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Nymphal feeding suppresses oviposition-induced indirect plant defense in rice

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Feeding and oviposition by phytophagous insects are both known to trigger defenses in plants. Whether these two defenses functionally interact remains poorly studied, although these interactions are likely important for pests with overlapping generations. Here we investigated the differences and interaction between feeding- and oviposition-induced plant defenses triggered by the brown planthopper (BPH, *Nilaparvata lugens*), which gregariously feeds and oviposits on rice. Analyses of host-plant transcriptomes, phytohormones, and direct and indirect defense compounds all show that BPH gravid females (GFs), but not nymphs and non-gravid females (NFs), strongly induce rice defenses. BPH nymphs and GFs prefer to feed on plants previously infested by nymphs over un-attacked plants, but are repelled by plants previously infested by GFs. Moreover, nymph feeding is found to reduce the attractiveness of rice plants to natural enemies and decrease egg parasitism by suppressing GF-induced volatiles that mediate indirect defenses in both growth chambers and paddies. Intergenerational interactions between oviposition- and feeding-induced plant defenses not only promote the development of the population of pest insects but may also contribute to the aggregation behavior of pest insects by suppressing oviposition-induced indirect plant defenses.

Plants provide essential nutrition, energy, and shelter for phytophagous insects. The selective pressures imposed by herbivores likely contributed to the evolution of plants' sophisticated defense systems. Upon perceiving herbivore-derived cues, such as elicitors in oral secretions and oviposition fluid, plants reprogram their transcriptomes, proteomes, and metabolomes to cope with the respective herbivore[1,2]. These changes are mediated by elaborate signaling networks, including specific receptor proteins, Ca^{2+} flux, kinase cascades, and hormone signaling systems, such as the jasmonates (jasmonic acid, JA, and derivatives, in particular, jasmonoyl Ile, JA-Ile); salicylic acid (SA); ethylene; and hydrogen peroxide (H_2O_2) signaling systems[3–6]. Plant defense can be categorized as direct or indirect based on whether the trait directly or indirectly decreases herbivores' impact on plant fitness[7]. Accordingly, direct defenses include the production of toxic, anti-digestive compounds, tough leaves, or trichomes; indirect defenses, in contrast, are plant traits that attract and improve foraging by predators or parasitoids of herbivores. Such traits are typically mediated by plant volatile organic compounds (VOCs)[8–10]. The sesquiterpene (*E*)-*β*-farnesene, which is produced by a taxonomically wide group of plants when its members are attacked by herbivores, is known to act as a synomone in attracting parasitoids[11,12].

Some herbivores have adopted ways to interfere with plant defenses threatening their fitness[13]. Herbivore suppression of plant

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defense is characterized as the decreased production of defense compounds accompanied by the greater fitness of herbivores. The phloem-feeding whitefly *Bemisia tabaci* can reduce the emission of volatile (*E*)-*β*-ocimene from one of its host plants, the lima bean *Phaseolus lunatus*, and thus interfere with the plant's indirect defense, likely by exploiting SA-signaling to dampen JA-signaling responses[14]. Parallel with the category of plant-pathogen interactions[15], components from herbivores that mediate the suppression of plant defense are also named effectors, which usually are proteins secreted from insect salivary glands and transferred into plants[16].

Feeding and oviposition are two essential components of insect-plant interactions, and plant responses to oviposition may determine future feeding damage. However, although feeding- and oviposition-induced plant responses have been widely studied[2,17–19], our knowledge about the interactions between plant responses to phytophagous insect feeding and oviposition has substantial gaps, as many phytophagous insects oviposit and feed on the same individual plants as part of their life cycle.

Brown planthopper (BPH), *Nilaparvata lugens*, is the most destructive insect pest on rice (an important staple crop worldwide), usually causing up to 40% rice yield loss if not controlled[20]. During feeding, BPH, a monophagous piercing-sucking herbivore, uses a stylet to penetrate intercellular spaces and reach the phloem sap. In addition to feeding, gravid females (GFs) can also cause serious damage to the rice plant when they lay eggs into rice tissues using their strong ovipositors. It has been reported that BPH GF infestation can induce complex responses in rice, including activating signaling pathways mediated by phytohormones, such as JA, reshaping transcriptome networks; and enhancing the production of volatile and non-volatile defense compounds[21,22], whereas BPH nymph infestation only weakly enhances levels of JA, JA-Ile, and H_2O_2[23,24]. Moreover, different elicitors and effectors have been reported in BPH saliva and oviposition fluids/eggs. In BPH saliva, for instance, several effectors (such as NlSEF1[23], NlEG1[24], calmodulin[25], and the C-terminal subunit of vitellogenins[26]) and several elicitors (such as NlG14[27] and a mucin-like protein (NlMLP)[28]) have been identified, whereas in BPH eggs only one elicitor, the N-terminal subunit of vitellogenins[29], has been reported. These results demonstrate that there may be a difference in the rice defense responses induced by BPH feeding and oviposition and that these two types of defenses may functionally interact. BPHs live gregariously on the lowest parts of rice plants among completely overlapping generations, meaning all developmental stages can co-occur on the same plant[30]. Moreover, single rice crops in China regularly experience from one to four overlapping BPH generations, depending on their locations, with more generations in the warmer south and fewer in the colder north[30]. However, because most studies have been conducted using insects at only a single developmental stage, the effects of these overlapping generations on host plants and on BPH itself, are largely unknown. This ambiguity is also found in other phytophagous insects with overlapping generations on hosts, such as aphids[31]. Here, we compared the responses of rice plants to infestations of BPH at different developmental stages: nymphs, GFs, or non-gravid female adults (NFs). Although all insects from three life stages feed on rice plants, only GFs will lay eggs into rice tissues, resulting in both feeding and oviposition damage. We systematically compared several aspects of a rice plant's responses to these different BPH elicitations, including changes in the plants' transcriptomes, phytohormones, and defense compounds, such as phenolamides, proteinase inhibitors, and volatiles. We also assessed subsequent BPH performance on previously infested plants. By comparing the responses of parasitic wasps to plants infested by GFs or by GFs together with nymphs (GFNs) in the laboratory and field, we found that aggregating cross-generations may benefit BPH by disarming plants' indirect defenses.

Results

BPH feeding and oviposition differently shaped transcriptomes of infested rice plants

To understand whether plants respond differently to insect feeding and oviposition, we first compared host-plant responses to the infestation of nymphs, GFs, and NFs. Notably, the anatomy of feeding is the same for NFs and GFs, as there is no ecdysis separating the two stages. To estimate the relative feeding effect of the three different groups, we first quantified the food intake of 3rd-instar nymphs, NFs, and GFs, using honeydew production[32]. The results showed that the amount of honeydew secreted by one nymph per day was about two-thirds of that of one NF, and one-third of that of one GF (Supplementary Fig. 1). Thus, we used a ratio of 3:2:1 to create rice plant treatments in which the feeding damage from the different BPH developmental stages was equivalent, namely, 30 3rd-instar nymphs, 20 NFs, and 10 GFs.

