Allelic diversity in an NLR gene *BPH9* enables rice to combat planthopper variation

Yan Zhao[a,1], Jin Huang[a,1], Zhizheng Wang[a,1], Shengli Jing[a], Yang Wang[a], Yidan Ouyang[b], Baodong Cai[c], Xiu-Fang Xin[d], Xin Liu[e], Chunxiao Zhang[a], Yufang Pan[a], Rui Ma[a], Qiaofeng Li[a], Weihua Jiang[a], Ya Zeng[a], Xinxin Shangguan[a], Huiying Wang[a], Bo Du[a], Lili Zhu[a], Xun Xu[e], Yu-Qi Feng[c], Sheng Yang He[d,f], Rongzhi Chen[a,2], Qifa Zhang (张启发)[b,2], and Guangcun He[a,2]

[a]National Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Wuhan 430072, China; [b]National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China; [c]Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China; [d]Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824; [e]Beijing Genomics Institute, Shenzhen 518083, China; and [f]Howard Hughes Medical Institute, Michigan State University, East Lansing, MI 48824

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Brown planthopper (BPH), *Nilaparvata lugens* Stål, is one of the most devastating insect pests of rice (*Oryza sativa* L.). Currently, 30 BPH-resistance genes have been genetically defined, most of which are clustered on specific chromosome regions. Here, we describe molecular cloning and characterization of a BPH-resistance gene, *BPH9*, mapped on the long arm of rice chromosome 12 (12L). *BPH9* encodes a rare type of nucleotide-binding and leucine-rich repeat (NLR)-containing protein that localizes to the endomembrane system and causes a cell death phenotype. BPH9 activates salicylic acid- and jasmonic acid-signaling pathways in rice plants and confers both antixenosis and antibiosis to BPH. We further demonstrated that the eight BPH-resistance genes that are clustered on chromosome 12L, including the widely used *BPH1*, are allelic with each other. To honor the priority in the literature, we thus designated this locus as *BPH1/9*. These eight genes can be classified into four allelotypes, *BPH1/9-1*, *-2*, *-7*, and *-9*. These allelotypes confer varying levels of resistance to different biotypes of BPH. The coding region of *BPH1/9* shows a high level of diversity in rice germplasm. Homologous fragments of the nucleotide-binding (NB) and leucine-rich repeat (LRR) domains exist, which might have served as a repository for generating allele diversity. Our findings reveal a rice plant strategy for modifying the genetic information to gain the upper hand in the struggle against insect herbivores. Further exploration of natural allelic variation and artificial shuffling within this gene may allow breeding to be tailored to control emerging biotypes of BPH.

brown planthopper | plant–insect interaction | CNL protein | allelotype | evolution

In nature, ever since plant-eating insects first arose ∼350 million years ago, plants and insects have been engaged in endless cycles of attack and counterattack (1, 2). During this process, insects have developed various capabilities to take food from plants, and, accordingly, plants have evolved numerous strategies against the insects, such as antixenosis that repels insects from its normal host and antibiosis that reduces insect survival, growth rate, or reproduction (3, 4). In agriculture, insect pests represent a major constraint that reduces crop yield and quality globally. A process analogous to the coevolution in nature also occurs in the agricultural system. The brown planthopper (BPH; *Nilaparvata lugens* Stål) is one of the most devastating insect pests of rice (*Oryza sativa* L.) that widely occurs in South, Southeast, and East Asia, as well as the South Pacific islands and Australia. This insect is believed to have undergone a host shift from *Leersia* plants to rice ∼0.25 million years ago (5, 6). After that, BPH evolved as a monophagous insect herbivore of the cultivated rice *O. sativa*. During the course of its coevolution with BPH, rice has evolved various resistance mechanisms against BPH, so that BPH was kept as a minor insect in the traditional rice-culturing system.

The ability of BPH to feed and establish populations on rice plants varies with rice varieties. In the last several decades, there has been a dramatic shift away from the planting of traditional rice cultivars to modern, high-yielding, but susceptible, rice cultivars with an emphasis on insecticides (7). Such a cropping system appears to have stimulated the buildup of the BPH populations, resulting in frequent outbreaks that seriously decreased rice yield. Host-plant resistance to BPH was first experimentally demonstrated in 1969 in the variety Mudgo, which carries the resistance gene *BPH1* (8). IR26 was the first modern variety with *BPH1* resistance to BPH and was released in 1973. Then, a number of BPH-resistant varieties with *BPH1* gene were released, which saved rice production from massive BPH damage (9). However, the resistance broke down in 1976 with the development of a new BPH population (biotype 2), which was apparently able to feed on *BPH1* plants to cause hopperburn. Varieties with *BPH2* showing effective resistance were then released and widely grown. In 1981, another BPH population (biotype 3) capable of overcoming resistance of *BPH2* was detected. It is now known that wide variation in virulence occurs in BPH populations (10, 11).

Significance

Insect pests represent a major constraint that reduces crop yield and quality globally. Host plant resistance is often used as a key tactic to control insect pests, but is frequently overcome by newly emerged insect populations. In nature, plants have developed various strategies for sustainable defense. In this work, we isolated a brown planthopper-resistance gene, *BPH9*, and show that alleles of this gene locus have been widely used in rice breeding and saved rice production from massive brown planthopper (BPH) damage. Allelic diversity in this gene locus has provided resistance to rice against different BPH populations. Manipulating allelic diversity of the gene may provide a strategy for developing resistant varieties to cope with evolving insect populations with new virulence variation.

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[1]Y. Zhao, J.H., and Z.W. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: qifazh@mail.hzau.edu.cn, rzchen@whu.edu.cn, or gche@whu.edu.cn.

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In the last several decades, 30 BPH-resistance genes were identified in the cultivated rice and wild *Oryza* species. Interestingly, most of these genes are mapped on several chromosome regions in clusters (12–14). The cluster on the long arm of chromosome 12 (12L) is the largest one that harbors eight BPH-resistance genes, including the most widely used *BPH1* and *BPH2* (12, 14). One gene, *BPH26*, has been cloned from this region (15).

