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Sakuranetin protects rice from brown planthopper attack by depleting its beneficial endosymbionts

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Plants produce chemical defenses that poison insect herbivores or deter their feeding, but herbivores are also accompanied by microbial endosymbionts crucial for their nutrition, reproduction, and fitness. Hence, plant defenses could target a herbivore's beneficial endosymbionts, but this has not yet been demonstrated. Here, we studied flavonoids that are induced when rice is attacked by a phloem-feeding pest, the brown planthopper (BPH), which harbors beneficial yeast-like symbionts (YLS) essential for insect nutrition, such as by remedying deficiencies in sterols. BPH attack dramatically increased sakuranetin accumulations in leaf sheaths and phloem exudates. Sakuranetin is an antifungal phytoalexin derived from the antibacterial precursor, naringenin, via catalysis of naringenin-*O*-methyltransferase (NOMT). When added to artificial diets, sakuranetin decreased BPH survivorship, suggesting that it functions as an induced defense. Mutation of *NOMT* abolished sakuranetin accumulation and increased BPH oviposition and hatching rates. High-throughput amplicon sequencing revealed that BPH fed on sakuranetin-deficient *nomt* lines were enriched in YLS with only minor changes in the bacterial endosymbionts, compared to those feeding on sakuranetin-rich wild-type (WT) plants. In-vitro feeding of sakuranetin suggested that this flavonoid directly inhibited the growth of YLS. BPH feeding on *nomt* lines accumulated higher cholesterol levels, which might be attributed to increases in the supply of sterol precursors from the YLS, while *nomt* lines suffered more damage than WT plants did from BPH herbivory. BPH-elicited accumulation of sakuranetin requires intact jasmonate (JA) signaling. This study reveals that rice uses a JA-induced antifungal flavonoid phytoalexin in defense against BPH by inhibiting its beneficial endosymbionts.

rice | sakuranetin | beneficial endosymbionts | jasmonate signaling | direct defense

Significance

Herbivorous insects commonly harbor beneficial microbial endosymbionts that provide nutritional benefits to their hosts. These microbes may be the targets of plant antiherbivore defense compounds, but such a role for plant defenses has not been previously demonstrated. Here, we found that rice attacked by its notorious pest, the phloem-feeding brown leafhopper (BPH), produces an antifungal flavonoid, sakuranetin, that targets the yeast-like beneficial endosymbionts of BPH. Abrogating sakuranetin biosynthesis in rice increases the reproductive performance and nutrition of BPH, and increases plant damage. We conclude that a plant's induced chemical defenses can target the beneficial endosymbionts of its insect attackers for protection.

Plants are frequently challenged by an array of abiotic and biotic stresses in their natural environments, among which insect herbivores constitute a very diverse threat. Plants employ multiple strategies to defend against herbivore attack. Defenses are either constitutively produced or only produced when plants are attacked (1). The latter, inducible defenses allow plants to forego costly defense production when the need is low and channel resources into other processes, such as growth or reproduction (2). The phytohormone jasmonate (JA) is the core regulator of plant inducible defenses against herbivores by directly mediating the biosynthesis of defensive proteins or specialized metabolites (1). Specialized metabolites comprise the most important and diverse component of a plant's defensive arsenal (3). The identification and functional analysis of herbivore-elicited specialized metabolites have been extensively studied in various plant species. The most well-studied sectors of specialized metabolism in plant–herbivore interactions are terpenoids, alkaloids, and glucosinolates. The alkaloid, nicotine, poisons the nicotinic acetylcholine receptors of neuromuscular junctions, blocking transmission, and impairing herbivore motor performance (4, 5). The volatile terpenes have direct repellent effects on some *Hemipteran* herbivores, and function as indirect defenses that attract the natural enemies of herbivores (6, 7). However, the action mode of most herbivory-elicited specialized metabolites remains largely unknown (3).

Microbial endosymbionts of some insect herbivores are known to play essential roles in plant–herbivore interactions. Bacterial symbionts in the oral secretions of chewing herbivores, for instance, can suppress plant JA-mediated defenses and increase herbivore performance (8–10). The gut bacteria from many herbivore species play important roles in the detoxification of ingested plant defensive metabolites (11), causing a negative effect on plant defense systems. In contrast, insect gut microbiomes can in some instances potentiate plant chemical defenses to enhance herbivore resistance (12). In addition, insect herbivores often harbor beneficial endosymbionts that play essential roles in their growth, development, reproduction, and stress resilience (13). Whether plant specialized metabolites can target these beneficial endosymbionts and thereby function as another form of direct defense, remains unknown (3).

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Flavonoids are ubiquitous across the plant kingdom. They are known as medicinal agents, nutritional compounds and pigments, and are involved in UV protection and plant responses to abiotic stresses (14). Some flavonoids can also act as phytoalexins in plant–microbe interactions. For instance, sakuranetin was first discovered in UV-irradiated rice leaves and rice blast fungus (*Magnaporthe oryzae*)-infected tissues and later identified as a phytoalexin against many fungal pathogens, while its precursor naringenin has antibacterial activity (15, 16). The accumulation of both flavonoids is variably induced among rice cultivars (15). In addition, some flavonoids are reported to be produced in response to herbivore attacks (17–20). Some studies have applied flavonoids to artificial diets and found decreases in the performance of phloem-sucking herbivores (17, 20). Maysin, a C-glycosyl flavone, in the silk of maize tassels, has long been known to confer resistance to the maize earworm. Enzymatic studies identified two flavonoid synthases that may be involved in maysin biosynthesis (21). However, genetic and ecological evidence is still required to clarify if flavonoids play roles in plant resistance to herbivores, and the underlying mechanisms remain to be investigated.

Here, we addressed the above questions in the well-studied brown planthopper (BPH, *Nilaparvata lugens*)-rice system. BPH is a destructive phloem-feeding herbivore in the paddy fields. Yeast-like symbionts (YLS) have long been identified as the most abundant endosymbionts in BPH, which are maternally transmitted via eggs through transovarial infections and are found in every developmental stage of BPH (22). YLS play vital roles in providing amino acids and sterol precursors, and in nitrogen recycling of their host (23–26). Our previous transcriptome analysis found that the phenylpropanoid pathway was particularly enriched in BPH-attacked rice plants (27), and some flavonoid biosynthetic genes were strongly up-regulated. By gene expression, chemical analysis, pharmacological approaches, and genetic analysis, sakuranetin was characterized as a defensive compound against BPH and found to be regulated by JA signaling. Given the antimicrobial activity of this methoxylated flavonoid, we explored its involvement in the maintenance of beneficial endosymbionts in BPH by amplicon sequencing and microbiome quantification. Our results demonstrate that sakuranetin is a JA-mediated inducible defense against BPH that directly targets its beneficial fungal endosymbionts.