To comprehensively evaluate rice plants' transcriptional responses to infestation by different types of BPHs, we performed a genome-wide transcriptome analysis using RNA-sequencing (RNA-seq), with untreated rice leaf sheaths as controls and 24 h infestations by BPHs as treatment (Fig. 1b). We identified 1219, 2645, and 4640 differentially expressed genes (DEGs; $P_{adj} < 0.05$ and fold changes > 2) in rice leaf sheaths exposed to nymphs, NFs, and GFs, in comparison with untreated controls, respectively (Fig. 1b and Supplementary Data 1). Principal co-ordinates analysis (PCoA) clearly showed that although infestation by nymphs and NF similarly affected the rice transcriptome, oviposition (infestation by GFs) elicited distinct responses compared with those elicited by only feeding (infestations by nymphs or NFs) (Fig. 1c). Among the 2533 DEGs that were uniquely differentially expressed in GF-infested rice plants (compared to untreated plants), 1732 genes were up-regulated and 801 genes were down-regulated. The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the 2533 DEGs showed that the up-regulated genes were mainly enriched in phytohormone and secondary metabolite biosynthesis, such as alpha-linolenic acid metabolism, terpenoid backbone biosynthesis, diterpenoid biosynthesis and pyruvate metabolism, and phenylpropanoid biosynthesis, and that the down-regulated genes were enriched in photosynthesis, carbon fixation, starch, and sucrose metabolism, and so on (Fig. 1d). These transcriptome data revealed that GF infestations, which included oviposition-induced damage, elicited a stronger transcriptional response in plants than feeding damage from nymphs and NFs did.

Infestation by gravid females, but not non-gravid females or nymphs, strongly induced plant defense responses

Some studies showed that BPH infestation of rice plants barely induced the jasmonate signaling pathway[21,33], but others showed that infested plants accumulated significantly more JA and JA-Ile[34]. We note that these studies used BPHs at different or ambiguous stages to infest rice, such as female adults without describing whether or not they were gravid. To evaluate if the dramatically distinct transcriptomic responses to BPH feeding and oviposition reflect differences in the types of defense being induced and whether the distinct jasmonate response is caused by oviposition, we quantified changes in phytohormones -- jasmonates (JA and JA-Ile), SA, ethylene, and H_2O_2 -- in response to infestation by different types of BPHs at multiple time points (Fig. 2a). Infestation by GFs significantly induced JA and JA-Ile levels in leaf sheaths within 3 h, reaching concentrations 24- and 157-fold higher than those of controls, respectively, after 72 h. In contrast, infestation by nymphs or by NFs did not significantly elevate jasmonate levels (Fig. 2b and c). These results matched the transcriptome data, namely, that GF infestation uniquely induced alpha-linolenic acid metabolism, which is the first step of jasmonate biosynthesis[35]. Infestation by all three stages of BPHs elevated SA concentrations, although GFs induced significantly higher concentrations at later stages of the infestation (Fig. 2d). Treatments involving nymphal infestation did not

significantly alter patterns of ethylene accumulation in the sampling chambers, which steadily increased in controls; in contrast, the rate of ethylene accumulation in treatments involving plants infested with GFs was significantly lower than that of controls (Fig. 2e). GFs induced significantly higher concentrations of H_2O_2 from 3 to 12 h after infestation; however, unlike the accumulation of the more stable jasmonate levels, the differences in this reactive metabolite vanished at 24 h. Neither nymphs nor NFs induced significant increases in H_2O_2 levels (Fig. 2f). In addition, in separate elicitation experiments, we varied the number of infesting nymphs (0-20 per plant) and GFs (0-10 per plant) and found that the magnitude of the SA and JA inductions by nymphs and/or GFs were linearly correlated with the number of infesting BPHs, showing a clear dosage-effect in BPH-induced rice defense responses (Supplementary Fig. 2).

