < 0.001$) and FBRR (Pearson's C = -0.76, $P < 0.001$).

Table 2.4. The adult mortality of *Nilaparvata lugens* on near-isogenic lines and pyramided lines carrying brown planthopper resistance genes.

Entry	Adult mortality (%)	
	Hadano-66	Koshi-2013
BPH2-NIL	68.9 ± 28.5 ^{abcd}	4.0 ± 8.9 ^{b}
BPH3-NIL	30.0 ± 38.0 ^{def}	0.0 ± 0.0 ^{b}
BPH17-NIL	59.0 ± 25.1 ^{abcde}	20.0 ± 14.1 ^{b}
BPH20-NIL	24.0 ± 22.7 ^{def}	4.0 ± 8.9 ^{b}
BPH21-NIL	36.0 ± 37.5 ^{cdef}	12.0 ± 17.9 ^{b}
BPH25-NIL	16.0 ± 15.8 ^{f}	16.0 ± 16.7 ^{b}
BPH26-NIL	50.0 ± 41.4 ^{abcdef}	4.0 ± 8.9 ^{b}
BPH32-NIL	14.0 ± 16.5 ^{f}	12.0 ± 17.9 ^{b}
BPH17-ptb-NIL	22.0 ± 19.9 ^{ef}	20.0 ± 20.0 ^{b}
BPH2+BPH17-PYL	75.0 ± 19.5 ^{abcd}	32.0 ± 17.9 ^{b}
BPH2+BPH25-PYL	87.5 ± 17.9 ^{ab}	12.0 ± 11.0 ^{b}
BPH2+BPH32-PYL	84.0 ± 16.7 ^{abc}	16.0 ± 16.7 ^{b}
BPH2+BPH17-ptb-PYL	84.0 ± 16.7 ^{abc}	16.0 ± 16.7 ^{b}
BPH3+BPH17-PYL	50.0 ± 32.5 ^{abcdef}	24.0 ± 16.7 ^{b}
BPH17+BPH21-PYL	58.3 ± 27.5 ^{abcdef}	16.0 ± 21.9 ^{b}
BPH20+BPH21-PYL	33.3 ± 22.2 ^{cdef}	24.0 ± 16.7 ^{b}
BPH20+BPH32-PYL	62.5 ± 38.5 ^{abcde}	36.0 ± 21.9 ^{b}
BPH21+BPH25-PYL	64.0 ± 26.1 ^{abcde}	24.0 ± 16.7 ^{b}
BPH21+BPH17-ptb-PYL	54.2 ± 35.0 ^{abcdef}	8.0 ± 17.9 ^{b}
BPH25+BPH17-ptb-PYL	68.0 ± 26.8 ^{abcde}	16.0 ± 16.7 ^{b}
BPH32+BPH17-ptb-PYL	62.5 ± 32.9 ^{abcde}	20.0 ± 14.1 ^{b}
BPH2+BPH3+BPH17-PYL	96.0 ± 8.9 ^{ab}	36.0 ± 38.5 ^{b}
BPH2+BPH32+BPH17-ptb-PYL	95.8 ± 8.9 ^{ab}	20.8 ± 20.1 ^{b}
BPH20+BPH21+BPH32-PYL	92.0 ± 11.0 ^{ab}	28.0 ± 26.8 ^{b}
'IR71033-121-15'	100.0 ± 0.0 ^{a}	44.0 ± 16.7 ^{ab}
'PTB33'	100.0 ± 0.0 ^{a}	36.0 ± 21.9 ^{b}
'Rathu Heenati'	100.0 ± 0.0 ^{a}	84.0 ± 35.8 ^{a}
'Taichung 65'	17.6 ± 16.7 ^{f}	5.0 ± 10.0 ^{b}

Values of parameter (mean± standard deviation) followed by the same letter are not significantly different between each brown planthopper population ($P < 0.05$, Tukey-Kramer multiple comparison tests).

3.5. Comparison of ADM for the Koshi-2013 population on the NILs and PYLs

T65 was susceptible to the Koshi-2013 population with ADM of 5.0%. 'Rathu Heenati' had the highest ADM among entries (84.0%) which was significantly higher than T65 (**Table 2.4**). The ADMs of the other donor parents, 'IR71033-121-15' (44.0%) and 'PTB33' (36.0%), were lower than that of 'Rathu Heenati'. The ADMs on the NILs were less than or equal to 20.0%. Among the PYLs, ADMs of *BPH2*+*BPH17*-PYL (32.0%), *BPH20*+*BPH32*-PYL (36.0%) and *BPH2*+*BPH3*+*BPH17*-PYL (36.0%) were highest. The ADMs on the six PYLs, *BPH3*+*BPH17*-PYL, *BPH20*+*BPH21*-PYL, *BPH21*+*BPH25*-PYL, *BPH32*+*BPH17-ptb*-PYL, *BPH2*+*BPH32*+*BPH17-ptb*-PYL and *BPH20*+*BPH21*+*BPH32*-PYL, ranged from 20% to 28%. The ADMs of the other PYLs, *BPH2*+*BPH32*-PYL, *BPH2*+*BPH17-ptb*-PYL, *BPH17*+*BPH21*-PYL, *BPH25*+*BPH17-ptb*-PYL, *BPH2*+*BPH25*-PYL and *BPH21*+*BPH17-ptb*-PYL, ranged from 8.0% to 16.0%.

3.6. Agronomic characteristic of the NILs and PYLs

Six agronomic traits: days to heading (DTH), panicle length (PL), culm length (CL), flag leaf length (LL), flag leaf width (LW) and panicle number per plant (PN) of the NILs and PYLs are presented in **Table 2.5**. DTH and PN of the NILs and PYLs were not significantly different from those of T65. The PL, CL, LL and LW were similar for NILs and T65, except that the *BPH2*-NIL had longer culms, *BPH25*-NIL had shorter panicles, and *BPH3*-NIL had wider flag leave. The PL, CL, LL and LW were not significantly different between the PYLs and T65, except that *BPH2*+*BPH17*-PYL, *BPH2*+*BPH25*-PYL, *BPH2*+*BPH32*-PYL, *BPH2*+*BPH17-ptb*-PYL, *BPH21*+*BPH25*-PYL, and *BPH2*+*BPH3*+*BPH17*-PYL had longer culms, *BPH21*+*BPH25*-PYL had longer flag leave,

Table 2.5. Agronomic traits of near-isogenic lines and pyramided lines for brown planthopper resistance genes.

Entry	Average of agronomic trait (AVE ± SD)					
	DTH (day)	CL (cm)	LL (cm)	LW (cm)	PL (cm)	PN
BPH2-NIL	104.0 ± 2.0	120.3 ±3.2 *	36.8 ±4.5	1.1 ± 0.0	23.8 ± 0.7	16.6 ± 3.2
BPH3-NIL	100.8 ± 1.3	104.3 ±4.8	30.2 ±3.3	1.3 ± 0.0 *	18.9 ± 1.0	16.2 ± 1.5
BPH17-NIL	98.6 ± 1.3	103.2 ±7.5	33.8 ±8.0	1.2 ± 0.1	23.4 ± 2.2	15.2 ± 3.1
BPH20-NIL	100.8 ± 1.6	94.2 ±5.6	29.7 ±4.5	1.1 ± 0.0	20.5 ± 2.0	14.8 ± 4.1
BPH21-NIL	100.8 ± 1.5	104.2 ±2.9	32.6 ±3.0	1.0 ± 0.1	20.9 ± 1.2	13.8 ± 2.6
BPH25-NIL	100.4 ± 0.9	100.0 ±1.9	26.7 ±2.8	1.1 ± 0.1	18.1 ± 1.9 **	17.6 ± 1.1
BPH26-NIL	98.4 ± 0.9	102.1 ±0.9	27.3 ±3.2	1.1 ± 0.0	22.0 ± 1.6	13.4 ± 1.8
BPH32-NIL	100.2 ± 1.8	97.4 ±1.5	29.3 ±1.0	1.2 ± 0.0	20.9 ± 0.9	15.2 ± 1.6
BPH17-ptb-NIL	99.0 ± 0.0	97.4 ±2.3	29.6 ±1.9	1.1 ± 0.0	20.7 ± 1.0	14.6 ± 1.7
BPH2+BPH17-PYL	104.6 ± 1.3	115.1 ±7.9 *	27.3 ±2.6	1.2 ± 0.0	19.8 ± 1.6	13.8 ± 2.0
BPH2+BPH25-PYL	103.0 ± 1.4	116.2 ±1.7 *	30.2 ±4.7	1.1 ± 0.1	21.0 ± 0.9	14.2 ± 1.6
BPH2+BPH32-PYL	104.8 ± 1.3	111.4 ±8.0 *	27.8 ±5.5	1.0 ± 0.0 **	19.6 ± 1.6	17.8 ± 3.7
BPH2+BPH17-ptb-PYL	100.8 ± 0.8	110.7 ±2.3 **	26.9 ±5.0	1.2 ± 0.1	23.0 ± 1.1	15.2 ± 2.7
BPH3+BPH17-PYL	102.4 ± 1.3	103.3 ±0.5	29.7 ±3.3	1.5 ± 0.1 *	18.8 ± 0.9	17.4 ± 2.8
BPH17+BPH21-PYL	98.0 ± 0.0	91.5 ±1.8	30.4 ±3.7	1.0 ± 0.1 *	21.9 ± 1.7	14.6 ± 1.5
BPH20+BPH21-PYL	102.8 ± 1.1	77.9 ±3.3	34.7 ±6.1	1.2 ± 0.0	23.2 ± 2.4	18.2 ± 2.3
BPH20+BPH32-PYL	105.2 ± 0.4	88.3 ±1.8	34.3 ±5.8	0.9 ± 0.1 *	19.3 ± 1.7	16.0 ± 5.2
BPH21+BPH25-PYL	102.8 ± 1.3	110.5 ±4.9 **	41.7 ±3.5 *	1.1 ± 0.1	23.0 ± 1.9	18.0 ± 3.6
BPH21+BPH17-ptb-PYL	102.8 ± 2.5	107.5 ±4.6	33.6 ±4.5	1.1 ± 0.1	22.4 ± 1.6	16.0 ± 2.6
BPH25+BPH17-ptb-PYL	99.6 ± 1.9	99.6 ±2.4	28.9 ±3.7	1.2 ± 0.1	19.9 ± 2.0	15.8 ± 4.8
BPH32+BPH17-ptb-PYL	99.0 ± 0.0	107.6 ±3.5	26.7 ±3.4	1.1 ± 0.0	20.4 ± 2.2	18.0 ± 1.6
BPH2+BPH3+BPH17-PYL	103.4 ± 2.9	119.0 ±2.6 *	31.4 ±2.2	1.1 ± 0.1	22.4 ± 0.7	16.0 ± 3.4
BPH2+BPH32+BPH17-ptb-PYL	100.6 ± 1.1	102.4 ±4.4	26.2 ±3.2	1.0 ± 0.1	19.1 ± 0.3	16.6 ± 5.2
BPH20+BPH21+BPH32-PYL	101.2 ± 0.4	91.7 ±3.1	26.6 ±4.5	1.0 ± 0.1	19.3 ± 1.4	17.8 ± 2.8
'Taichung 65'	99.2 ± 0.4	91.4 ±2.7	29.7 ±4.2	1.1 ± 0.1	21.2 ± 0.7	14.6 ± 1.5

DTH: days to heading, CL: culm length, PL: panicle length, LL: flag leaf length, LW: flag leaf width, PN: panicle number per plant. *P<0.01, **P<0.05 (Dunnett's test with the control is 'Taichung 65').

BPH2+*BPH32*-PYL, *BPH17*+*BPH21*-PYL and *BPH20*+*BPH32*-PYL had narrower flag leave, and *BPH3*+*BPH17*-PYL had wider flag leave.

4. Discussion

The seven developed NILs carried BPH resistance genes on the short arm of chromosome 4 (*BPH17*-NIL, *BPH20*-NIL and *BPH17*-*ptb*-NIL), on the short arm of chromosome 6 (*BPH3*-NIL and *BPH32*-NIL) and on the long arm of chromosome 12 (*BPH2*-NIL and *BPH21*-NIL). One of the resistance genes on chromosome 12, *BPH2*, was identified from 'ASD7' which used as a donor parent for many modern resistant varieties (e.g., 'IR36', 'IR42' and so on) (Athwal *et al*. 1971, Khush and Virk 2005). *BPH2* from 'ASD7' is identical to *BPH26* in DNA sequence and resistance level (Tamura *et al*. 2014). *BPH2* from 'ASD7' was resistant against Hatano-66 (synonym of Hadano-66) but susceptible to Nishigoshi-05, a BPH population collected in Koshi, Kumamoto Pref. in 2005 (Myint *et al*. 2009 a). 'PTB33' was reported to carry one dominant and one recessive gene (Sidhu *et al*. 1978) those were confirmed to be *BPH2* and *BPH3* by conventional genetic analysis (Angeles *et al*. 1986). However, there was no report of the exact location of *BPH2* from 'PTB33'. In our study, *BPH2*-NIL had similar resistance patterns to *BPH2* on 'ASD7': *BPH2*-NIL was highly resistant (ADM of 68.9%) against the Hadano-66 population but less effective (ADM of 4.0%) against the recently collected population, Koshi-2013. Moreover, *BPH2*-NIL ('PTB33') and *BPH26*-NIL had similar resistance levels against both Hadano-66 and Koshi-2013, suggesting that 'PTB33', 'ADR52' and 'ASD7' might harbor the same resistance gene. Further sequence analysis for *BPH2* from 'PTB33' is necessary to understand its genetic basis. Another gene on chromosome 12, *BPH21,* was identified from 'IR71033-121-15', an introgression line derived from *O. minuta,* and estimated between two markers, S12094A and B122, on the long arm of

chromosome 12 (Rahman *et al*. 2009). Recently, *BPH21* has been reported to be allelic to *BPH26* (Zhao *et al*. 2016) and *BPH18* (Xiao *et al*. 2016 a) based on amino acid sequences. Both *BPH18* and *BPH26* were isolated and located at 22.9 Mbp on chromosome 12 (Ji *et al*. 2016, Tamura *et al*. 2014). Therefore, we estimated that the location of *BPH21* is around 22.9 Mbp on chromosome 12, and the region carrying *BPH21* from 'IR71033-121-15' was selected using RM1246 (19.2 Mbp) and RM28493 (23.3 Mbp) in this study.

The *BPH17* locus on chromosome 4S from 'Rathu Heenati' has been reported by Sun *et al*. (2005). *BPH17* was mapped between two markers, RHD9 (6.2 Mbp) and RHC10 (7.0 Mbp), on chromosome 4S and isolated by Liu *et al*. (2014). The amino acid sequence and chromosomal location of *BPH17* from 'Rathu Heenati' were the same as that of *BPH17-ptb* from 'PTB33' (Liu *et al*. 2014). In this study, resistance of *BPH17*-NIL and *BPH17-ptb*-NIL against the Hadano-66 populations differed, however, both NILs had similar effects on the Koshi-2013 population. The different resistant levels might be because the loci were derived from different accessions or varieties of rice. Therefore, the amino acid sequences of 'PTB33' and 'Rathu Heenati' on *BPH17* locus should be determined for future research. Additionally, *BPH20* was detected between two markers, B42 (8.7 Mbp) and B44 (8.9 Mbp) on chromosome 4 (Rahman *et al*. 2009). Two NILs for *BPH17* and *BPH20* on the genetic background of 9311 variety developed by Xiao *et al*. (2016 a) showed different resistance levels against a BPH population from China. In our study, the resistance levels of *BPH17* and *BPH20* were different in both MSST and antibiosis tests against the Hadano-66 population and in antibiosis tests against the Koshi-2013 population, which corresponded well with previous research by Xiao *et al*. (2016 a). Therefore, the genes on chromosome 4S of 'IR71033-121-15', 'PTB33' and 'Rathu Heenati' might be different. To confirm this, further sequence analyses are needed for the three loci *BPH17, BPH17-ptb* and *BPH20*.

Among six genes/QTLs have been identified on the short arm of chromosome 6 of *O. sativa* and its wild relatives (Fujita *et al*. 2013, Ren *et al*. 2016), *BPH3* and *BPH32* have been widely introduced to elite rice cultivars to improve BPH resistance and were related to durable and broad-spectrum resistance in 'PTB33' and 'Rathu Heenati' (Khush and Virk 2005, Jairin *et al*. 2007 a). In previous research, *BPH3* was mapped on chromosome 6 between two markers, RM19291 (1.2 Mbp) and RM8072 (1.4 Mbp) (Jairin *et al*. 2007 a). *BPH32* from 'PTB33' was identified at the same location as *BPH3* from 'Rathu Heenati', but the amino acid sequence of *BPH3* was not identical to that of *BPH32* (Ren *et al*. 2016). In our study, the resistance levels of the *BPH3*-NIL were slightly different from those of the *BPH32*-NIL suggesting that *BPH3* might be different from *BPH32*. A comparison of amino acid sequences between *BPH3* and *BPH32* would be necessary to confirm whether these resistance genes are different.

