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Map-based Cloning and Characterization of a Brown Planthopper Resistance Gene *BPH26* from *Oryza sativa* L. ssp. *indica* Cultivar ADR52

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The brown planthopper (BPH) is the most serious insect pest of rice in Asia. The *indica* rice cultivar ADR52 carries two BPH resistance genes, *BPH26* (BROWN PLANTHOPPER RESISTANCE 26) and *BPH25*. Map-based cloning of *BPH26* revealed that *BPH26* encodes a coiled-coil-nucleotide-binding-site-leucine-rich repeat (CC-NBS-LRR) protein. *BPH26* mediated sucking inhibition in the phloem sieve element. *BPH26* was identical to *BPH2* on the basis of DNA sequence analysis and feeding ability of the *BPH2*-virulent biotype of BPH. *BPH2* was widely incorporated in elite rice cultivars and was well-cultivated in many Asian countries as a favorable gene resource in rice breeding against BPH. However, *BPH2* was rendered ineffective by a virulent biotype of BPH in rice fields in Asia. In this study, we suggest that *BPH2* can be reused by combining with other BPH resistance genes, such as *BPH25*, to ensure durable resistance to BPH.

Plants have various mechanisms to afford protection against herbivorous insects¹. However, varieties of highly productive crops suitable for multi-fertilizer cultivation lose their inherent protective mechanisms against insect-feeding. Development and utilization of resistant crop varieties will promote environment-friendly agricultural practices involving limited use of pesticides. Many insect-resistant crop species have been screened, and their application in breeding programs is being investigated².

Rice is one of the most widely grown crops worldwide. The brown planthopper (BPH), *Nilaparvata lugens* Stål (Homoptera: Delphacidae), is the most serious insect pest of rice in Asia. BPH is a monophagous sucking insect that mainly feeds on rice (*Oryza sativa* L.)^{3,4}. These insects cause direct damage by sucking sap from the phloem of susceptible rice varieties and indirect damage by transmitting viral diseases⁵. When the density of BPH remarkably increases, many rice plants die and, eventually, “hopperburn” appears in the rice paddy. Large amounts of insecticides are used to control BPH. However, BPH showing resistance to insecticides such as imidacloprid have emerged⁶, requiring the development of other control methods as an alternative to insecticide use.

Rice varieties resistant to BPH have been screened on a large scale at the International Rice Research Institute (IRRI)⁷. The resistance gene from the *indica* cultivar Mudgo was named *Bph1* and that from the *indica* cultivar ASD7 was named *bph2*. The *indica* cultivar IR26 harboring the *Bph1* gene was bred at IRRI and used as the first commercial cultivar for controlling BPH. However, a *Bph1*-virulent biotype of BPH gradually rendered the resistance gene *Bph1* ineffective⁸. Next, some cultivars such as IR42 harboring *bph2* were released for BPH control. However, they were also rendered ineffective by the appearance of a new virulent BPH biotype⁹.

BPH resistance genes were screened from not only *O. sativa* L., which is the main host plant of BPH but also other *Oryza* species, and currently, more than 30 BPH resistance loci have been reported¹⁰. Breeding of resistant rice cultivars for controlling BPH necessitates the discovery of new gene sources and the development of control methods to better retain the effect of resistance genes. Myint *et al.*¹¹ found two BPH resistance genes in the *indica*

