Identification and characterization of *Bph14*, a gene conferring resistance to brown planthopper in rice

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Planthoppers are highly destructive pests in crop production worldwide. Brown planthopper (BPH) causes the most serious damage of the rice crop globally among all rice pests. Growing resistant varieties is the most effective and environment-friendly strategy for protecting the crop from BPH. More than 19 BPH-resistance genes have been reported and used to various extents in rice breeding and production. In this study, we cloned *Bph14*, a gene conferring resistance to BPH at seedling and maturity stages of the rice plant, using a map-base cloning approach. We show that *Bph14* encodes a coiled-coil, nucleotide-binding, and leucine-rich repeat (CC-NB-LRR) protein. Sequence comparison indicates that *Bph14* carries a unique LRR domain that might function in recognition of the BPH insect invasion and activating the defense response. *Bph14* is predominantly expressed in vascular bundles, the site of BPH feeding. Expression of *Bph14* activates the salicylic acid signaling pathway and induces callose deposition in phloem cells and trypsin inhibitor production after planthopper infestation, thus reducing the feeding, growth rate, and longevity of the BPH insects. Our work provides insights into the molecular mechanisms of rice defense against insects and facilitates the development of resistant varieties to control this devastating insect.

herbivore | insect-resistance gene | CC-NB-LRR protein | antibiosis | salicyclic acid signaling

Rice (*Oryza sativa* L.) is a primary staple food crop for billions of people worldwide. To ensure global food security for continuing population growth, it is vital to control the various insect pests that damage rice (1). Among the herbivorous rice insects, the brown planthopper (BPH) (*Nilaparvata lugens* Stål) is the most destructive pest to rice production. Brown planthopper is a rice-specific herbivore and sucks the phloem sap of rice plants through its stylet mouthparts. Light planthopper infestation reduces plant height, growth vigor, and the number of productive tillers, whereas heavy infestation causes complete drying of the crop, a condition known as "hopperburn" (2). BPH also serves as a vector that transmits rice grassy stunt virus and ragged stunt virus, which are serious diseases in the tropical region (3). In recent years, BPH infestations have intensified across Asia, causing heavy rice yield losses (1). As the popular rice varieties are susceptible to planthoppers, farmers depend solely on chemical pesticides for controlling this insect, which are expensive in terms of labor, cost, and the environment. In addition, overuse of pesticides destroys the natural predators and leads to the insect developing resistance, which results in pest resurgence (4). The most economical and environment-friendly strategy to control this insect is to grow genetically resistant rice varieties (5).

The host resistance of rice against BPH was first reported for the variety Mudgo in 1969 (6). Most studies indicated that the resistant rice varieties suppressed the weight gain of insects and maintained low BPH populations across multiple generations and in a large rice production area (7, 8). In addition, the ratios of predators to BPH insects were most often highest in the highly resistant varieties and lowest in susceptible varieties. The yields of the resistant varieties were significantly higher than those of susceptible varieties (9). Therefore, resistant varieties are important "insurance" for farmers against BPH in integrative pest management. Up to now, 19 BPH-resistance genes have been identified and assigned to rice chromosomes in cultivated and wild rice species (10) and some have been incorporated into rice varieties and released in rice production (8, 9), but none of these BPH-resistance genes has been cloned thus far.

Molecular responses of plants to herbivores are strongly correlated with the mode of feeding and the degree of tissue damage at the feeding site. For the chewing insects that cause extensive damage to plant foliage, it is clear now that the elicitors like volicitin in the oral secretions of insects trigger the direct and indirect defenses (11, 12), via a wound-signaling pathway in which jasmonic acid (JA) plays a central role (13). In contrast, less is known about molecular responses of plants to sucking insects that produce little tissue damage to the host plant. The only insect-resistance gene that has been cloned in plants is the *Mi* gene of tomato (14) and most studies on plant defense responses to phloem-feeding insects have focused on aphids and whiteflies (13, 15, 16). The results of these studies suggest that plant defenses against aphids and whiteflies are similar to those against pathogens. The interaction between rice and BPH has the potential to serve as a model system for understanding the molecular basis of plant defense against phloem-feeding insects. Expression profiles showed that the genes significantly affected by BPH feeding in rice covered a wide range of functional categories, including metabolism, cellular transport, macromolecular degradation, signal transduction, and plant defense (17–19). These changes in RNA levels suggest that responses of rice to BPH feeding are more similar to pathogen-defense responses than to chewing insect defenses. Although differences in transcript levels between the compatible and incompatible interactions were detected, the BPH-resistance gene-mediated molecular mechanism still remains unknown.