An improvement of rice resistance levels against BPH is necessary since many genes have become less effective against BPH across Asia (Horgan *et al*. 2015). In this study, we developed 15 PYLs carrying two or three genes for BPH resistance. The developed PYLs tended to increase resistance against the two BPH populations, Hadano-66 and Koshi-2013. Among the 15 PYLs, 12 and nine PYLs had higher ADMs than corresponding NILs against Hadano-66 and Koshi-2013, respectively; ten PYLs had lower FBRR compared to corresponding NILs in the MSST against the Hadano-66 population. For example, *BPH2*+*BPH32*-PYL (84.0%) and *BPH2*+*BPH32*+*BPH17-ptb*-PYL (95.8%) had higher resistance levels than those of the *BPH2*-NIL (68.9%), *BPH32*-PYL (14.0%) and *BPH17-ptb*-PYL (22.0%) in antibiosis tests against the Hadano-66 population. The ADMs of *BPH2*+*BPH17*-PYL (32.0%) and *BPH2*+*BPH3*+*BPH17*-PYL (36.0%) were higher than those of *BPH2*-NIL (4.0%), *BPH3*-NIL (0%) and *BPH17*-NIL (20.0%) against the Koshi-2013 population. Additionally, the FBRR of *BPH2*+*BPH17* (42.3%) was lower

than for *BPH2*-NIL (67.6%) and *BPH17*-NIL (58.8%). However, the effectiveness of the PYLs was not consistently higher than that of the corresponding NILs. The effect of PYLs was influenced by specific interactions between gene loci, the specific BPH populations and the screening methods. For example, the resistance level of *BPH3+BPH17*-PYL (50.0%) and *BPH17+BPH21*-PYL (58.3%) was not higher than that of *BPH17*-NIL (59.0%) in antibiosis tests against the Hadano-66 population. *BPH2+BPH25*-PYL (87.5%) showed higher ADM against the Hadano-66 population in comparison to *BPH2*-NIL (68.9%) and *BPH25*-NIL (16.0%), while the ADM of *BPH2+BPH25*-PYL (12.0%) was lower than that of *BPH25*-NIL (16.0%) against the Koshi-2013 population. A similar tendency has been reported for gene combinations between *BPH1* and *BPH2* (Sharma *et al*. 2004); *BPH18* and *BPH32*; *BPH20* and *BPH32*; *BPH2*, *BPH18* and *BPH32* (Jena *et al*. 2017).

In a previous study, virulence of the BPH population collected in 2005 from Japan had increased compared with the virulence of BPH collected in 1966 (Myint *et al*. 2009 a). Through antibiosis tests, we evaluated BPH resistance against the populations collected in 1966 (Hadano-66) and 2013 (Koshi-2013). Both represent BPH arriving as migrants to Japan. The Hadano-66 population was virulent to T65 (with no resistance gene) but avirulent to all plants with resistance genes, including 'Mudgo' (*BPH1*), 'ASD7' (*BPH2*), 'Rathu Heenati' (*BPH3* and *BPH17*), 'Babawee' (*BPH4*), 'Chin Saba' (*BPH8*), 'Balamawee' (*BPH9*) and two NILs, *BPH25*-NIL and *BPH26*-NIL (Myint *et al*. 2009 a, Myint *et al*. 2009 b). In the present study, most of the NILs, all of the PYLs and the donor parents are still effective against the Hadano-66 population. In contrast, all of NILs and most of the PYLs were susceptible to the Koshi-2013 population, suggesting that BPH recently arriving from China to Japan have greater virulence than was evident about 50 years ago (i.e., 1966). Among the PYLs, two PYLs, *BPH20+BPH32*-PYL and

BPH2+BPH3+BPH17-PYL, had relatively higher resistance, suggesting that PYLs with combinations of these genes are likely to provide good resistance against current BPH population (Koshi-2013). Furthermore, finding new resistance gene sources will be necessary to improve resistance against BPH populations as these gain virulence. The resistance levels of 'PTB33', 'Rathu Heenati' and 'IR71033-121-15' were higher than those of the PYLs (*BPH2+BPH32+BPH17-ptb*-PYL, *BPH3+BPH17*-PYL and *BPH20+BPH21*-PYL, respectively), suggesting that 'PTB33', 'Rathu Heenati' and 'IR71033-121-15' might also contain other BPH resistance gene(s). The other genetic factor(s) for BPH resistance can be revealed through analyzing the segregating population derived from crosses between the developed PYLs and their donor parents in future studies.

5. Summary

To survey BPH virulence patterns, seven NILs each with a single BPH resistance gene (*BPH2*-NIL, *BPH3*-NIL, *BPH17*-NIL, *BPH20*-NIL, *BPH21*-NIL, *BPH32*-NIL and *BPH17-ptb*-NIL) and fifteen PYLs carrying multiple resistance genes were developed with the genetic background of the *japonica* rice variety, T65, and assessed resistance levels against two BPH populations (Hadano-66 and Koshi-2013 collected in Japan in 1966 and 2013, respectively). Many of the NILs and PYLs were resistant against the Hadano-66 population but were less effective against the Koshi-2013 population. Among PYLs, *BPH20+BPH32*-PYL and *BPH2+BPH3+BPH17*-PYL had relatively high BPH resistance against Koshi-2013. The NILs and PYLs developed in this research will be useful to monitor BPH virulence prior to exploiting resistant rice varieties and improve rice resistance to BPH in the context of regionally increasing levels of virulence.

Chapter 3

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

1. Introduction

Since the late 1960s, the improvement of host plant resistance in rice has been one of the strategies to reduce BPH damage. Among more than 40 loci for BPH resistance, seven genes (*BPH6*, *BPH7*, *BPH15*, *BPH27*, *BPH28*(t), *BPH33*, and *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, Qiu *et al*. 2014, Yang *et al*. 2004, Wu *et al*. 2014). 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). The mapping of BPH resistance genes has facilitated the introduction of resistance genes using 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 favourable 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, 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, Li *et al*. 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*. 2007 b, 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*. 2007 c, 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'. 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. *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*. (2007 a) 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, 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 NILs for *BPH2* (*BPH2*-NIL), *BPH17-ptb* (*BPH17-ptb*-NIL), and *BPH32* (*BPH32*-NIL) have been developed on the genetic background of *japonica* cultivar T65. 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.

2. Materials and methods

2.1. Development of populations for QTL analysis and 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 (**Figure 3.1**). Ninety-six BC_4F_2 plants from each crossing were used to QTL analysis of BPH resistance genes. The BC_4F_2 plants were evaluated for BPH resistance using antibiosis (adult mortality) and genotyped by several SSR markers

around tentative locations of genes. Plants carrying *BPH2* were genotyped using SSR markers around location of *BPH26.* Plants carrying *BPH17-ptb* were genotyped with SSR markers around location of *BPH17.* Several SSR markers around *BPH32* were utilized for genotyping population with *BPH32.* After that, the plants carrying the recombinants around position of *BPH2*, *BPH17-ptb*, and *BPH32* were selected. Homozygous recombinant chromosome substitution lines were developed from selected recombinant plants. 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.

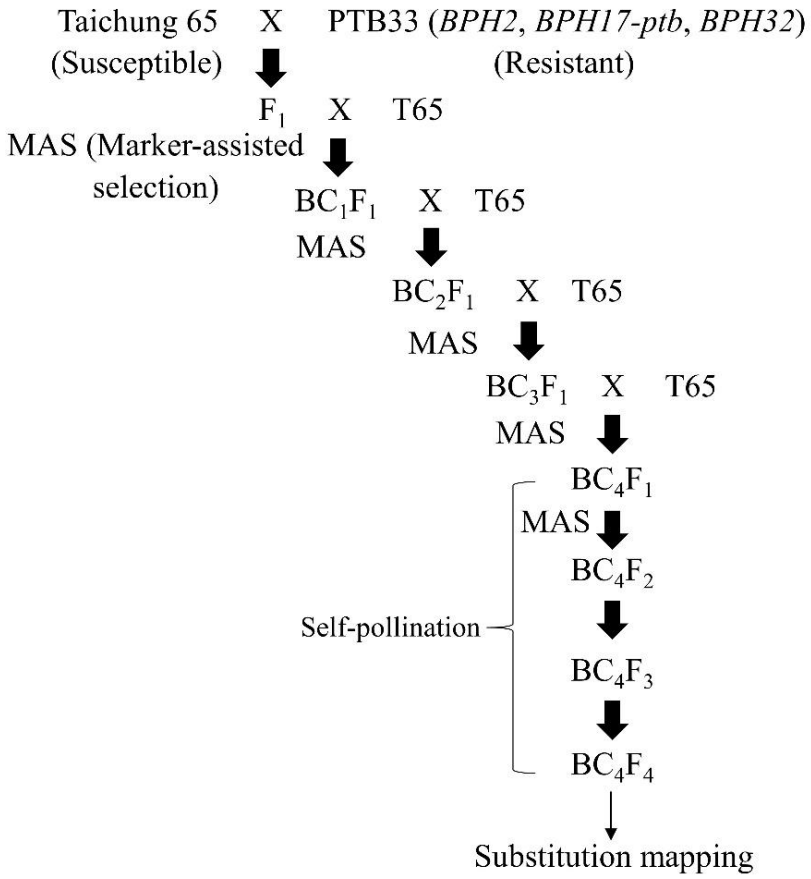


Figure 3.1. The breeding scheme for development of substitution mapping populations for *BPH2*, *BPH17-ptb*, and *BPH32*.

2.2. 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. Six SSR markers, RM277, RM1246, RM28493, RM1103, S12091B, and RM5479 on chromosome 12 L, were used for genotyping BC_4F_2 and recombinant BC_4F_3 plants segregating at *BPH2* (**Table 3.1**). 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) (**Table 3.1**).

Table 3.1. The SSR markers used for mapping of *BPH2*, *BPH17-ptb* and *BPH32*

Marker	Resistance gene tagged	Chr.	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Physical position (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
RM37268[c]	*BPH2*	12	TACACCCACCCACATACGTCAGC	GTCGTACTCCCGGATCTTCTTCC	23,275,244
RM28493[c]	*BPH2*	12	ACCGTTAGATGACACAAGCAACG	GGTTAGCAAGACTGGAGGAGACG	23,279,853
RM5479[b]	*BPH2*	12	CTCACCATAGCAATCTCCTGTGC	ACTTCGTTCACTTGCATCATGG	24,446,205
RM1103[b]	*BPH2*	12	GTCGGTGTGTACTCCGTGTTTGG	CATATGCAGTGGTCAGTGGAGTGG	23,606,775
S12091B[f]	*BPH2*	12	GGCTTTCTTCCTCACACTGC	CGAGGACGAGATGAGACGA	23,685,715
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[g]	*BPH17*-ptb	4	GGTAAGGTTGGGCGGTAG	AGTGAAGGGTGAGGGTGG	6,597,076
WH2[g]	*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[h]	*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] Liu *et al*. (2014) and [h] Yang *et al*. (2004). The physical position of marker was the physical location of forward primer for each marker obtained from The Rice Annotation Project Database (Sakai *et al*. 2013) based on the 'Nipponbare' genome sequence. Chr.: Chromosome.

2.3. 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.

2.4. 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 × 2.5 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 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. The experiments were performed in triplicates.

MMTS described by Jairin *et al*. (2007 b) 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 20 nymphs per tiller. Ten days after infestation (DAI), the DSs of the substitution lines and parents were determined.

2.5. Antibiosis on feeding rates

The feeding rates of BPH on the NILs were determined following the methods described by Heinrich *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).

2.6. 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.

2.7. 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:

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

2.8. Statistical analysis

The mean values of the DS 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 DSs 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.

3. Results

3.1. Detection of QTLs for BPH resistance on populations segregating at BPH2, BPH17-ptb and BPH32.

The segregation populations for *BPH2*, *BPH17-ptb* and *BPH32* was evaluated for BPH resistance individually against Hadano-66. Susceptible control, T65, had 24.0% of BPH ADM at 5 DAI, while the donor parent, 'PTB33' had 74.0% of ADM (**Figure 3.2**). Three populations showed the continuous frequency distributions for ADMs of BPHs on the F_2 plants. The QTL analysis was performed using data of F_2 plants phenotypes and genotypes of SSR markers surrounding target gene's locations. However, no QTL for BPH resistance was detected.

3.2. 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*, 13 plants carrying recombinant events that occurred between the two markers, RM277 and RM5479, on chromosome 12 were selected (**Figure 3.3**). Ten BC_4F_4 lines carrying different sizes of 'PTB33' substituted chromosomal segments were developed (**Figure 3.4**). 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',

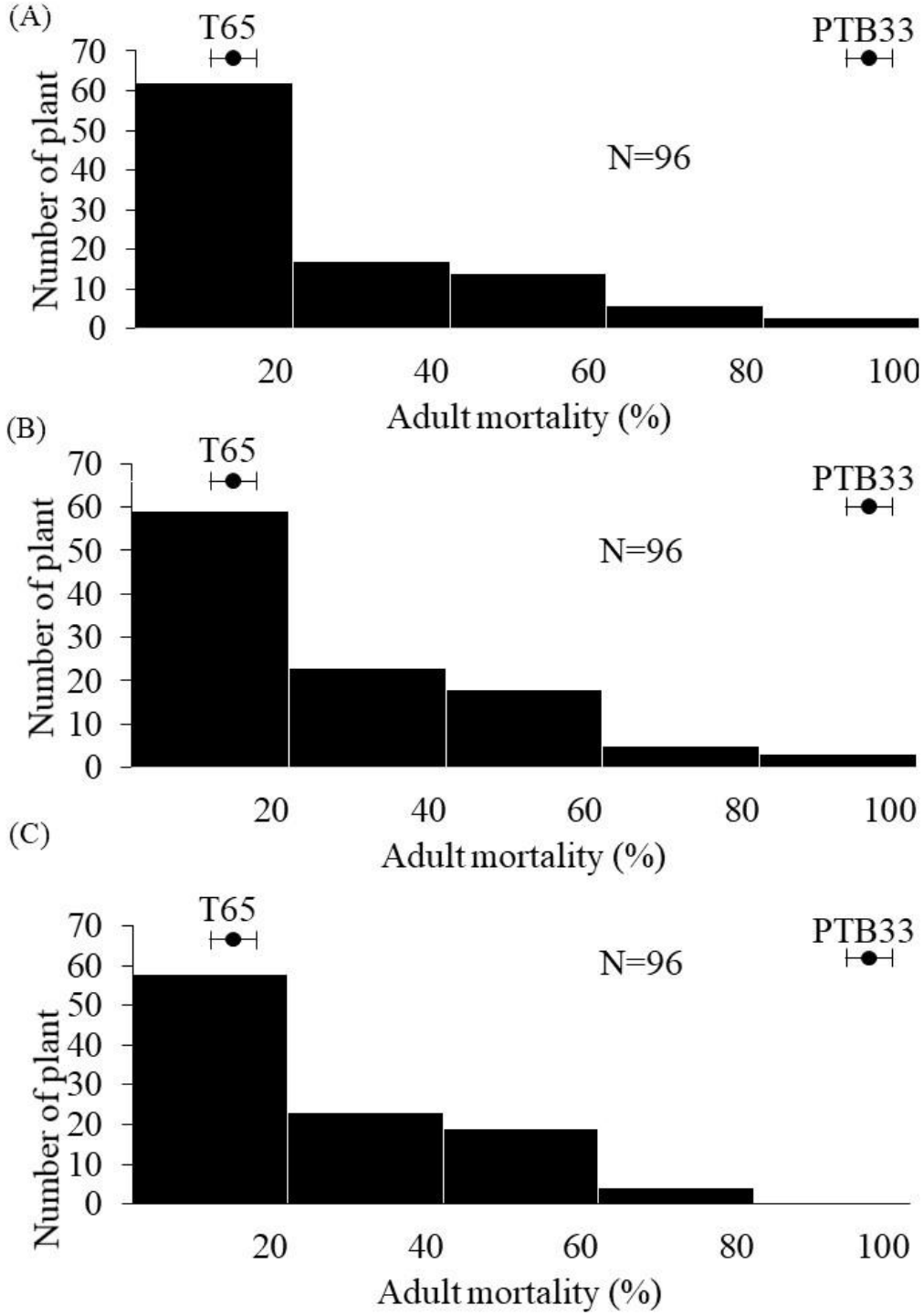


Figure 3.2. Frequency distributions of Koshi-2013 *Nilaparvata lugens* Stål. adult mortality on the BC_4F_2 populations carrying *BPH2* (A), *BPH17-ptb* (B) and *BPH32* (C).