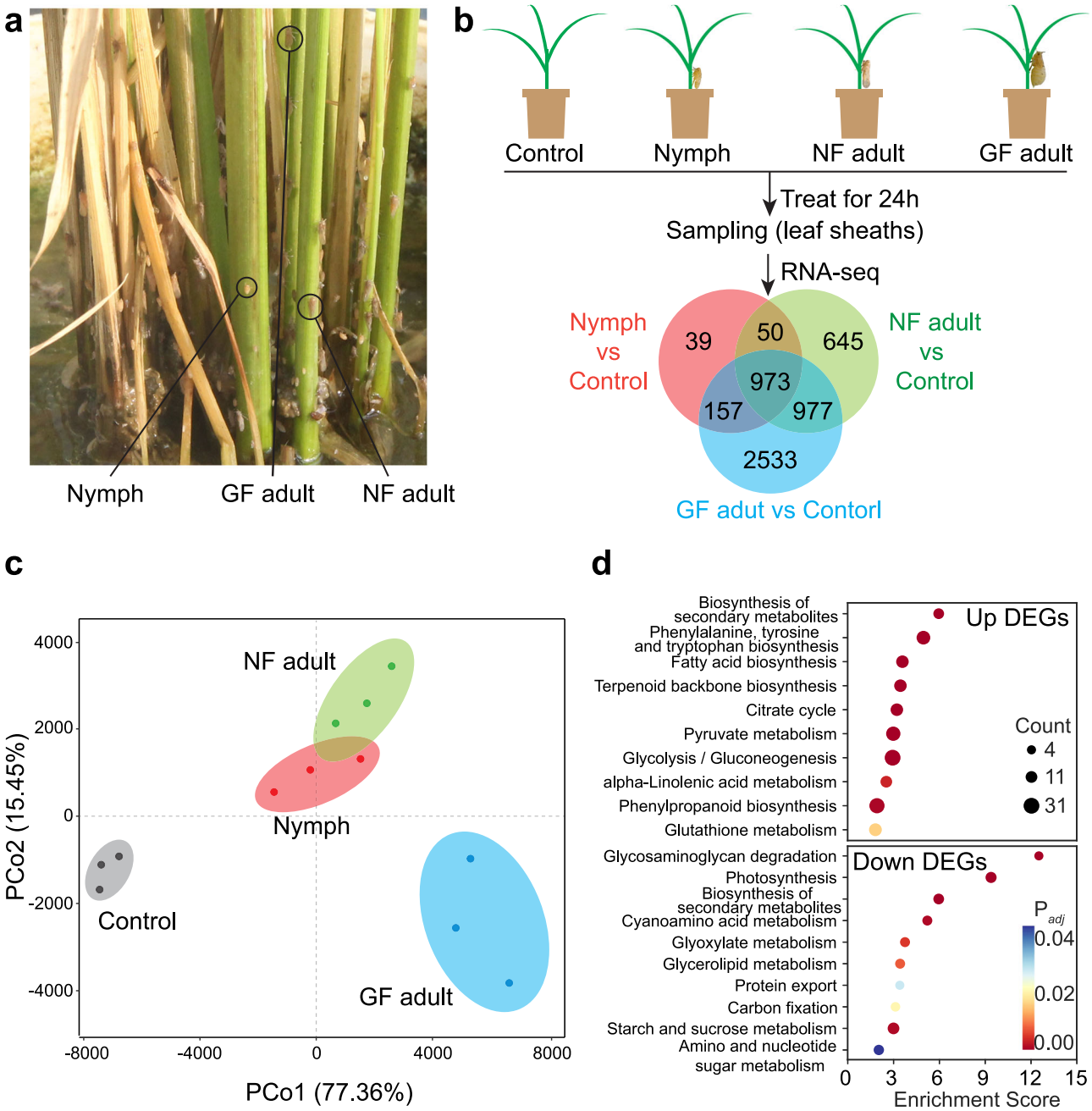

Fig. 1 | Infestation of BPH at various stages differentiate host-plant transcriptomes. a BPHs of different developmental stages commonly co-occur in rice fields. A representative image of an infested rice crop, showing feeding nymphs, non-gravid females (NFs), and gravid females (GFs). **b** Sample preparation and data analysis used to compare rice transcriptomes induced by different stages of BPHs. The Venn diagram quantifies the overlap of DEGs in the three types of BPHs-treated rice samples, with absolute |fold change | > 2 and P_{adj} value < 0.05 as cut-off values. The raw data (counts) of each gene was statistically analyzed by the negative binomial distribution of DEseq, followed by the *P*-value adjustment using the Benjamini-Hochberg method. **c** Principal coordinates analysis (PCoA) of the gene expression profiles reveals the similarity of rice-plant responses to nymph and NF feeding, while PCo1, which explains 77.4% variance, describes the dramatic effect of oviposition from GFs. **d** KEGG enrichment analysis of the 2553 DEGs (both up-regulated and down-regulated) in GF-infested rice plants but not in those infested by nymphs or NFs. The top 10 enriched KEGG pathways for up-regulated and down-regulated genes are shown in the upper and lower panels, respectively. The circle size indicates the proportion of DEGs for the indicated KEGG term, while the circle color indicates the significance of the enrichment term. Source data are provided as a Source Data file.

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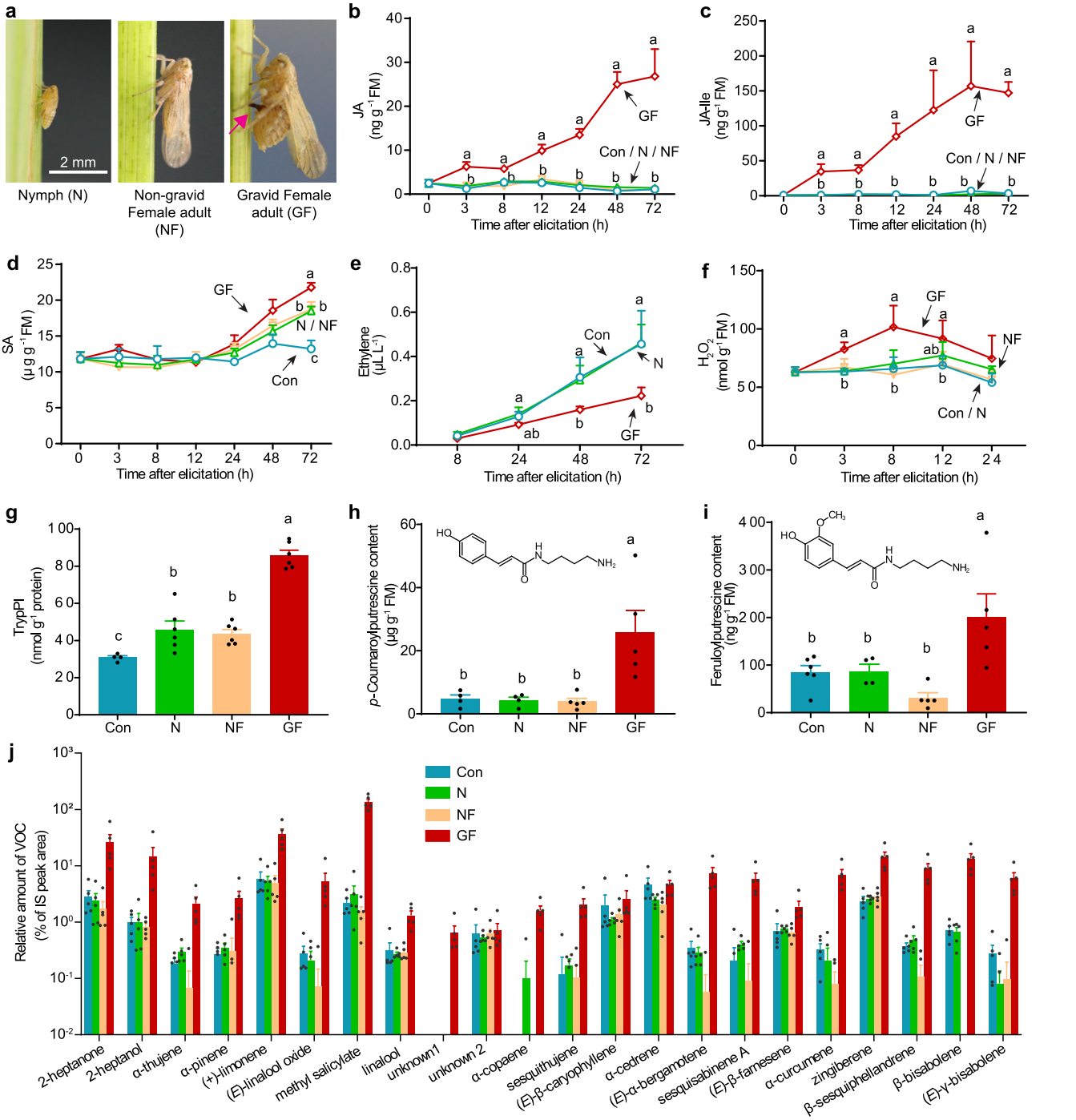