Results

Sakuranetin Biosynthesis in Rice Is Activated by BPH Attack. The most abundant flavonoids in rice are derived from the core precursor, naringenin (Fig. 1*A*). To evaluate if these highly abundant metabolites are involved in herbivore resistance, the expression of BPH-elicited flavonoid biosynthetic genes in Nipponbare variety was analyzed. A total of five naringenin metabolic genes were identified, of which, the transcripts of naringenin-*O*-methyltransferase (*NOMT*) were significantly elevated in plants under BPH attack, whereas the transcript levels of *UGT707A3*, *FNS* and *F2H* were reduced in BPH-attacked plant tissue compared with control tissue, and *CYP75B3* transcripts were only mildly increased at 72 h after BPH infestation (*SI Appendix*, Fig. S1 *A–E* and Fig. 1*B*). Next, we quantified the accumulations of naringenin and its major derivatives in BPH-attacked leaf sheaths of Nipponbare variety. Naringenin levels increased at 48 and 72 h after BPH attack (Fig. 1*C*), suggesting that the upstream phenylpropanoid pathway was activated. Consistent with the gene expression inferences, the concentrations of the NOMT product, sakuranetin, increased continuously during 72 h of BPH attack (Fig. 1*D*), while levels in unattacked controls were below the limits of detection. Levels of all other naringenin derivatives (apigenin, apigenin-7-*O*-β-glucoside, eriodictyol-7-*O*-β-glucoside and naringenin-7-*O*-β-glucoside) decreased in response to BPH attack (Fig. 1 *E–H*).

Sakuranetin Decreases BPH Performance in Artificial Diets and in Rice Plants. BPH feeds mainly on rice phloem sap with its stylets. To determine if these insects would be exposed to flavonoids when feeding on rice, flavonoid concentrations in rice Nipponbare phloem sap were determined. Sakuranetin was detected in phloem sap and its concentration increased dramatically after BPH attack (*SI Appendix*, Fig. S2*A*), attaining levels that were ten times higher than those of naringenin and other flavonoids (*SI Appendix*, Fig. S2 *B–F*). Based on these measurements, BPH feeding areas of leaf sheath and the rate of rice phloem sap exudation, we estimated the physiological concentrations of BPH-elicited sakuranetin levels in the phloem sap to be 0.5 to 1 μg mL^{-1} (28). To evaluate if these concentrations of sakuranetin could influence BPH performance, sakuranetin-feeding assays using artificial diets (AD) were conducted. The survival rate of BPH nymphs fed on 0.5 μg mL^{-1} sakuranetin-containing AD was significantly lower than those fed control AD (Fig. 2*A*). Collectively, these results suggest that sakuranetin could function as a rice defense against BPH attack.

To evaluate if sakuranetin affects BPH performance in vivo, *NOMT* mutants were constructed and used in BPH bioassays. Two types of homozygous mutants were constructed in the Nipponbare rice variety by CRISPR-Cas9-based genome editing, both of which resulted in premature stop codons in the *O*-methyltransferase domain of the NOMT gene (*SI Appendix*, Fig. S3*A*). BPH-elicited flavonoid levels were quantified in these lines. As expected, the *nomt* mutants did not produce sakuranetin in response to BPH attack (Fig. 2*B*), while levels of naringenin and other naringenin derivatives did not differ from WT levels (Fig. 2*C* and *SI Appendix*, Fig. S3 *B–E*). The survival rate of BPH nymphs fed on WT and *nomt* lines did not differ, but BPH developmental rates on *nomt* mutants were significantly shorter than those on WT plants (*SI Appendix*, Fig. S4 *A* and *B*). Moreover, when freshly emerged female and male BPH adult pairs were allowed to feed on the plants, a pair produced approximately 70 additional eggs (20% increase) when feeding on *nomt* mutants than that on WT plants (Fig. 2*D*). The hatching rate of BPH eggs on *nomt* mutants was also increased by 30% compared with those on WT plants (Fig. 2*E* and *SI Appendix*, Fig. S4*C*). These results indicate that BPH-induced sakuranetin production significantly decreases BPH performance on rice.

Sakuranetin Alters the Abundance of BPH Fungal Endosymbionts. The bacterial and fungal symbionts of BPH are known to be important for the growth, development, and reproduction of this important pest of rice (26, 29). Given that sakuranetin is described to have strong antifungal pathogen activity (15), we asked if sakuranetin could directly affect the endosymbionts in BPH and performed 16S RNA and ITS sequencing of BPHs fed on sakuranetin-deficient lines and WT plants. Partial least squares discriminant analysis (PLS-DA) of all bacterial community revealed that the eight samples of *nomt*- and WT-fed samples were not well separated ($P = 0.094$, PERMANOVA analysis), but the opposite was true for all fungal community (Fig. 3*A*, *SI Appendix*, Fig. S5*A*, and $P = 0.034$, PERMANOVA analysis). The differentially enriched fungal endosymbionts between BPH fed on sakuranetin-deficient lines and WT plants were identified at the genus level. Among these, *Cordyceps* and *Candida* were the most enriched taxa (proportion > 0.1%) and their abundances were much higher in *nomt*-fed than in WT-fed BPH (Fig. 3*B*). The Ascomycetes YLS is the well-known dominant fungal endosymbiont in BPH, belonging to the genus *Cordyceps* (22, 30, 31). Consistent with