A

Plant number	Markers (Mbp)						
	RM277 (18.32)	RM1246 (19.17)	RM3726 (23.28)	RM28493 (23.29)	RM1103 (23.61)	S12091B (23.69)	RM5479 (24.45)
1	1	1	1	1	1	1	1
49	3	3	1	1	1	1	1
16	1	1	1	1	3	3	3
46	1	1	1	1	3	3	2
88	1	1	1	1	1	1	3
86	1	1	3	3	3	3	3
96	2	2	1	1	1	1	1
11	2	2	2	2	2	3	3
82	2	2	2	2	2	2	3
22	2	2	3	3	3	3	3
102	3	3	1	1	1	1	1
90	3	3	2	2	2	2	2
43	3	3	3	3	2	2	2
71	3	3	3	3	3	3	1
21	2	2	2	2	2	2	2

B

Line	Markers (Mbp)																	
	RM277 (18.32)	RM1246 (19.17)	RM28305 (20.00)	RM28346 (20.99)	RM28396 (21.77)	RM28404 (21.89)	RM28424 (22.40)	RM28433 (22.60)	RM28449 (22.69)	ID-28L4 (22.88)	ID-174 (22.91)	ID-161 (22.94)	ID-161-2 (22.95)	RM3726 (23.28)	RM28493 (23.29)	RM1103 (23.61)	S12091B (23.69)	RM5479 (24.45)
T65	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1 - 1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16 - 1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2
24 - 2	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
9 - 3	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
2 - 2	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
5 - 3	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
1 - 3	2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
3 - 3	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
6 - 3	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17 - 4	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1
19 - 3	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1
10 - 1	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1
11 - 1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1
17 - 1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1
21 - 6	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
PTB33	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Figure 3.3. Selection of homozygous recombinant plants for substitution mapping of *BPH2*. A: BC_4F_2 plants carrying recombination events occurred around location of *BPH2*. B: All homozygous recombinant plants for *BPH2* at BC_4F_3. Denote of marker genotypes - 1: Taichung 65 homozygous, 2: PTB33 homozygous, 3: heterozygous.

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 (**Figure 3.4**). 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

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

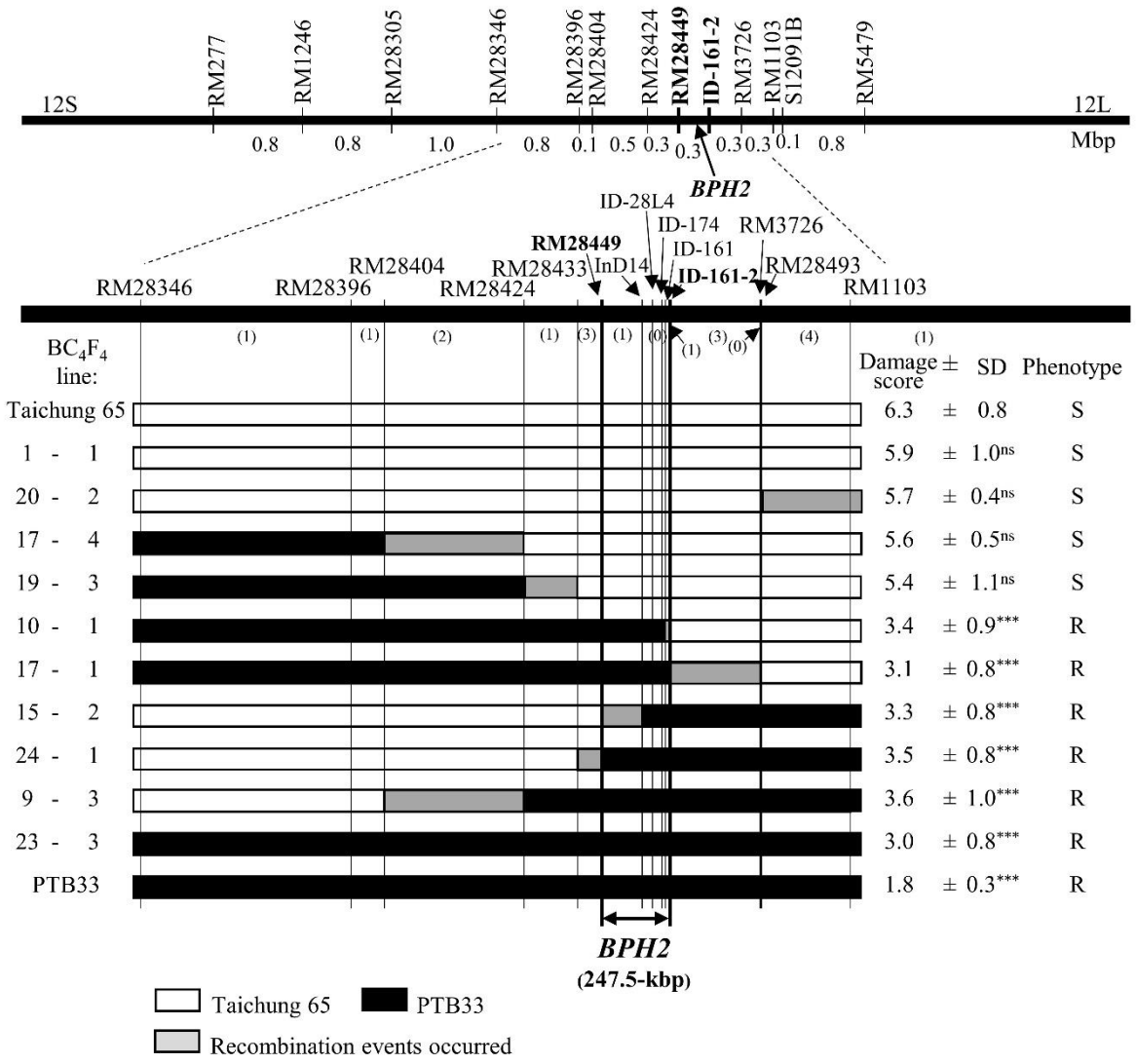


Figure 3.4. The substitution map of *BPH2* on chromosome 12. Upper line indicates the physical position of DNA markers around location of *BPH2* on chromosome 12L. Vertical bars indicate the positions of DNA markers and the numbers below the upper bar indicate physical distance between markers. The numbers 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.

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 (**Figure 3.5**). Four lines homozygous for 'PTB33' segments encompassing overlapping regions of *BPH17-ptb* were developed from the 11 selected recombinant plants (**Figure 3.6**). 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 (**Figure 3.6**).

A

Plant number	Markers (Mbp)		
	C61009 (2.43)	RM8213 (4.42)	B40 (8.21)
5	3	3	1
7	1	1	3
14	2	2	3
23	3	3	2
45	3	3	2
51	2	2	3
60	3	1	3
64	3	3	1
69	3	3	1
73	3	3	2
90	3	3	1

B

Line	Markers (Mbp)															
	C61009 (2.43)	RM8213 (4.42)	RM16460 (5.32)	RM3658 (5.57)	RM1305 (5.62)	RM16479 (5.94)	RM16480 (6.00)	RM16482 (6.02)	RM3471 (6.28)	RHD3 (6.60)	RM16508 (7.00)	RM16514 (7.21)	MS5 (7.25)	RM6156 (7.86)	RM16531 (7.94)	B40 (8.21)
T65	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
43 - 6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
44 - 5	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	
46 - 5	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	
47 - 1	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	
44 - 4	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
PTB33	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Figure 3.5. Selection of homozygous recombinant plants for substitution mapping of *BPH17-ptb*. A: BC_4F_2 plants carrying recombination events occurred around location of *BPH17-ptb*. B: All homozygous recombinant plants for *BPH7-ptb* at BC_4F_3. Denote of marker genotypes - 1: Taichung 65 homozygous, 2: PTB33 homozygous, 3: heterozygous.

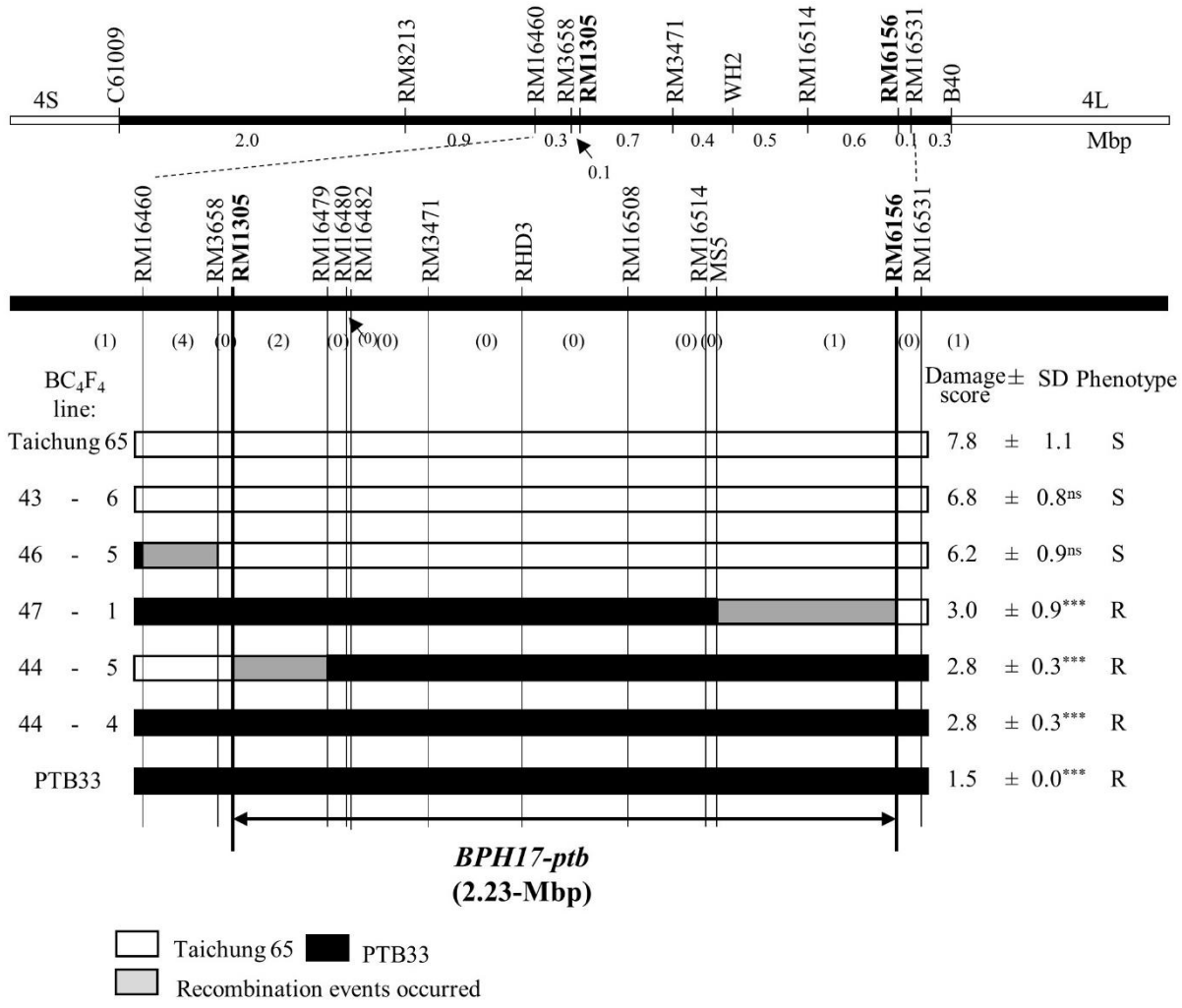


Figure 3.6. The substitution map of *BPH17-ptb* on chromosome 4. Upper line indicates the physical position of DNA markers around location of *BPH17-ptb* on chromosome 4S. Vertical bars indicate the position of DNA markers and the numbers below the upper line indicate physical distance between markers. 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.

From 96 BC_4F_2 plants for *BPH32,* ten plants with recombination events between RM6775 and RM19341 were selected (**Figure 3.7**). 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 (**Figure 3.8**). 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 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 (**Figure 3.8**).

A

Plant number	Markers (Mbp)					
	RM6775 (0.21)	S00310 (0.22)	RM508 (0.44)	RM586 (1.48)	RM588 (1.61)	RM19341 (1.76)
22	2	2	2	2	2	3
56	2	2	3	3	3	3
59	3	3	1	1	1	1
68	3	3	3	3	3	2
72	3	3	3	2	2	2
74	2	2	3	1	1	1
87	3	3	3	3	3	2
91	3	3	3	3	3	2
100	3	3	2	2	2	2
103	3	3	3	3	3	2

B

Line	Markers (Mbp)										
	RM6775 (0.21)	S00310 (0.22)	RM508 (0.44)	RM19288 (1.18)	RM19291 (1.22)	RM19296 (1.30)	RM589 (1.38)	RM19311 (1.46)	RM586 (1.48)	RM588 (1.61)	RM19341 (1.76)
T65	1	1	1	1	1	1	1	1	1	1	1
36 - 1	1	1	1	1	1	1	1	1	1	1	1
40 - 1	1	1	1	1	1	1	1	1	1	1	2
42 - 1	1	1	1	2	2	2	2	2	2	2	2
37 - 1	1	1	2	2	2	2	2	2	2	2	2
36 - 3	2	2	1	1	1	1	1	1	1	1	1
34 - 1	2	2	2	1	1	1	1	1	1	1	1
35 - 1	2	2	2	2	2	2	2	2	2	2	1
35 - 3	2	2	2	2	2	2	2	2	2	2	2
PTB33	2	2	2	2	2	2	2	2	2	2	2

Figure 3.7. Selection of homozygous recombinant plants for substitution mapping of *BPH32*. A: BC_4F_2 plants carrying recombination events occurred around location of *BPH32*. B: All homozygous recombinant plants for *BPH32* at BC_4F_3. Denote of marker genotypes - 1: Taichung 65 homozygous, 2: PTB33 homozygous, 3: heterozygous.

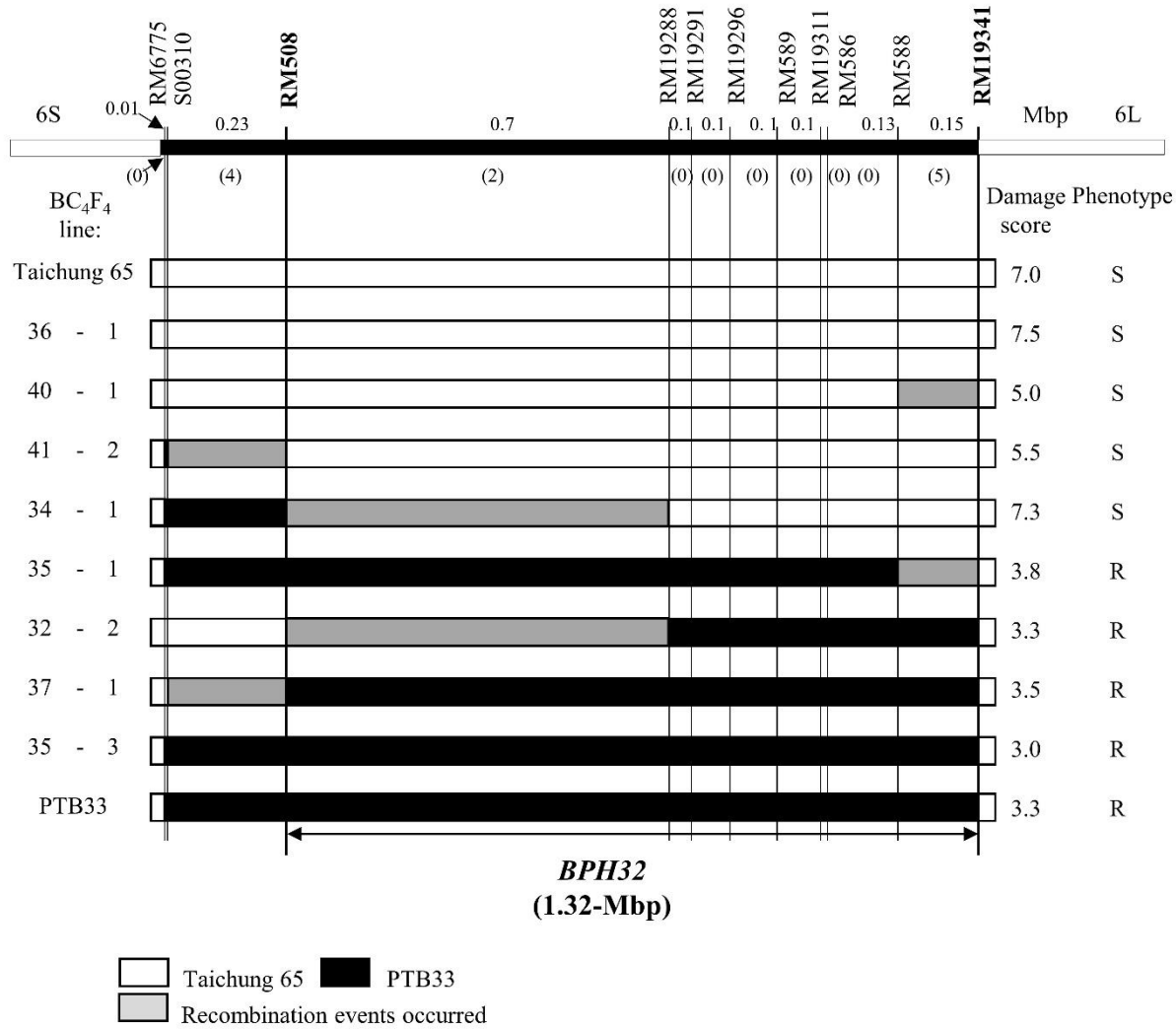


Figure 3.8. The substitution map of *BPH32* on chromosome 6. Vertical bars indicate the position of DNA markers. The numbers above the top bar indicate physical distance between the markers and the below ones indicate the number of recombinants. White rectangles are 'Taichung 65' homozygous; black rectangles are 'PTB33' homozygous; grey rectangles are the position where recombinant events occurred. S: susceptible; R: resistant.