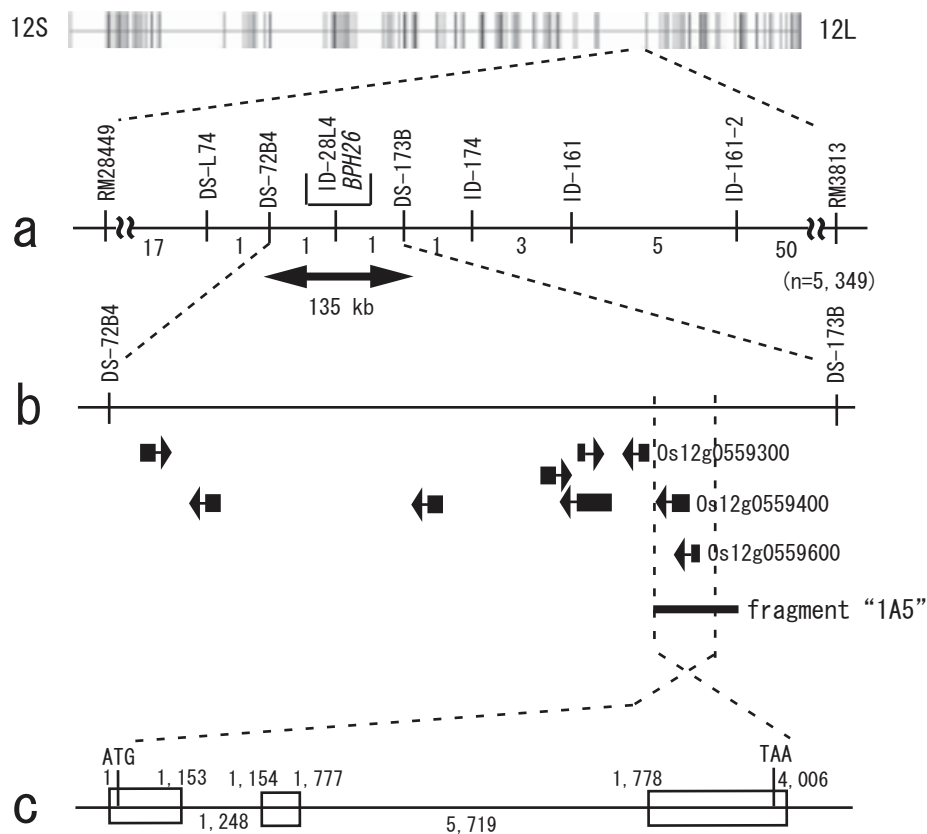


Figure 1 | High-resolution genetic map of *BPH26*. (a) Genetic linkage map of 5,349 BC₆F₃ plants showing the relative position of *BPH26* with the single-sequence repeat, insertion-deletion, and single nucleotide polymorphism markers on chromosome 12. The numbers under the linkage map indicate the number of recombinants in the adjacent marker intervals. Framework maps are quoted from Harushima *et al.*⁸. (b) Putative genes in the candidate region whose presence is predicted by the Rice Annotation Project Database. A fragment 1A5 shows the genomic DNA of ADR52 which was used for the functional complementation test. (c) The structure of *BPH26* on the genomic DNA of ADR52.

cultivar ADR52 and named them *BPH25* and *BPH26*. *BPH25* is located on the short arm of chromosome 6, and *BPH26* is located on the long arm of chromosome 12. Many BPH resistance genes, such as *Bph1*, *bph2*, *Bph9*, *Bph10*, *Bph18(t)*, and *Bph21(t)*, have been mapped to the long arm of chromosome 12¹⁰. *Bph1*^{12,13}, *bph2*^{12,14,15}, *Bph9*^{16–18}, and *BPH26* were found in *O. sativa* L. varieties; *Bph10*, in *O. australiensis*¹⁹ and *O. officinalis*²⁰; *Bph18(t)*, in *O. australiensis*²¹; and *Bph21(t)*, in *O. minuta*²². The long arm of chromosome 12 is considered to be one of the most important locations for conferring resistance against BPH in *Oryza* species. However, there are no reports on the molecular cloning of the BPH resistance gene in this region. In this study, we isolated *BPH26* from the long arm of chromosome 12 of the *indica* cultivar ADR52 (*O. sativa* L.). Till date, only a few resistance genes against sap-sucking insects, such as the aphid resistance gene *Mi* (*Mi-1.2*) from tomato^{23,24} and the BPH resistance gene *Bph14*²⁵, have been cloned and their nucleotide sequences have been reported. Although *Bph14* originated from the wild rice variety *O. officinalis* on chromosome 3 has already been isolated as a BPH resistance gene using introgression lines^{25–27}, none of the BPH resistance genes has been isolated from *O. sativa* L., which is the main host plant of BPH.

Furthermore, we confirmed that *BPH26* is identical to *BPH2*, which was rendered ineffective by a virulent biotype of BPH in the Asian rice fields. Herein, we discuss the possibility for reusing *BPH2*.

Results

Delimitation of a candidate genomic region of *BPH26*. High-resolution genetic maps of *BPH26* were constructed using 5,349 BC₆F₃ individuals (Fig. 1a). Seventy-nine plants showing recombination between two markers (RM28449 and RM3813) were selected,

and the BPH resistance of these plants was analyzed based on the survival rate of BPH in a test tube. Eventually, we determined the candidate genomic region of *BPH26* in a 135-kb region between two flanking markers, DS-72B4 and DS-173B (Fig. 1a). Nine putative genes were predicted in the corresponding candidate region of Nipponbare by using a rice annotation project database (RAP-DB; Fig. 1b). Among them, the amino acid sequences of Os12g0559300, Os12g0559400, and Os12g0559600 were homologous to those of NBS-LRR type disease resistance proteins (Supplementary Table S1 online). Two putative genes (Os12g0559400 and Os12g0559600) were predicted to encode parts of one nucleotide-binding site-leucine-rich repeat (NBS-LRR) protein, although one putative gene (Os12g0559300) was considered to be incomplete and was non-functional. One bacterial artificial chromosome (BAC) clone 14H01 (153 kb), containing Os12g0559400 and Os12g0559600, was selected from a BAC genomic library of ADR52, and the corresponding region was sequenced (Supplementary Fig. S1 online). One gene that was expected to code the NBS-LRR protein was also present on the corresponding sequences of the BAC clone 14H01, as revealed by the rice genome automated annotation system (RiceGAAS). Rapid amplification of cDNA ends (RACE)-PCR was performed, and cDNA encoding coiled coil-nucleotide binding site-leucine rich repeat (CC-NBS-LRR) protein was cloned and sequenced, using primers designed from the sequence data of the BAC clone 14H01A.

Functional complementation with the candidate gene in transgenic rice. A genomic sub-clone library of about 20 kb was constructed from the BAC clone 14H01. A clone containing a 15.6-kb DNA fragment (fragment 1A5) with the full length of the candidate