Here, we isolated *BPH9* from 12L via map-based cloning strategy. We showed that *BPH9* is allelic to *BPH26*, and the other BPH-resistance genes in the cluster are alleles of *BPH9*/*26*. This gene locus showed wide sequence diversity in the rice germplasm. This finding has significant implications for coevolution of the host–pest interactions and also for breeding of resistance varieties.

Results

Characterization of BPH Resistance of NIL-*BPH9*. *BPH9* from an *indica* rice variety Pokkali (International Rice Genebank Collection no. 108921) (4, 16) was introgressed into the BPH-susceptible, high-yielding *indica* variety 9311 through successive back-crossing. The resulting near-isogenic line NIL-*BPH9* showed strong resistance to BPH at the seedling, tillering, and mature stages (Fig. 1*A* and *SI Appendix*, Fig. S1 *A–C*). NIL-*BPH9* displayed resistance to BPH biotypes 1, 2, and 3, indicating that *BPH9* offers broad-spectrum resistance to BPH (*SI Appendix*, Fig. S1*D*). Furthermore, *BPH9* did not affect the agronomic performance of the rice plant (*SI Appendix*, Fig. S2). These results demonstrated that *BPH9* is a valuable resource for rice breeding. A two-host choice test showed that BPH insects preferred to settle on 9311 than on NIL-*BPH9* plants; thus *BPH9*, had an antixenosis effect (Fig. 1*B*). Furthermore, BPH insects fed on NIL-*BPH9* plants showed significantly lower survival rate, body weight gain, and honeydew excretion (which is an indirect measure of phloem consumption) than those on 9311 plants; thus, *BPH9* also exhibited antibiosis effects (Fig. 1*C* and *SI Appendix*, Fig. S1 *E* and *F*).

Map-Based Cloning of BPH9. To map the *BPH9* gene, we developed an F_2 mapping population derived from a cross between 9311 and Pokkali. The *BPH9* locus explained 77.8% of the resistance variation in the F_2 population and was mapped between molecular markers RM28486 and RM28438 (Fig. 1*D*) within the BPH-resistance gene cluster on chromosome 12L. Analysis of 3,000 BC_3F_2 plants delimited *BPH9* to the genomic region flanked by InD2 and RsaI (Fig. 1*E* and *SI Appendix*, Table S1). Further analysis of 10,000 BC_5F_2 plants using the flanking markers InD2 and RsaI identified another four recombinants, which further narrowed the target region. To obtain the sequence information of *BPH9* region, we constructed a fosmid genomic library of Pokkali, and two clones covering the *BPH9* region were identified and sequenced (Fig. 1*F*). It was found that, in the Pokkali genome, the *BPH9* region flanked by InD2 and RsaI was 68 kb in length and contained two pairs of transposable elements in opposite directions (*SI Appendix*, Fig. S3, Pok). The corresponding regions in 9311 and Nipponbare were 47 and 51 kb, respectively (signal.salk.edu/cgi-bin/RiceGE) (*SI Appendix*, Fig. S3, Nip and 9311).

Among the eight putative genes annotated in this region in Pokkali, two leucine-rich repeat-containing genes showing sequence difference from 9311 were considered as candidates for *BPH9* thus designated *R1* and *R2* (*SI Appendix*, Fig. S3, Pok). We obtained the full-length cDNA of *R2* using rapid amplification of cDNA ends (RACE), but did not detect the expression of *R1*. The *R1* and *R2* genomic fragments were transformed into the BPH-susceptible *indica* variety Kasalath (*SI Appendix*, Fig. S4). Transgenic plants containing the candidate gene *R1* showed no resistance to BPH and died within a few days of BPH infestation (*SI Appendix*, Fig. S5). This gene was excluded from further analysis. By contrast, transgenic lines derived from independent T_0 transformants with *R2* genomic construct showed high levels of resistance to BPH (Fig. 1*G*). Complementation with the *R2* cDNA construct under the control of its native promoter showed the same results (Fig. 1*H*). The BPH resistance of *R2*-transgenic lines was also confirmed by measuring survival rate, weight gain, honeydew excretion, and a two-host choice test of BPH individuals (*SI Appendix*, Fig. S6 *A–D*). These results indicated that *R2* confers BPH resistance and is the *BPH9* gene.

BPH9 Encodes an Endomembrane-Localized Nucleotide-Binding and Leucine-Rich Repeat Protein That Induces Cell Death. *BPH9* was predicted to encode 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 (Fig. 2*A*), a rare type of nucleotide-binding and leucine-rich repeat (NLR) gene having partially duplicated NBS domains (17) (*SI Appendix*, Fig. S7). Many