Previously, we mapped two major BPH-resistance genes in rice B5, *Bph14* on the long arm of chromosome 3 and *Bph15* on the shorter arm of chromosome 4 (20). These loci also showed resistance to the whitebacked planthopper, *Sogatella furcifera* Horváth (21). *Bph14* showed a stable resistance in different genetic backgrounds and thus is valuable in development of resistant rice varieties. This study sought to clone *Bph14* by a map-based strategy, revealing that it encodes a coiled-coil,

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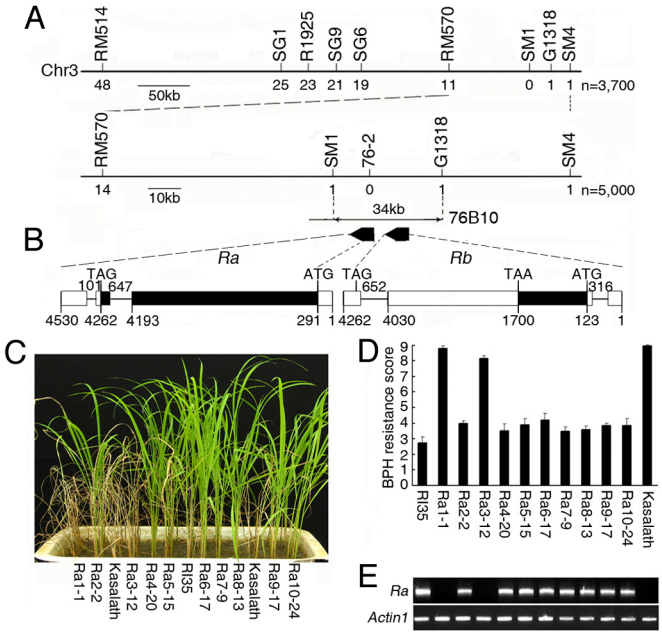

Fig. 1. Map-based cloning and complementation tests of the planthopper-resistance gene *Bph14*. (*A*) Fine mapping of the *Bph14* locus. The *Bph14* locus is located within a 34-kb region of chromosome 3, which contains two predicted genes. Numbers under the linkage map indicate the number of recombinants detected between the molecular markers and the *Bph14* locus. (*B*) Structure of *Ra* and *Rb*. The three exons in each gene are boxed; the black boxes show the ORFs. (*C*) BPH-resistance test of the *Bph14*-transgenic and susceptible wild-type (WT) rice. RI35, resistant parental rice; Kasalath, susceptible WT rice; Ra1–Ra10, *Bph14*-transgenic T_2 lines. (*D*) BPH-resistance scores of the *Bph14*-transgenic rice at the seedling stage. The lower scores indicate the higher resistance to the insect. Data are means ± SD ($n = 60$ plants). (*E*) RT-PCR analysis showing the expression of *Ra* in the transgenic T_2 lines.

nucleotide-binding, and leucine-rich repeat (CC-NB-LRR) protein. *Bph14* is strongly expressed in vascular bundles where the BPH feeds and confers resistance to the BPH insects. In the *Bph14*-mediated resistance, the salicylic acid (SA) signaling pathway is activated.

Results

Map-Based Cloning of *Bph14*. To isolate the *Bph14* gene, we developed an F_2 mapping population derived from a cross between RI35, a recombinant inbred line containing the *Bph14* locus from B5 (22), and Taichuang Native 1 (TN1), a BPH-susceptible *indica* variety (Fig. S1*A*). The resistant and susceptible plants segregated in a 3:1 ratio (72:28; $\chi^2 = 0.48$; $P > 0.90$) in the F_2 population, indicating that the *Bph14* gene as a single Mendelian factor conferred the BPH resistance in RI35 (Fig. S1*B*). We set out to fine map *Bph14* by analyzing 3,700 plants from the F_2 population and 5,000 plants from an F_5 population of the same cross. This exercise delimited the *Bph14* gene to a 34-kb region flanked by markers SM1 and G1318 (Fig. 1*A*). Two predicted genes encoding putative resistance proteins, designated *Ra* and *Rb*, respectively, were identified after sequencing clone 76B10 in this region from the genomic library of resistant rice B5 (Fig. 1*B*).

To determine which gene was *Bph14*, we performed the complementation tests. The 9.6- and 9.1-kb genomic fragments containing the predicted *Ra* and *Rb* genes with their native promoters, respectively, were transferred into the BPH-susceptible *indica* variety Kasalath. The T_2 families were then examined for BPH resistance, using the bulked seedling test (Fig. 1*C*). We found that BPH resistance cosegregated with *Ra* in the transgenic population. The T_2 transgenic lines expressing *Ra* from different independent T_0 transformants, which were homozygous for the transgene, clearly showed high resistance to the BPH and survived, whereas the wild-type plants were killed by BPH insects (Fig. 1 *C–E*). The transgenic plants carrying *Rb* were also susceptible and killed by the BPHs in the test. In addition, we used RNA interference (RNAi) to suppress the expression of *Ra* in the RI35 rice plants. The RNAi-transgenic lines were susceptible and were killed by the BPHs in the tests (Fig. S2). Thus, we concluded that *Ra* confers the resistance phenotype and is the *Bph14* gene.