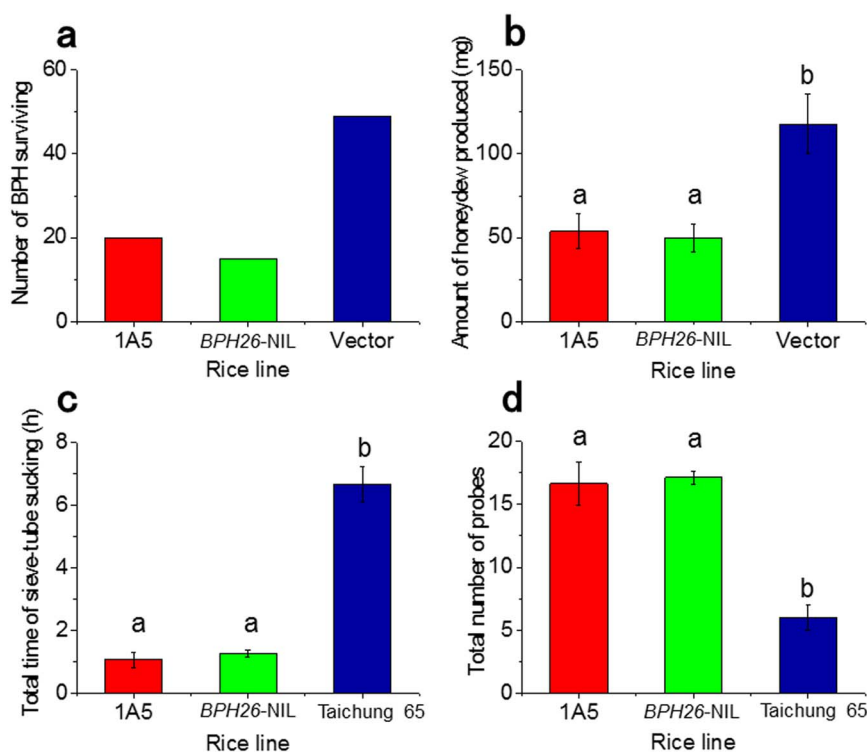


Figure 2 | Functional complementation test of candidate genes. T₁ lines that have been PCR validated to harbor the gene were used in the bioassays. The *BPH2*-avirulent biotype was used in these experiments. (a) The survival number of brown planthopper (BPH) on the three rice lines. The 1A5 line is the transgenic plant with the predicted gene on the DNA fragment 1A5 in the background of Taichung 65. The vector line is the transgenic plant with only vector fragment in the background of Taichung 65. *BPH26-NIL* is the near isogenic line with *BPH26* in the genetic background of Taichung 65. In all, sixty last instar larvae that had been fasted for 24 h were released on each rice line for 5 days. (b) The amount of honeydew excreted by BPH females on the three rice lines. Values represent mean and SE ($n = 10$). Means labeled with the same letters are not significantly different by Tukey–Kramer test ($P < 0.05$). (c) The total duration of phloem ingestion on the three rice lines. The feeding behavior of BPH females was measured using an AC-EPG system for 10 h. Values represent mean and SE ($n = 10$). Means labeled with the same letters are not significantly different by Tukey–Kramer test ($P < 0.05$). (d) The total number of probes. The feeding behavior of BPH females was measured using an AC-EPG system for 10 h. Values represent mean and SE ($n = 10$). Means labeled with the same letters are not significantly different by Tukey–Kramer test ($P < 0.05$).

gene was selected (Fig. 1b). There was only one gene predicted on the fragment 1A5, as revealed by BLASTN search. The clone was transformed into the susceptible cultivar Taichung 65. Of the five independent transgenic lines tested, all transgenic lines with fragment 1A5 showed resistance to BPH (Supplementary Fig. S2 online). The result of the complementation test using one of those five lines (line name, “1A5”) was compared to those of the near isogenic line carrying *BPH26* (line name, “*BPH26-NIL*”) and vector control (line name, “Vector”; Fig. 2). The BPH survival was significantly decreased on the transgenic “1A5” in comparison with that on the “Vector” control, and was equivalent to that on the “*BPH26-NIL*” (chi-square test²⁸; Fig. 2a). Similarly, the amount of honeydew excreted on “1A5” and “*BPH26-NIL*” was smaller than that on “Vector” control (Fig. 2b). The feeding activities of BPH were investigated using an alternating current electric penetration graph (AC-EPG). The total hours of sucking sieve-tube on “1A5” and “*BPH26-NIL*” were suppressed around to only 1 h compared to 7 h of sucking on the susceptible cultivar Taichung 65 during the 10-h exposure to BPH (Fig. 2c). Furthermore, the numbers of probes on “1A5” and “*BPH26-NIL*” were higher than those on the susceptible cultivar Taichung 65 (Fig. 2d). These data suggest that phloem ingestion was inhibited and probing behavior was repeated on “1A5” and “*BPH26-NIL*.” Therefore, the gene on fragment 1A5 was identified as the resistance gene *BPH26*, because the “1A5” line exhibited the same resistance as the original line “*BPH26-NIL*.”

Sequence analysis of the *BPH26* gene. Comparison of the cDNA and genomic DNA sequences revealed that there are three exons and

two introns (Fig. 1c). The deduced open reading frame (ORF) of *BPH26* encoded a protein of 1,218 amino acids. The cDNA and amino acid sequences are shown in Supplementary Fig. S3 online and Fig. 3, respectively. Motifs present in the protein encoded by *BPH26* were assessed on the basis of the report of Zhou *et al.*²⁹. *BPH26* also contained many kinds of motifs, e.g., CC motif; motifs characteristic of the NBS domain; and LRR motifs, which are found in other CC-NBS-LRR proteins (Fig. 3).

Next, the deduced amino acid sequence of *BPH26* was compared with those in other rice varieties (Supplementary Fig. S4 online). There was a nonsense mutation in the allele of Nipponbare and Taichung 65, which are both susceptible *japonica* varieties. *Indica* rice variety Babawee and Rathu Heenati do not possess the resistance gene on chromosome 12^{30–32}. The nucleotide sequence was identical between Rathu Heenati and Babawee, and there was no nonsense mutation in their sequences. There were many substitutions and losses of amino acid sequences when the alleles of ADR52 were compared with those of Rathu Heenati. Amino acid substitutions tended to be mainly found in the LRR domain (Supplementary Fig. S4 online).