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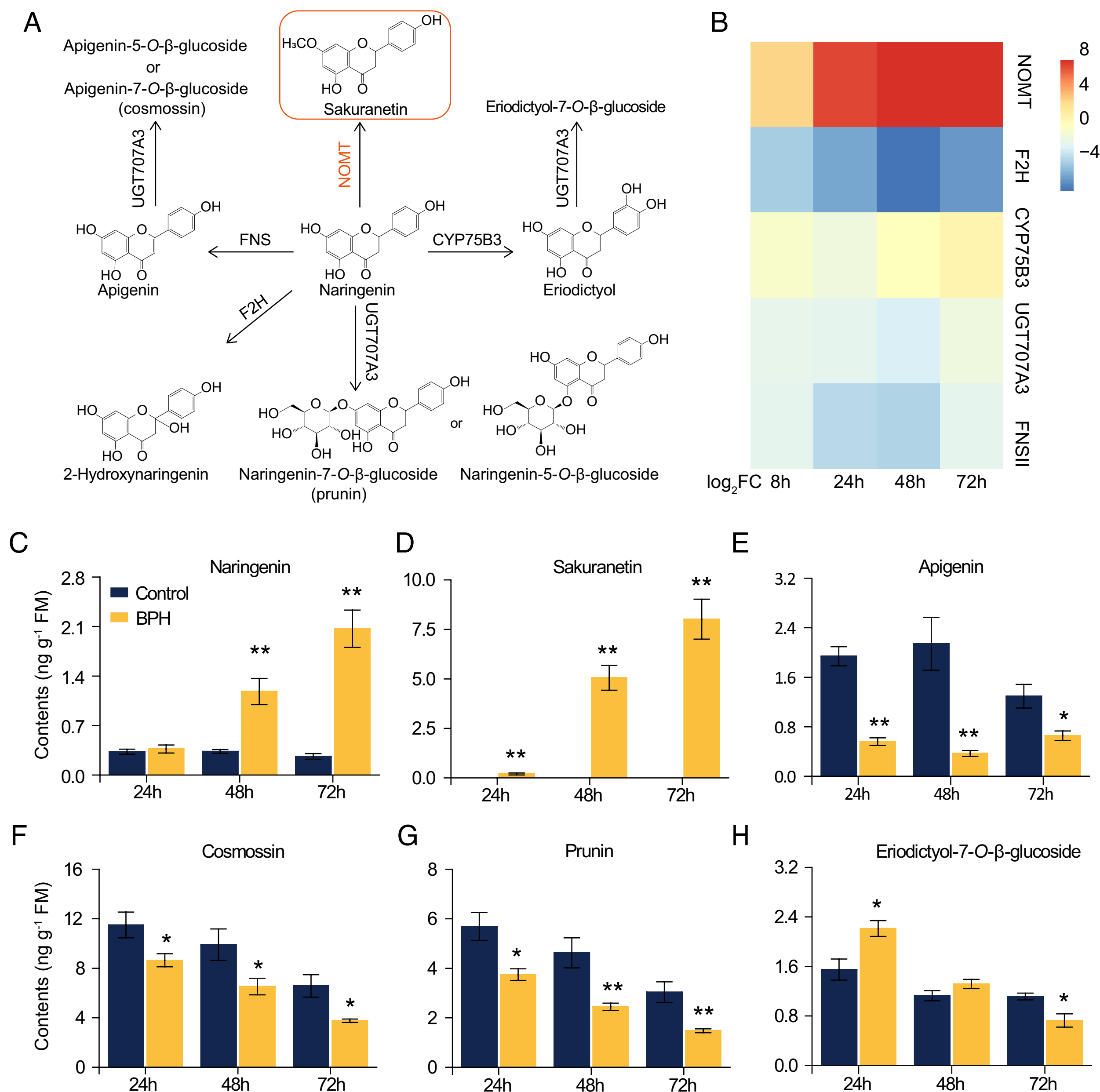

Fig. 1. BPH attack on rice induces an increase in sakuranetin biosynthesis and accumulation. (*A*) The biosynthetic pathway of major flavonoids derived from naringenin in rice. CYP75B3, flavonoid 3′-hydroxylase; FNS, flavone synthase; F2H, flavanone 2-hydroxylase; NOMT, naringenin-*O*-methyltransferase; UGT707A3, uridine diphosphate-dependent glucosyltransferases. (*B*) Heatmap represents the transcript levels of key genes involved in naringenin-derived flavonoids biosynthesis in BPH-treated plants compared with control plants. Gene expression levels were inferred from transcript abundances quantified by qRT-PCR. See *SI Appendix*, Fig. S1 for detailed results. FC, fold change. Color gradient represents the relative transcript abundance: numbers indicate $\log_2$FC. Mean concentrations (± SE, n = 5) of naringenin (*C*), sakuranetin (*D*), apigenin (*E*), cosmossin (*F*), prunin (*G*), and eriodictyol-7-*O*-β-glucoside (*H*) in BPH-attacked and control plants. Asterisks indicate significant differences in BPH-attacked plants compared with controls (**P* < 0.05; ***P* < 0.01; Student's *t* test).

previous studies, the proportion of fungal symbionts of *Cordyceps* in BPH was above 80%. Furthermore, the sequence abundances of this YLS were higher in *nomt*-fed than in WT-fed BPH (Fig. 3*C*). The abundance of *Candida carpophila*, the most abundant *Candida* species identified from the sequencing data, showed a similar pattern (Fig. 3*D*). Four bacterial genera were also differentially enriched in *nomt*-fed vs. WT-fed BPH, including the dominant community *Arsenophonus*. These enrichments were modest compared to those of the fungal taxa (*SI Appendix*, Fig. S5*B*).

Sakuranetin Decreases the Number of Beneficial Endosymbionts in BPH. The YLS in BPH are known to participate in amino acid and sterol biosynthesis and nitrogen recycling (26). Some *Candida* species are also reported to be beneficial endosymbionts in many insect species (32). According to the ITS sequencing data, sakuranetin ingestion may inhibit the growth of beneficial endosymbionts in BPH. To test this hypothesis, endosymbiont abundances were quantified in the different treatments using microscopy and qPCR with specific primers. The number of YLS was significantly increased in BPH fed on *nomt* mutants compared with that in BPH fed on WT plants and the relative abundance increased (Fig. 4 *A*–*E*). In contrast, the number of YLS was significantly reduced in BPH fed on sakuranetin-containing AD compared to control AD, and the relative abundance decreased (Fig. 4 *F*–*J*). The relative abundance of *C. carpophila* in BPH also increased when BPH were fed on sakuranetin-deficient lines and

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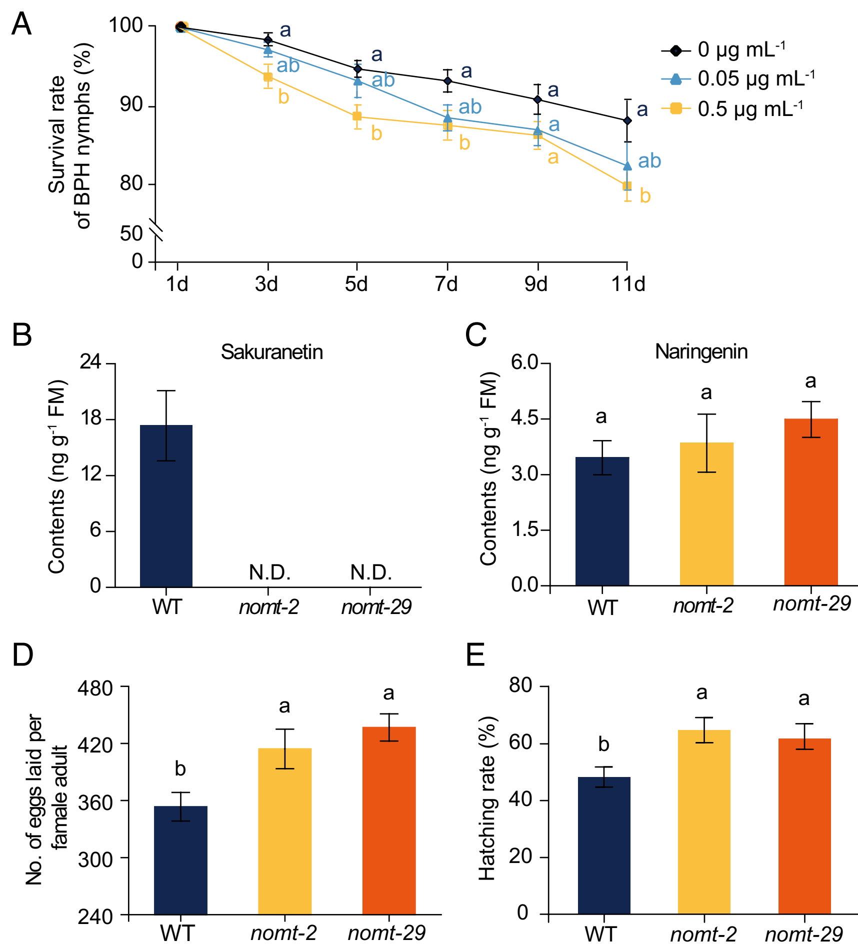