***Bph14* Encodes a Unique CC-NB-LRR Protein.** Comparison of the genomic and cDNA sequences of the *Bph14* and *Rb* genes revealed that both consist of three exons and two introns (see Fig. 1*B*), but *Rb* is interrupted by a premature stop codon. The *Bph14* gene encodes a putative 1,323-aa protein containing a CC-NB-LRR motif (Fig. S3), which shares similarity with proteins encoded by a number of genes for resistance to several diseases (23). *Bph14* shares 83% sequence identity with its allele (*Os03g0848700*) in Nipponbare. Phylogenetic analysis revealed that Bph14 is closely related to other rice homologs and is divergent from the majority of known plant disease resistance proteins in other species (Fig. 2*A*).

To investigate whether the resistance of *Bph14* is due to the coding sequence or the transcription level, we analyzed the sequences of the *Bph14* gene for 21 rice varieties, including 10 *indica* (4 of which were resistant cultivars carrying different BPH-resistance genes), 7 *japonica* varieties, and 4 accessions of wild rice, *Oryza rufipogon* (Table S1). Through comparison of the coding sequences between *Bph14* and its alleles, we found that the central motifs of the CC and NB domains are well conserved among the diverse rice materials (Fig. S4 *A* and *B*), but in the LRR domain 54 residues and two deletions of *Bph14* are unique (Fig. S4*C*).

RT-PCR analysis showed that the transcripts were present in all of the rice varieties (Fig. S5*A*). The real-time PCR data further confirmed that the transcript levels were not significantly different in most of the varieties (Fig. S5*B*). In addition, the expression of *Bph14* was enhanced by BPH feeding and was not significantly different between the resistant plant RI35 and the susceptible plant Kasalath (Fig. 2*B*). These results further support the idea that the sequence variations in the coding region, not the allelic transcription levels, account for the gene being functional in insect resistance.

Expression Analysis of *Bph14*. Because BPH insects usually aggregate on the lower parts of rice plants and ingest the phloem sap, we thus studied the tissue specificity of *Bph14* expression. We found that *Bph14* was expressed constitutively in leaf sheaths, leaf blades, and roots (Fig. 2*C*). Then we examined *Bph14* activity in more detail, using transgenic plants carrying the fusion construct of the *Bph14* promoter region and the *GUS* reporter gene. The expression of GUS in transgenic plants was detected mainly in the vascular tissue of various organs, including leaf sheaths and leaf blades (Fig. 2 *E*, *G*, and *I*). In cross-sections of these organs, GUS activity was strongly detected in the parenchyma cells bordering xylem vessels and sieve tubes (Fig. 2 *F*, *H*, and *J*). Such an expression pattern is consistent with the role of *Bph14* in recognizing BPH attack in phloem cells. To determine the subcellular localization of the Bph14 protein, the coding region of *Bph14* fused to modified green fluorescent protein (GFP) at the N-terminal end was expressed under the control of the *CaMV 35S* promoter. Fluorescence was detectable in the cytoplasm in onion epidermal cells following particle bombardment (Fig. 2*D*).

***Bph14* Confers an Antibiosis Resistance to Planthopper.** The transgenic rice plants that express *Bph14* survived after BPH infes-