Identification of *BPH26* as *BPH2*. The map positions of both *BPH26* and *BPH2* seemed to overlap with each other on the long arm of chromosome 12. Nucleotide and amino acid sequences of *BPH26* were entirely identical to those of ASD7 and IR1154–243 (Supplementary Fig. S4 online), which are known to harbor the *BPH2* gene^{12,14,15}. The expression analysis by reverse transcriptase (RT)-PCR was conducted using *BPH26-NIL*, Taichung 65, ASD7,



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MEATAVSIGRSVLKGALGFAKSTLVEEVSLQLGVQRDQAFIRDELEMMNSFLMAANDEKD 60
DNKVVRTWVKQVRDVAYDVEDCLQDFAVRLGGKSSTWWLSPHTLWERRRIAKQMKELRGK 120
      non-TIR (QLRD)                               Coiled-Coil
VEDVSQRNMRYQLIKGSKPTVATNVTPSSTARATMSGAEHERWQHEKAIDHLVRLVKTKV 180
DERRVIAVWGTSGDIREMSIVGGAYDHLKRNNKFECYAWVNLMHPLNPTKLLQTIVRQFY 240
VRSLQEAGKATPSCQILSSMLIKEDQGLGFRVLRSLMKEDHLNDEFNKYLSDKCYLIVL 300
NDLSTAEWVKQIKMLFPDNKKGSRIVFTQQVEVASFCARTEEVAPEHMQLFADQTYAF 360
HCKGAKDGVDSMEDSSNLNEDTTYNAVEGKSLTRTYSMVTAFKESEIVGRVDEIKEIHEL 420
ISKGSQQLEKISVWGMGGIGKTTLIQNVYRSEKVKKMFDKHACVTIMRPFNLNDLLMSLV 480
      P-loop                               RNBS-I
RQLEDSKTSGGKELVSILEGKYYLIVLDDVLFTEWDIAIESYFPATETGSRRIITRHE 540
      Kinase-2                               RNBS-II
IAKHCSGDQQGKMYQLNRLGDNDAKNLFKAKVKFESVNLDDQDLELIKEAKPILKKNGL 600
      RNBS-III                               GLPL
PLAIVTIGGFLASRPKTTLEWRKLNHEHISAELETNPGLEAIRAVLNISYDGLPYHLKSCF 660
      RNBS-IV                               RNBS-V
LYLSIFPEDDKISRKRLVLRWCAEGYSRELWDKSAEEIANNYFFELIDRSMILPTQKSTY 720
      RNBS-VI
SNRGADSQVHDMREIAISKSKEENLVLRLGGRRLHNHDIVRHLISITNSEDWETDVG 780
      MHDV                               NL17
ELKTTVDMSRIRSLTVFGWRPIFISDKMRLLRVLDLEDTEGVRNHHIKQIGELLHRLYL 840
SLRGCTHIAYMPDSLGNLRQLETLDVRDTEFILRLPKTITNLCKLKYLRASKDLNFEYEGIR 900
EKLPELMRNRLCIFTAALLGLCLACSASAIGKFDFEINTRDVCTMCCCSILPSIAMRLQG 960
NGVVAPRGLRIRRLTALHTLGVVDISWQPSILQDIKRLIQLRKLEVTGVNKKNSKFLFA 1020
LAALSQLESLSLFSKWKPGIWGCLDAEEKFSPKLNKTLKIQGNLVELPKWIGKLNILVK 1080
LKLSKSRDKDHDAAIQVLGMLPNLTILCLPRKSFHSLEGGELNFSEGSFKSLVLELDFS 1140
GSKGVTFFQQGAFRKLLELLL SVHSEEVQSKLSGLEFLQSIKEVQIDGYCPNEEGLKDDL 1200
VQLSQNPKKPFLKIDGYF

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Figure 3 | The deduced amino acid sequence of BPH26. Typical motifs with rice R proteins are shown in blue letters, and the leucine-rich repeat (LRR) domain is underlined in red.

and Norin-PL4. Norin-PL4 included a *BPH2* originated from IR1125-243 in the background of the *japonica* cultivar Asominori¹⁵. Actin primers were used in the control amplification. The expressions of the *BPH26* gene were observed in all the varieties tested (Fig. 4a). Taichung 65 was considered to have lost resistance because the normal *BPH26* protein had not been translated due to a nonsense mutation, although gene expression was observed. Gene expressions were also observed in ASD7 and Norin-PL4, both of which have the *BPH2* gene. The feeding abilities of BPH on *BPH26*-NIL and Norin-PL4 were investigated using a *BPH2*-virulent biotype of BPH. The chi-square test²⁸ revealed that there was no difference between the numbers of survivors on *BPH26*-NIL and Norin-PL4 (Fig. 4b). This result indicated that the feeding capability of the *BPH2*-virulent biotype on the plants harboring *BPH26* was almost the same as that on the plants harboring *BPH2*. Thus, *BPH26* could be considered to be identical to *BPH2* on the basis of the results of map positioning, gene sequence analysis, gene expression analysis, and feeding ability of the *BPH2*-virulent biotype.