Fig. 2. Sakuranetin decreases BPH performance in artificial diets and *in planta.* (*A*) Mean survival rate (± SE, n = 9) of BPH nymphs fed artificial diet containing 0 to 0.5 μg mL^{-1} sakuranetin. Twenty-five freshly hatched BPH nymphs were released to a glass cage containing different artificial diets. The number of surviving nymphs was recorded each day. Letters indicate significant differences among different sakuranetin concentrations ($P < 0.05$, one-way ANOVA followed by LSD test). Mean concentrations (± SE, n = 5) of sakuranetin (*B*) and naringenin (*C*) in *nomt* mutants and WT plants at 48 h after BPH infestation. Mean number (± SE, n = 18) of eggs laid per BPH adult female (*D*) and hatching rate (± SE, n = 12) of BPH eggs (*E*) on *nomt* mutants and WT plants. For BPH fecundity assays, a pair of newly emerged BPH female and male adults were allowed to oviposit on the leaf sheath of each plant for 10 d. For BPH hatching assays, ten gravid BPH females were allowed to oviposit on each plant for 24 h. N.D., not detected. Letters indicate significant differences among mutants and WT plants ($P < 0.05$, Kruskal–Wallis test followed by a Dunn's posttest for BPH fecundity assays; one-way ANOVA followed by LSD test for BPH hatching assays).

decreased when BPH were fed on sakuranetin-containing AD (Fig. 4 *K* and *L*). Although the abundance of YLS is very high in BPH, this endosymbiont cannot be cultured in vitro (22). Given that YLS could be maternally transmitted via eggs, the direct antifungal activity of sakuranetin on YLS was evaluated using fresh BPH eggs. The fresh eggs were homogenized with phosphate-buffered saline (PBS, PH = 7.4), the YLS in which can survive to at least 48 h. Sakuranetin supplementation (0.5 μg mL^{-1}) significantly reduced the number of YLS in BPH egg homogenate after 36-h incubation (Fig. 4*M*). In addition, the number of YLS was also decreased in the intact fresh eggs placed on the filter paper soaked with sakuranetin solution compared with the control solution after 4 d (*SI Appendix*, Fig. S6). The direct effects of sakuranetin on *C. carpophila* and a closely related species of YLS, *Hypomyces chrysospermus* (33), were also examined. Augmenting media with sakuranetin reduced the growth of *C. carpophila* and *H. chrysospermus* in a dose-dependent manner (*SI Appendix*, Fig. S7). These results demonstrate that sakuranetin could directly target the beneficial endosymbionts in BPH.

To investigate how these endosymbionts might benefit BPH, the concentration of cholesterol was quantified in BPHs fed on *nomt* mutant and WT plants, which harbor more or fewer fungal symbionts, respectively. The biosynthesis of this important sterol requires a basic sterol precursor from YLS, which is modified by BPH (26, 34). Cholesterol levels were found to be 26% higher in BPH fed on sakuranetin-free plants than in BPH fed WT plants (Fig. 4*N*), suggesting a mechanism for how sakuranetin could decrease BPH performance.

Sakuranetin Is a JA-Mediated Inducible Defensive Compound against BPH. Finally, to determine if sakuranetin actually protects rice plants from BPH damage, we conducted a plant survival assay. When exposed to equal numbers of BPHs, the *nomt* mutants with undetectable sakuranetin were more severely damaged than WT plants (Fig. 5 *A* and *B*), consistent with the inference that sakuranetin functions as a direct defense against BPH by targeting its endosymbionts.

To determine the mechanisms underlying sakuranetin induction, we manipulated the defense hormone JA using another high-sakuranetin-accumulating variety, XS11. Our previous research had found that BPH-elicited expression of *NOMT* was decreased in JA biosynthesis (*aoc*) and signaling (*myc2*) mutants (27). Here, we measured sakuranetin accumulation in six JA mutants and MeJA treatments (*SI Appendix*, Fig. S8*A*). In addition to the previously produced *aoc*, *myc2*, *coi1*, *coi2* mutants (32, 35), the JA-Ile biosynthetic gene, JASMONATE RESISTANT1

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Fig. 3. Sakuranetin alters the fungal endosymbiont community in BPH. (*A*) Partial least squares discriminant analysis (PLS-DA) of fungal endosymbionts in BPHs fed *nomt* mutants (no sakuranetin) and WT (sakuranetin-rich) plants. Eight biological replicates were used. (*B*) The abundance of fungal endosymbionts in BPHs fed *nomt* mutants and WT plants at the genus level. Mean abundance (± SE, n = 8) of (*C*) Ascomycetes yeast-like symbionts (YLS) and *Candida carpophila* (*D*) in BPHs fed *nomt* mutants and WT plants by ITS sequencing. Asterisks indicate significant differences between BPHs fed on *nomt* mutants and WT plants (*$P < 0.05$; **$P < 0.01$; Wilcoxon rank-sum test). Horizontal lines are medians, boxes show the upper and lower quartiles, and whiskers show the full data range.

(*JAR1*), and signaling gene, mediator complex subunit 25 (*MED25*), were also mutated by CRISPR-Cas9 based genome editing. Both mutants produce premature stop codons (*SI Appendix*, Fig. S8 *B* and *C*). The sakuranetin contents in leaf sheath were increased after MeJA treatment, but were differentially decreased in JA mutants under BPH attack (*SI Appendix*, Fig. S8 *D* and *E*). Naringenin levels tracked sakuranetin levels in these plant lines, while other derivatives were differentially regulated by JA and its signaling components (*SI Appendix*, Fig. S9). Together these results indicate that the accumulation of BPH-elicited sakuranetin is dependent on JA signaling in rice.

Discussion

The biosynthetic pathway of the major flavonoids in rice is being gradually elucidated (36), allowing us to explore their biological functions. Here, we studied the methylated flavanone sakuranetin in high-accumulating rice varieties (Nipponbare and XS11), which has been previously established to be a phytoalexin, an inducible antifungal defense. Several lines of evidence are consistent with the inference that sakuranetin protects rice from brown planthopper (BPH) attack by targeting the beneficial endosymbionts of BPH. First, sakuranetin dramatically accumulated in BPH-attacked leaf sheaths, while other naringenin-derived flavonoids did not. The induction of its biosynthesis by BPH was found to be regulated by the defense-related hormone, JA. Second, sakuranetin decreased BPH damage to rice plants. Third, BPH performance was lower on WT rice than on lines where sakuranetin biosynthesis was blocked; BPH performance was also decreased when this insect fed on a sakuranetin-containing artificial diet. Fourth, sakuranetin reduced the abundance of BPH fungal endosymbionts, which are thought to furnish the insect with amino acids, sterol precursors, and nitrogen recycling capability. In support of this proposition, sakuranetin reduced the cholesterol content of BPH, which may be a consequence of the low titer of the endosymbiont. While the previously described chemical defenses of plants generally act on the herbivores themselves, this study reveals that plant chemical defenses can also impact herbivores by targeting their beneficial endosymbionts.