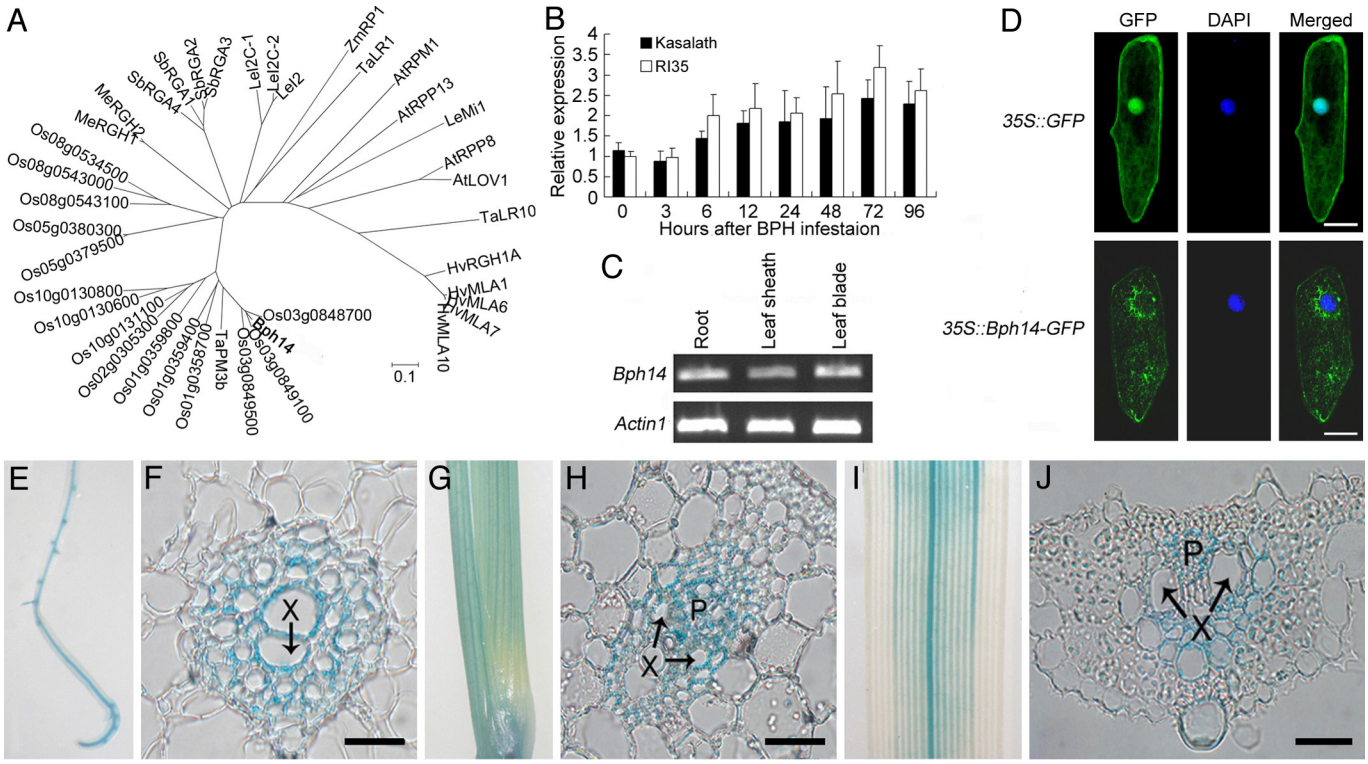

Fig. 2. Molecular characterization of *Bph14*. (*A*) Phylogenetic relationships of Bph14 homologs in rice (Os), wheat (Ta), cassava (Me), potato (Sb), tomato (Le), maize (Zm), barley (Hv), and *Arabidopsis* (At). (Scale bar, 0.1 amino acid substitutions per site.) (*B*) Time-dependent expression of *Bph14* and its alleles in the resistant and susceptible plants after BPH infestation. The mean is based on the average of three biological repeats calculated. (*C*) Expression analysis of *Bph14* in the root, leaf sheath, and leaf blade of rice by RT-PCR. (*D*) *Bph14* subcellular localization. The *35S*::*GFP* (*Upper*) and *35S*::*Bph14-GFP* (*Lower*) fusion genes were transiently expressed in onion epidermal cells. The Bph14-GFP fusion protein is localized in the cytoplasm. (*E–J*) *Bph14* promoter–GUS expression pattern in transgenic rice plants. GUS express in the vascular system of root (*E*), leaf sheath (*G*), and leaf blade (*I*). Cross-sections of root (*F*), leaf sheath (*H*), and leaf blade (*J*) indicated that *Bph14* is strongly expressed in the parenchyma cells bordering xylem vessels and sieve tubes. X, xylem; P, phloem. (Scale bars: *D*, 50 μm; *F*, *H*, and *J*, 20 μm.)

tation at the seedling stage (see Fig. 1 *C–E*). At the maturing stage, the wild-type plants showed symptoms of stem chlorosis, leaf wilting, reduced fertility, and even death of the whole plant after infestation by BPH, whereas the *Bph14*-transgenic plants were healthy (Fig. 3*A*). Generally, plants may employ two resistance strategies against herbivores: antixenosis that affects insect settling, colonization, or oviposition and antibiosis that reduces insect feeding, growth rate, or survival (24). To explore the resistance mechanism of the *Bph14*-transgenic plants, we investigated the responses of the BPH insects feeding on the resistant *Bph14*-transgenic and susceptible wild-type plants in terms of host choice, feeding activities, honeydew excretion, population growth rate, nymph survival, and fecundity (Table S2 and Fig. 3). In host choice tests, there was no significant difference in numbers of BPH nymphs that settled on the plants between the transgenics and wild type when observed from 3 to 48 h after infestation (Fig. 3*B*). Neither was there a difference in number of eggs found on the plants (Fig. 3*C*). We also recorded in detail the feeding behavior of BPHs on rice plants in real time, employing the electronic penetration graph (EPG) analysis (25). The EPG data showed that there were no significant differences between the transgenic plants and the wild type in the time from the beginning of plant penetration to the first phloem ingestion and the duration of the first phloem ingestion. However, the duration of nonprobing and penetration was significantly longer and the duration of phloem ingestion was clearly shorter on the transgenic plants, compared with the wild-type plants (Fig. 3*D*). These results showed that BPH feeding was inhibited on the transgenic plants. Honeydew excretion, a simple measurable indicator of BPH feeding activity, was found to be lower on the transgenic plants compared with that on the wild-type plants, consistent with the data of EPG (Fig. 3*E*). The population growth rate of the insects on the transgenic plants was only one-fifth of that on the wild-type plants (Fig. 3*F*). There was a pronounced decrease in the survival rate of the insects on the transgenic plants (Fig. 3*G*). Taken together, these results demonstrate that *Bph14* confers a resistance that reduces the feeding, growth rate, and longevity of the BPH insects.