Phylogenetic analysis. Phylogenetic relationships were investigated among the CC-NBS-LRR proteins, such as BPH14 and the proteins encoded by blast-disease-resistance genes in *Oryza* species (Fig. 5). *BPH26* was more closely related with some disease-resistance proteins than with BPH14. The amino acid sequences of the main motifs in BPH26, BPH14, and PIB were compared. In CC-NBS domain, the amino acid sequences of the main motifs were well-conserved, although none of the motifs had the same amino acid sequences between BPH26 and BPH14 (Supplementary Fig. S5 online). The structures of LRR domains of BPH26, BPH14, and

PIB were more different than those of CC-NBS domains (Supplementary Fig. S6 online).

Histochemical detection of *BPH26* expression. The expression site of *BPH26* was analyzed using the *in situ* hybridization method (Fig. 6). *BPH26* showed relatively stronger expression in the inner leaf than in the outer leaf of the leaf sheath (Fig. 6a). Furthermore, *BPH26* showed relatively stronger expression in vascular bundles than in other tissues in the leaves of the middle layer of the leaf sheath (Fig. 6b).

Discussion

The host-plant resistance gene *BPH2*, which conferred resistance to BPH, was widely incorporated in elite rice cultivars, and was well-cultivated in many Asian countries as a favorable gene resource in rice breeding against BPH³³. The BPH resistance gene *BPH26* was found in the *O. sativa* L. ssp. *indica* cultivar ADR52 and mapped on the rice chromosome 12. In the present study, map-based cloning of the BPH resistance gene *BPH26* showed that *BPH26* is identical to *BPH2* on the basis of DNA sequence analysis and feeding ability of the *BPH2*-virulent biotype of BPH. The protein encoded by *BPH26* was a CC-NBS-LRR protein. The sequence of *BPH26* derived from ADR52 was completely identical to one from ASD7, which carried the resistance allele of *BPH2*. On the other hand, there was a nonsense mutation in the sequence of the susceptible cultivar Taichung 65. The aphid resistance gene *Mi* from tomato^{23,24} and BPH resistance gene *BPH14* from wild rice²⁵ have been reported to encode a CC-NBS-LRR-receptor kinase-like protein. Although only a few resistance genes against sap-sucking insects have been cloned, genes

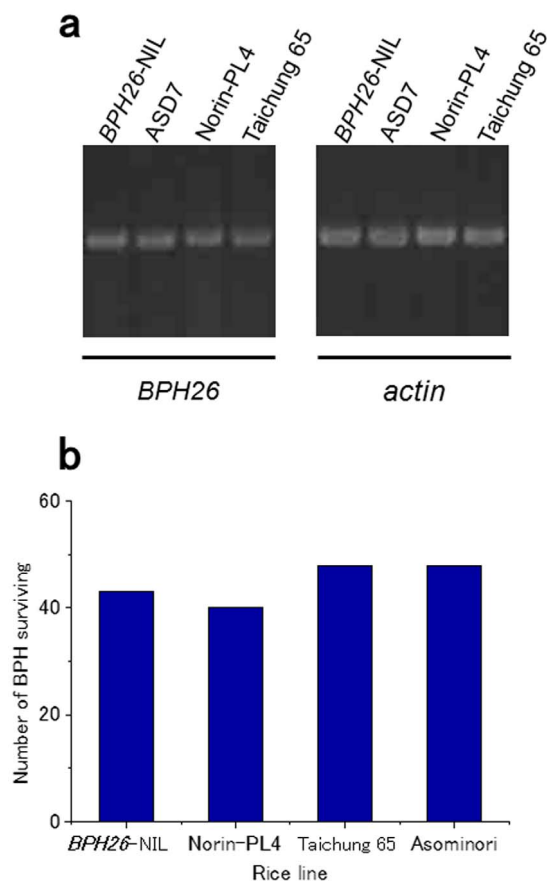


Figure 4 | The comparisons of *BPH26* and *BPH2*. (a) Gene expression analysis of *BPH26* in some rice varieties by using reverse transcriptase-PCR. Actin primers were used in the control amplification. The gels have been run under the same experimental conditions. Full-length gel is presented in Supplementary Figure S7. (b) The feeding abilities of the *BPH2*-virulent biotype of brown planthopper (BPH) on *BPH26*-near isogenic line and Norin-PL4 harboring the *BPH2* gene. Total of sixty last instar larvae that had been starved for 24 h were released on each rice line for 5 days.

belonging to the NBS-LRR family might confer resistance to sap-sucking insects, in addition to conferring disease resistance.

The *R* genes of the NBS-LRR family, such as *BPH26*, are considered to be receptors related to signal perception and transduction. *BPH26* plants showed resistance by inhibiting sucking from the phloem, as revealed by the analysis of AC-EPG. Many studies also indicated that the inhibition of sucking occurred in the phloem of the resistant *O. sativa* L. variety^{34–36}. In the case of a non-host barnyard grass, probing of BPH was interrupted before the stylets reached the sieve elements of the phloem because the barnyard grass constitutively contains trans-aconitic acid, which acts as an antifeedant of BPH, in the non-phloem tissues such as parenchyma^{37–39}. *BPH14* originated from the wild rice *O. officinalis* mediated sucking inhibition in the phloem²⁵. *O. sativa* L. and *O. officinalis* seem to have the same mechanism of resistance, i.e., defense in the phloem, against BPH. The *R* genes of the NBS-LRR family against BPH might mediate sucking inhibition in the phloem sieve element.