The yeast-like symbiont (YLS) is the most abundant endosymbionts in BPH, occupying 22% of the abdominal volume (31). Depleting YLS by heat treatment (35 °C) significantly reduced the growth of BPH resulting in lower body weight and delayed emergence (24). Moreover, YLS-depleted BPH lose their ability to recycle nitrogen, which results in the accumulation of high concentrations of uric acid. The biosynthetic genes of ten essential amino acids and steroids are not present in the BPH genome but are contained in the YLS genome, suggesting the additional roles for YLS in meeting the nutritional needs of BPH (26). Here, we show that sakuranetin accumulates in the phloem sap and thus, is likely assimilated by BPH during the feeding process. In vitro and in vivo BPH feeding assays indicated that sakuranetin negatively affects the abundance of YLS in BPH (Fig. 4). The depletion of YLS in BPH could in turn affect the growth and development of BPH. In line with this inference, BPH performance is negatively correlated with the concentration of sakuranetin (Fig. 2). The fecundity of BPH and the hatching rate of BPH eggs were reduced on WT plants versus sakuranetin-free plants, leading to reduced plant damage (Fig. 5 *A* and *B*). In addition to YLS, *Candida carpophila* is also suppressed by sakuranetin. *Candida* endosymbionts are known to play nutritional roles in various insect species (32). But further study is required to elucidate the role of *C. carpophila* in BPH. Sakuranetin

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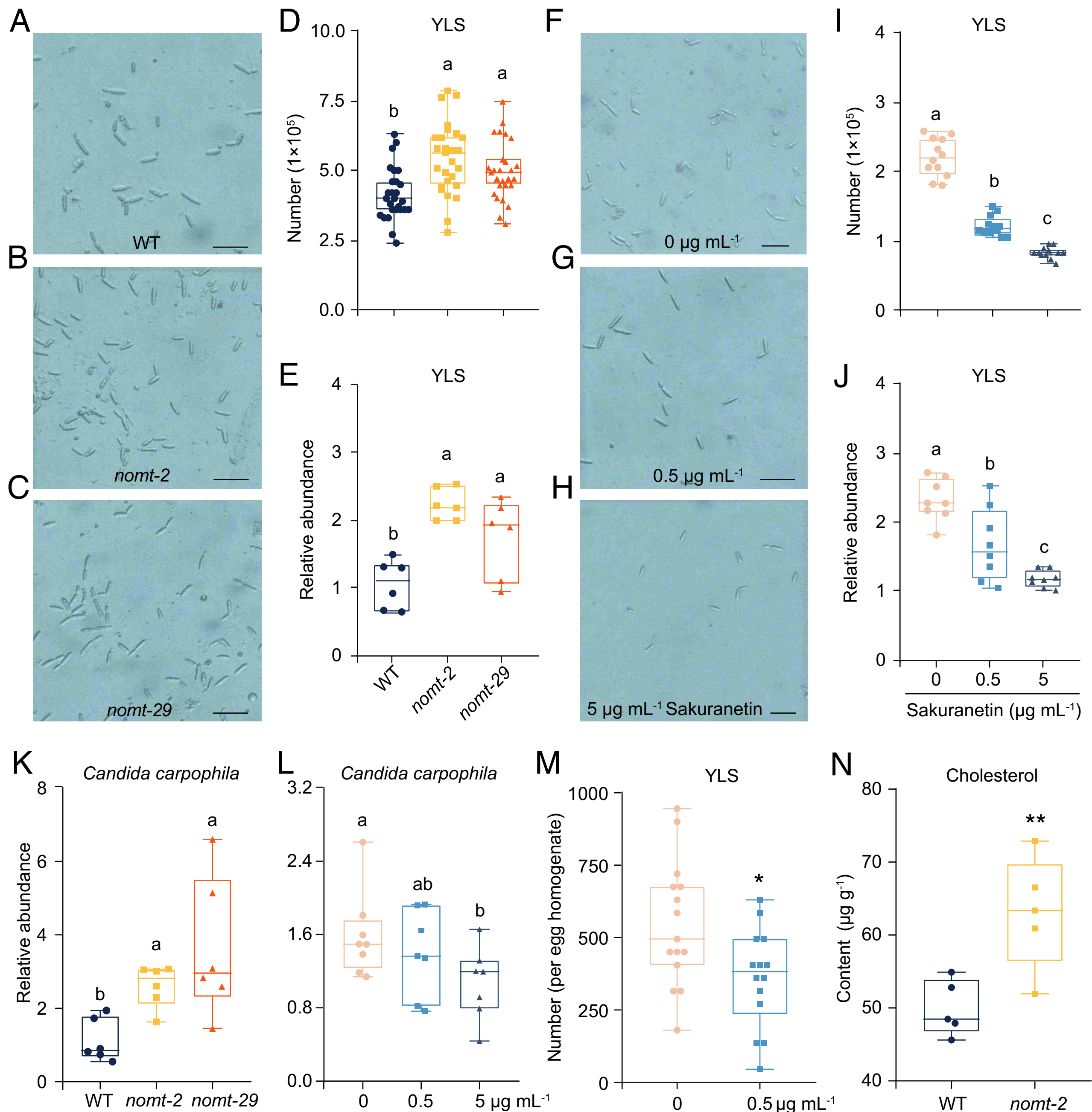

Fig. 4. Sakuranetin reduces the abundance of beneficial endosymbionts in BPH. (*A*–*C*) The morphology of YLS in fat bodies of BPHs fed on *nomt* mutants and WT plants. (Scale bars, 25 μm.) Mean number (n = 27) (*D*) and relative abundance (n = 6) (*E*) of YLS in BPH fed on *nomt* mutants and WT plants. Letters indicate significant differences among mutants and WT plants ($P < 0.05$, one-way ANOVA followed by LSD test). (*F*–*H*) The morphology of YLS in BPH fed on artificial diets containing 0 to 5 μg mL^{-1} sakuranetin. (Scale bars, 25 μm.) Mean number (n = 12) (*I*) and relative abundance (n = 8) (*J*) of YLS in BPH fed artificial diets containing 0 to 5 μg mL^{-1} sakuranetin. Letters indicate significant differences among different sakuranetin concentrations ($P < 0.05$, one-way ANOVA followed by LSD test). (*K*) Mean relative abundance (n = 6) of *Candida carpophila* in BPH fed on *nomt* mutants and WT plants. Letters indicate significant differences among mutants and WT plants ($P < 0.05$, one-way ANOVA followed by LSD test). (*L*) Mean relative abundance (n = 7 to 8) of *C. carpophila* in BPH fed artificial diets containing 0 to 5 μg mL^{-1} sakuranetin. Letters indicate significant differences among different sakuranetin concentrations ($P < 0.05$, one-way ANOVA followed by LSD test). (*M*) Mean number (n = 15) of YLS in one BPH homogenate treated with 0.5 μg mL^{-1} sakuranetin or control solution for 36 h. The fresh BPH eggs (within 3 h after oviposition) were homogenized with PBS buffer. Asterisks indicate significant differences between different treatments (*$P < 0.05$; Student's *t* test). (*N*) Mean concentrations (± SE, n = 5) of cholesterol in BPH fed on *nomt* mutants and WT plants. Asterisks indicate significant differences between *nomt* mutants and WT plants (**$P < 0.01$; Student's *t* test). Horizontal lines are medians, boxes show the upper and lower quartiles, and whiskers show the full data range.