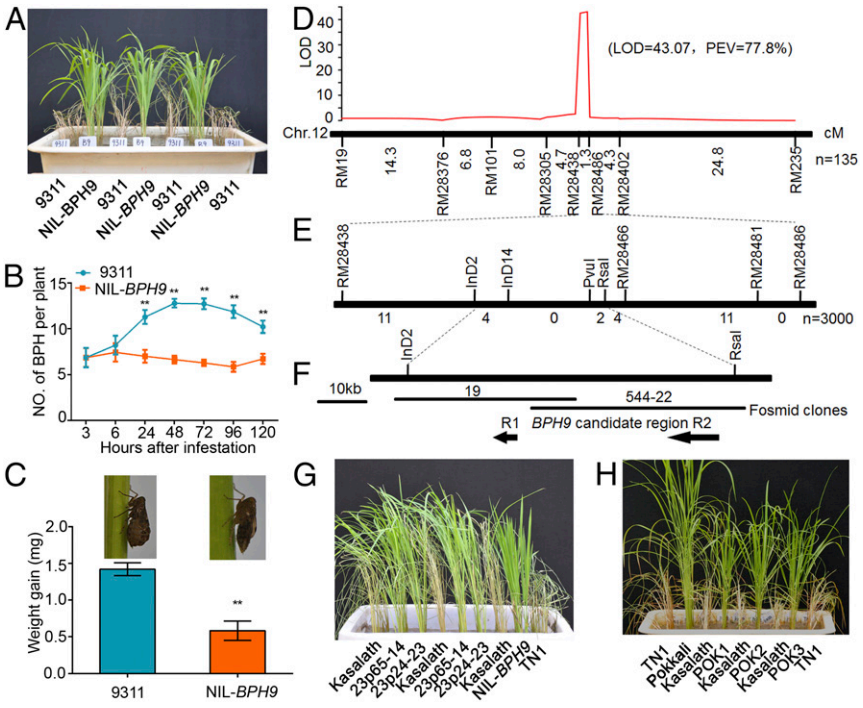

Fig. 1. Map-based cloning and functional characterization of *BPH9*. (*A*) NIL-*BPH9* shows a high level of resistance to BPH at the seedling stage. (*B*) Two-host choice test showing more BPH insects settling on 9311 than on NIL-*BPH9* plants (n = 15). (*C*) Body weight gain and images of BPH insects feeding on NIL-*BPH9* and 9311 plants for 2 d. All data are means ± SEM. $^{**}P < 0.01$ (Student's t test). (*D*) Mapping of *BPH9* to the interval between the molecular markers RM28486 and RM28438 on chromosome 12L. LOD, logarithm of odds; PEV, phenotypic variance explained by the locus. (*E*) Delimiting *BPH9* to the genomic region flanked by InD2 and RsaI. The numbers below the line indicate the numbers of recombinants between adjacent markers. (*F*) Identifying and sequencing of the two fosmid clones of Pokkali genomic library to obtain the 68-kb sequence of the *BPH9* region and identifying *R1* and *R2* as candidates for *BPH9*. (*G*) BPH-resistance assay of transgenic lines harboring the *BPH9*-genomic region (15.6 kb). The lines 23p65-14 and 23p24-23 are two independent transgenic lines. (*H*) BPH resistance assay of transgenic lines harboring the *BPH9* cDNA construct. POK1–POK3 are three independent transgenic lines. Kasalath and TN1, susceptible variety.

nucleotide differences were detected between *BPH9* and susceptible alleles, causing amino acid substitutions, deletions, or protein truncation (Fig. 2*A* and *SI Appendix*, Fig. S7). Expression of *BPH9* was detected in all of the tissues assayed at very low levels, with relatively high expression in leaf sheaths and florets (*SI Appendix*, Fig. S8*A*). The expression level of *BPH9* was stable, even upon BPH infestation, and no significant difference was observed between NIL-*BPH9* and 9311 (*SI Appendix*, Fig. S8*B*), suggesting that nucleotide polymorphisms might be mainly responsible for the resistance difference between resistant and susceptible alleles. P_{BPH9}::*GUS* transgenic lines showed that the gene was mainly expressed in the parenchyma cells bordering xylem vessels and sieve tubes (Fig. 2*B*), the site of BPH feeding. GUS activity was not significantly enhanced by BPH infestation (*SI Appendix*, Fig. S8*C*). Coexpressing *BPH9:GFP* or *BPH9:YFP* fusion gene with the nuclear, peroxisome, and plastid markers in rice protoplast excluded the possibility of colocalization of BPH9 with these organelles (*SI Appendix*, Fig. S9 *A–C*). Further colocalization experiments showed that BPH9-YFP overlapped with a series of endomembrane organelle markers, including endoplasmic reticulum (ER), Golgi apparatus (GA), and exocyst-positive organelle (EXPO) (Fig. 2*C*).

When BPH9 was overexpressed in rice protoplasts, the viability of rice protoplasts was obviously reduced, indicating that it caused a cell death phenotype (*SI Appendix*, Fig. S10). This cell death phenotype is different from the situation of *BPH14*, a previously cloned NLR-type BPH-resistance gene containing a single NB domain (18), which did not induce such cell death (*SI Appendix*, Fig. S10). To validate this result, we further coexpressed *BPH9* with the firefly luciferase (LUC) reporter gene in rice protoplasts (19). The expression of *BPH9* led to a significant reduction in LUC activity in rice protoplasts, also suggesting induction of cell death (Fig. 2*D*), whereas expression of *BPH14* had no effect on LUC activity (Fig. 2*D*).

Activation of Salicylic Acid- and Jasmonic Acid-Signaling Pathways in *BPH9* Plants. To better understand the molecular mechanism underlying resistance to BPH mediated by *BPH9*, we conducted a genome-wide microarray analysis of NIL-*BPH9* and 9311 at the early stages (3 and 6 h) of BPH infestation (*SI Appendix*, Fig. S11*A*). Gene Ontology (GO) analysis revealed strong correlation of hormone pathways, especially salicylic acid (SA) and jasmonic acid (JA), with resistance of *BPH9*. Genes related to defense pathways were enriched in uninfested NIL-*BPH9* plants, suggesting that the plants might be in a primed state (*SI Appendix*, Fig. S11*B*). After BPH infestation, more GO terms corresponding to defense and hormone pathways were up-regulated in NIL-*BPH9* plants (*SI Appendix*, Fig. S11 *C* and *D*). We then confirmed by quantitative PCR that genes involved in SA (*OsPAL* and *OsICS1*; refs. 20 and 21) and JA biosynthesis (*OsLOX, OsAOS2*, and *OsJAmyb*; refs. 20 and 22) and signaling responses (*OsPR10*; ref. 23), as well as ones related to defense, were differentially expressed between NIL-*BPH9* and 9311 and also in comparison of post- vs. pre-BPH infestation (*SI Appendix*, Fig. S12). Measurement of phytohormone levels demonstrated that SA increased substantially 1 h after BPH infestation and throughout the infestation in NIL-*BPH9*, whereas no increase was detected in 9311 (*SI Appendix*, Fig. S13*A*). JA and its conjugate JA-Ile also increased rapidly after BPH infestation in NIL-*BPH9*, but the overall level was lower in NIL-*BPH9* than in 9311, probably because of the antagonistic relationship of SA and JA (*SI Appendix*, Fig. S13 *B* and *C*; refs. 24 and 25). No significant difference between NIL-*BPH9* and 9311 was observed for other hormones (*SI Appendix*, Fig. S13 *D–F*). Because plant hormones play pivotal roles in the regulation of defense-signaling networks, the above results suggest that *BPH9* may allow NIL plants to maintain a primed state that quickly activates SA- and JA-signaling pathways for rapid response to BPH feeding.