In further investigation of the possible mechanism of *Bph14*-mediated resistance, we observed that callose was deposited abundantly on sieve plates and the cell walls of vascular tissue in the *Bph14*-transgenic plants (Fig. 3*H*), which is an important defense mechanism that prevents planthoppers from ingesting phloem sap (25). We thus examined the expression patterns of callose synthase-encoding genes and β-1,3-glucanase genes, using real-time PCR. Three callose synthase-encoding genes (*GSL1*, *GSL5*, and *GSL10*) were clearly up-regulated in both the wild-type and transgenic rice plants after BPH infestation. However, the expression of *GNS5* and *GNS9*, the genes encoding callose-hydrolyzing enzyme β-1,3-glucanase, was slightly down-regulated in the transgenic plants, which prevented callose from decomposing and kept the sieve tubes occluded (Fig. S6). In addition, the expression of the Bowman-Birk trypsin inhibitor genes (26) was enhanced in the transgenic plants, whereas the expression of the trypsin gene (27) was suppressed to a greater degree in the BPH insects that fed on the transgenic plants compared to those that fed on the wild-type plants (Fig. S7).

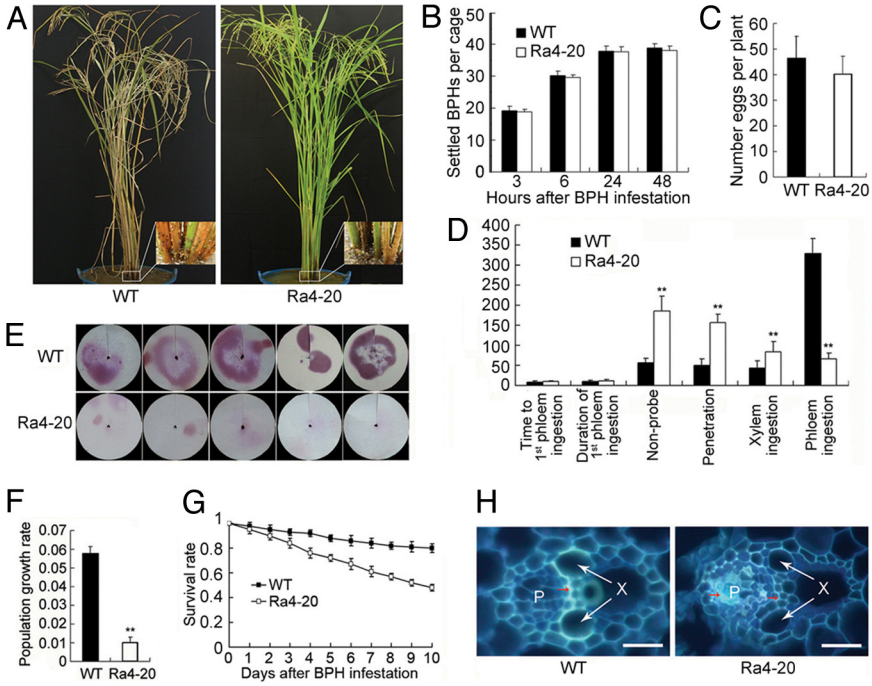

Fig. 3. Characterization of insect resistance in *Bph14*-transgenic rice. (*A*) Planthopper-resistance test of the *Bph14*-transgenic and wild-type rice at the mature stage. Magnified views show the locations of BPH feeding. (*B*) Settling of BPH in a host choice test. (*C*) BPH fecundity. (*D*)Total duration of electronic penetration graph (EPG) waveform types for the BPH over an 8-h recording period. (*E*) Honeydew excretion on filter paper. The size of the honeydew area and the intensity of the honeydew color correspond to the BPH feeding activity. (*F*) BPH population growth rate. (*G*) BPH survival rate. The number of surviving BPHs per plant was significantly lower on resistant plants than on wild-type plants 3 days after infestation ($P = 0.0038$). (*H*) Induced callose deposition (red arrows) in the vascular bundle indicated by bright blue fluorescence. X, xylem; P, phloem. (Scale bar, 20 μm.) WT, the susceptible wild-type rice; Ra4–20, the resistant homozygous *Bph14*-transgenic rice. Data are means ± SD ($n = 10$). **, $P < 0.01$.