is well-known as a phytoalexin with antifungal activity against some fungal plant pathogens (15). Although it could not be tested against YLS, since this symbiont cannot be cultured in vitro (22), sakuranetin is clearly effective against YLS in the fresh BPH egg homogenate and intact eggs (Fig. 4*M* and *SI Appendix*, Fig. S6). Moreover, sakuranetin showed direct antifungal activity against *C. carpophila* and a closely related endosymbiont species of YLS, *H. chrysospermus* (*SI Appendix*, Fig. S7) (33). These results suggest that sakuranetin could directly target the YLS of BPH, and in turn affect BPH performance. Although the abundance of two bacterial genera is higher in *nomt*-fed BPH than WT-fed BPH, the proportion of them is less than 0.1%. The biological functions of these bacteria in BPH remain unknown. *Arsenophonus* is the dominant community member in our tested BPH, the proportion of which is higher than 99%.

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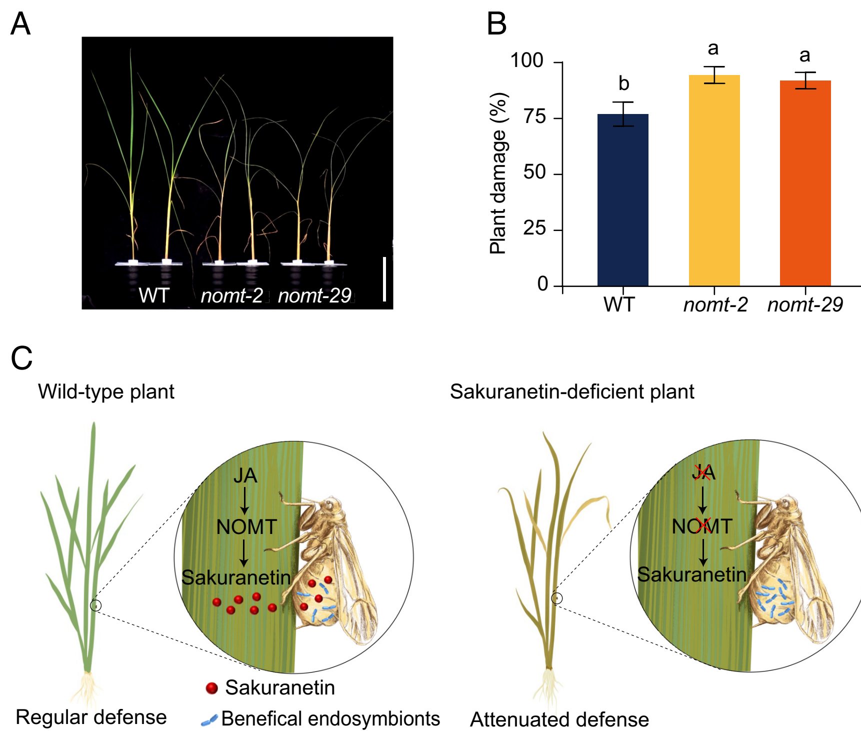

Fig. 5. The performance of *nomt* mutants and WT plants exposed to BPH: a model of sakuranetin function as a direct defense. The performance (*A*) and mean damage percentage (± SE, n = 12) (*B*) of *nomt* mutants and WT plants 18 d after BPH infestation. (Scale bar, 12 cm.) Letters indicate significant differences among mutants and WT plants ($P < 0.05$, one-way ANOVA followed by LSD test). (*C*) A model of the direct defensive function of sakuranetin in rice resistance to BPH. Biosynthesis of sakuranetin is regulated by JA signaling, which is activated by BPH attack. Sakuranetin decreases BPH performance by inhibiting growth and performance of beneficial BPH endosymbionts. The red cross indicates that the expression of the genes controlling this trait are blocked.

However, there was only a 0.14% proportion difference between BPH fed on *nomt* and WT plants. This modest change may not affect the function of this bacteria in BPH.

The transcript levels of *NOMT* and concentrations of sakuranetin were extremely low in unattacked plants, but increased dramatically in response to BPH infestation, indicating that sakuranetin is an inducible defensive compound in rice. In contrast, the biosynthesis of other naringenin-derivatives was reduced in BPH-attacked plants, suggesting that flavonoid metabolic flux is altered when plant defense is required. Sakuranetin is also induced in rice upon infection by fungi, like the rice blast pathogen, where it is activated by the JA signaling cascade (37). Our results indicated that sakuranetin formation induced by the herbivore BPH is also dependent on JA signaling (*SI Appendix*, Fig. S8). Infection of the rice blast fungus is somewhat similar to herbivory since the turgor pressure produced in the appressorium allows the penetration peg to rupture the rice cuticle (38). Thus, it is not surprising that the plant response to both enemies is orchestrated by JA.

JA-elicited naringenin and sakuranetin concentrations are known to vary considerably among rice cultivars, being high in the Nipponbare japonica varieties used here and low in the Kasalath cultivars (15). The variation has arisen several times independently since the domestication of rice and is thought to reflect the varying selection pressures of fungal and bacterial pathogens, as naringenin and sakuranetin are thought to be effective phytoalexins against bacterial and fungal pathogens, respectively (15). The discovery that sakuranetin is an effective defense against the beneficial fungal endophytes of BPH, suggests that the variations in these flavonoid phytoalexins need to be reconsidered and may have been influenced by herbivore pressure, such as that of the BPH, as well as by pathogens.

In summary, BPH infestation results in the activation of JA signaling, which in turn mediates the biosynthesis of sakuranetin. Sakuranetin in the phloem sap is then taken up by BPH during feeding, reducing the abundance of the beneficial endosymbionts (e.g., YLS) in BPH, and so decreases the performance of this insect (Fig. 5*C*). BPH requires YLS for growth on rice and hence this insect-fungal association is an example of a "holobiont"; in this context, sakuranetin is a "direct" defense, though it may be considered to affect the herbivore BPH only indirectly. Other plant species may well defend against herbivores by also targeting their symbionts, and the large variety of defense compounds having antibacterial activity should now be considered in this light as well as in having a protective role against pathogens. In the same way, various natural and synthetic antimicrobial substances may have value as pest control agents by targeting endogenous symbionts of insects.