3.3. 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 percentage of settling insect, feeding rate on honeydew area, and PFWL. 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 (**Figures 3.9A, 3.9B, 3.9C**). 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 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.

For antibiosis by honeydew test, the area of honeydew excreted was 70.34 mm^2 for T65 and 8.23 mm^2 for 'PTB33' (**Figure 3.9D**). 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.

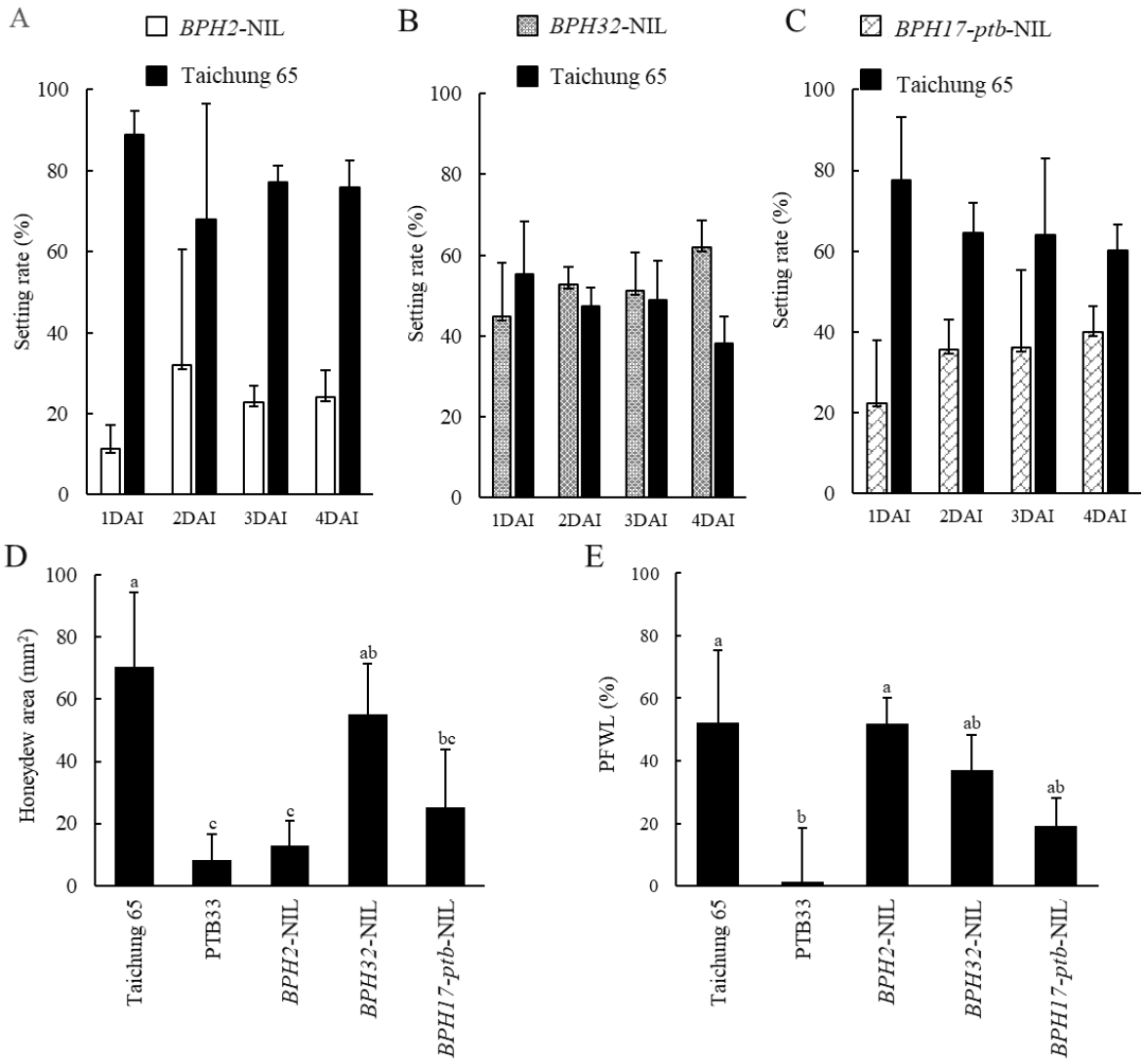


Figure 3.9. Antixenosis, antibiosis, and tolerance level of *BPH2*-NIL, *BPH17-ptb*-NIL, and *BPH32*-NIL against Hadano-66. (A) The percentages of insects settling on *BPH2*-NIL, (B) *BPH32*-NIL and (C) *BPH17-ptb*-NIL at 1 - 4 days after infestation (DAI). (D) Honeydew area excreted by insect feeding. (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.

The tolerance of the NILs was measured as the percentage of PFWL due to BPH (**Figure 3.9E**). 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.

4. 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 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*. 2009 b, Myint *et al*. 2012). 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*. 2009 b). The effectiveness of *BPH25* and *BPH26* against Hadano-66 was stronger than those of Isahaya-99 and Nishigoshi-05 (Myint *et al*. 2009 a). 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) (**Figure 3.10**). 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’

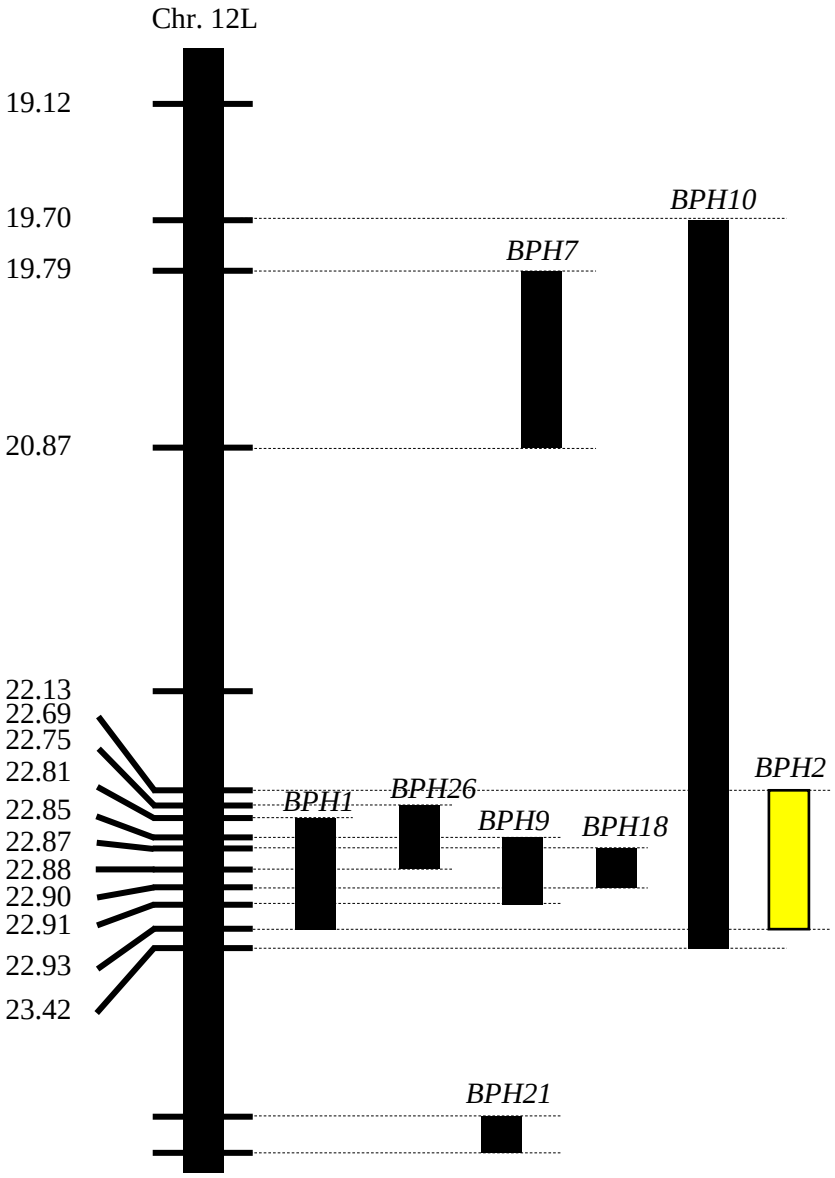


Figure 3.10. Physical locations of brown planthopper resistance genes on the long arm of chromosome 12.

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 5.62 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. 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) (**Figure 3.11**). Among these, *BPH17* (*BPH3* in Liu *et al*. 2014), which was identified from 'Rathu Heenati' on chromosome 4, had amino acid sequences that are identical to *BPH17-ptb* of 'PTB33' (Liu *et al*. 2014). However, there is possibility that the delimited region of *BPH17-ptb* 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*. (2007 b) and Ren *et al*. (2016). This result confirms the presence of *BPH32* on the *BPH32*-NIL plants developed in a previous study.

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*. 2007 b, Jena *et al*. 2017, 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*. (2007 b), *BPH32* was evaluated using the plant at the tillering stage and the Thai BPH population. Therefore, in

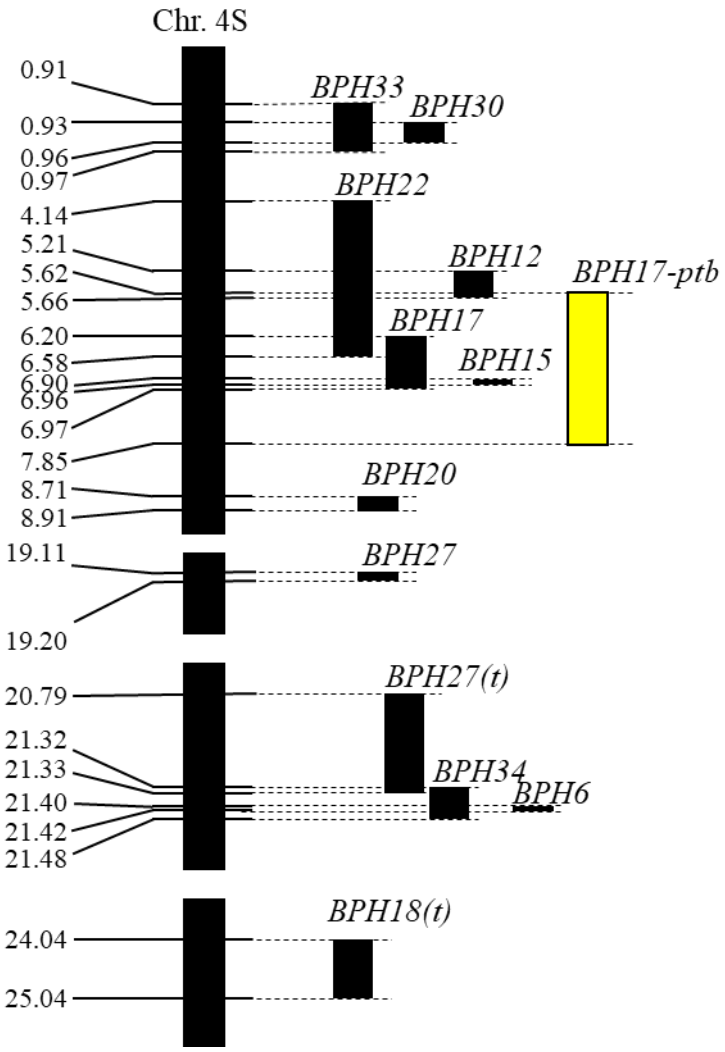


Figure 3.11. Physical locations of brown planthopper resistance genes on chromosome 4.

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 12 L (*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 paid 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 chapter.

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 (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. 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 PYLs with different resistance mechanisms is crucial for understanding the effect of different mechanisms to strengthen resistance level.

5. Summary

Characterization of location and resistance mechanism (antibiosis, antixenosis and tolerance) can accelerate the deployment of genes in breeding program. Although *BPH2*, *BPH17-ptb* and *BPH32* have been identified on ‘PTB33’, their exact locations and resistance mechanism remain unclear. This experiment focused on revealing exact locations and type of resistance mechanism of these three genes. *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. *BPH2* and *BPH17-ptb* showed 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’.

Chapter 4

Genetic analysis for brown planthopper resistance in *indica* cultivar 'Rathu Heenati' and two accessions of *O. nivara*

1. Introduction

Oryza genus belongs to Gramineae family that can distribute from 53 ^{o}N to 40 ^{o}S latitude. Based on morphology and genetics, the gene pool of rice with AA genome was classified into five wild species- *O. rufipogon* (including *O. nivara*), *O. glumaepatula, O. meridionalis, O. barthii, O. longistaminata*- and two cultivated species:- *O. sativa* and *O. glaberrima*. *O. sativa* is domesticated from *O. rufipogon* in Asia, while *O. glaberrima* is independently domesticated from *O. barthii* in Western Africa. *O. rufipogon* consists of perennial and annual plants, and the annual type is called as *O. nivara* in some cases. *O. nivara* is one of closest wild relatives of *O. sativa* and mainly distributed in South and Southeast Asia (Grillo *et al*. 2008). *O. nivara* and *O. sativa* have many common morphological characteristics including annual life cycle, selfing type of mating system, the erect and semi-erect tillers, a compact panicle shape, and small anther length (<2.5 mm). *O. nivara* carry many valuable characteristics against biotic stress: bacterial blight resistance, BPH resistance, green rice leafhopper resistance, and grassy stunt virus resistance (Heinrichs *et al*. 1985). Because *O. nivara* has the AA genome as same as *O. sativa*, it is possible to introduce desirable traits from *O. nivara* into cultivated rice (Jacquemin *et al*. 2013).

The host plant resistance of rice has been regarded as an effective means for reducing BPH damage. Among more than 46 BPH resistance loci identified, more than half of genes are originated from wild rice. *BPH10, BPH18,* and *qBPH4.2* have been identified from *O. australiensis* (Hu *et al*. 2015 b, Ishii *et al*. 1994, Ji *et al*. 2016). *BPH11*,

BPH12, *BPH13*, *BPH14*, *BPH15*, *qBPH3,* and *qBPH4* are from *O. officinalis* (Du *et al*. 2009, Hirabayashi *et al*. 1999, Hu *et al*. 2015 a, Qiu *et al*. 2012, Renganayaki *et al*. 2002, Yang *et al*. 2004). *BPH18*(t), *BPH19*(t), *BPH22*(t), *BPH23*(t), *BPH27*, *BPH29*, and *BPH30* have been detected from *O. rufipogon*. Two BPH resistance genes, *BPH20* and *BPH21*, were identified using an introgression line from *O. minuta*. Other genes, *BPH22*(t) and *BPH34* were detected from *O. glaberrima* and *O. nivara*, respectively. Therefore, wild rice is an important source for BPH resistance genes.

From the late of 1960s, study for host plant resistance has been started, and many cultivars carrying resistance genes were released. However, the effectiveness of resistance genes have decreased because of the increasing of BPH virulence. For example, in Japan, many single BPH resistance genes are effective against BPH population collected in 1966 but susceptible to one collected in 2013. Several previous studies described that many BPH resistance genes have lost their resistance against latest BPH populations. Horgan *et al*. (2015) reported that only six of 39 varieties were consistently resistance against BPHs in a multi-nation study in Asia. Therefore, identification of novel resistance genes in new genetic resource is crucial for dealing with the widely and extremely devastation of BPH.