The sieve element can be plugged to prevent the erosion of the phloem fluid after damage⁴⁰, and sieve tube occlusion in the phloem was suggested to contribute to the inhibition against sucking insects⁴¹. In legumes, crystalline P-protein (forisome), which is contained in the sieve elements, is responsible for the plugging of sieve elements^{40,42}. In the case of monocotyledons, sieve element plastids are suggested to promote occlusion in sieve-plate pores, similar to the

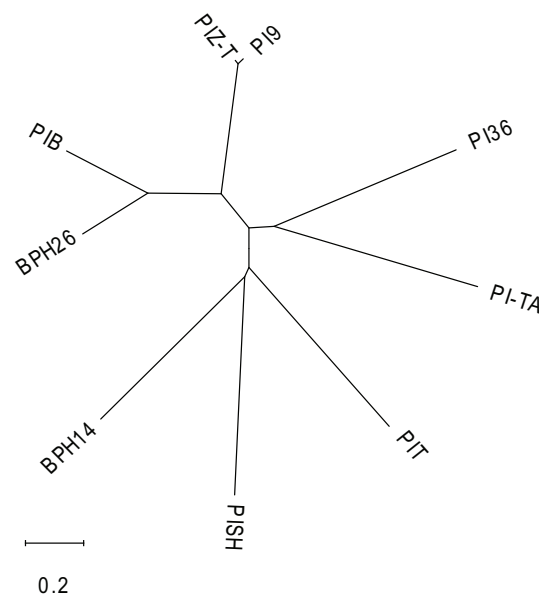


Figure 5 | Phylogenetic tree of some disease and pest resistance proteins in *Oryza* species. Sequences were aligned by MEGA6 program⁵¹, and a full-length amino acid sequence was used for clustering.

P-protein^{43,44}. *BPH14* has been inferred to mediate the deposition of callose (β -1,3 glucan polymer) in phloem cells^{25,27}. Further studies are required to confirm the defense mechanism mediated by *BPH26* in the phloem of *O. sativa* L.

There are about 480 predicted NBS-LRR genes in the rice genome²⁹, of which some have been identified as disease-resistance genes. Phylogenetic analysis of *BPH26*, *BPH14*, and some blast-resistance proteins revealed that *BPH26* was closely related to many blast-resistance proteins rather than *BPH14*. The *indica* variety Engkatek that harbored the blast-resistance gene *PYRICULARIA ORYZAE RESISTANCE B (Pib)* did not show resistance to BPH (data not shown). When the amino acid sequences of *BPH26*, *BPH14*, and other blast-resistance proteins were compared, typical consensus sequences between the two BPH resistance proteins (*BPH26* and *BPH14*) were not found in the main motifs of the CC-NBS domain and LRR domain (Supplementary Figs. S5 and S6 online). It is necessary to analyze in more detail not only the amino acid sequence but also their three-dimensional structures that separate blast resistance and BPH resistance. *BPH26* showed relatively strong expression in the vascular bundle of young leaves inside the leaf sheath, as revealed by *in situ* hybridization analysis. This suggests that *BPH26* is especially required in the vascular bundles where BPH often attacks to feed. *BPH14* has been suggested to be remarkably expressed in the vascular bundles via the β -glucuronidase (GUS) reporter system²⁵. Expression of *R* gene in the vascular bundles might be important for BPH resistance.

Initially, *BPH2* was reported as a recessive gene¹². However, Murai *et al.* reported that *BPH2* was a dominant gene by analyzing Norin-PL4 that harbored *BPH2* from IR1154-243 in the genetic background of a susceptible japonica cultivar. *BPH26* has also been reported as a dominant gene¹¹. Cultivars containing the resistance allele of *BPH2*, such as IR42, were bred in Southeast Asia to control BPH. However, a virulent biotype of BPH appeared, and the resistance afforded by *BPH2* was gradually rendered ineffective⁹. Every year, BPH populations that propagated in Southeast Asia such as northern Vietnam and the Philippines migrate via south China and Taiwan to Japan in early summer^{45,46} and cannot overwinter in Japan. Therefore, the strains of BPH that migrate to Japan are affected by the resistant cultivars cultivated in Southeast Asia. Myint *et al.*¹¹ reported that *BPH26-NIL* showed resistance to the BPH strain collected in Japan