Materials and Methods

Plant Materials and Growth Condition. The japonica rice varieties, Nipponbare (Nip) and XiuShui 11 (XS11) both high sakuranetin producing cultivars (Fig. 1*D* and *SI Appendix*, Fig. S8*E*) (15) were used as wild-type (WT) plants and the genetic backgrounds for the transformations. The rice mutants, *aoc-2*, *myc2-5*, *coi1-2*, and *coi2-1* were produced in the XS11 background and screened as previously described (27, 35). Seeds were germinated in the petri dish with water in an illuminated incubator (28 ± 2 °C). Nine-day-old seedlings were transferred in hydroponic cultivation as described previously (27). Plants were used for experiments after 20 to 25 d.

Insect Rearing and Fungal Cultivation. A colony of BPH was originally provided by the Chinese National Rice Research Institute (Hangzhou, China) and reared on TN1 rice seedlings in a controlled climate room at 26 ± 2 °C and 80% relative humidity for more than 30 generations. *Candida carpophila* was obtained from China General Microbiological Culture Collection Center (accession number: 2.1868), and cultured on Yeast Malt medium (Coolaber). *Hypomyces chrysospermus* was kindly provided by Liping Sun from Kunming University of Science and Technology, and cultured on potato dextrose medium (Solarbio).

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Generation and Characterization of Genome-Edited Plants. The target sequences of each gene were introduced into pLYsgRNA-OsU6b or pLYsgRNA-OsU3 to yield rice U6b or U3 promoter-driven single-guide RNA (sgRNA). The sgRNA expression cassette was then introduced into plant CRISPR-Cas9 binary vector pYLCRISPR/Cas9Pubi-H (39). For *NOMT* gene, two targets were used, and the T-DNA was inserted into the Nip genetic background using *Agrobacterium tumefaciens*-mediated transformation. For *MED25* and *JAR1* genes, one target was used and the T-DNA was inserted into the XS11 genetic background. The positive transgenic plants were screened by targeting DNA sequencing and hygromycin gene identification.

Plant Treatments and Sampling. For BPH treatments, plants were infested with 10 to 15 gravid female adults that were confined in a glass cage (diameter 4 cm, height 8 cm, with 48 small holes, diameter 0.8 mm). Plants with an empty cage were used as controls. The outermost two leaf sheaths were collected at the indicated time points. For MeJA treatments, 25-d-old plants were transferred in hydroponic cultivation containing 100 μM MeJA. Plants in hydroponic cultivation with solvent were used as controls. Plants were collected at the indicated time points.

For phloem sap collection, the EDTA-facilitated exudation method was used. Each plant was infested with 10 gravid female adults. Nontreated plants were used as controls. The shoots of BPH-treated and control plants were cut at the base of the stem with a sharp razor blade, and immediately submerged in a large volume exudation buffer (10 mM HEPES, 20 mM EDTA, adjusted to pH = 7.5 with NaOH). The shoots were then transferred to 50-mL tubes containing 3 mL fresh exudation buffer and placed in a dark, humid chamber (relative humidity > 90%) for 1 h to prevent self-sealing. After washing with distilled water, the shoots were transferred to a new 50-mL tube containing 5 mL double-distilled water for collecting the phloem exudates. After collection for 8 h, exudates were dried by vacuum freeze dryer and dissolved in 200 μL 70% methanol. The precise mass of the shoot was recorded.

Previous study found that the rate of phloem sap exudation in rice plant is around 1.5 to 2.2 $\mu L\ h^{-1}$ (28). We collected for 8 h in this study, suggesting a yield of 12 to 17.6 μL phloem exudates. The concentration of sakuranetin shown in *SI Appendix*, Fig. S2A (1.5 $\mu g\ g^{-1}$ FM) was normalized by the fresh mass (FM) of the shoot we used. Since the average shoot mass is 1.9 g, the total sakuranetin yield for the 8 h collection is 2.85 μg. Considering the volume of phloem exudates, the concentrations of sakuranetin in our collection are 0.16 to 0.24 $\mu g\ mL^{-1}$. As described above, we confined BPH in the leaf sheath of a plant, which approximately accounts for 25 % of the shoot. Finally, we estimated the physiological concentrations of BPH-elicited sakuranetin levels in the phloem sap to be 0.5 to 1 $\mu g\ mL^{-1}$.

RNA Extraction and Quantitative RT-PCR Analysis. Total RNA was isolated using the FastPure Universal Plant Total RNA Isolation Kit (Vazyme) according to the manufacturer's instructions. One microgram of total RNA for each sample was reverse transcribed using the HiScript II Q RT SuperMix (Vazyme). qRT-PCR was performed on the CFX96 Touch (BioRad) using ChamQ SYBR qPCR Master mix (Vazyme). Five independent biological samples were collected and analyzed. Primers used for qRT-PCR are listed in *SI Appendix*, Table S1. The rice *Ubi* mRNA was used as an internal control.

Flavonoid and Cholesterol Measurements. Approximately 80 mg material (precise mass was recorded) was ground and extracted twice with 800 μL 70% methanol. The supernatant was combined and evaporated by a vacuum freeze dryer. The dried sample was dissolved with 120 μL 70% methanol and analyzed by a liquid chromatography-tandem mass spectrometry system configured with an electrospray ionization source (Agilent 6460). The amounts of each compound were calculated using a standard curve method (40).

The concentration of cholesterol in BPH was quantified as described previously (41). In brief, ten BPH female adults were weighed (~10 mg) and transferred into a 1.5-mL tube containing two zirconia beads (2 mm diameter, 95%). After homogenization, 0.5 mL methanol, 0.5 mL chloroform and 10 μg cholestane (internal standard) were added to each tube and vortexed vigorously. Next, 0.45 mL H_2O was added to each tube and vortexed again. After phase separation, the lower chloroform layer was transferred into a 20-mL glass vial. To hydrolyze the sterol esters, 8 mL 70% methanol containing 5% NaOH was added and incubated at 60 °C for 2 h. Then, 3 mL H_2O was added to the solution and thrice extracted with hexane. The hexane extracts were combined in a new vial, evaporated, redissolved 1 mL dry hexane and derivatized with 100 μL 1-(trimethylsilyl) imidazole. The reaction was terminated by adding 300 μL 70% methanol and 100 μL hexane. After separation, the hexane fraction was thrice washed with 70% methanol and analyzed by GC-MS (Thermo Fisher Scientific) with a DB-5MS column (Agilent). The cholesterol was identified and quantified by derivatized standards.