Rathu Heenati, an *indica* variety originated from Sri Lanka, is being widely used as donor for BPH resistance. Two BPH resistance genes, *BPH3* and *BPH17* from 'Rathu Heenati', have been detected on chromosomes 6 and 4, respectively. In comparison of *BPH17* sequencing among *Oryza* genus with AA genome, *BPH17* from 'Rathu Heenati' has the highest similarity for sequences compared to that of *O. nivara* (Liu *et al*. 2014). This variety showed strong and durable resistance against many BPH populations in Asia. Although *BPH3* was overcome by "biotype 4", which is prominent in South and Southeast Asia, 'Rathu Heenati' has resistance against all "biotypes" of BPH (Horgan *et al*. 2015, Prahalada *et al*. 2017). In the chapter 2, the PYL for *BPH3* and *BPH17* (*BPH3+17*-PYL)

from ‘Rathu Heenati’ were developed and characterized for BPH resistance. The BPH resistance level of *BPH3+17*-PYL was higher than either *BPH3*-NIL or *BPH17*-NIL but significantly lower than that of ‘Rathu Heenati’. The difference in the resistance between ‘Rathu Heenati’ and *BPH3+17*-PYL suggests that ‘Rathu Heenati’ has other unknown genetic factors related to BPH resistance. In addition, two accessions of *O. nivara*, IRGC 89073 (from Laos) and IRGC 93005 (from Cambodia), have strong resistance against the Koshi-2013 (**Figure 4.1**). The resistance genes from these two accessions might have great potential for BPH resistance. Therefore, in this chapter, we aimed to identify new BPH resistance genes from ‘Rathu Heenati’ and two accessions of *O. nivara*, IRGC 89073 and IRGC 93005.

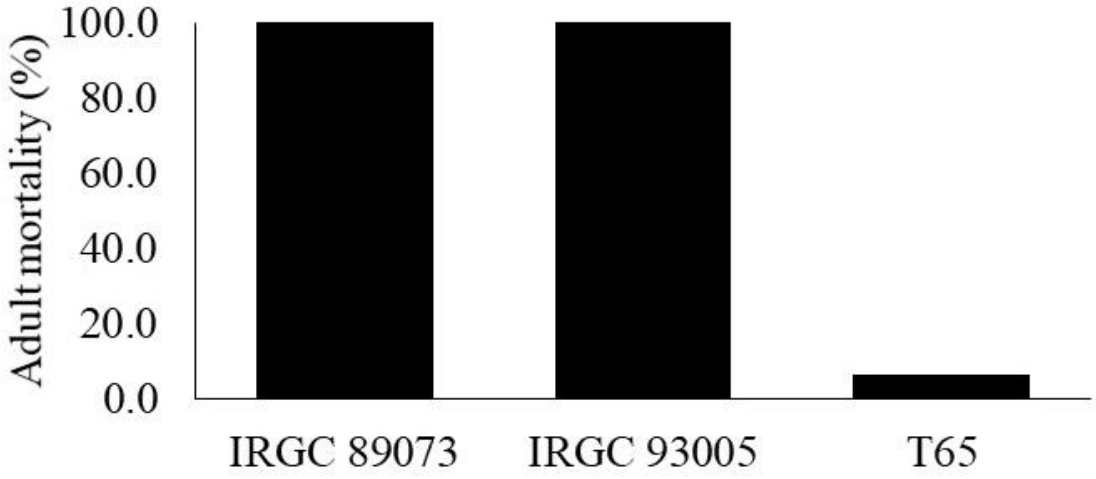


Figure 4.1 Adult mortality of Koshi-2013 (*Nilaparvata lugens* Stål) on two *O. nivara* accessions (IRGC 89073 and IRGC 93005) and 'Taichung 65' (T65) at 5 days after infestation.

2. Materials and methods

2.1. Plant materials

IRGC 89073 and IRGC 93005, two accessions of *O. nivara*, were provided by IRRI. IRGC 89073 and IRGC 93005 were crossed to susceptible variety, T65 and the F_1 plants were subsequently backcrossed with T65 (**Figure 4.2A**). Two hundred forty-six BC_1F_1 plants derived from a cross between IRGC 89073 and T65 and 190 BC_1F_1 plants derived from a cross between IRGC 93005 and T65 were individually evaluated for BPH resistance against Koshi-2013. The BC_1F_1 plants were used for bulked segregant analysis (BSA) and QTL analysis.

For developing population for genetic analysis on 'Rathu Heenati', the PYL carrying two genes *BPH3* and *BPH17* (*BPH3+17*-PYL) was crossed with T65 and the F_1 plants were self-pollinated for collecting F_2 seeds. To conduct QTL analysis for BPH resistance, 165 F_2 plants were used (**Figure 4.2B**).

2.2. Bulked segregant analysis.

Among 246 BC_1F_1 derived from a cross between IRGC 89073 and T65, 12 BC_1F_1 plants with more than 80% of ADM at 5 DAI were selected as extremely resistant group and other 12 BC_1F_1 plants with 0% of ADM at 5 DAI were selected as extremely susceptible group for bulked segregant analysis (BSA). The bulked DNA of extremely resistant group and that of extremely susceptible group were genotyped by 384 SSR markers which are distributed evenly on 12 rice chromosomes. To estimate the associated regions for BPH resistance by BSA, DNA markers with homozygous for extremely susceptible group and heterozygous for extremely resistance group were selected.

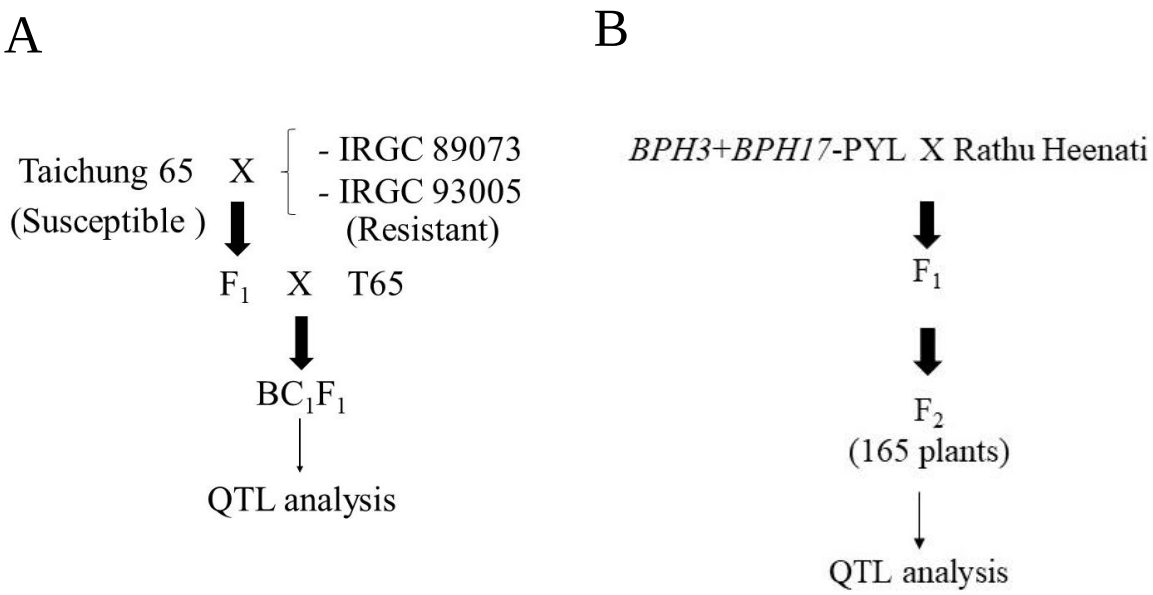


Figure. 4.2 Breeding schemes for development of segregating populations derived from crosses between IRGC 89073, IRGC 93005 and 'Taichung 65' (A), *BPH3*+*BPH17*-PYL and 'Rathu Heenati' (B)

Similarly, among 190 BC_1F_1 plants derived from a cross between IRGC 93005 and T65, six plants with more than 80% of ADM at 5 DAI were chosen as extremely resistant group. Other eight plants with 0% of ADMs at 5 DAI were selected as extremely susceptible group for BSA. The bulked DNA of resistant and that of susceptible group were genotyped with 384 SSR markers which are distributed evenly on 12 rice chromosomes. To estimate the associated regions for BPH resistance by BSA, the DNA markers with homozygous for extremely susceptible group and heterozygous for extremely resistant group were selected.

Totally 388 SSR markers distributed on 12 rice chromosomes were tested for polymorphism between donor varieties (IRGC 89073, IRGC 93005, and 'Rathu Heenati') and recurrent parent, T65. The markers visually distinguished between each donor and recurrent parent were classified as polymorphic markers (**Table 4.1**).

2.3. DNA extraction and genotyping

The total DNA of parental plants, bulked DNA of resistance and susceptible groups, as well as BC_1F_1, and F_2 plants were extracted from fresh leaves by potassium acetate (Dellaporta *et al.* 1983). The genotyping of SSR markers was performed by polymerase chain reaction (PCR) as mentioned in Chapter 2. PCR products were separated on 4% agarose gel and DNA bands were detected by ethidium bromide 0.02% under ultraviolet light. The F_2 plants derived from a cross between 'Rathu Heenati' and *BPH3+BPH17*-PYL were genotyped by 120 SSR polymorphic markers.

2.4. BPH population and evaluation of BPH resistance

The insect collected in Kumamoto Prefecture, Japan in 2013 (Koshi-2013) was used for evaluation of BPH resistance of all BC_1F_1 populations and parental varieties. Koshi-2013 was provided by Kyushu Okinawa Agricultural Research Center, National Agriculture and Food Research Organization in Japan (KOARC, NARO) and maintained at Faculty of Agriculture, Saga University under conditions of 25 ^oC and 16/8 h of light/dark.

Thorough the antibiosis test, the resistance levels of BC_1F_1 plants from IRGC 89073 and IRGC 93005 and F_2 plants from 'Rathu Heenati' were evaluated. These seeds were individually sown in 220 mL plastic cups. At 30 DAS, the plants were trimmed to around 20 cm height and covered by the plastic tubes with ventilators. Five adult female BPHs with small abdomens were added to each tube. The tubes were covered by mesh to prevent insects from escaping. Five days after infestation (DAI), the mortality of adult BPHs was calculated as an indicator for BPH resistance.

3. Results

3.1. Polymorphic tests between IRGC 89073, IRGC 93005, 'Rathu Heenati' and T65

Among 384 SSR markers used for polymorphic test between IRGC 89073 and T65, 252 markers (65.6%) showed polymorphism (**Table 4.1**). The number of polymorphic markers on each chromosome were ranged from 10 to 33. The highest number of polymorphism markers (33 markers) was chromosome 4, while the lowest number of polymorphic markers (10 markers) was chromosome 10. Among 384 SSR markers, 251 markers were polymorphic between IRGC 93005 and T65. From and 11 to 31 polymorphic

Table 4.1. Number of polymorphic markers between ‘Taichung 65’ and BPH resistant donors

Chromosome	Number of markers tested	Number of polymorphic markers		
		IRGC 89073	IRGC 93005	Rathu Heenati
1	43	31	30	18
2	28	31	16	13
3	45	25	26	27
4	55	33	31	30
5	36	21	23	17
6	36	23	22	20
7	25	15	21	16
8	31	20	23	22
9	20	12	14	10
10	18	10	11	8
11	24	14	17	9
12	27	17	17	14
Total	388	252	251	204

markers were detected on each chromosome. Chromosome 10 had the least number of polymorphic markers (11) while chromosome 4 had highest number of polymorphic markers (31). There were 204 polymorphic markers between 'Rathu Heenati' and T65 among 384 SSR tested. The number of polymorphic markers was lowest on chromosome 10 (eight markers).

3.2. Resistance levels of BC_1F_1 plants from cross between IRGC 89073 and T65

At 5 DAI, 64% of ADMs on IRGC 89073 was lower than that of 'Rathu Heenati' (88%) and higher than that of T65 (6%) (**Figure 4.3A**). The ADMs of the BC_1F_1 population were continuously distributed from 0% to 100%, suggesting that IRGC 89073 has several QTLs controlling BPH resistance.

The polymorphic markers between IRGC 89073 and T65 were utilized to detect the associated chromosomal regions for BPH resistance. Nine markers were detected as the assumed regions related to BPH resistance from IRGC 89073. These were RM3709 on chromosome 1, S00310, RM1369, and RM400 on chromosome 6, RM444 on chromosome 9, RM5348, RM1375, and RM5620 on chromosome 10 and RM5582 on chromosome 11 (**Table 4.2**).

3.3. Resistance level of BC_1F_1 plants of IRGC 93005 and T65

At 5DAI, 62% of ADMs on IRGC 93005 was lower than that of 'Rathu Heenati' (83.5%) and higher than that of T65 (11%) (**Figure 4.3B**). The ADMs of the BC_1F_1 population were continuously distributed from 0% to 100%, suggesting that IRGC 93005 also contains several QTLs controlling BPH resistance.

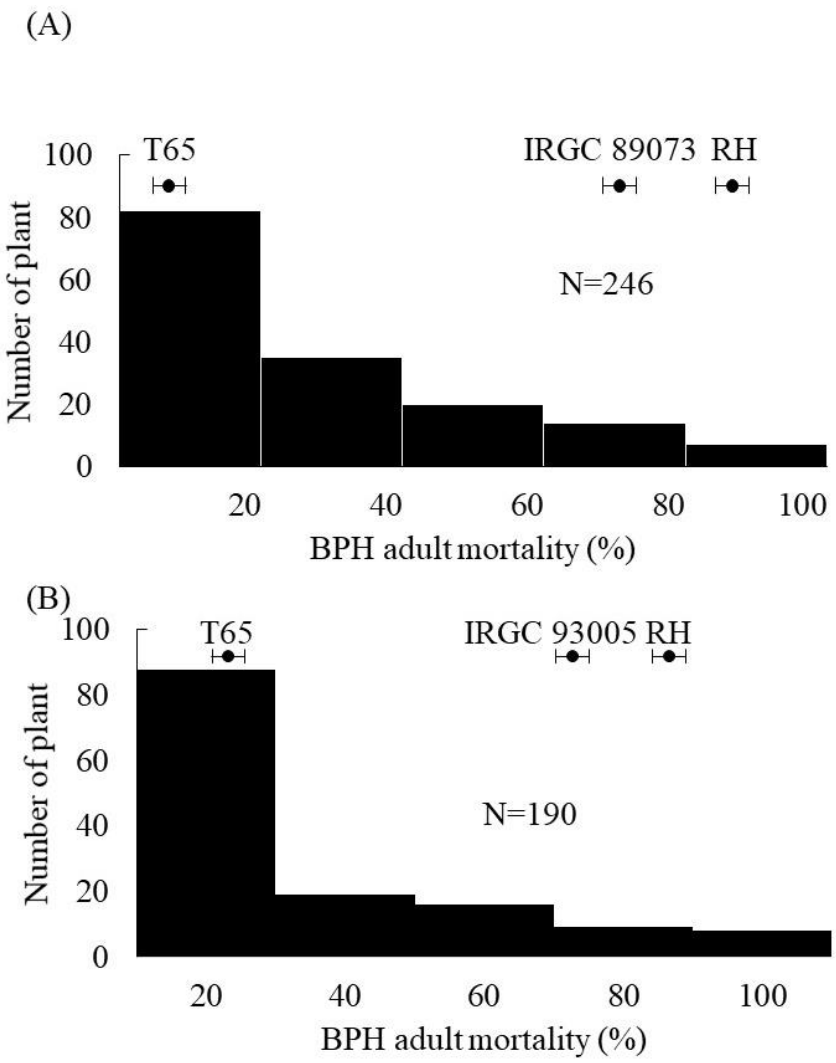


Figure 4.3. Frequency distributions of adult mortalities of Koshi-2013 at 5 DAI on BC_1F_1 populations derived from crosses between T65 and IRGC 89073 (A), and T65 and IRGC 93005 (B).

Table 4.2. The estimated regions for BPH resistance on IRGC 89073.

Marker	Chromosome	Physical location (bp)
RM3709	1	31,941,787
S00310	6	214,278
RM1369	6	1,562,658
RM400	6	28,049,016
RM444	9	5,872,126
RM5348	10	8,080,499
RM1375	10	16,202,087
RM5620	10	16,959,928
RM5582	11	17,713,298

After genotyping of two bulked DNAs of susceptible and resistant group from the BC_1F_1 plants of IRGC 93005 and T65, the markers showed polymorphism between two groups were detected. Six assumed regions related to BPH resistance in IRGC 93005 were detected: around RM5389 and RM6321 (35.7 - 42.9 Mpb) on chromosome 1, around RM3916 to RM348 (28.7 - 32.7 Mbp) on chromosome 4, around RM5579 to RM6313 (1.9-28.1 Mbp) on chromosome 5, around RM5985 (21.6 Mbp) on chromosome 8. Other two regions were around RM1328 to RM7048 (9.2 - 16.9 Mbp) on chromosome 9 and around RM1986 (21.3 Mbp) on chromosome 12 (**Table 4.3**).