Fig. 2 | Infestation by gravid BPHs, but not by non-gravid adults or nymphs, strongly induced rice-plant defense. a Representative images of different stages of BPHs, in which a GF's ovipositor puncturing a leaf sheath is indicated by a magenta arrow. BPH nymphs (N), non-gravid female adults (NFs), and gravid female adults (GFs) were allowed to infest separate rice plants; uninfested plants grown under the same conditions served as controls (Con). **b**–**f** The contents (mean + SE; $n = 4$ - 8) of JA (**b**), JA-Ile (**c**), SA (**d**), ethylene (**e**), and H_2O_2 (**f**) in leaf sheaths of plants at different times after they were individually infested by 10 GFs, 20 NFs, or 30 nymphs (N), or kept unmanipulated (Con). **g**–**i** The contents (mean + SE; $n = 5$ - 6) of TrypPI (**g**), *p*-coumaroylputrescine (**h**), and feruloylputrescine (**i**) in leaf sheaths of plants 3 d after they were individually infested by 10 GFs, 20 NFs, or 30 nymphs (N), or kept unmanipulated (Con). **j** Relative amounts (mean + SE; $n = 5$) of volatile organic compounds (VOCs) were quantified after 8 h of sampling the headspace of plants 24 h after they were individually infested by 10 GFs, 20 NFs, or 30 nymphs (N), or kept unmanipulated (Con). Different letters indicate significant differences among different treatments ($P < 0.05$, one-way ANOVA followed by Tukey's HSD post-hoc tests). Results of the statistical analysis of relative VOC abundance are shown in Supplemental Table 1. All of the statistical values are shown in the source data. Source data are provided as a Source Data file.

Phytohormone signaling induces the accumulation of many defense compounds in infested rice plants, like trypsin protease inhibitors (TrypPIs), phenolamides, and VOCs, all of which have been reported to be defense compounds against BPH and to be regulated by JA and ethylene signaling pathways[21,34,36,37]. We, therefore, quantified TrypPIs, phenolamides (*p*-coumaroylputrescine and feruloylputrescience), and VOCs in or from plants after infestation by different stages of BPHs. Consistent with the transcriptome data that GF

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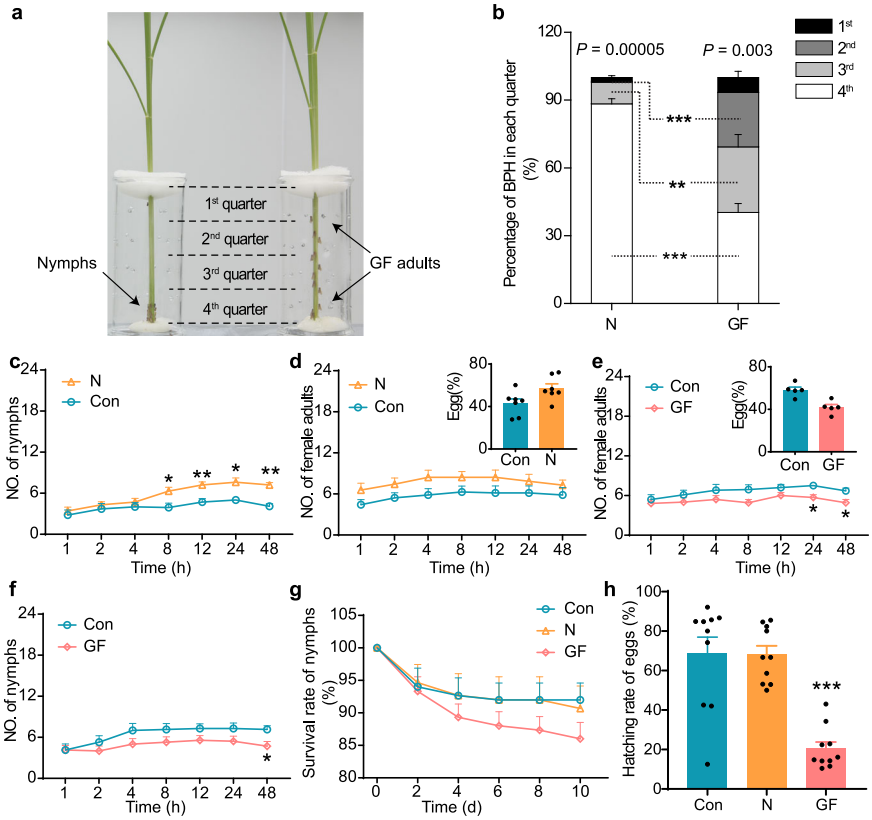

Fig. 3 | Pre-infestation by BPH nymphs and gravid adult females affected subsequent BPH performance differently. a, **b** A representative image (**a**) and percentages (mean + SE; $n = 8$) (**b**) of BPH nymphs (N) and gravid females (GF) on each quarter-section of the rice stem at 48 h after being released. The *P*-value on each column indicates the probability that BPH nymphs (N) or GF evenly distribute on rice shoots using the Friedman rank sum test. **c**–**f** Numbers (mean + SE; $n = 7$ - 10) of nymphs (**c** and **f**) or GFs (**d** and **e**) on each plant at 1–48 h after 15 BPHs were exposed to paired plants [Con vs N (**c** and **d**) or Con vs GF (**e** and **f**)]. Inserts in (**d** and **e**): percentages (mean + SE; $n = 5$ or 7) of eggs laid by 15 GFs on each plant. N, plants that were pre-infested by 30 nymphs; GF, plants that were pre-infested by 10 GFs; Con, un-infested plants. **g** Survival rates (mean + SE; $n = 10$) of nymphs at indicated time points on plants that had been individually pre-treated with 30 nymphs (N) or 10 GFs, or kept un-infested (Con) for 24 h. **h** Hatching rates (mean + SE; $n = 10$) of BPH eggs on plants that had been individually pre-treated with 30 nymphs (N) or 10 GFs, or kept un-infested (Con) for 24 h. Asterisks indicate significant differences between two treatments (**b**) or between control and pre-infested plants (**c**–**h**) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Wilcoxon's signed-rank tests for **b** to **f**, with *P*-values of each group being corrected by the false discovery rate method; one-way ANOVA followed by Tukey's HSD post-hoc tests for **g** and **h**. All of the statistical values are shown in the source data. Source data are provided as a Source Data file.

infestation strongly up-regulated genes related to secondary metabolite biosynthesis (compared to NF and nymph infestation), in plants infested by GFs, TrypPI activity was 2.9-fold higher than that in control plants, and 1.96-fold higher than that in nymph- or NF-infested plants (Fig. 2g). The two phenolamides were significantly induced by GF infestation but not infestation by nymphs or NFs (Fig. 2h and i). Moreover, GF infestation dramatically increased the emission of VOCs (Fig. 2j and Supplementary Fig. 3): emission of 19 of the 22 most abundant VOCs was significantly induced by GF infestation (Supplementary Table 1), whereas infestation by nymphs or NFs did not significantly induce any of the 22 VOCs. These results indicate that infestation by GFs strongly induced plant defense responses, likely due to the oviposition on rice plants of 256 eggs on average over 72 h (laid by 10 GFs) (Supplementary Fig. 4).