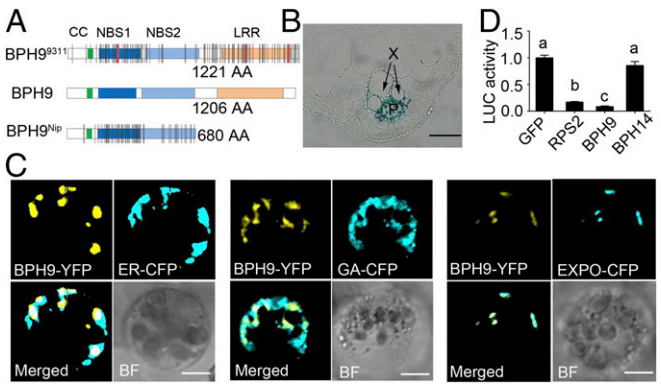

Fig. 2. Molecular characterization of *BPH9*. (*A*) *BPH9* and susceptible alleles in 9311 ($BPH9^{9311}$) and Nipponbare ($BPH9^{NIP}$) showing substantial variation in single-nucleotide polymorphisms (SNPs; black lines) and insertion/deletions (InDels; red lines), with detail shown in *SI Appendix*, Fig. S7. (*B*) Histochemical staining of P_{BPH9}::*GUS* reporter transgenic lines showing *BPH9* expression in parenchyma cells bordering the vascular bundle. (Scale bar, 500 μm.) P, phloem; X, xylem. (*C*) Colocalization of BPH9-YFP fluorescence with the endomembrane marker-CFP. BF, bright field; Endoplasmic reticulum (ER) marker, AtWAK2-HDEL-CFP; Exocyst-positive organelle (EXPO) marker, EXO70E2-CFP; Golgi apparatus (GA) marker, Man49-CFP. (Scale bars, 5 μm.) (*D*) Relative LUC activity in rice protoplasts expressing *BPH9* and *BPH14* with *RPS2* as the positive control. Different characters above the bar indicate a significant difference ($P < 0.05$ by one-way analysis of variance and LSD test as post hoc analysis). Data are means ± SEM.

The Eight BPH-Resistance Genes on Chromosome 12L Are Multiple Alleles of the Same Locus. In addition to *BPH9*, seven BPH-resistance genes (*BPH1*, *BPH2*, *BPH7*, *BPH10*, *BPH18*, *BPH21*, and *BPH26*) have been reported on chromosome 12L (12, 14, 26–30). Among them, *BPH26* has been cloned, and *BPH2* is identical to *BPH26* (ref. 15; GenBank accession no. AB910360.1). Sequence information for *BPH18* is also available in the National Center for Biotechnology Information database (GenBank accession nos. KF890252 and AIE17464.1). *BPH9* shares 91.32% and 96.05% nucleotide sequence identity with *BPH26* and *BPH18*, respectively (*SI Appendix*, Fig. S14*A*). Together with their chromosome position and genomic sequence, these data confirmed that *BPH9, BPH2/26*, and *BPH18* are alleles. To test whether the remaining four BPH-resistance genes (*BPH1*, *BPH7*, *BPH10*, and *BPH21*) in this cluster are also alleles of *BPH9/26*, the full-length cDNAs of the gene corresponding to *BPH9* were obtained from the varieties with which these four genes were originally mapped. We found that the nucleotide and deduced amino acid sequences in rice varieties harboring *BPH1*, *BPH10*, and *BPH21* were identical to *BPH18*, whereas the sequence from T12 (harboring *BPH7*) indicated a distinct allele (*SI Appendix*, Fig. S14*B*; refs. 26–30). Thus, our findings demonstrated that the eight genes in this cluster are multiple alleles of the same locus, and, to honor the priority in the literature, we designate this locus *BPH1/9*. These alleles can be classified into four allelotypes based on their sequences, *BPH1/9-1*, *-2*, *-7*, and *-9* (Fig. 3*A*).

To identify the features of the resistance alleles *BPH1/9-1*, *-2*, and *-7*, we again used the rice protoplast transient expression assay system to analyze the effect of these alleles on the expression of the LUC reporter gene. The results indicated that the expression of these three allelotypes also induced cell death in rice protoplasts. It is interesting that the susceptible allele in Nipponbare also caused a moderate degree of cell death (Fig. 3 *B* and *C* and *SI Appendix*, Fig. S15). When transiently expressed in rice protoplast, these three allelotypes showed the same subcellular localization as BPH1/9-9 (*SI Appendix*, Fig. S16). Together, these results suggested that four resistant allelotypes *BPH1/9-1*, *-2*, *-7*, and *-9* occurring on chromosome 12L had similar characteristics to BPH.

We further tested the effects of these resistant allelotypes against different BPH biotypes by measuring the insect bodyweight gain on plants carrying different allelotypes. All four types of alleles conferred strong resistance to BPH biotype 1 and a wild BPH population (Fig. 3*D* and *SI Appendix*, Fig. S17). BPH biotype 2 was a *BPH1*-virulent population (10). As expected, all