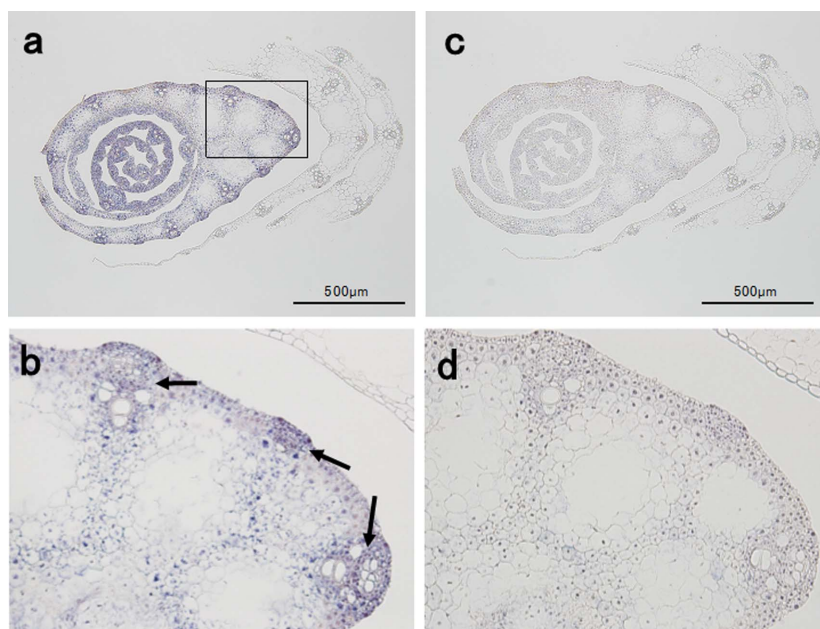


Figure 6 | The results of *in situ* hybridization of *BPH26*. Near isogenic lines with *BPH26* at the 5- to 6-leaf stages were used for *in situ* hybridization analysis. Cross-sections of the leaf sheath are shown. (a): antisense probe of *BPH26* m-RNA, (b): enlarged photograph of a square in (a), (c): sense probe of *BPH26* m-RNA (blank sample), (d): enlarged photograph of (c).

in 1989 (strain-Chikugo-89). However, it did not show resistance to the strain collected in 2006 (strain-Japan-KG-06). “Japan-KG-06” is considered to be a *BPH2*-virulent biotype, because cultivars with the *BPH2* gene have been used in Southeast Asia, and the virulent biotype has been dominated in these regions. Nevertheless, *BPH26* showed resistance to BPH “Japan-KG-06” because of the coexistence of *BPH25*, although neither *BPH26* nor *BPH25* showed resistance to this strain¹¹. Thus, the *BPH2* gene, which has lost its resistance, can be reused along with other genes, such as *BPH25*.

Of more than 30 BPH-resistance loci reported, some genes are likely to be located in similar positions¹⁰. Controlling BPH by using BPH resistance genes might become difficult if all BPH resistance genes are rendered ineffective by respective virulent biotypes, because of the limited number of BPH resistance genes. Our findings might encourage the breeding of rice varieties containing insect-resistance genes.

Methods

Gene nomenclature. We follow the new gene nomenclature system for rice⁴⁷. The gene full name of resistance to BPH is *BROWN PLANTHOPPER RESISTANCE*, and a gene symbol is an abbreviation of the gene full name such as *BPH* and written in italics and all capital letters.

Plant materials. The BPH-resistant *indica* cultivar ADR52 and the susceptible *japonica* cultivar Taichung 65 were used as parental lines for obtaining a *BPH26* mapping population. The F₁ plants from this cross were backcrossed for 5 rounds with Taichung 65 through marker-assisted selection (MAS), and BC₆F₂ population was obtained to develop NILs carrying *BPH26* gene in a Taichung 65 genetic background⁴⁸. Heterozygous individuals in the *BPH26* region on chromosome 12 were selected from the BC₆F₂ population and self-pollinated to collect a large-scaled segregating population (BC₆F₃). The BC₆F₃ population was used for fine-scaled linkage mapping. Recombinant BC₆F₃ individuals were selected by MAS and self-pollinated to confirm the phenotype in the progeny. The BC₆F₆ seeds carrying *BPH26* gene in a Taichung 65 genetic background (*BPH26*-NIL; line name, “TBPH101”) were used for molecular analysis and bioassays.

The rice cultivar, ADR52, was originally obtained from the International Rice Research Institute, the Philippines and has been maintained in Kyushu University. The rice varieties, ASD7, IR1154–243, Rathu Heenati, Babawee, Norin-PL4, and Asominori (which are stocked at the Rice Genome Resource Center), were used for sequencing the exon regions of *BPH26* and for bioassays.

BPH (*N. lugens* Stål) rearing. BPH adults (*BPH2*-avirulent biotype) were collected in Osaka Prefecture, Japan, in 1973. A stock colony of BPH was reared successively on rice seedlings at our institute at 25 ± 2°C and 70 ± 5% relative humidity under a

16:8 h light/dark photoperiod. The BPH was reared on the sensitive cultivar Koshihikari. BPH collected from Shimane Prefecture, Japan, in 1983, were used for selecting a *BPH2*-virulent biotype that can attack ASD7 carrying *BPH2*. The *BPH2*-virulent biotype was also reared on the sensitive cultivar Koshihikari, and its ability to feed on ASD7 was monitored at regular intervals.

Bioassays. Survival rate of BPH. A germinating seedling was placed in a test tube (diameter, 1 cm; height, 18 cm) with absorbent cotton moistened with liquid fertilizer, and the test tube was secured with a cotton plug. It was incubated for 13 days at 25°C under a 16-h light:8-h dark cycle. The two individuals of last instar larvae that had been fasted for 24 h on a moist filter paper were released in each test tube, and the numbers of live BPH were recorded 5 days after releasing. The tests were repeated 30 times (the total number of BPH was 60), and the number of living BPH was summed accordingly. Chi-square tests²⁸ were used to compare the number of individuals surviving in the two different groups.

Electronic monitoring of feeding. The feeding activities of BPH on some rice lines were investigated using an AC-EPG as previously described³⁹. An oscillator (IEM-04; Tsukuba Rica Seiki Inc.) was set at 0.5 V and 500 Hz, and all recordings were acquired over 12 h. The waveforms during probing behavior of each insect for 10 h from the start of the first probe were analyzed. The waveforms were interpreted on the basis of a previous report³⁹. Each variety of plant was tested 10 times by using fresh insects and fresh plants. The data on the total time of phloem ingestion and the numbers of probe were analyzed using Tukey–Kramer test²⁸.