Nymph and GF infestations affect subsequent BPH performance differently

Consistent with reports that in the field BPHs like to live in aggregates at the bottom of rice plants[30,32], both BPH nymphs and GFs were clearly gathering at the lowest parts of rice plants when they were released into glass cylinders enclosing stems. However, compared to nymphs, GFs distributed themselves more uniformly across stems (Fig. 3a and b). As GFs, but not nymphs, strongly induced host-plant defenses, we hypothesized that GFs disperse in order to evade plant defenses induced by the oviposition of other GFs, whereas nymphs are able to benefit from spatial aggregation within host plants, probably by suppressing plant defense via secreting effectors during feeding. If this hypothesis is correct, then we would also expect that, when given a choice, BPHs would avoid plants elicited by GFs but not those elicited by nymphs.

To examine this inference, BPH preferences for un-infested plants versus those that were infested beforehand either by nymphs or by GFs, were compared. After being released into a cylinder with a pair of plants -- one un-infested and one previously infested -- BPH nymphs were most frequently found on previously nymph-infested plants, and GFs also tended to feed and lay eggs on previously nymph-infested plants, although no significant difference was detected (Fig. 3c and d). However, GFs, which were less frequently observed, laid fewer eggs on previously GF-infested plants than on un-infested plants (Fig. 3e). Similarly, nymphs were marginally less often found on rice plants infested by GFs than on un-infested plants (Fig. 3f). Survival rate from egg hatching to emergence is a reliable predictor of host-plant direct defense[21]. Nymphal survival rates were not clearly affected by prior nymphal infestation but tended to be lowest on stems previously infested by GFs (Fig. 3g). Similarly, egg-hatching rates were significantly reduced by prior GF infestation but not by prior nymphal infestation (Fig. 3h). From these results, we conclude that GF infestation decreases host-plant suitability to BPHs that subsequently feed on the same plants, but nymph infestation does not.

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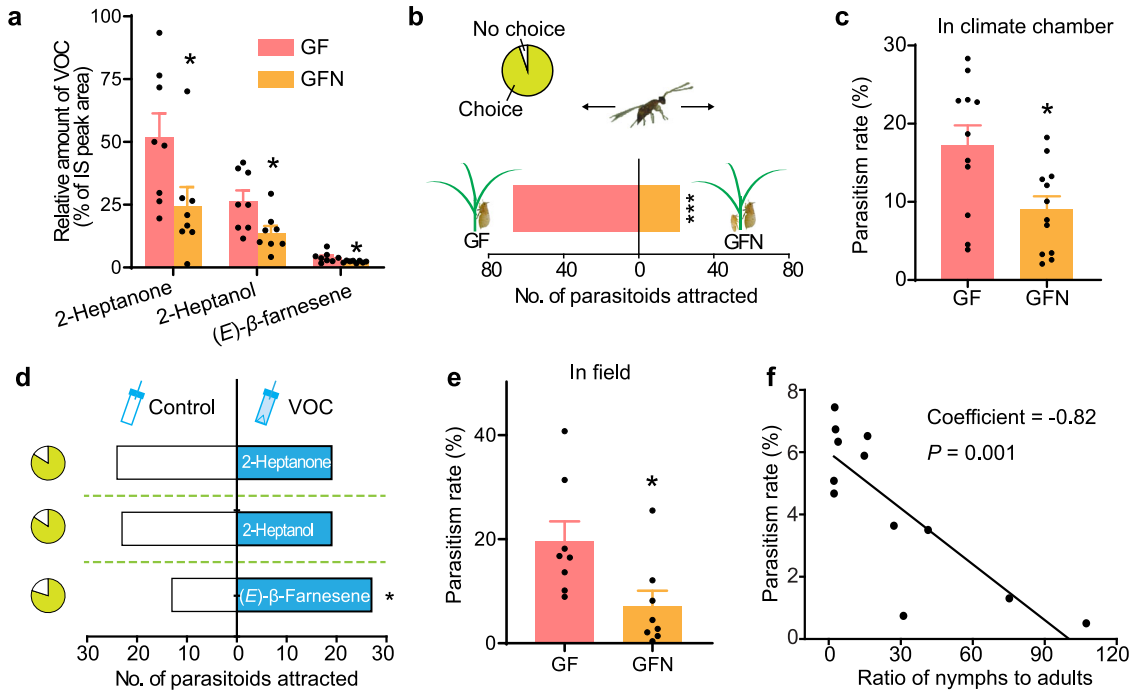

Fig. 4 | Feeding by BPH nymphs suppresses VOC-mediated indirect defenses induced by GF-infestations in the laboratory and field. a Relative amounts (mean + SE; $n = 8$) of three VOCs, 2-heptanone, 2-heptanol, and (*E*)-β-farnesene, in the headspace of rice plants previously infested by 10 GFs or 10 GFs together with 30 3rd-instar nymphs (GFNs) for 24 h. **b** Number (mean + SE; $n = 8$) of *Anagrus nilaparvatae* female adults attracted by VOCs emitted from plants that had been infested by 10 GFs or 10 GFs together with 30 3rd-instar nymphs (GFNs) for 24 h. The pie chart indicates the proportion of wasps that made a choice. **c** Parasitism rates (mean + SE; $n = 11$ - 12) of BPH eggs by *A. nilaparvatae* in climate chambers on plants that had been infested by 10 GFs or 10 GFs together with 30 3rd-instar nymphs (GFNs) for 24 h. **d** Number of female wasps attracted by pure VOC standards in solvent (2-heptanone, 5 mg L^{-1}; 2-heptanol, 2 mg L^{-1}; and (*E*)-β-farnesene, 2 mg L^{-1}) or solvent controls. **e** Parasitism rates (mean + SE; $n = 8$) of BPH eggs by *A. nilaparvatae* in the field on plants that had been infested by 10 GFs or 10 GFs together with 30 3rd-instar nymphs (GFNs) for 24 h. **f** Correlation between ratios of nymphs to adults and the percentages of egg parasitism by *A. nilaparvatae* in the field. Asterisks indicate significant differences between different treatments (*$P < 0.05$; ***$P < 0.001$; Student's *t* test for **a**, **c**, and **e**; chi-square goodness-of-fit test for **b** and **d**). Pearson correlation was used for **f**. All of the statistical values are shown in the source data. Source data are provided as a Source Data file.