These results indicate that callose deposition and trypsin inhibitor production prevent BPH insects from continuously ingesting and digesting phloem sap in the *Bph14*-transgenic plants.

The SA Pathway Was Activated in the *Bph14*-Mediated Insect Resistance. Plant defense responses to insects include the activation of pathways dependent on SA and JA/ethylene signaling molecules (13, 15, 16). To investigate the pathway involved in *Bph14*-mediated resistance, we examined the transcript levels of defense-responsive genes, which are known to function in SA- and JA/ethylene-dependent pathways during disease resistance in rice (28) (Fig. 4). There was no significant difference between the transgenic and the wild-type plants in transcript levels of the JA synthesis-related genes *LOX* (lipoxygenase) and *AOS2* (allene oxide synthase 2) (13, 28) in 24 h after BPH infestation. At all subsequent time points, however, transcript levels of these genes were substantially lower in the transgenics than in wild-type plants. In addition, the ethylene signaling pathway receptor gene *EIN2* (ethylene insensitive 2) (29) accumulated faster and at higher levels in the wild type than in transgenics. These results suggested that BPH feeding induced the defenses in the susceptible plants associated with a JA/ethylene-dependent pathway. However, transcript levels of the SA synthesis-related genes *EDS1* (enhanced disease susceptibility 1), *PAD4* (phytoalexin deficient 4), *PAL* (phenylalanine ammonia-lyase), and *ICS1* (isochorismate synthase 1) (28) were higher in the transgenic plants than in wild-type plants after BPH infestation, suggesting that *Bph14* may activate an SA-dependent resistance pathway after BPH feeding. *NPR1* (homolog of *Arabidopsis* nonexpressor of pathogenesis-related genes 1) is a key regulator of SA-dependent systemic acquired resistance (SAR) that was found to enhance the expression of *PR1b* (basic pathogenesis-related gene 1) (30). *PR1b* is suggested to be effective in inhibiting pathogen growth, multiplication, and/or spread and is responsible for the SAR in plants (31). The transcript level of *NPR1* was also higher in the transgenic than in the wild-type plants. However, the higher transcript level of *NPR1* did not result in stronger

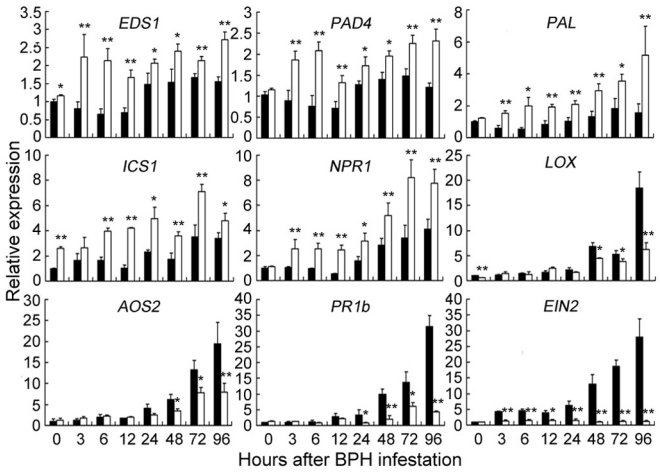

Fig. 4. Expression patterns of plant defense-response genes. *EDS1*, *PAD4*, *PAL*, and *ICS1* are the SA synthesis-related genes. *NPR1* is a key regulator of SA-dependent systemic acquired resistance. *LOX* and *AOS2* are the JA synthesis-related genes. *PR1b* is pathogen-related gene 1 in rice. *EIN2* is the ethylene signaling pathway receptor gene. Rice *Actin1* was used as reference control. Expression of genes was quantified relative to the value obtained from 0-h susceptible samples. Solid bars, the wild-type rice; open bars, the resistant homozygous *Bph14*-transgenic rice (Ra4–20). In all panels, the mean is based on the average of three biological repeats calculated. *, $P < 0.05$; **, $P < 0.01$. One-way ANOVA was used to generate the *P* values.

induction of *PR1b*, suggesting that the transcript level of *PR1b* was regulated by JA/ethylene signal in rice after BPH infestation. These results demonstrate that *Bph14* activates a *NPR1*-dependent, but *PR1*-independent, resistance after BPH feeding.