Internal Transcribed Spacer (ITS) and16S rRNA Sequencing. BPH samples were sterilized in 75% ethanol solution for 2 min and washed with sterile distilled water and phosphate-buffered saline (PBS, PH = 7.4), respectively, for three times. Total DNA was extracted using the MagAttract® PowerSoil® Pro DNA Kit (Qiagen) according to the manufacturer's instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo). The V3-V4 region of the bacterial 16S rRNA gene was amplified with barcoded primer pair 338F and 806R, while the fungal ITS region ITS1F-2R was amplified with the barcoded primer pair ITS1F and ITS2R. The primers used for PCR are listed in *SI Appendix*, Table S1. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen) according to the manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega).

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). After demultiplexing, the sequencing was quality filtered with fastp (https://github.com/OpenGene/fastp) and merged with FLASH (https://ccb.jhu.edu/software/FLASH/index.shtml) (42, 43). Then, Raw sequencing data were analyzed by the Quantitative Insights into Microbial Ecology toolkit 1.9.1 (http://qiime.org/install/index.html). The resulted sequences were clustered into operational taxonomic units (OTUs) at 97% similarity cutoff, and chimeric sequences were identified and removed (44, 45). The taxonomic information of representative sequences from the OTUs was determined by the RDP Classifier 2.1.3 (https://sourceforge.net/projects/rdp-classifier/) against the SILVA 16S rRNA database (V138, http://www.arb-silva.de) for bacteria, and the UNITE ITS database (8.0, http://unite.ut.ee/index.php) and *Ascomycetes* YLS ITS sequence (NCBI accession number: JF732894.1) for fungi, both at a confidence level of 70%. The data were analyzed on the online platform of Majorbio Cloud Platform (www.majorbio.com) (46).

BPH Performance Bioassays. For sakuranetin feeding assays, twenty-five newly hatched BPH nymphs were fed on artificial diets containing different concentrations (0 to 0.5 $\mu g\ mL^{-1}$) of sakuranetin in a double-ended open glass cylinder (diameter 4 cm, height 8 cm, with 48 small holes, diameter 0.8 mm) as described (40). The artificial diet was renewed every day. The number of surviving nymphs in each cylinder was recorded every day. Nine replicates were used for each treatment.

BPH performances on different lines were evaluated by the fecundity of adult females, the hatching rate of eggs and the survival rate of nymphs. BPH nymphs or adults were confined in a glass cage (diameter 4 cm, height 8 cm, with 48 small holes, diameter 0.8 mm). For BPH fecundity assays, a pair of newly emerged BPH female and male adults were allowed to oviposit on the leaf sheath of each plant for 10 d. The number of eggs laid by each female adult was counted under a microscope at 10× magnification. Eighteen biological replicates were used.

For the BPH hatching assays, ten gravid BPH females were allowed to oviposit on each plant for 24 h. After removing all BPHs, the newly hatched nymphs were counted every day, until no new nymphs were found. The unhatched eggs from the oviposited leaf sheath were counted under a microscope. Twelve biological replicates were used.

For BPH survival assays, fifteen newly hatched BPH nymphs were allowed to feed on each plant. The number of surviving BPHs was recorded every day. Fourteen biological replicates were used.

For plant survival assays, twenty-five gravid BPH females were allowed to infest each plant, twelve plants were used for each genotype. The degree of plant damage (ratio of dead leaves number/ total leaves number, %) was recorded and photographed after 10 d.

Determination of Antimicrobial Activity. For the antimicrobial activity of sakuranetin against YLS, the fresh BPH eggs were collected (within 3 h after oviposition) and used for two assays. Approximately 2,000 eggs were homogenized with 900 μL phosphate-buffered saline (PBS, PH = 7.4) and 30 μL homogenate was transferred to each 1.5-mL centrifuge tube. Next, 0.5 $\mu g\ mL^{-1}$ sakuranetin

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or control solution was added into each tube and incubated at 26 °C in the dark for 36 h. After ten times dilution, ten microliters homogenate was pipetted into a clean blood cell counting plate, and the number of YLS was counted under a microscope at 300× magnification. The other intact eggs were directly placed on a filter paper socked with 0.5 μg mL^{-1} sakuranetin or control solution in a petri dish and incubated at 26 to 28 °C under 14-h light for 4 d. Ten normally developing eggs (with embryonic red eye spots) were then homogenized with 50 μL PBS, and the number of YLS was counted.

For the antimicrobial activity of sakuranetin against *Candida carpophila*, the stock cultures of *C. carpophila* were inoculated into Yeast Malt medium (YM), and incubated at 28 °C for 24 h with shaking at 200 rpm. One microliter of overnight cultures was inoculated into 1 mL fresh YM medium containing 0, 0.05, 0.5, 5, 10, or 20 μg mL^{-1} sakuranetin in a 1.5-mL centrifuge tube. The tube was incubated at 28 °C in the dark, and the OD_{600} was determined using a BioPhotometer (Eppendorf D30).

For the antimicrobial activity of sakuranetin against *Hypomyces chrysospermus*, the stock strains of *H. chrysospermus* were cultured on potato dextrose agar (PDA) at 28 °C in the dark. Seven days after incubation, one plug (diameter 7 mm) was prepared from the extended front area of mycelium, and inoculated onto PDA containing various concentrations of sakuranetin (0, 0.05, 0.5, 5, 10, or 20 μg mL^{-1}) in a petri dish. The diameter of the colony was measured at 0, 36, and 72 h after incubation in the dark.

Quantification of Fungal Endosymbionts in BPH. BPH female adults were sterilized in 75% ethanol solution for 2 min and washed with sterile distilled water and phosphate-buffered saline (PBS, PH = 7.4) respectively, for three times. Three BPHs were placed into a 1.5-mL centrifuge tube containing 0.5 mL PBS. The samples were homogenized on a tissue grinder using 0.3-mm diameter steel beads. Ten microliters homogenate was pipetted into a clean blood cell counting plate, and the number of YLS was counted under a microscope at 300× magnification.

For quantification of the abundance of endosymbionts in BPH, total DNA of BPH samples was isolated using a DNeasy® Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. The relative DNA abundance of each endosymbiont was quantified by qRT-PCR on the CFX96 Touch (BioRad) using ChamQ SYBR qPCR Master mix (Vazyme). Primers targeting ITS region of YLS and IGS region of *Candida carpophila* were used for qRT-PCR. The beta-actin of BPH was used as an internal control. All primers are listed in Supplemental *SI Appendix*, Table S1. Six to eight biological replicates were used.

Data, Materials, and Software Availability. Sequence data from this work can be found in The Rice Annotation Project under the following accession numbers: NOMT (Os12g0240900), F2H (Os06g0102100), UGT707A3 (Os07g0503500), FNS II (Os04g0101400), CYP75B3 (Os10g0320100), MYC2 (Os10g0575000), AOC (Os03g0438100), MED25 (Os09g0306700), JAR1 (Os05g0586200), COI1a (Os01g0853400), COI1b (Os05g0449500); COI2 (Os03g0265500). The raw 16S rRNA and ITS sequencing data reported in this paper have been deposited in the Genome Sequence Archive at the BIG Data Center (http://bigd.big.ac.cn/gsa), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession no. CRA010011 (47) and CRA010012 (48).

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