Nymphal infestation suppresses GF-induced VOC emissions and attenuates indirect defenses of rice in the laboratory and field

Based on the phenomenon that BPHs at different developmental stages aggregate on plants in the field[30,32] (Fig. 1a) and on our result that nymphal infestations make plants more susceptible to subsequent BPH feedings (Fig. 3), we hypothesized that nymphal infestation suppresses GF-induced plant defenses, providing both an intergenerational benefit for the BPH community and an advantage to aggregating. To test this hypothesis, we first compared defense responses by quantifying defense phytohormones and compounds in plants infested either by GFs or by **GFs** together with **n**ymphs (GFNs). No significant difference was observed in levels of JA, JA-Ile, SA, ethylene, and H_2O_2 between GF-infested plants and GFN-infested plants (Supplementary Fig. 5). Three direct defense compounds -- TrypPIs, *p*-coumaroylputrescine, and feruloylputrescine -- were also quantified. Although the level of feruloylputrescine decreased in GFN-infested plants compared with that in GF-infested plants, neither TrypPIs nor *p*-coumaroylputrescine were significantly affected (Supplementary Fig. 5a–c). We also compared the VOCs emitted from plants infested by GF and GFN. Infestations that included nymphs had significantly reduced amounts of 2-heptanone, 2-heptanonol, and (*E*)-β-farnesene in their headspace, compared with plants infested only by GFs (Fig. 4a and Supplementary Table 2), although the inclusion of nymphal infestation did not significantly influence the number of eggs laid by GFs (Supplementary Fig. 4).

We then measured the effect of GF- or GFN-infestation on the performance of BPHs subsequently feeding on the same plants and the behavioral response of *Anagrus nilaparvatae*, a wasp that parasitizes BPH eggs and is known to use BPH-induced VOCs to locate BPH eggs[20]. Bioassays revealed that survival rates of nymphs and hatching rates of eggs did not differ between GF- and GFN-infested plants (Supplementary Fig. 6). However, VOCs emitted from GFN-infested rice were less attractive to *A. nilaparvatae* than those from GF-infested plants. Specifically, the number of parasitoids preferring volatiles emitted from GF-infested plants was 3-fold the number preferring volatiles from GFN-infested plants (Fig. 4b). Eggs on GFN-infested plants also suffered consistently less parasitism than those on GF-infested plants in climate chambers (Fig. 4c), although there was no difference in the number of BPH eggs laid on the two treated plants (Supplementary Fig. 7). The three VOCs that were suppressed by nymphal infestation were selected as candidates potentially mediating the reduction in parasitoid attraction observed. We first quantified the amount of the three compounds using external standard curves and found that the emission rates of 2-heptanone, 2-heptanol, and (*E*)-β-farnesene from GF-infested plants and GFN-infested plants were 1.86 ± 0.32, 0.57 ± 0.09, 0.31 ± 0.05, and 0.91 ± 0.26, 0.30 ± 0.06, 0.19 ± 0.01 ng min^{-1}, respectively. Choice assays at physiological concentrations revealed that (*E*)-β-farnesene significantly attracted *A. nilaparvatae* (Fig. 4d). To evaluate if the infestation-induced difference in VOCs released was sufficiently robust to influence parasitism rates under field conditions, we released rice plants infested with GF or GFN into a paddy field at the end of September when the rice plants are at the heading stage, and quantified parasitism rates on BPH eggs. No difference was observed between the number of BPH eggs laid on GF-infested plants and those laid on GFN-infested plants (Supplementary Fig. 7); however, compared with only GF infestation, additional nymphal infestation decreased parasitism by *A. nilaparvatae* by 59% (Fig. 4e). By mining data from field surveys[22] conducted in 2014, we

found a strong negative correlation (Pearson correlation co-efficient = − 0.82, $P = 0.001$) between the egg parasitism rate and the ratio of nymphs to adults; specifically, the egg parasitism rate decreased as the ratio of nymphs to adults increased in the BPH population (Fig. 4f). These results show that nymphal infestation suppressed GF-induced indirect defense, i.e., decreasing host-plant attractiveness to egg parasitoids.

To elucidate the molecular mechanism underlying the nymphal infestation suppression of GF-induced indirect defenses, we compared the transcriptomes of rice plants in response to the infestation of GF and GFN. Consistent with the previous result that the nymphal infestation weakly induced plant defense, we only identified 30 DEGs in leaf sheaths of GFN-infested plants, in comparison with leaf sheaths of GF-infested plants, including 16 up-regulated and 14 down-regulated genes (Supplementary Table 3). Among these DEGs, we did not find genes that are involved in the biosynthesis of VOCs. However, we observed that a gene encoding a non-specific lipid transfer protein and two genes encoding laccases were significantly down-regulated in GFN-infested plants (Supplementary Table 3); some members of these two gene families have been reported to be related to volatile emission[38]. These results indicate that the suppression of GF-induced indirect defenses brought about by nymphal infestation might be a result of impaired volatile emission rather than biosynthesis.

Discussion

In this study, we found that infestation of rice plants by GFs, most probably via oviposition, dramatically reshapes rice-plant transcriptomes and phytohormone-signaling networks, induces plant direct and indirect defenses, and decreases the suitability of host plants for subsequent BPHs. Intriguingly, nymphal feeding suppresses GF-induced indirect defenses in rice, decreasing both the attractiveness of GF-infested plants to egg parasitoids and the parasitism of BPH eggs in climate chambers and paddy fields. Our research reveals host-plant-mediated interactions between the overlapping generations of pests that result from two plant defense-eliciting behaviors of insect herbivores, namely feeding and ovipositing on host plants, and their likely consequences for the performance of pest populations.