3.4. Resistance level of F_2 plants from a cross between 'Rathu Heenati' and BPH3+BPH17-PYL

'Rathu Heenati' showed strong resistance against Koshi-2013 with 82.0% of ADM. *BPH3+17*-PYL had lower resistance level (55.0 % of ADM) compared to 'Rathu Heenati' (**Figure 4.4**). The recurrent parent T65 had 16.6 % of ADM. The frequency of ADMs on 165 F_2 plants showed in a continuous distribution, suggesting that there are several QTLs for BPH resistance in 'Rathu Heenati' other than *BPH3* and *BPH17*. However, we have not identified any QTL for BPH resistance from F_2 population derived from a cross between 'Rathu Heenati' and *BPH3+17*-PYL.

Table 4.3. The assumed regions related to BPH resistance from IRGC 93005

Marker	Chromosome	Physical location
RM5389	1	35,726,691
RM5536	1	41,160,287
RM6321	1	42,916,592
RM3785	4	24,222,926
RM3916	4	28,733,281
RM3781	4	28,758,188
RM5503	4	30,397,705
RM3276	4	30,715,761
RM3534	4	31,198,068
RM6909	4	32,094,591
RM348	4	32,869,438
RM5579	5	1,855,946
RM4691	5	6,941,334
RM289	5	7,807,804
RM249	5	10,676,235
RM6841	5	22,657,455
RM6545	5	24,789,344
RM7446	5	24,815,813
RM3348	5	24,948,976
RM3170	5	27,796,435
RM6360	5	27,924,665
RM6313	5	28,120,199
RM5985	8	21,584,459
RM1328	9	9,152,293
RM7390	9	10,417,793
RM7048	9	16,881,647
RM1986	12	21,282,462

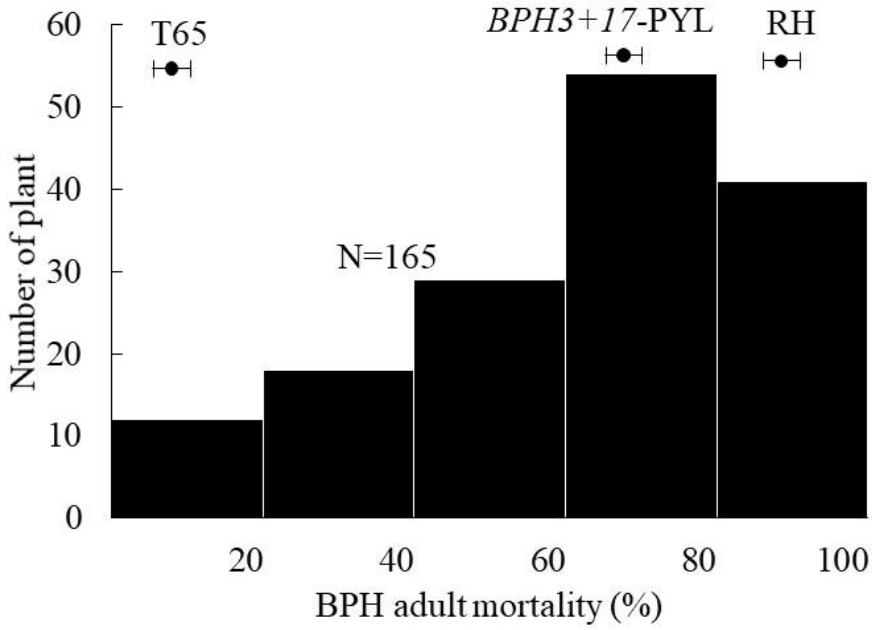


Figure 4.4 The frequency distribution of BPH adult mortality at 5 days after infestation on F_2 plants from cross between *BPH3+17*-PYL and 'Rathu Heenati' (RH).

4. Discussion

The chapter 2 showed that many resistance genes have lost effect against the BPH collected recently in Japan, Koshi-2013. In near future, the number of effective resistance genes might be reduced due to the increase of BPH virulence and lack of a proper management tactic for preventing adaptation. Therefore, conservation of the available gene sources and discovering new BPH resistance genes are fundamental for continuing to deal with BPH damage.

Several studies on screening of BPH resistance showed that many *O. nivara* accessions carrying strong resistance against BPHs. Recently, *BPH34* was identified from an *O. nivara* accession, IRGC 104646. *BPH34* showed resistance against "biotype 4" collected in Punjab, India (Kumar *et al*. 2019). Madurangi *et al*. (2015) found several *O. nivara* accessions carrying *BPH2*, *BPH10* or *BPH13* could resistance against BPH populations collected in Sri Lanka. In this study, two *O. nivara* accessions showed strong resistance against Koshi-2013 in antibiosis test. Due to the similarity in virulence of BPHs from Japan, Northern Vietnam and Eastern China, the resistance gene(s) from these two accessions are effective and promising for BPHs from Japan, Eastern China and Northern Vietnam (Horgan *et al*. 2015, Saxena Barrion 1985).

Based on the frequency distributions of BPH adult mortalities on BC_1F_1 populations derived from crosses between T65 / IRGC 89073 and T65 / IRGC 93005, two accessions might contain multiple QTLs for BPH resistance. Through BSA, several estimated regions were localized for both accessions, but we did not conclude which locations were related to BPH resistance because multiple genetic factors might be related to BPH resistance from these two accessions. Additionally, the resistance level of most of BC_1F_1 individuals were low and unstable. This might be resulted from the heterozygous

genotypes of BC_1F_1 population in which the recessive genes are not phenotypically expressed. Therefore, in future study, the plants that showed high resistance need to be backcrossed to the recurrent parent (T65), for developing of backcross inbred lines (BILs). Using the BILs, it is possible to increase replications of phenotypic data. In addition, the recessive genes can be identified by the BILs.

Recent reports indicated that plants with multiple resistance genes can resistance against strong virulence BPHs which easily adapt to the monogenic lines with single BPH resistance genes. Although *BPH3*-NIL and *BPH17*-NIL are not individually effective against Koshi-2013, their donor parent, 'Rathu Heenati', has the strong resistance to this BPH populations. In frequency distribution of BPH adult mortality on F_2 populations derived from a cross between *BPH3*+*BPH17*-PYL and 'Rathu Heenati', the majority of F_2 plants had higher resistance level than *BPH3*+*BPH17*-PYL. These results suggested that resistance of BPH from 'Rathu Heenati' is controlled by unknown genetic factors other than *BPH3* and *BPH17*. However, we have not identified any QTLs for BPH resistance from this F_2 population. There might be two reasons related to failure detection of QTL. Firstly, because the poor amplifications of some markers and the non-available of polymorphic markers on some locations, the F_2 plants were genotyped by only 96 SSR markers. The average interval between two markers on the whole genome was approximately 4.4 Mbp. However, larger intervals were on chromosome 4 (around the centromere), chromosomes 9, chromosome 10 and chromosome 12. Therefore, increase of marker density is crucial to reduce the effect of recombination occur between the marker and the QTL, which might be a factor related to failure detection of QTL. Secondly, 'Rathu Heenati' has been reported to carry multiple QTLs on chromosomes: 1, 3, 6, 7, 9, 10 and 12 other than *BPH3* and *BPH17* (Shabanimofrad *et al*. 2017). Therefore, development of

chromosome segment substitution lines (CSSLs) can dissect the genetics of BPH resistance from ‘Rathu Heenati’.

Chapter 5. General discussion

Rice production needs to be raised 26 % (approximately 116 million tons) until 2035 to feed growing populations (Seck *et al*. 2012). Elimination of BPH can promote rice production since yield loss by BPH is approximately three million tons of rice every year (2007). Identification of new resistance genes as well as resistance mechanisms are important to facilitate the application of host plant resistance to solve BPH problem.

Development of NILs using genetic background of susceptible varieties can pave the way to study all relevant aspects of resistance genes before utilizing for controlling the BPH. Seven NILs carrying different genes (*BPH2*-NIL, *BPH3*-NIL, *BPH17*-NIL, *BPH17-ptb*-NIL, *BPH20*-NIL, *BPH21*-NIL and *BPH32*-NIL), from donors: 'PTB33', 'Rathu Heenati', or 'IR71033-121-15', were developed. These NILs could be the advanced materials for both studies of gene performance as well as remove crossing barriers. 'PTB33' and 'Rathu Heenati' are strong photoperiod-sensitive varieties, which do not flower under long-day conditions. Therefore, the developed NILs on genetic background of less photoperiod-sensitive cultivar, T65, can facilitate breeding of varieties carrying resistance genes. On the other hand, due to BPH virulence is fluctuated by time and location, effectiveness of resistance genes on different BPH populations can be changed over time. The NILs are useful materials for monitoring the changing in virulence of BPH from different rice cultivated areas.

Through evaluation against Hadano-66 and Koshi-2013, seven genes: *BPH2, BPH3, BPH17, BPH17-ptb, BPH20, BPH21* and *BPH32* are vulnerable against BPH in monogenic lines. Therefore, conservation of these genes against BPH adaptation should be considered when utilizing these genes in field condition. On the other hand, the PYLs

carrying two or three genes generally enhance resistance levels in plants compared with the NILs. The BPH resistance levels of PYLs with three genes (*BPH2+BPH3+BPH17*-PYL, *BPH2+BPH32+BPH17-ptb*-PYL and *BPH20+BPH21+BPH32*-PYL) were stronger than those of the NILs and PYLs. Therefore, more attention should be paid to check the effects of other gene combinations.

'PTB33' has multiple genes for BPH resistance and showed strong and durable resistance level against BPH. Therefore, the understanding of resistance level and genetic factors related to resistance in 'PTB33' is important to utilize this characteristic in rice breeding. Through substitution mapping, the location of *BPH2* and *BPH17-ptb* were revealed that facilitate the utilization of these genes in breeding. The confirmation of *BPH32* in *BPH32*-NIL is necessary to use this material in further studies and for breeding. Moreover, the different resistance mechanisms harboured by *BPH2, BPH17-ptb* and *BPH32* may intensify BPH resistance in 'PTB33'. Therefore, it is important to characterize resistant mechanisms of the PYLs carrying resistant genes from PTB33, *BPH2+BPH17-ptb*-PYL, *BPH2+BPH32*-PYL, *BPH17-ptb+BPH32*-PYL and *BPH2+BPH17-ptb+BPH32*-PYL, to reveal gene interaction in PTB33.

'Rathu Heenati' has strong resistance against current BPH population in Japan. Therefore, these varieties are valuable materials against BPHs with strong virulence. In a previous study, ten QTLs for BPH resistance have been detected on seven chromosomes of 'Rathu Heenati': 1, 3, 6, 7, 9, 10 and 12 (Shabanimofrad *et al*. 2017). Among those, four QTLs on chromosomes 1, 3, 6, 7 were detected by standard seedbox screening test and two QTLs on chromosomes 6 and 9 were identified by the antibiosis and honeydew test. The other four QTLs on chromosomes 3, 6, 10 and 12 were detected by antixenosis test. In Kamolsukyeunyong *et al*. (2019), two QTLs on chromosome 4S and 4L were detected by using BILs population from Rathu Heeanti and 'KDML105'' variety. These studies

suggested that the BPH resistance from ‘Rathu Heenati’ is controlled by multiple QTLs with different resistance mechanism. Therefore, in further studies, the CSSLs on genetic background of susceptible variety are crucial for dissection of genetic factors controlling BPH resistance in this variety. It would be essential to evaluate resistance of the CSSLs using several resistance evaluation methods for antibiosis, antixenosis, tolerance, and/or standard seedbox screening test.

Recently, resistance gene source from wild rice has attracted scientists. Many rice accessions from *O. nivara* carry strong resistance against BPHs, especially the strong virulence populations from South Asia (Akanksha *et al*. 2019, Kumar *et al*. 2018, Madurangi *et al*. 2010, 2012, 2013, Sandhu *et al*. 2021). However, only a small number of BPH resistance loci have been detected from *O. nivara* (Akanksha *et al*. 2019, Kumar *et al*. 2018). Therefore, there is a need for screening resistance donors and conducting genetic analysis for BPH resistance from *O. nivara*. In this study, two accessions indicated strong resistance against Koshi-2013; however, the BPH resistance genes have not been detected. In future studies, QTL analysis will be necessary to detect BPH resistance genes from these *O. nivara* accessions.

Summary

Brown planthopper is one of the most extremely devastating insect pests of rice in Asia. Identifications of genetic location and characterizations for BPH resistance level as well as gene behaviour are important for exploiting genes in rice breeding.

The major objectives of this research are: 1) develop the materials for resistance genes characterization as well as monitoring the BPH virulence; 2) understand exact location and resistance mechanisms of several BPH resistance genes; and 3) identify novel genes for BPH resistance. As a result, seven NILs carrying a single gene and fifteen PYLs carrying multiple resistance genes on T65 genetic background were developed (Chapter 2). All the NILs and PYLs have high similarity in genetic background and agronomic traits to current parent, T65. Many of the NILs and PYLs were resistant against the Hadano-66 population but were less effective against Koshi-2013 population. The PYLs had higher resistance level than the NILs. Among PYLs, *BPH20+BPH32*-PYL and *BPH2+BPH3+BPH17*-PYL had relatively high BPH resistance against Koshi-2013.

In Chapter 3, the locations of *BPH2, BPH17-ptb* and *BPH32* were detected through substitution mapping. *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’.

In conclusion of this dissertation, these are possible areas for further research with regards to understanding of BPH resistance gene location and their effectiveness:

1. Resistance levels of BPH resistance genes decreased against BPH populations collected recently. Therefore, a strategy for releasing the BPH resistance genes is necessary to efficiently use without causing adaptation of BPH.
2. The combinations of multiple genes can enhance the resistance level for plants. Therefore, more attention should be paid to the development of PYLs, especially the PYLs with three genes, in the strategy of resistance improvement.
3. High mapping of *BPH17-ptb* is necessary to determine more precise location of this genes on chromosome 4S.
4. The experiments for IRGC 89073, IRGC 93005 and ‘Rathu Heenati’ need to be continued to identify the QTL for BPH resistance on these variety/accessions.

Abstract in Japanese

イネ (*Oryza sativa* L.) は、重要な作物の 1 つであり、アジア全域で栽培されている。イネの害虫であるトビイロウンカ (BPH、*Nilaparvata lugens* Stål.) は、アジア全域のイネ生産に深刻な被害を与えてる。一部のイネがもつトビイロウンカ抵抗性の利用は、この昆虫による被害を軽減するための効果的で経済的な手段と見なされている。これまでに、栽培品種や近縁野生種から、40 個 以上のトビイロウンカ抵抗性遺伝子が同定されている。これらの抵抗性遺伝子を効率的かつ持続的に運用するには、各抵抗性遺伝子の位置情報の明確化、抵抗性機構の解明が必要である。

第 1 章では、日本型品種台中 65 号(T65)の遺伝的背景に単一の BPH 抵抗性遺伝子を持つ 7 つの準同質遺伝子系統 (NIL: near-isogenic line) (*BPH2*-NIL、*BPH3*-NIL、*BPH17*-NIL、*BPH20*-NIL、*BPH21*-NIL、*BPH32*-NIL と *BPH17-ptb*-NIL) を作出した。そして、2 つまたは 3 つの遺伝子を保有する 15 系統 の集積系統 (PYL: Pyramided lines)を作出した。NIL と PYL を用いて、1966 年 (Hadano-66) と 2013 年 (Koshi-2013) に、日本で収集された 2 つのトビイロウンカ集団に対する抵抗性に関して評価した。NIL と PYL の多くは、Hadano-66 集団に対して抵抗性を示したが、Koshi-2013 集団に対してはあまり効果がなかった。PYL の中で、*BPH20*+*BPH32*-PYL と *BPH2*+*BPH3*+*BPH17*-PYL は、 Koshi-2013 に対して比較的高いトビイロウンカ抵抗性を示した。

第 2 章では、T65 と NIL を交雑した分離集団を用いて、*BPH2*、*BPH17-ptb*、および *BPH32* 各染色体上に位置付けた。具体的に、第 12 染色体の RM28449 と ID-161-2 の間（約 247.5 kbp）の間に *BPH2* を特定した。*BPH17-ptb* は、第 4 染色体の RM1305 と RM6156 の間に、*BPH32* は、第 6 染色体の RM508 と RM19341 の間に、それぞれ特定した。また、*BPH2*、*BPH17-ptb*、および *BPH32* の抵抗性機構を解明するために、抗生作用、好寄生性、および耐性に関して評価した。*BPH2* は、抗生作用と好寄生性を示したが、*BPH32* は耐性を示した。*BPH17-ptb* は、抗生作用、好寄生性、および耐性を示した。本研究で作出した NIL や PYL は、トビイロウンカの加害性の違いを判別するための材料として利用できる。また、イネのトビイロウンカ抵抗性を改良するための育種素材としての利用が期待できる。

References

Akanksha S., Lakshmi V. J., Singh A. K., Deepthi Y., Chirutkar P. M., Ramdeen, Balakrishnan D., Sarla N., Mangrauthia S. K. and Ram T., (2019) Genetics of novel brown planthopper *Nilaparvata lugens* (Stål) resistance genes in derived introgression lines from the interspecific cross *O. sativa* var. Swarna × *O. nivara*. J. Genet. 98: 113.