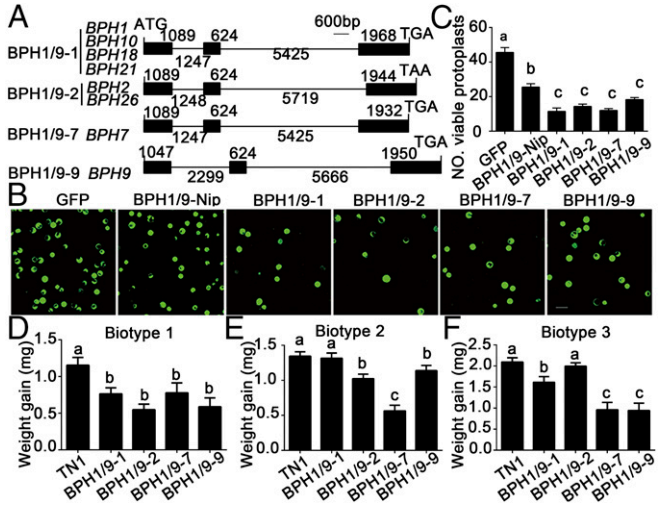

Fig. 3. The eight BPH-resistance genes and their diverse levels of resistance to BPH. (*A*) Structures of the four allelotypes identified from the eight genes from chromosome 12L. Shaded boxes indicate exons, and lines represent introns. Numbers indicate the lengths (bp) of the corresponding exons and introns. (*B*) Images of fluorescein diacetate (FDA)-stained viable rice protoplasts transformed with *GFP* or *BPH1/9* allelotypes. Photographs were taken 20 h after transfection. (Scale bar, 20 μm.) (*C*) Numbers of viable rice protoplasts transformed with *GFP* or *BPH1/9* allelotypes were scored after FDA-staining. Average values and SEs were calculated from three independent experiments, and at least 10 randomly selected microscopy fields were scored per experiment ($n = 10$). (*D–F*) Evaluation of resistance of rice plants carrying different *BPH1/9* allelotypes to BPH biotype 1 (*D*), biotype 2 (*E*), and biotype 3 (*F*). Body weight gains of BPH insects were measured after feeding on rice plants. Different characters above the bars indicate a significant difference by one-way analysis of variance and LSD test as post hoc analysis. All data are means ± SEM. For *D–F*, at least 30 BPH insects were used for analysis.

allelotypes, except for *BPH1/9-1*, conferred resistance to biotype 2 (Fig. 3*E*). BPH biotype 3 was a *BPH2*-virulent population. Apparently, biotype 3 insects gained more weight on *BPH1/9-2*–containing plants than on other resistant plants, indicating improved feeding ability (Fig. 3*F*). The above results demonstrated that the allelic variants of this BPH-resistance gene locus on chromosome 12L confer diverse levels of resistance to BPH biotypes.

Allelic Variation of *BPH1/9* in Rice Populations. An analysis of nucleotide variation in the coding region of *BPH1/9* locus in 117 rice varieties and landraces (*SI Appendix*, Table S2) detected 520 polymorphic sites and 21 haplotypes (Fig. 4*A*). The *indica* varieties showed a wide diversity in *BPH1/9* with 19 haplotypes including the four resistant allelotypes. It is noteworthy that the haplotype H20 (allelotype *BPH1/9-1*) was widely distributed in landraces and improved varieties (*SI Appendix*, Table S2). The significant positive Tajima's D and Fu and Li's D values indicated a clear signature of balancing selection imposed on the *BPH1/9* locus in the *indica* group (*SI Appendix*, Table S3). By contrast, the *BPH1/9* sequences in *japonica* varieties were highly conserved (*SI Appendix*, Tables S2 and S3) by encoding a truncated protein without an LRR domain, because of a premature stop codon (2041T) in exon3 (Fig. 2*A*), as reported (15). Notably, this site was G (2041G) in the wild relative species *Leersia* and most of the *indica* group; thus, it is likely the original base at this site. It mutated to T and later became fixed in the *japonica* group. Significant negative Tajima's D and Fu and Li's D values indicated that the *japonica* group had undergone either purifying selection or population expansion. The higher π value indicated that nucleotide diversity was much higher in the LRR domain than in the CC and NBS domains (Fig. 4*B* and *SI Appendix*, Table S3). Amino acid analysis of the resistance alleles also revealed that their variation occurred mainly within the LRR region (Fig. 4 *B* and *C* and *SI Appendix*, Fig. S14). Further analysis of the indicated resistant and susceptible variants identified potential functional polymorphisms in 14 amino acids, most of which also reside in LRR region (Fig. 4*C*). We infer that the LRR domain may play a crucial role in determining the resistance difference.

The LRR domain of BPH9 is encoded by exon3 (*SI Appendix*, Fig. S18*A*). We detected an additional fragment, separated by the two pairs of transposable elements, having high sequence identity to exon3 of *BPH9* (this fragment is hereafter referred to as "B9E3H"; Fig. 4*D*). The candidate gene *R1* is a partial sequence of B9E3H (*SI Appendix*, Fig. S3). Fragments homologous to exon3 are also present in 9311 and Nipponbare, both which are susceptible to BPH, and in all of the sequenced varieties that carry the four resistant allelotypes (Fig. 4*D*). Detailed analysis of exon3 and the homologous fragments revealed that there is a patchwork pattern of sequence combinations. A contiguous fragment (~600 bp) in exon3 of *BPH1/9-7*, which is the major region distinguishing *BPH1/9-7* from *BPH1/9-1*, is identical to the fragment B9E3H (Fig. 4*E*). A contiguous segment identical to B9E3H was also present in *BPH1/9-2* (Fig. 4*E*). Additional homologous fragments were found in wild rice *O. barthii*, including two homologs of exon3, two homologs of exon1, and one homolog of exon2 (*SI Appendix*, Fig. S18*B*). The polymorphic sites in exon1 that differentiate *BPH1/9-9* from other resistant alleles share most nucleotides with exon1 and its reverse homologous fragment (E1R) in *O. barthii* (*SI Appendix*, Fig. S19). This patchwork distribution suggested that sequence exchanges between the coding sequences and their corresponding homologous fragments may have been involved in the evolution of this gene locus. It may also be possible that such additional homologous sequences serve as repositories for recombination, conversion, and unequal crossing over, and thus for the generation of sequence diversity leading to novel resistant alleles (31, 32).