Located at the bottom of many food chains, plants have evolved the ability to discriminate among attackers and mount tailored defense responses, which in turn lead to dramatically different responses to infestation by different herbivores[39]. Here, we found that the infestation of BPH GFs strongly induced defense responses in rice, including a change in the transcript levels of genes related to phytohormone and secondary metabolite biosynthesis and to primary metabolite catabolism, and an increase in defense-related signaling molecules (JA, JA-Ile, SA, and H_2O_2) and compounds, such as TrypPIs, and VOCs, whereas infestation by BPH nymphs or NFs induced minor defense responses. Notably, the enriched pathways of up-regulated DEGs that GF infestation uniquely induced (compared to untreated plants) matched levels of phytohormones and defense compounds in GF-infested plants (Figs. 1 and 2). Similarly, infestation by GFs rather than nymphs of *Sogatella furcifera*, another rice planthopper species, induced dramatic plant defense responses[40]. It has been well documented that plant defenses induced by herbivore infestation mainly depend on chemical signals, such as elicitors and effectors derived from herbivore oral secretions and oviposition fluids, and on damage patterns that the herbivore causes over time (which may influence the type and level of damage-associated molecular patterns released), both of which influence defense signaling and shape the production of defense compounds and resistance of plants to herbivores[1,10]. As a piercing-sucking insect, BPH passes its needle-shaped stylet through intercellular spaces and sucks phloem sap[32]. This feeding style can cause subtle damage to plants. In addition, and unlike nymphs, BPH GFs also damage plants when laying eggs: they puncture rice tissues using their strong ovipositors, which heavily damage plants, and then lay eggs inside, mainly in the ridges of leaf sheaths and the main veins of leaves[27]. Therefore, the main reason why rice plants respond more strongly to infestation by BPH GFs than to infestation by BPH nymphs is likely owing to the additional plant damage caused by BPH GF oviposition and to elicitors in oviposition fluids. The difference in elicitors and effectors between the saliva of BPH nymphs and that of GFs might also be a reason. Recently, our study showed that the N-terminal subunit of vitellogenin (VgN) from both saliva and the egg surface of BPH, functions as an elicitor inducing rice defenses; moreover, VgN from the egg surface, together with the damage caused by BPH oviposition, induces the production of JA and JA-Ile in rice, whereas VgN from the saliva of BPH, together with BPH feeding, does not[29]. This VgN work highlights the importance of understanding the combined effects of damage levels, elicitors, and effectors on BPH-induced rice defenses. Further research should seek to identify specific elicitors in BPH oviposition fluids and elucidate their roles in the defense responses of rice induced by the oviposition of GFs.

Our study showed that infestation by BPH nymphs decreased both the levels of certain volatiles induced by BPH GFs and the attractiveness of GF-infested rice plants to an egg parasitoid (Fig. 4). This decrease was not because infestation by BPH nymphs suppressed the oviposition activity of GFs, as the number of BPH eggs laid on GF-infested plants was similar to the number laid on GFN-infested plants (Supplementary Fig. 5). Therefore, the suppression of GF-induced defense responses by nymphal feeding may be due to effectors in oral secretions. In BPH, several effectors, such as NlEG1[24], NlSEF1[23], and calmodulin[25], have been identified in salivary glands at different developmental stages, including the nymphal stages. Both JA- and ethylene-signaling pathways in rice have been reported to regulate the emission of herbivore-induced volatiles[21,41,42]. However, we did not detect pronounced differences in levels of JA, JA-Ile, or ethylene between rice plants infested by GFs or GFNs (Supplementary Fig. 5). We also did not find differences in the transcript levels of genes related to the biosynthesis of volatile compounds between GF-infested plants and GFN-infested plants (Supplementary Table 3). However, we observed that the transcript levels of two laccase genes and a non-specific lipid transfer protein gene were significantly down-regulated in GFN-infested plants compared to GF-infested plants (Supplementary Table 3). Laccase is a copper-containing polyphenol oxidase that can oxidize monolignols and is deeply involved in lignin biosynthesis and cell wall formation[43]. The lignification and microstructure of cell walls significantly affect the emission of volatiles[44,45]. Moreover, non-specific lipid transfer proteins have recently been demonstrated as intrinsic members that promote VOC emission[38]. The data suggest that the suppression of GF-induced rice volatile emissions may result from the interference of nymphal infestation with the process of VOC emission rather than with VOC biosynthesis. Using mutants of these downregulated genes may elucidate the exact mechanism underlying nymphal manipulation of GF-induced rice VOC emission.

In nature, many herbivorous insects occur with overlapping generations. Although some research has reported cross-generational intraspecific competition of herbivore insects[46], in our understanding, no study has investigated how cross-generational infestation by insects shapes plant defenses and consequently influences the insect community. Our studies showed that infestation by BPHs at different life stages induced distinct plant responses. Interestingly, BPH offspring benefits when plants are infested by nymphs because nymphal infestation suppresses the emission of rice volatiles induced by egg laying and thereby decreases the attractiveness of plants to the parasitoid *A. nilaparvatae* (Fig. 4). Notably, most of the previous studies on the volatile-mediated attraction of natural enemies were conducted only in the laboratory using artificial olfactometer choice assays. Here, the census of natural populations confirms that the inhibition by nymphal infestation of egg-laying-induced indirect defense works well in natural conditions (Fig. 4f). Moreover, this inhibition may cause a

decrease of more than 10% in the parasitism rate of BPH eggs (Fig. 4e, f), meaning an increase of more than 10% in the hatching rate of eggs. The hatching rate of BPH eggs is one of the main factors that influence the population dynamics of BPHs[47]. It has been reported that a 10% decrease in the hatching rate of eggs in the fourth generation results in reductions (21.1% and 7.2%, respectively) in the density of the peak total BPH population in the fifth generation and in the density of the parasitoid population[47]. Therefore, a decrease in the parasitism rate of BPH eggs will probably have a relatively large effect on the population density of BPH, especially in places such as Hainan, China, where from 10 to 12 BPH generations occur per year[30]. These results demonstrate that cross-generational intraspecific cooperation may occur in BPH, an herbivorous insect with overlapping generations that lives gregariously; this phenomenon may not only help BPH populations to thrive but may also contribute to the aggregation behavior of BPHs. Future work should investigate whether this phenomenon also occurs in interactions between other combinations of plants and overlapping generations of insects.

In conclusion, our research shows that BPH infestation -- more likely via oviposition by BPH GFs rather than feeding by nymphs or adults -- strongly induces rice defenses. Moreover, BPH nymph infestation suppresses GF-induced defenses, which benefits the offspring of the herbivore population and may contribute to aggregation behavior. Our study provides an important example of how a specialist herbivore and problematic agricultural pest overcomes host-plant defenses via intergenerational gregariousness and cooperation.

Methods

Plants

The rice (*Oryza sativa*) variety used in this study is Xiushui 11, a *japonica* variety. Pre-germinated seeds were cultured in plastic bottles (diameter 8 cm, height 10 cm) in a greenhouse (28 ± 2 °C, 60–70% relative humidity, and 14 h light phase). Ten-day-old seedlings were transferred to 20 L hydroponic boxes with rice nutrient solution[48]. After 30 days, seedlings were individually transferred into opaque 400 mL plastic pots with the nutrient solution. Plants were used for experiments 4 to 5 days post-transplantation.