Alam S. N. and Cohen M. B., (1998) Detection and analysis of QTLs for resistance to the brown planthopper, *Nilaparvata lugens*, in a doubled-haploid rice population. Theor. Appl. Genet. 97: 1370–1379.

Ali M. P., Alghamdi S. S., Begum M. A., Anwar Uddin A. B. M., Alam M. Z. and Huang D., (2012) Screening of rice genotypes for resistance to the brown planthopper, *Nilaparvata lugens* Stål. Cereal Res. Commun. 40: 502–508.

Angeles E. R., Khush G. S. and Heinrichs E. A., (1986) Inheritance of resistance to planthoppers and leafhoppers in rice. *In* IRRI (ed.) Rice Genetics, IRRI, Los Baños, Philippines, pp. 537–549.

Athwal D. S., Pathak M. D., Bacalangco E. H. and Pura C. D., (1971) Genetics of resistance to brown planthoppers and green leafhoppers in *Oryza sativa* L. Crop Sci. 11: 747–750.

Catindig J. L. A., Arida G. S., Baehaki S. E., Bentur J. S., Cuong L. Q., Norowi M., Rattanakarn W., Sriratanasak W., Xia J. and Lu Z., (2009) Situation of planthoppers in Asia. *In*: Heong, K. L. and B. Hard (eds.) Planthoppers: new threats to the sustainability of intensive rice production systems in Asia. IRRI, Los Baños, Philippines, pp. 191–220.

Cha Y. S., Ji H., Yun D. W., Ahn B. O., Lee M. C., Suh S. C., Lee C. S., Ahn E. K., Jeon Y. H., Jin I. D. *et al*., (2008) Fine mapping of the rice *BPH1* gene, which confers resistance to the brown planthopper (*Nilaparvata lugens* Stal), and development of STS markers for marker-assisted selection. Mol. Cells 26: 146–151.

Chen J. W., Wang L., Pang X. F. and Pan Q. H., (2006) Genetic analysis and fine mapping of a rice brown planthopper (*Nilaparvata lugens* Stål) resistance gene *BPH19*(t). Mol. Gen. Genomics 275: 321-329.

Claridge M. F. and Hollander J. D., (1982) Virulence to rice cultivars and selection for virulence in populations of the brown planthopper *Nilaparvata lugens*. Ent. Exp. Appl. 32: 213–221.

Cohen M. B., Alam S. N., Medina E. B. and Bernal C. C., (1997) Brown planthopper, *Nilaparvata lugens*, resistance in rice cultivar IR64: mechanism and role in successful *N. lugens* management in Central Luzon, Philippines. Entomol. Exp. Appl. 85: 221–229.

Dawe D. and Timmer C. P. (2012) Why stable food prices are a good thing: lessons from stabilizing prices in Asia. Glob. Food Secur. 1: 127-133

Dellaporta S. L., Wood J. and Hicks J. B., (1983) A plant DNA minipreparation: Version II. Plant Mol. Biol. Report 1: 19–21.

Du B., Zhang W., Liu B., Hu J., Wei Z., Shi Z., He R., Zhu L., Chen R., Han B. *et al*., (2009) Identification and characterization of *BPH14*, a gene conferring resistance to brown planthopper in rice. Proc. Natl. Acad. Sci. U.S.A. 106: 22163–22168.

Du B., Chen R., Guo J. and He G., (2020) Current understanding of the genomic, genetic, and molecular control of insect resistance in rice. Mol. Breed. 40: 24.

Dyck V. A., Misra B. C., Alum S., Chen C. N., Hsieh C. Y. and Rejesus R. S., (1979) Ecology of the brown planthopper in the tropics. *In*: IRRI (ed.) Brown planthopper:

Threat to rice production in Asia. IRRI, Los Baños, Philippines. pp. 61–100.

Ferrater J. B., Naredo A. I., Almazan M. L. P., de Jong P. W., Dicke M. and Horgan F. G., (2015) Varied responses by yeast-like symbionts during virulence adaptation in a monophagous phloem-feeding insect. Arthropod. Plant Interact. 9: 215–224.

Fujita D., Myint K. K. M., Matsumura M. and Yasui H., (2009) The genetics of host-plant resistance to rice planthopper and leafhopper. *In*: Heong, K. L. and B. Hard (eds.) Planthoppers: New threats to the sustainability of intensive rice production systems in Asia. IRRI, Los Baños, Philippines, pp. 389–399.

Fujita D., Kohli A. and Horgan F. G., (2013) Rice resistance to planthoppers and leafhoppers. CRC. Crit. Rev. Plant Sci. 32: 162–191.

Guo J., Xu C., Wu D., Zhao Y., Qiu Y., Wang X., Ouyang Y., Cai B., Liu X., Jing S. *et al*., (2018) *BPH6* encodes an exocyst-localized protein and confers broad resistance to planthoppers in rice. Nat. Genet. 50: 297–306.

Grillo M. A., Li C., Fowlkes A. M., Briggeman T. M., Zhou A., Schemske D. W. and Sang T., (2009) Genetic architecture for the adaptive origin of annual wild rice, *Oryza nivara*. Evolution 63: 870–883.

He J., Liu Y., Liu Y., Jiang L., Wu H., Kang H., Liu S., Chen L., Liu X., Cheng X. *et al*., (2013) High-resolution mapping of brown planthopper (BPH) resistance gene *BPH27*(t) in rice (*Oryza sativa* L.). Mol. Breed. 31: 549–557.

Heinrichs E. A., Medrano E. G. and Rapusas H. R., (1985) Brown planthopper, whitebacked planthopper, green leafhopper, and zigzag leafhopper. *In*: Heinrichs, E. A., Medrano F. G. and Rapusas H. R. (eds.) Genetic evaluation for insect resistance in rice, International Rice Research Institute, Los Baños, Philippines, pp. 71–170.

Hirabayashi H., Kaji R., Angeles E. R., Ogawa T., Brar D. S. and Khush G. S., (1999)

RFLP analysis of a new gene for resistance to brown planthopper derived from *O. officinalis* on rice chromosome 4. Breed. Sci. 1: 48.

Horgan F. G., Ramal A. F., Bentur J. S., Kumar R., Bhanu K. V., Sarao P. S., Iswanto E. H., Van Chien H., Phyu M. H., Bernal C. C. *et al*., (2015) Virulence of brown planthopper (*Nilaparvata lugens*) populations from South and South East Asia against resistant rice varieties. Crop Prot. 78: 222–231.

Horgan F. G., Crisol-Martínez E., Almazan M. L. P., Romena A., Ramal A. F., Ferrater J. B. and Bernal C. C., (2016) Susceptibility and tolerance in hybrid and pure-line rice varieties to herbivore attack: biomass partitioning and resource-based compensation in response to damage. Ann. Appl. Biol. 169: 200-213.

Horgan F. G. (2017) Insect herbivores of rice: Their natural regulation and ecologically based management. *In:* Chauhan B. S. and Mahajan K. J. G (ed.) Rice production worldwide. Springer Nature. Gewerbestrasse 11, 6330 Cham, Switzerland. pp. 279-303.

Horgan F. G., Cruz A. P., Bernal C. C., Ramal A. F., Almazan M. L. P. and Wilby A., (2018) Resistance and tolerance to the brown planthopper, *Nilaparvata lugens* (Stål), in rice infested at different growth stages across a gradient of nitrogen applications. Field Crop Res. 217: 53–65.

Horgan F. G., (2018) Integrating gene deployment and crop management for improved rice resistance to Asian planthoppers. Crop Prot. 110: 21–33.

Horgan F. G., Almazan M. L. P., Vu Q., Ramal A. F., Bernal C. C., Yasui H. and Fujita D., (2019) Unanticipated benefits and potential ecological costs associated with pyramiding leafhopper resistance loci in rice. Crop Prot. 115: 47–58.

Hou L., Yu P., Xu Q., Yuan X., Yu H., Wang Y., Wang C., Wan G., Tang S., Peng S. *et al*.,

(2011) Genetic analysis and preliminary mapping of two tecessive tesistance genes to brown planthopper, *Nilaparvata lugens* Stål in rice. Rice Sci. 18: 238–242.

Hu J., Li X., Wu C., Yang C., Hua H., Gao G., Xiao J. and He Y., (2012) Pyramiding and evaluation of the brown planthopper resistance genes *BPH14* and *BPH15* in hybrid rice. Mol. Breed. 29: 61–69.

Hu J., Xiao C., Cheng M., Gao G., Zhang Q. and He Y., (2015 a) Fine mapping and pyramiding of brown planthopper resistance genes *QBPH3* and *QBPH4* in an introgression line from wild rice *O. officinalis*. Mol. Breed. 35: 3.

Hu J., Xiao C., Cheng M. X., Gao G. J., Zhang Q. L. and He Y. Q., (2015 b) A new finely mapped *Oryza australiensis*-derived QTL in rice confers resistance to brown planthopper. Gene 561: 132–137.

Hu J., Chang X., Zou L., Tang W. and Wu W., (2018) Identification and fine mapping of *BPH33*, a new brown planthopper resistance gene in rice (*Oryza sativa* L.). Rice 11: 55.

Huang D., Qiu Y., Zhang Y., Huang F., Meng J., Wei S., Li R. and Chen B., (2013) Fine mapping and characterization of *BPH27*, a brown planthopper resistance gene from wild rice (*Oryza rufipogon* Griff.). Theor. Appl. Genet. 126: 219–229.

International Rice Genome Sequencing Project (IRGSP), (2005) The map-based sequence of the rice genome. Nature 436: 793–800.

IRRI, (2014) Standard evaluation system (SES) for rice, 5th edn. IRRI, Los Baños, Philippines.

Ishii T., Brar D. S., Multani D. S. and Khush G. S., (1994) Molecular tagging of genes for brown planthopper resistance and earliness introgressed from *Oryza australiensis* into cultivated rice, *O. sativa*. Genome 37: 217–221.

Jackson M. T., (1997) Conservation of rice genetic resources: the role of the International Rice Genebank at IRRI. Plant Mol. Biol. 35: 61–67.

Jacquemin J., Bhatia D., Singh K. and Wing R. A., (2013) The international *Oryza* map alignment project: development of a genus-wide comparative genomics platform to help solve the 9 billion-people question. Curr. Opin. Plant Biol. 16: 147–156.

Jairin J., Teangdeerith S., Leelagud P., Phengrat K., Vanavichit A. and Toojinda T., (2007 a) Physical mapping of *BPH3*, a brown planthopper resistance locus in rice. Maejo Int. J. Sci. Technol. 1: 166–177.

Jairin J., Teangdeerith S., Leelagud P., Phengrat K., Vanavichit A. and Toojinda T., (2007 b) Detection of brown planthopper resistance genes from different rice mapping populations in the same genomic location. ScienceAsia 33: 347–352.

Jairin J., Phengrat K., Teangdeerith S., Vanavichit A. and Toojinda T., (2007 c) Mapping of a broad-spectrum brown planthopper resistance gene, *BPH3*, on rice chromosome 6. Mol. Breed. 19: 35–44.

Jena K. K., Hechanova S. L., Verdeprado H., Prahalada G. D. and Kim S. R., (2017) Development of 25 near-isogenic lines (NILs) with ten BPH resistance genes in rice (*Oryza sativa* L.): production, resistance spectrum, and molecular analysis. Theor. Appl. Genet. 130: 2345–2360.

Ji H., Kim S. R., Kim Y. H., Suh J. P., Park H. M., Sreenivasulu N., Misra G., Kim S. M., Hechanova S. L., Kim H. *et al*., (2016) Map-based cloning and characterization of the *BPH18* gene from wild rice conferring resistance to brown planthopper (BPH) insect pest. Sci. Rep. 6: 34376.

Kabir M. A. and Khush G. S., (1988) Genetic analysis of resistance to brown planthopper in rice (*Oryza sativa* L.). Plant Breed. 100: 54–58.

Kamolsukyeunyong W., Ruengphayak S., Chumwong P., Kusumawati L., Chaichoompu E., Jamboonsri W., Saensuk C., Phoonsiri K., Toojinda T. and Vanavichit A., (2019) Identification of spontaneous mutation for broad-spectrum brown planthopper resistance in a large, long-term fast neutron mutagenized rice population. Rice 12: 16.

Ketipearachchi Y., Kaneda C. and Nakamura C., (1998) Adaptation of the brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), to BPH resistant rice cultivars carrying *BPH8* or *BPH9*. Appl. Entomol. Zool. 33: 497–505.

Khush G. S. and Virk P. S., (2005) IR varieties and their impact. IRRI. Los Baños, Philippines. pp. 12.

Kogan M. and Ortman E. F., (1978) Antixenosis - a new term proposed to define Painter's "Nonpreference" modality of resistance. Bull. Ent. Soc. Am. 24: 175-176.

Kumar K., Sarao P. S., Bhatia D., Neelam K., Kaur A., Mangat G. S., Brar D. S. and Singh K., (2018) High-resolution genetic mapping of a novel brown planthopper resistance locus, *BPH34* in *Oryza sativa* L. X *Oryza nivara* (Sharma and Shastry) derived interspecific F_2 population. Theor. Appl. Genet. 131: 1163–1171.

Kuno E., (1979) Ecology of the brown planthopper in temperate regions. *In*: IRRI (ed.) Brown planthopper: Threat to rice production in Asia. IRRI, Los Baños, Philippines. pp. 45–60.

Lakshminarayana A. and Khush G. S., (1977) New genes for resistance to the brown planthopper in rice. Crop Sci. 17: 96–100.

Li J., Chen Q., Wang L., Liu J., Shang K. and Hua H., (2011) Biological effects of rice harboring *BPH14* and *BPH15* on brown planthopper, *Nilaparvata lugens*. Pest Manag. Sci. 67: 528–534.

Li Z., Xue Y., Zhou H., Li Y., Usman B., Jiao X., Wang X., Liu F., Qin B., Li R. *et al*., (2019) High-resolution mapping and breeding application of a novel brown planthopper resistance gene derived from wild rice (*Oryza. rufipogon* Griff). Rice 12: 41.

Liu G., Yan H., Fu Q., Qian Q., Zhang Z., Zhai W. and Zhu L., (2001) Mapping of a new gene for brown planthopper resistance in cultivated rice introgressed from *Oryza eichingeri*. Chinese Sci. Bull. 46: 1459–1462.

Liu Y., Wu H., Chen H., Liu Y., He J., Kang H., Sun Z., Pan G., Wang Q., Hu J. *et al*., (2014) A gene cluster encoding lectin receptor kinases confers broad-spectrum and durable insect resistance in rice. Nat. Biotechnol. 33: 301–305.

Liu Y., Chen L., Liu Y., Dai H., He J., Kang H., Pan G., Huang J., Qiu Z., Wang Q. *et al*., (2016) Marker assisted pyramiding of two brown planthopper resistance genes, *BPH3* and *BPH27* (t), into elite rice cultivars. Rice 9: 27.

Lynn B., Dupo B. and Barrion A. T., (2009) Taxonomy and general biology of delphacid planthoppers in rice agroecosytems. *In*: Heong, K. L. and B. Hard (eds.) Planthoppers: new threats to the sustainability of intensive rice production systems in Asia. IRRI, Los Baños, Philippines. pp. 3–156.

Madurangi S. A. P., Ratnasekera D., Hemachandara P. V. and Senanayake S. G. J. N., (2010) Evaluation of brown planthopper *Nilaparvata lugens* (Stål) resistance in *Oryza nivara* wild rice accessions found in Sri Lanka. Proceeding of the 15th International Forestry and Environment Symposium, University of Sri Jayewardenepura, Sri Lanka, pp. 172–175.

Madurangi S. A. P., Ratnasekera D. and Senanayake G., (2012) Evaluation of resistance of wild rice ; *Oryza nivara* to feeding by brown planthopper [Nilaparvata lugens

(Stal)] populations. Section I - Biodiversity conservation and management. Proceedings of the International Forestry and Environment Symposium, University of Sri Jayewardenepura, Sri Lanka.