Discussion

Allelic variation is considered to be an important driving force in evolution. Mutations in a gene may have very large effects on the phenotype by altering the structure, function, or expression level of the encoded protein, which may be necessary for competitive advantage and survival (33). In crops, superior alleles for higher grain yield have been priorities for selection during the domestication and breeding of cereals, whereas superior alleles of disease- and insect-resistance genes may have been left untapped in wild species or landraces (34, 35). Exploring allelic variation in resistance genes is therefore essential in modern crop improvement programs. In this study, we cloned *BPH9* and found it to be an allele of *BPH26* (15). Exploring the allelic diversity of this gene locus in rice germplasm showed that there is a wide range of allelic variation in this gene, and the eight BPH-resistance genes (*BPH1*, *BPH2*, *BPH7*, *BPH9*, *BPH10*, *BPH18*, *BPH21*, and *BPH26*) previously mapped on chromosome 12L are actually multiple alleles of the same gene (Fig. 3*A*). Our results showed that allelic variation is an important strategy for rice to combat BPH biotype variation.

Allelic variation is obviously a conserved and effective genetic mechanism for plants to coevolve with bioinvaders. Several disease-resistance gene clusters have been reported as being of multiple resistance alleles at a single locus, such as the *Pi9* locus in rice, the *L* locus in flax, and the *Rpp8* locus in *Arabidopsis* (36–38). The mapped BPH-resistance genes are mostly clustered on several chromosome regions, and ~50% of these genes aggregate in clusters on chromosomes 4S and 12L (7). In this study, we showed that insect-resistance genes clustered on chromosome 12L are actually multialleles. For those BPH-resistance genes on chromosome 4S, at least two, *BPH3* and *BPH15*, have been identified to encode lectin receptor kinases (13, 39). Rice appears to adapt the same strategy in the coevolution war to insect pests as to pathogens. It is speculated that the clustering of related R genes can increase the opportunity for genetic exchange that could act as reservoirs of mutational variation (40). No additional NBS-LRR–encoding genes were found within the 500-kb region surrounding *BPH1/9* in the reference genome sequence of

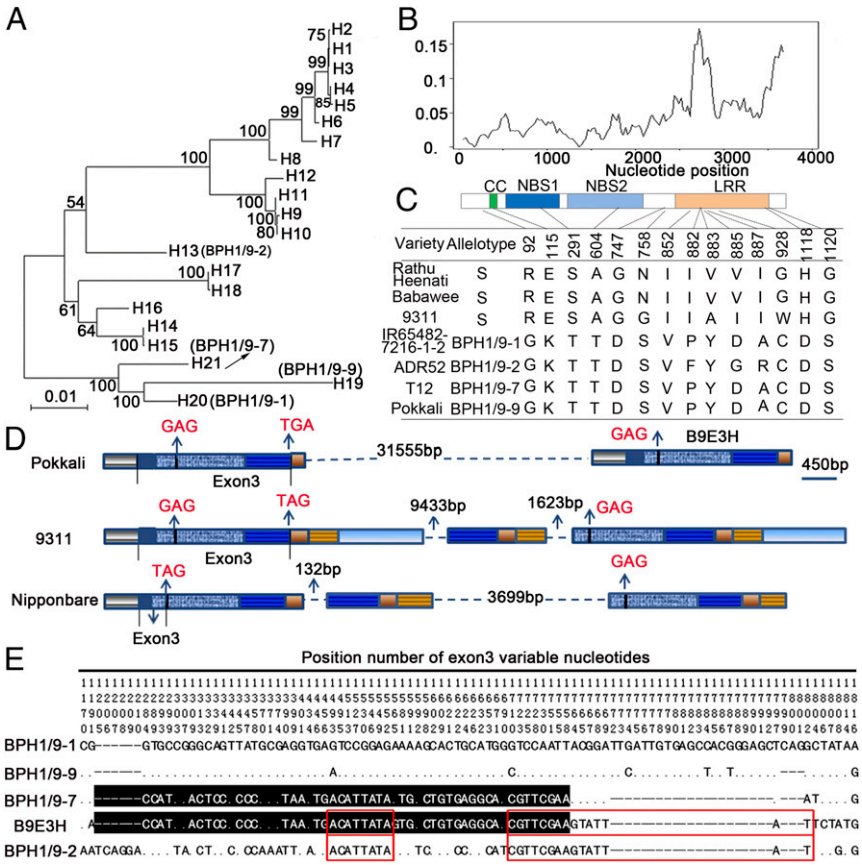

Fig. 4. Evolutionary analysis of *BPH1/9*. (*A*) Phylogenetic tree of the *BPH1/9* haplotypes. (*B*) Sliding-window plot of nucleotide diversity (π) in the *BPH1/9* coding region in all rice varieties sequenced. (*C*) Potential functional polymorphic sites in BPH1/9 detected by comparing resistant and susceptible alleles with no consensus amino acid. Positions indicated are relative to the first amino acid in BPH9. (*D*) *BPH1/9* exon3 and its homologous fragments in varieties of Pokkali, 9311, and Nipponbare. Identical colors indicate sequence with high identity. Exon3 interval is indicated by short lines. B9E3H is separated from exon3 by a pair of transposable elements (~31,555 bp) in Pokkali. In 9311 and Nipponbare, two incomplete exon3 homologous fragments were found, but not separated by large transposable elements from exon3. (*E*) Sequence patchwork in exon3 of *BPH9* alleles and its homologous fragment B9E3H. Only polymorphic sites are shown. The numbers presented vertically at the top indicate position number of exon 3 variable nucleotides. Dots represent nucleotides identical to those in the reference sequence of *BPH1/9-1*. Gaps in the alignment are represented by dashes. Black shaded letters indicate uninterrupted sequence stretches of identity sequence affiliations between *BPH1/9-7* and B9E3H. Red boxes indicate uninterrupted sequence affiliations between *BPH1/9-2* and B9E3H.

9311 and Nipponbare (signal.salk.edu/cgi-bin/RiceGE). Thus, they do not belong to a duplicated gene family. However, homologous fragments of *BPH1/9* exons exist, which seems to play a role in generating the alleles similar to physically linked paralogs (Fig. 4*E* and *SI Appendix*, Fig. S18). It is noteworthy that the average nucleotide diversity of the LRR region (π = 0.07928, θ = 0.05131) at the *BPH1/9* locus is much higher than that of the other domains and other studies (41). The LRR domains are thought to mediate direct or indirect interactions with pathogen-derived molecules that mediate recognition specificity (37), and the highly variable LRR domain may evolve to counteract the high degree of genetic variability in BPH. Further identification of BPH effectors would benefit the elucidation of interaction mechanism.