Discussion

At present, insect pests are becoming more epidemic and destructive as a result of changes in climate and crop systems (32). Despite the importance of the development of resistant varieties to control insects, knowledge of the function of the insect-resistance gene and the molecular mechanism of host resistance is very limited in the literature. This study identified *Bph14* by map-based cloning and elucidated the molecular basis of *Bph14*-mediated resistance against planthoppers in rice.

Our results reveal that the BPH-resistance gene *Bph14* is a member of the CC-NB-LRR disease resistance gene family, which is known to mediate resistance through direct or indirect recognition of pathogen-associated molecular patterns (PAMPs) or pathogen effectors (23, 33). The *Bph14*-mediated resistance mechanism to BPH is fundamentally similar to defense mechanisms against pathogens. First, BPH is a typical piercing-sucking insect. When feeding on the rice plant, the BPH inserts its stylets into the phloem sieve element, forming a feeding sheath, and ingests plant fluids (34), which produces little physical injury to rice. There is an intimate and prolonged interaction between the insect stylets and plant cells. The stylet actions of BPH infestation are similar in some ways to infection and intercellular hyphae growth of fungal pathogens and invasion of a nematode's stylet (33–35) and thus may similarly be perceived by the plants as pathogen infection, leading to similar responses (18). Second, *Bph14* encodes a NB-LRR protein and carries a unique LRR domain. Studies on alleles of the *L* and *P* genes in flax showed that the LRR domain provided pathogen recognition specificity (36). The unique LRR domain of *Bph14* may function to recognize the invading insects, thus inducing defense response (Fig. S5), although the extent of similarity between the defense responses to BPH mediated by *Bph14* and those to pathogens remains an interesting issue. Third, our results showed that the defense-signaling pathway induced by BPH is most commonly activated by pathogens. The transcripts of the SA synthesis-related genes accumulated faster and at higher levels in the *Bph14*-transgenic plants than in the wild-type plants, suggesting that *Bph14* activated an SA-dependent pathway (Fig. 4). In plant/pathogen interaction, SA stimulates expression of defense-response genes and promotes the development of systemic acquired resistance, which confers a broad-range resistance to pathogens (13, 33). Fourth, there are similar defense mechanisms against pathogens, nematodes, and insects. Callose deposition and protease inhibitors are induced in *Bph14*-mediated resistant rice, which are the frequent mechanisms of plant defense against pathogens (37, 38). Taken together, the nature of the *Bph14* gene, early host/insect recognition, activation of the plant defense signaling pathway, and callose deposition and protease inhibitor production show the commonality in the mechanisms of plant defense against pathogens and planthoppers.

The commonality in the mechanisms of plant defense against pathogens, nematodes, and insects should be highlighted. It has been known for a long time that rice resistance to BPH could be overcome by a new "biotype" of BPH, which is a virulent strain of the insect with the ability to survive on and damage the previously resistant varieties (39). Although the precise nature of BPH biotype and rice resistance is not clear, their interaction is analogous to "gene-for-gene" interaction of the plant/pathogen systems (39, 40). Identification of *Bph14* in this study makes it clear that a CC-NB-LRR gene mediates such a specific gene-for-gene resistance in rice to BPH. To develop a sound strategy for breeding of resistant rice varieties, it is necessary to identify not only the genes governing resistance of rice, but also the corresponding factors governing virulence in BPH. In plant/pathogen interaction, a number of PAMPs and pathogen effectors have been defined (33). Whether the biotypes of the insects are governed by substances like the pathogen effectors remains to be identified. With the knowledge about the nature of the BPH biotype, the response of the insect populations to resistance genes of rice and the extent of occurrence of specific virulence can be assessed.

It is interesting that the *Mi-1* gene from tomato also belongs to the NB-LRR family of resistance genes (14). Although *Bph14* and *Mi* belong to the same gene family, they show a distant relationship (Fig. 2*A*). *Mi* confers both antixenosis and antibiosis resistance to potato aphid (41), and antibiosis resistance to planthoppers is identified for *Bph14*. Both *Bph14*- and *Mi*-mediated resistance involve the SA signaling pathway (15), and the transcript levels of *PR1* in the resistant *Mi* plants accumulated faster and at higher amounts than in the susceptible *mi* plants after aphid infestation (42), whereas *Bph14* activates a *PR1*-independent resistance pathway. *Mi* is a dual purpose gene, which confer resistance to nematodes and aphids (14). Whether *Bph14* has a function against nematodes should be tested in the future.

BPH is a monophagous herbivore in the sense that its development can be completed only on rice. It was suggested that a host shift for BPH from *Leersia* to rice happened probably less than 0.25 Myr ago (43). As a result, resistance genes must have evolved in the rice genome to protect plant growth and reproduction. As new biotypes of BPH emerged to overcome the host resistance, a number of resistance genes would evolve in rice to reduce the BPH damage. Actually, a number of resistance genes have been mapped on the chromosomes in landrace varieties and wild rice species (10). Identification and characterization of these BPH-resistance genes will help us to understand that rice resistance coevolved with the BPH biotype and to develop resistant rice varieties efficiently.