Honeydew assays. For quantitative analysis of honeydew, modified Parafilm sachet technique was used⁴⁹. A Parafilm sachet was attached to the leaf sheath of each seedling at the 5- to 6-leaf stage. A female BPH within 2 days after emergence was then enclosed in a Parafilm sachet. After 48 h of incubation at 25°C under a 16-h light:8-h dark cycle, each insect was removed, and the sachet was weighed before and after the removal of honeydew. Each line of plants was tested 10 times by using fresh insects and fresh plants. The data was analyzed using Tukey–Kramer test²⁸.

DNA markers for mapping. The candidate region of *BPH26* was elucidated using single sequence repeat (SSR) markers reported by the International Rice Genome Sequencing Project⁵⁰. Furthermore, newly developed insertion-deletion (InDel) and single nucleotide polymorphism (SNP) markers in the candidate region were used to determine regions having a tight linkage to the resistance gene. These new markers were developed using the direct sequences of both Taichung 65 and ADR52 genomic DNA. SNP was detected by direct sequencing of the DNA amplified by PCR. The primers for markers are shown in Supplementary Table S2 online.

RNA extraction and analysis. Total RNA was extracted from the shoots and leaf blades by using a NucleoSpin RNA II (Macherey-Nagel). Genomic DNA was removed using TURBO DNA-free™ Kit. RT-PCR assay was conducted using one-step RT-PCR kit (Quick Master Mix; TOYOBO) according to the manufacturer’s instructions. PCR analysis consisted of 40 cycles by using primers shown in Supplementary Table S2 online. RNA extraction and RT-PCR were repeated three



times for each sample. To isolate the cDNA of *BPH26* from ADR52, ASD7, and Norin-PL4, RACE-PCR was performed using a SMARTer RACE cDNA Amplification Kit (Clontech). RACE-PCR products were cloned and sequenced.

Sequence comparisons of BPH26 among some rice varieties. Three exon regions of *BPH26* from some rice varieties were amplified by PCR using the genomic DNA as template. PCR analyses were conducted using KOD-Plus neo (TOYOBO) and the primers shown in Supplementary Table S2 online. The PCR products were cloned using Target clone™ Plus (TOYOBO) and sequenced to compare the alleles from some rice varieties. In addition to the genomic sequences, cDNA sequences from ADR52, ASD7, and Norin-PL4 were also used for sequence comparisons. Amino acid sequences were deduced from these nucleotide sequences.

Phylogenetic analysis. The phylogenetic analysis was conducted using the MEGA6 program⁵¹. A neighbor-joining (NJ) method was then applied to produce a phylogenetic tree⁵². The evolutionary distances were computed using the Poisson correction method⁵³ and are in the units of the number of amino acid substitutions per site.

Complementation test. A BAC library of ADR52 genomic DNA was constructed as described previously⁵⁴, and a BAC clone (14H01) covering the *BPH26* gene was selected using DNA markers tightly linked to *BPH26*. A genomic sub-clone library of about 20 kb was constructed from 14H01. Restriction fragments of 14H01 after digestion with *Sau3AI* were gel purified and cloned into the *BamHI* site of binary plasmid vector pPZP2H-lac⁵⁵ by ligation. Recombinants were cloned into the *Escherichia coli* DH5 α strain, and the clones that contained a fragment with the candidate *BPH26* gene were selected by colony PCR and sequenced. The selected recombinants were purified and introduced into *Agrobacterium* strain EHA101⁵⁶ to infest calluses of susceptible cultivar Taichung 65. Plants regenerated from hygromycin-resistant calluses were grown in growth chambers. DNA from T₁ plants was collected from leaf tips. T₁ plants that were PCR validated to harbor the candidate *BPH26* gene were selected using primers complementary to the insertion site of the pPZP2H-lac (Supplementary Table S2 online) and used in the biological assays. For analysis of the expression levels and copy numbers of *Bph26* in the transgenic lines, real-time quantitative RT-PCR were performed using a Light Cycler 480 Instrument (Roche) in accordance with the manufacturer's instructions. Total RNA for the analysis of gene expression was extracted from the leaf sheath of 14-d-old seedlings by using NucleoSpin RNA II (Macherey-Nagel), and a PrimeScript RT reagent Kit (Takara) was used for reverse transcription. DNA for analysis of the copy number was extracted from the leaves of the same 14-d-old seedlings used for RNA extraction. The gene-specific primers used for *Bph26* and an internal standard gene are shown in Supplementary Table S2 online.

In situ hybridization analysis. NIL plants carrying *BPH26* in a Taichung 65 background at the 5- to 6-leaf stages were used for *in situ* hybridization analysis of *BPH26* m-RNA. NIL plants that did not experience sucking from BPH were used for this analysis. *In situ* hybridization was conducted according to the methods described previously⁵⁷. The *BPH26* antisense probe was synthesized by *in vitro* transcription by using SP6 RNA polymerase from a 250-bp fragment that was a part of the *BPH26*-specific 3'UTR sequences amplified using PCR (Supplementary Table S2 online).

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Author contributions

Y.T. designed and performed the experiments and wrote the manuscript. H.Y. developed the materials, provided advice on the experiments, and improved the manuscript. M.H. supported bioassays and cloning of cDNA. H.Y. and M.Y. analyzed the structure of genes. A.T. provided advice on the experiments and phylogenetic analysis. J.W. designed BAC clone analysis. N.S. designed *in situ* hybridization analysis. All authors reviewed the manuscript.

Additional information

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