Madurangi S. A. P., Ratnasekera D., Senanayake S. G. J. N., Samarasinghe W. L. G. and Hemachandra P. V, (2013) Antixenosis and antibiosis effects of *Oryza nivara* accessions harbouring *BPH2* gene on brown planthopper [*Nilaparvata lugens* (Stal)]. J. Natl. Sci. Found. Sri Lanka 41: 147–154.

McCouch S. R., Teytelman L., Xu Y., Lobos K., Clare K., Walton M., Fu B., Maghirang R., Li Z., Xing Y. *et al*., (2002) Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). DNA Res. 9: 199–207.

Milovanovic V. and Smutka L., (2017) Asian countries in the global rice market. Acta Univ. Agric. Silvic. Mendelianae Brun. 65: 679–688.

Mochida O. and Okada T., (1979) Taxonomy and biology of *Nilaparvata lugens* (Hom., Delphacidae). *In*: IRRI (ed.) Brown planthopper: Threat to rice production in Asia. IRRI, Los Baños, Philippines. pp. 21–44.

Mohanty S. K., Panda R. S., Mohapatra S. L., Nanda A., Behera L., Jena M., Sahu R. K., Sahu S. C. and Mohapatra T., (2017) Identification of novel quantitative trait loci associated with brown planthopper resistance in the rice landrace Salkathi. Euphytica 213: 38.

Muthayya S., Sugimoto J. D., Montgomery S. and Maberly G. F., (2014) An overview of global rice production, supply, trade, and consumption. Ann. N. Y. Acad. Sci. 1324: 7–14.

Myint K. K. M., Yasui H., Takagi M. and Matsumura M., (2009 a) Virulence of long-term laboratory populations of the brown planthopper, *Nilaparvata lugens* (Stål), and

whitebacked planthopper, *Sogatella furcifera* (Horváth) (Homoptera: Delphacidae), on rice differential varieties. Appl. Entomol. Zool. 44: 149–153.

Myint K. K. M., Matsumura M., Takagi M. and Yasui H., (2009 b) Demographic parameters of long-term laboratory strains of the brown planthopper, *Nilaparvata lugens* Stål, (Homoptera: Delphacidae) on resistance genes, *BPH20*(t) and *BPH21*(t) in rice. J. Fac. Agric. Kyushu Univ. 54: 159–164.

Myint K. K. M., Fujita D., Matsumura M., Sonoda T., Yoshimura A. and Yasui H., (2012) Mapping and pyramiding of two major genes for resistance to the brown planthopper (*Nilaparvata lugens* [Stål]) in the rice cultivar 'ADR52'. Theor. Appl. Genet. 124: 495–504.

Naeemullah M., Sharma P. N., Tufail M., Mori N., Matsumura M., Takeda M. and Nakamura C., (2009) Characterization of brown planthopper strains based on their differential responses to introgressed resistance genes and on mitochondrial DNA polymorphism. Appl. Entomol. Zool. 44: 475–483.

Nemoto H., Ikeda R. and Kaneda C., (1989) New genes for resistance to brown planthopper, *Nilaparvata lugens* Stål, in rice. Japan J. Breed. 39: 23–28.

Oerke E. C., (2006) Contenary review: Crop losses to pests. J. Agric. Sci. 144: 31–43.

Painter R. H., (1951) Insect resistance in crop plants. The Macmillan Company, New York. pp. 81.

Pathak M. D. and Khush G. S., (1979) Studies of varietal resistance in rice to the brown planthopper at the IRRI. *In*: IRRI (ed.) Brown planthopper: Threat to rice production in Asia. IRRI, Los Baños, Philippines. pp. 285–301.

Pathak M. D. and Khan Z. R., (1994) Insect pests of rice. IRRI, P.O. Box 933, 1099 Manila, Philippines.

Peñalver Cruz A., Arida A., Heong K. L. and Horgan F. G., (2011) Aspects of brown planthopper adaptation to resistant rice varieties with the *BPH3* gene. Entomol. Exp. Appl. 141: 245–257.

Prahalada G. D., Shivakumar N., Lohithaswa H. C., Sidde Gowda D. K., Ramkumar G., Kim S. R., Ramachandra C., Hittalmani S., Mohapatra T. and Jena K. K., (2017) Identification and fine mapping of a new gene, *BPH31* conferring resistance to brown planthopper biotype 4 of India to improve rice, *Oryza sativa* L. Rice 10: 41.

Prasad R., Shivay Y. S. and Kumar D. (2017) Current status, challenges, and opportunities in rice production. *In:* Chauhan B. S. and Mahajan K. J. G (ed.) Rice production worldwide. Springer Nature. Gewerbestrasse 11, 6330 Cham, Switzerland. pp. 1-32.

Qiu Y., Guo J., Jing S., Zhu L. and He G., (2010) High-resolution mapping of the brown planthopper resistance gene *BPH6* in rice and characterizing its resistance in the 9311 and Nipponbare near isogenic backgrounds. Theor. Appl. Genet. 121: 1601–1611.

Qiu Y., Guo J., Jing S., Tang M., Zhu L. and He G., (2011) Identification of antibiosis and tolerance in rice varieties carrying brown planthopper resistance genes. Entomol. Exp. Appl. 141: 224–231.

Qiu Y., Guo J., Jing S., Zhu L. and He G., (2012) Development and characterization of *japonica* rice lines carrying the brown planthopper-resistance genes *BPH12* and *BPH6*. Theor. Appl. Genet. 124: 485–494.

Qiu Y., Guo J., Jing S., Zhu L. and He G., (2014) Fine mapping of the rice brown planthopper resistance gene *BPH7* and characterization of its resistance in the 93-11 background. Euphytica 198: 369–379.

Rahman M. L., Jiang W., Chu S. H., Qiao Y., Ham T. H., Woo M. O., Lee J., Khanam M.

S., Chin J. H., Jeung J. U. *et al*., (2009) High-resolution mapping of two rice brown planthopper resistance genes, *BPH20*(t) and *BPH21*(t), originating from *Oryza minuta*. Theor. Appl. Genet. 119: 1237–1246.

Reissig W. H., Heinrichs E. A., Litsinger J. A., Moody K, Fiedler L., Mew T. W. and Barrion A. T., (1986) Illustrated guide to integrated pest management in rice in tropical Asia. IRRI, Los Baños, Philippines. pp. 411.

Ren J., Gao F., Wu X., Lu X., Zeng L., Lv J., Su X., Luo H. and Ren G., (2016) *BPH32*, a novel gene encoding an unknown SCR domain-containing protein, confers resistance against the brown planthopper in rice. Sci. Rep. 6: 37645.

Renganayaki K., Fritz A. K., Sadasivam S., Pammi S., Harrington S. E., McCouch S. R., Kumar S. M. and Reddy A. S., (2002) Mapping and progress toward map-based cloning of brown planthopper biotype-4 resistance gene introgressed from *Oryza officinalis* into cultivated rice, *O. sativa*. Crop Sci. 42: 2112–2117.

Sakai H., Lee S. S., Tanaka T., Numa H., Kim J., Kawahara Y., Wakimoto H., Yang C. C., Iwamoto M., Abe T. *et al*., (2013) Rice annotation project database (RAP-DB): An integrative and interactive database for rice genomics. Plant Cell Physiol. 54: e6(1-11).

Sandhu R. K. and Sarao P. S., (2021) Evaluation of antixenosis resistance in wild rice accessions against brown planthopper, *Nilaparvata lugens* (Stål). Int. J. Trop. Insect Sci. 41: 65–73.

Saxena R. C. and Barrion A. A., (1985) Biotypes of the brown planthopper *Nilaparvata lugens* (Stål) and strategies in deployment of host plant resistance. Insect Sci. Applic. 6: 271–289.

Seck P. A., Diagne A., Mohanty S. and Wopereis M. C. S. (2012) Crops that feed the

world 7: Rice. Food Sec. 4: 7–24.

Shabanimofrad M., Rafii M. Y., Ashkani S., Hanafi M. M., Adam N. A., Harun A. R., Latif M. A., Miah G., Sahebi M. and Azizi P., (2017) Mapping of QTLs conferring resistance in rice to brown planthopper, *Nilaparvata lugens*. Entomol. Exp. Appl. 162: 60–68.

Sharma P. N., Torii A., Takumi S., Mori N. and Nakamura C., (2004) Marker-assisted pyramiding of brown planthopper (*Nilaparvata lugens* Stål) resistance genes *BPH1* and *BPH2* on rice chromosome 12. Hereditas 140: 61–69.

Sidhu G. S. and Khush G. S., (1978) Genetic analysis of brown planthopper resistance in twenty varieties of rice, *Oryza sativa* L. Theor. Appl. Genet. 53: 199–203.

L., Su C., Wang C., Zhai H. and Wan J., (2005) Mapping of a major resistance gene to the brown planthopper in the rice cultivar 'Rathu Heenati'. Breed. Sci. 55: 391–396.

Sun L. H., Wang C. M., Su C. C., Liu Y. Q., Zhai H. Q. and Wan J. M., (2006) Mapping and marker-assisted selection of a brown planthopper resistance gene *BPH2* in rice (*Oryza sativa* L.). Acta Genet. Sin. 33: 717–723.

Tamura Y., Hattori M., Yoshioka H., Yoshioka M., Takahashi A., Wu J., Sentoku N. and Yasui H., (2014) Map-based cloning and characterization of a brown planthopper resistance gene *BPH26* from *Oryza sativa* L. ssp. *indica* cultivar 'ADR52'. Sci. Rep. 4: 5872.

Temnykh S., DeClerck G., Lukashova A., Lipovich L., Cartinhour S. and McCouch S., (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): Frequency, length variation, transposon associations, and genetic marker potential. Genome Res. 11: 1441–1452.

Velusamy R., Heinrichs E. A. and Medrano F. G., (1986) Greenhouse techniques to

identify field resistance to the brown planthopper, *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae), in rice cultivars. Crop Prot. 5: 328–333.

Wang H., Shi S., Guo Q., Nie L., Du B., Chen R., Zhu L. and He G., (2018) High-resolution mapping of a gene conferring strong antibiosis to brown planthopper and developing resistant near-isogenic lines in 9311 background. Mol. Breed. 38: 107.

Wang Y., Cao L., Zhang Y., Cao C., Liu F., Huang F., Qiu Y., Li R. and Luo X., (2015) Map-based cloning and characterization of *BPH29*, a B3 domain-containing recessive gene conferring brown planthopper resistance in rice. J. Exp. Bot. 66: 6035–6045.

Wu H., Liu Y., He J., Liu Y., Jiang L., Liu L., Wang C., Cheng X. and Wan J., (2014) Fine mapping of brown planthopper (*Nilaparvata lugens* Stål) resistance gene *BPH28*(t) in rice (*Oryza sativa* L.). Mol. Breed. 33: 909–918.

Xiao C., Hu J., Ao Y. T., Cheng M. X., Gao G. J., Zhang Q. L., He G. C. and He Y. Q., (2016 a) Development and evaluation of near-isogenic lines for brown planthopper resistance in rice cv. 9311. Sci. Rep. 6: 38159.

Xiao Y., Li J., Yu J., Meng Q., Deng X., Yi Z. and Xiao G., (2016 b) Improvement of bacterial blight and brown planthopper resistance in an elite restorer line Huazhan of *Oryza*. Field Crop. Res. 186: 47–57.

Yang H., You A., Yang Z., Zhang F., He R., Zhu L. and He G., (2004) High-resolution genetic mapping at the *BPH15* locus for brown planthopper resistance in rice (*Oryza sativa* L.). Theor. Appl. Genet. 110: 182–191.

Yang L., Li R. B., Li Y. R., Huang F. K., Chen Y. Z., Huang S. S., Huang L. F., Liu C., Ma Z. F., Huang D. H. *et al*., (2012) Genetic mapping of *BPH20*(t) and *BPH21*(t) loci conferring brown planthopper resistance to *Nilaparvata lugens* Stål in rice

(*Oryza sativa* L.). Euphytica 183: 161–171.

Yang M., Cheng L., Yan L., Shu W., Wang X. and Qiu Y., (2019) Mapping and characterization of a quantitative trait locus resistance to the brown planthopper in the rice variety 'IR64'. Hereditas 156: 1–9.

Yara A., Phi C. N., Matsumura M., Yoshimura A. and Yasui H., (2010) Development of near-isogenic lines for *BPH25*(t) and *BPH26*(t), which confer resistance to the brown planthopper, *Nilaparvata lugens* (Stål.) in indica rice 'ADR52'. Breed. Sci. 60: 639–647.

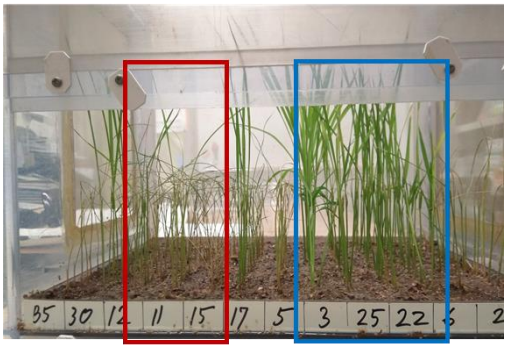
Young N. D. and Tanksley S. D., (1989) Restriction fragment length polymorphism maps and the concept of graphical genotypes. Theor. Appl. Genet. 77: 95–101.

Zhao Y., Huang J., Wang Z., Jing S., Wang Y., Ouyang Y., Cai B., Xin X. F., Liu X., Zhang C. *et al*., (2016) Allelic diversity in an NLR gene *BPH9* enables rice to combat planthopper variation. Proc. Natl. Acad. Sci. U.S.A. 113: 12850–12855.

Supplementary: Photos of experiments

Brown planthopper (*Nilaparvata lugens* (Stål))

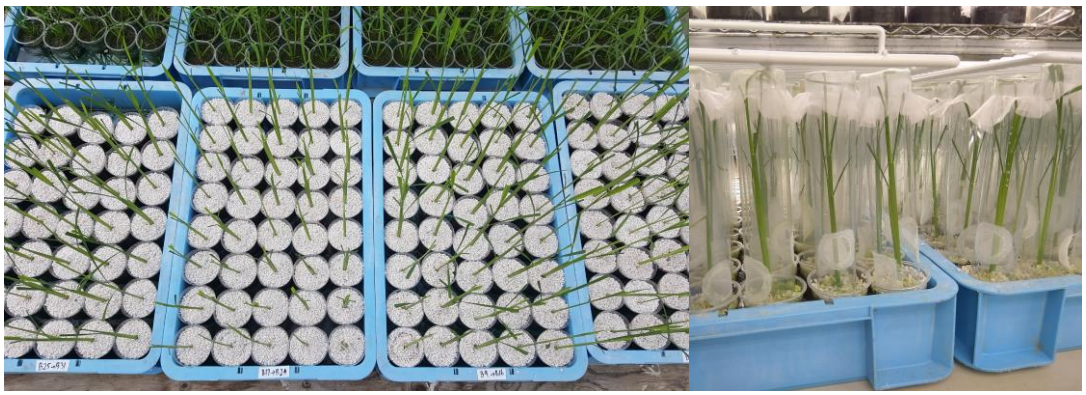
“Hopperburn” occurred in Saga city paddy field (2019)



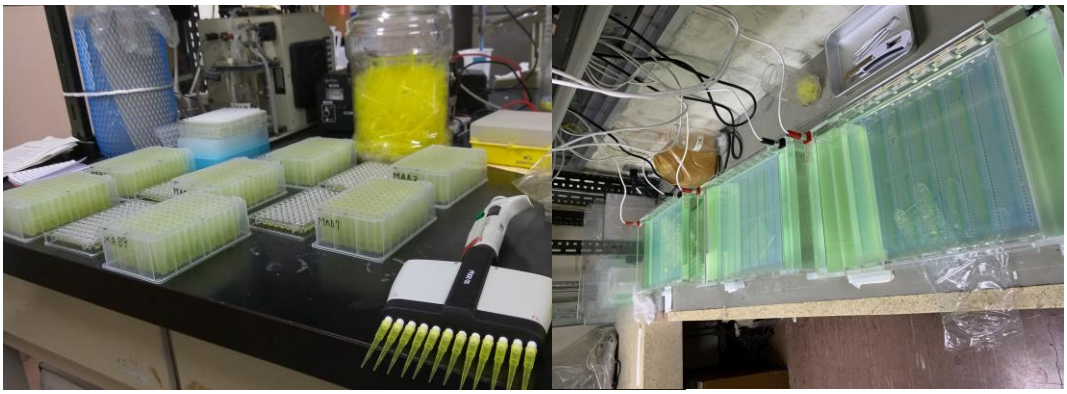
Non-resistant plants Resistant plants

Modified seedbox screening test

Field infestation by BPH



Antibiosis test



DNA extraction and PCR

Rice transplantation


Rice at various developmental stages


Rice flowering

Rice crossing


Rice harvesting