Our results clearly validated the usefulness of the *Bph14* gene in control of planthopper in rice. Transforming the *Bph14* gene into a susceptible rice variety results in a resistance effect that significantly suppresses the feeding and reduces the growth rate and longevity of BPH insects. Introgression of *Bph14* into elite hybrid rice varieties has been shown to yield lines with satisfactory resistance to the BPH in the field (10). The identification of *Bph14* greatly facilitates the development of rice varieties with resistance to BPH, thus simultaneously reducing pesticide usage and decreasing economic and environmental costs.

Methods

Plant and Insect Materials. The 21 varieties of cultivated rice used in this study are listed in Table S1. The BPH insects used for infestation were collected from rice fields in Zhejiang Province, China, and maintained on TN1 plants. The resistance to BPH of the mapping populations and transgenic plants was evaluated using methods described in *SI Methods*. All of the experiments were repeated over 10 replicates.

Map-Based Cloning of *Bph14*. We used the flanking markers RM514 and SM4 to screen 3,700 F_2 plants and obtained 49 recombinants. The *Bph14* gene was located in a 120-kb region between the markers RM570 and G1318. For further fine mapping, we selected the F_2 plants in which the region around *Bph14* was heterozygous and other regions were derived from TN1. These plants were self-pollinated and selected until the F_5 generation. The F_5 population consisting of 5,000 plants was used for the high-resolution mapping and *Bph14* was defined on a 34-kb region between SM1 and G1318 (see Fig. 1*A*). The primers of these markers are listed in Table S3.

Complementation and Knockdown Tests. A 9.6-kb DNA fragment, digested by XbaI and KpnI, containing the entire *Ra* coding region, and a 9-kb DNA fragment, digested by XmaI, containing *Rb*, were independently inserted into the binary vector pCAMBIA1301 for the complementation test. These constructs were transformed into rice Kasalath, using an *Agrobacterium*-mediated method (44). Two

copies of a 342-bp fragment of *Ra* cDNA were amplified by PCR using primers RNAi1 and RNAi2 and inserted at inverted repeats into the pKANNIBAL vector to generate a hairpin RNAi construct, which was then cloned into the binary vector pCU BamHI site (45). This construct was subsequently transformed into RI35. Plants regenerated from hygromycin-resistant calli (T_0 plants) were grown, and T_1 seeds were obtained after self-pollination. T_1 transformants were selected on the basis of PCR for the transgene and cultivated to set T_2 seeds that were collected for resistance evaluation.

Subcellular and Tissue Localization. The *Bph14* coding sequence amplified by PCR using the primers Bph14-GFPF and Bph14-GFPR was cloned into the downstream of the *CaMV 35S* promoter and in frame with GFP in the modified vector pCAMBIA1302. The resulting plasmid DNA was bombarded into onion epidermal cells, using a helium biolistic device (Bio-Rad PDS-1000). Samples were examined by confocal laser-scanning microscopy (Olympus FV1000).

A 2-kb *Bph14* promoter region upstream of the ATG start codon was amplified by PCR using the primers PromoterF and PromoterR and cloned into the vector pCAMBIA1391 to obtain a *Bph14* promoter–GUS fusion construct. Transgenic plants carrying this construct were generated as described above. GUS activity in transgenic plants was detected by histochemical assay.

Expression Analysis. Seeds of the *Bph14*-transgenic and wild-type plants were sown in 9-cm diameter plastic cups. At the four-leaf stage, plants were infested with eight second instar nymphae per plant and sampled after 0, 3, 6, 12, 24, 48, 72, and 96 h. All treatments, each with three biological replicates, were terminated at the same time. Total RNA was extracted from the leaf sheaths and the BPHs, using TRIzol reagent (Invitrogen), and then converted into first-strand cDNA. Expression of *Bph14* and other genes involved in BPH feeding responses was amplified by quantitative RT-PCR, using an RG-6000 rotary analyzer (Corbett Research). The sequences of the primers are listed in Table S3. The measurements were obtained using the relative quantification method (46).

Histochemistry and Microscopy. Rice plant seedlings at the four-leaf stage were each infested with 10 BPHs. Leaf sheaths were fixed with formalin–acetic acid–alcohol fixative solution at 4 °C overnight, dehydrated, and embedded in paraffin (Sigma). The tissues were sliced into 10-μm sections with a microtome and fixed to microscope slides, then stained with 0.1% aniline blue in 0.15 M K_2PHO_4 for 5 min, and examined under a BX51 (Olympus) fluorescence microscope (25).

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