Our results suggest that *BPH1/9* is the most important locus in resistance evolution and resistance variety breeding because the widely used BPH-resistance genes *BPH1* and *BPH2* are resistance alleles of this locus. On the basis of our results and knowledge currently available about BPH-resistance genes on chromosome 12L, we propose that allelotype *BPH1/9-1* (including previously reported *BPH1*, *BPH10*, *BPH18*, and *BPH21*) evolved early in the wild progenitor of rice based on two lines of evidence. First, *BPH10* and *BPH18* were introgressed from the wild rice species *O. australiensis* (E genome) (29, 42) and *BPH21* from *O. minuta* (BC genome) (30), respectively. Second, the allelotype *BPH1/9-1* (H20) was found to be the most widespread among the four resistant allelotypes in rice populations (*SI Appendix*, Table S2). This allelotype efficiently resists the most common form of the BPH population (biotype 1). Other resistant allelotypes would have evolved later as a consequence of the emergence of new BPH biotypes.

What is especially interesting is that genetic improvement for BPH resistance in the course of modern rice breeding has reproduced the evolution process in the nature. *BPH1* is the first identified BPH resistance gene, and the resistance was broken down by the BPH biotype 2. Then, *BPH2* was identified to resist BPH biotype 2, but the resistance was overcome by BPH biotypes 3 later (10, 11). In this study, we showed that *BPH1/9-7* and *-9* are alternative allelotypes in this locus that confer resistance to BPH biotypes 1, 2, and 3. Importantly, these resistant allelotypes do not affect the agronomic traits of rice, as we showed in *BPH1/9-9*. Further exploration of natural allelic variation and artificial shuffling in this gene will allow breeding to be tailored to control emerging biotypes of BPH in the future.

Subcellular localization experiments showed that *BPH1/9* alleles localized to the endoplasmic reticulum, the Golgi apparatus, and EXPO, highlighting the endomembrane system (ES) as a prominent battlefield for rice–BPH interaction. ES has recently attracted much attention in regulating immune receptor activation, signal transduction, and execution of multiple defense responses, including pathogen-induced programmed cell death, cell wall synthesis, and callose deposition (43, 44). Because of its pivotal role for plants, some pathogens cleverly target their

effectors to ES to interfere with secretory and endocytic trafficking to promote successful infection (45). Previous studies have shown that disease-resistance protein L6 and M in flax and RPP1 in *Arabidopsis* also localize to the ES (46). In a recent report, the blast effector AVR-Pii forms a complex with rice *OsExo70-F2/F3*, which is required for Pii-dependent blast resistance, suggesting a role for this EXO70 paralog as a decoy or helper in Pii/Avr-Pii interaction (47). These observations suggest that the components of the secretory system are monitored by NLRs. It can be inferred that *BPH1/9-9* and its alleles may act as a supervisor residing in the ES to recognize the feeding of BPH. Once perceiving the signals of BPH, the secretory-trafficking system can deliver enzymes to deposit callose, thicken cell wall, or produce defense compounds (43). Because plant resistance response is a series of complicated and multilayered orchestration of defense strategies, wide distribution of resistance proteins in the ES would contribute to the rapid activation of these downstream responses. It is expected that more details and mechanisms will be elucidated in the near future and eventually facilitate the development of BPH-resistant Green Super Rice (48).

Materials and Methods

Plant materials and brown planthopper are described in *SI Appendix, SI Materials and Methods*. Details of the methods used are provided in *SI Appendix, SI Materials and Methods*, including BPH resistance evaluations, the process of map-based cloning of *BPH9*, complementation tests of *BPH9,* RNA isolation, quantitative RT-PCR analysis and RACE, microarray analysis, GUS histochemical staining, protoplast transient expression assays, sequencing and phylogenetic analysis, and agronomic performance test.

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1. Rausher MD (2001) Co-evolution and plant resistance to natural enemies. *Nature* 411(6839):857–864.
2. Labandeira C (2007) The origin of herbivory on land: Initial patterns of plant tissue consumption by arthropods. *Insect Sci* 14(4):259–275.
3. Smith CM, Clement SL (2012) Molecular bases of plant resistance to arthropods. *Annu Rev Entomol* 57:309–328.
4. Qiu YF, et al. (2011) Identification of antibiosis and tolerance in rice varieties carrying brown planthopper resistance genes. *Entomol Exp Appl* 141(3):224–231.
5. International Rice Research Institute (1979) *Brown Planthopper: Threat to Rice Production in Asia* (International Rice Research Institute, Los Baños, The Philippines).
6. Jones PL, Gacesa P, Butlin RK (1996) Systematics of brown planthopper and related species using nuclear and mitochondrial DNA. *Syst Assoc Spec Vol* 53:133–148.
7. Heinrichs EA, Mochida O (1984) From secondary to major pest status: The case of insecticide-induced rice brown planthopper, *Nilaparvata lugens*, resurgence. *Prot Ecol* 7(2/3):201–218.
8. Pathak MD, Cheng CH, Fortuno ME (1969) Resistance to *Nephotettix impicticeps* and *Nilaparvata lugens* in varieties of rice. *Nature* 223(5205):502–504.
9. Khush GS, Virk PS (2005) *IR Varieties and Their Impact* (International Rice Research Institute, Los Baños, Philippines).
10. Claridge MF, Hollander JD (1980) The "biotypes" of the rice brown planthopper, *Nilaparvata lugens*. *Entomol Exp Appl* 27(1):23–30.
11. Kobayashi T (2016) Evolving ideas about genetics underlying insect virulence to plant resistance in rice-brown planthopper interactions. *J Insect Physiol* 84:32–39.
12. Cheng X, Zhu L, He G (2013) Towards understanding of molecular interactions between rice and the brown planthopper. *Mol Plant* 6(3):621–634.
13. Liu Y, et al. (2015) A gene cluster encoding lectin receptor kinases confers broad-spectrum and durable insect resistance in rice. *Nat Biotechnol* 33(3):301–305.
14. Jena KK, Kim SM (2010) Current status of brown planthopper (BPH) resistance and genetics. *Rice (N Y)* 3(2-3):161–171.
15. Tamura Y, et al. (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.
16. Nemoto H, Ikeda R, Kaneda C (1989) New genes for resistance to brown planthopper, *Nilaparvata lugens* Stål, in rice. *Jap Jour Breed* 39:23–28.
17. Zhou T, et al. (2004) Genome-wide identification of NBS genes in japonica rice reveals significant expansion of divergent non-TIR NBS-LRR genes. *Mol Genet Genomics* 271(4):402–415.
18. Du B, et al. (2009) Identification and characterization of Bph14, a gene conferring resistance to brown planthopper in rice. *Proc Natl Acad Sci USA* 106(52):22163–22168.
19. Li X, et al. (2005) Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. *Proc Natl Acad Sci USA* 102(36): 12990–12995.
20. Duan L, Liu H, Li X, Xiao J, Wang S (2014) Multiple phytohormones and phytoalexins are involved in disease resistance to *Magnaporthe oryzae* invaded from roots in rice. *Physiol Plant* 152(3):486–500.
21. Qiu D, et al. (2007) OsWRKY13 mediates rice disease resistance by regulating defense-related genes in salicylate- and jasmonate-dependent signaling. *Mol Plant Microbe Interact* 20(5):492–499.
22. Lee MW, Qi M, Yang Y (2001) A novel jasmonic acid-inducible rice myb gene associates with fungal infection and host cell death. *Mol Plant Microbe Interact* 14(4): 527–535.
23. Hwang SH, Lee IA, Yie SW, Hwang DJ (2008) Identification of an OsPR10a promoter region responsive to salicylic acid. *Planta* 227(5):1141–1150.
24. Lee A, et al. (2004) Inverse correlation between jasmonic acid and salicylic acid during early wound response in rice. *Biochem Biophys Res Commun* 318(3):734–738.
25. De Vleesschauwer D, Gheysen G, Höfte M (2013) Hormone defense networking in rice: Tales from a different world. *Trends Plant Sci* 18(10):555–565.
26. Cha YS, 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(2):146–151.
27. Qiu YF, Guo JP, Jing SL, Zhu LL, He GC (2014) Fine mapping of the rice brown planthopper resistance gene *BPH7* and characterization of its resistance in the 93-11 background. *Euphytica* 198(3):369–379.
28. Thi Lang N, Chi Buu B (2003) Genetic and physical maps of gene *Bph10* controling brown plant hopper resistance in rice (*Oryza sativa* L.). *Omonrice* 11:35–41.
29. Jena KK, Jeung JU, Lee JH, Choi HC, Brar DS (2006) High-resolution mapping of a new brown planthopper (BPH) resistance gene, *Bph18*(t), and marker-assisted selection for BPH resistance in rice (*Oryza sativa* L.). *Theor Appl Genet* 112(2):288–297.
30. Rahman ML, 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(7):1237–1246.
31. Leister D (2004) Tandem and segmental gene duplication and recombination in the evolution of plant disease resistance gene. *Trends Genet* 20(3):116–122.
32. Parniske M, et al. (1997) Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the Cf-4/9 locus of tomato. *Cell* 91(6):821–832.
33. Kloosterman B, et al. (2013) Naturally occurring allele diversity allows potato cultivation in northern latitudes. *Nature* 495(7440):246–250.
34. Konishi S, et al. (2006) An SNP caused loss of seed shattering during rice domestication. *Science* 312(5778):1392–1396.
35. Tanksley SD, McCouch SR (1997) Seed banks and molecular maps: Unlocking genetic potential from the wild. *Science* 277(5329):1063–1066.
36. McDowell JM, et al. (1998) Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the RPP8 locus of *Arabidopsis*. *Plant Cell* 10(11):1861–1874.
37. Ellis JG, Lawrence GJ, Luck JE, Dodds PN (1999) Identification of regions in alleles of the flax rust resistance gene L that determine differences in gene-for-gene specificity. *Plant Cell* 11(3):495–506.
38. Liu J, et al. (2011) Genetic variation and evolution of the Pi9 blast resistance locus in the AA genome *Oryza* species. *J Plant Biol* 54:294–302.
39. Cheng X, et al. (2013) A rice lectin receptor-like kinase that is involved in innate immune responses also contributes to seed germination. *Plant J* 76(4):687–698.
40. Bergelson J, Kreitman M, Stahl EA, Tian D (2001) Evolutionary dynamics of plant R-genes. *Science* 292(5525):2281–2285.
41. Yang S, et al. (2008) Genetic variation of NBS-LRR class resistance genes in rice lines. *Theor Appl Genet* 116(2):165–177.
42. Ishii T, Brar DS, Multani DS, Khush GS (1994) Molecular tagging of genes for brown planthopper resistance and earliness introgressed from *Oryza australiensis* into cultivated rice, *O. sativa*. *Genome* 37(2):217–221.
43. Ben Khaled S, Postma J, Robatzek S (2015) A moving view: Subcellular trafficking processes in pattern recognition receptor-triggered plant immunity. *Annu Rev Phytopathol* 53:379–402.
44. Inada N, Ueda T (2014) Membrane trafficking pathways and their roles in plant-microbe interactions. *Plant Cell Physiol* 55(4):672–686.
45. Frei dit Frey N, Robatzek S (2009) Trafficking vesicles: Pro or contra pathogens? *Curr Opin Plant Biol* 12(4):437–443.
46. Takemoto D, et al. (2012) N-terminal motifs in some plant disease resistance proteins function in membrane attachment and contribute to disease resistance. *Mol Plant Microbe Interact* 25(3):379–392.
47. Fujisaki K, et al. (2015) Rice Exo70 interacts with a fungal effector, AVR-Pii, and is required for AVR-Pii-triggered immunity. *Plant J* 83(5):875–887.
48. Zhang Q (2007) Strategies for developing green super rice. *Proc Natl Acad Sci USA* 104(42):16402–16409.