Insects

The BPH colony was originally obtained from rice fields in Hangzhou, China, and subsequently maintained on TN1 (an *indica* rice variety susceptible to BPH) seedlings in a controlled climate chamber at 26 ± 2 °C, 12 h light phase and 80% relative humidity for at least 30 generations prior to use. Third-instar nymphs were directly taken from the colony for experiments. Late 5th-instar nymphs (final instar) were transferred into new cages with fresh TN1 seedlings, and then the newly-emerged (within 8 h after emergence) adults were divided into two groups: the first group was all female adults that were used as non-gravid females (NFs); the second group was transferred into new cages with a male:female ratio of 2:1, and the females were used as gravid females (GFs) 3 days after emergence. A laboratory colony of *A. nilaparvatae* was started from individuals trapped in rice fields in Hangzhou, China. The colony was propagated from BPH eggs laid on TN1 seedlings. Female wasps were used for experiments less than 24 hours after emergence.

Plant treatments

For BPH treatments, rice plants were randomly assigned to groups and individually confined in glass cylinders (diameter 4 cm, height 8 cm; on the wall of the cylinder with 48 small holes, diameter 0.8 mm) with round sponges covering the tops as shown in Fig. 3a. For each plant, 10 GFs, 20 NFs, 30 3rd-instar nymphs or 10 GFs together with 30 3rd-instar nymphs (GFNs) were introduced (see details in each experiment), except the experiments about the effect of BPH density on the biosynthesis of JA and SA in rice (Supplementary Fig. S2). For these, the numbers of BPHs per plant (0-20 nymphs per plant or 0-10 GFs per plant) were specifically indicated. Plants with empty cylinders were used as controls.

cDNA library generation and RNA sequencing

Rice plants were individually infested with nymphs, NFs, GFs, and GFN for 24 h as described above. Whole leaf sheaths covered by the glass cylinder from five individual plants were combined as a biological replicate, and each treatment had three replicates. Total RNA was extracted with TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and purified using RNeasy spin columns (Qiagen, Valencia, CA, USA). Library construction and RNA sequencing were performed by Novogene company in Beijing, China. The rice reference genome version of "rice_uga_oryza_sativa_version_7_0" was used to align the RNA-seq reads, using the software of HISAT2 with default parameters. The quantile analysis of counts of each gene was conducted using the software "freatureCounts" with default parameters. DEGs were filtered by $P_{adj} < 0.05$, and absolute fold changes > 2; the different significances were analyzed by the negative binomial distribution of DEseq, followed by the *P*-values adjustment using the Benjamini-Hochberg method. Gene annotations were derived from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu). Principal coordinates analysis and KEGG enrichment analysis were performed using online tools of Novogen (https://magic.novogene.com/customer/main#/small-tools/) with default parameters.

JA, JA-Ile, SA, and H_2O_2 analysis

Plants (one plant per pot) were randomly assigned to groups of control and BPH treatments, followed by infestation with BPHs as described above. Whole leaf sheaths covered by the glass cylinder from individual plants were harvested at 0, 3, 8, 12, 24, 48, or 72 h following the start of BPH infestation. For the experiment about the effect of BPH density on the production of these signals in rice, treated leaf sheaths were harvested at 48 h after the start of infestation. Each treatment at each time interval had 4 to 8 biological replicates. Samples were collected, immersed immediately in liquid nitrogen, and then stored at − 80 °C until extraction and analysis. Samples were ground in liquid nitrogen, aliquoted (about 100 mg each sample), and extracted for JA, JA-Ile, and SA analysis using an LC/MS/MS system configured with an electrospray ionization source (Agilent 6460, CA, USA), following a previously described method[49].

For H_2O_2 analysis, samples were harvested following the same procedure used for JA and SA analysis. The H_2O_2 concentrations were determined using the AmplexRed Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) as previously described[50].

Ethylene analysis

Rice plants were individually covered with sealed glass cylinders (height, 50 cm; internal diameter, 4 cm). Ethylene production was determined at 8, 24, 48 and 72 h following the start of the treatment, by taking 5 ml of headspace from the cylinder using a syringe. The ethylene samples were analyzed using a gas chromatograph (GC) as previously described[51]. Each treatment had 8 biological replicates.

Trypsin proteinase inhibitor (TryPI) analysis

Plants (one plant per pot) were randomly assigned to groups of control and BPH treatments, followed by infestation with BPHs as described above. Whole leaf sheaths covered by the glass cylinder from individual plants were harvested 3 days after the start of the treatment. The activity of TryPI was measured using a radial diffusion assay as previously described[52]. Each treatment had 4 - 6 biological replicates.

Collection, isolation, and identification of volatile compounds

The collection, isolation, and identification of rice headspace volatiles were carried out using the method previously described by Lou et al.[53].

Plants were individually treated by BPHs for 24 h as described above, and BPHs were then removed. The volatiles from the headspace of differently treated plants were individually collected from 10 am to 6 pm. Each treatment had 5 biological replicates. Volatile compounds were identified by comparing retention times and mass spectra with authentic standards. The relative amount of each compound from the 8 h trapping was expressed as the percentage of peak area relative to the internal standard (ethyl decanoate). Three compounds (2-heptanone, 2-heptanol, and (*E*)-β-farnesene) were quantified using an external standard curve with levels ranging from 0.625 to 10 ppm.

BPH preference assay

Rice plants were individually pre-treated for 24 h by BPH nymphs, gravid females, or controls as stated above. BPHs were removed after treatments, and two plants (one treated vs. one control) were transplanted into one pot and confined within a glass cylinder. Each cylinder received 15 nymphs or gravid females for the preference assay. The number of BPHs on each plant was counted at 1, 2, 4, 8, 12, 24, and 48 h after BPH release. Eggs on each plant were counted 48 h after BPH release, using a microscope as previously described[21].

BPH nymph survival assay

Rice plants were individually pre-treated by different types of BPHs for 24 h as described above, and then exposed to 15 newly hatched BPH nymphs within a glass cylinder. The number of surviving nymphs on each plant was recorded every other day until 10 days after the release of the experimental nymphs. Each treatment had 10 biological replicates.

BPH egg hatch assay

Rice plants were individually treated by different types of BPHs or controls for 24 h as described above; however, in this experiment, the sealing sponges were moved to the middle of cylinders, meaning that the lower half of each plant within the cylinder was infested with BPHs or kept non-infested. After this, 10 gravid females were exposed to the upper half of control or treated rice plants within the cylinder for 12 h and then removed. We counted the newly hatched nymphs on each plant every day until no new nymphs were found for three days in a row. The number of unhatched eggs on each plant was counted under a microscope, and then the hatching rate of BPH eggs was calculated. This experiment had 10 biological replicates.

We also investigated the effect of nymphal infestation on the hatching rate of BPH eggs. Plants were individually infested with 10 GFs or 10 GFs together with 30 3rd-instar nymphs (GFNs) for 24 h and then removed. After this, following the process described above, the hatching rate of BPH eggs on plants was calculated. This experiment had 10 biological replicates.

Wasp preference assay

Responses of *A. nilaparvatae* females to rice volatiles and three volatile compounds (2-heptanone, 2-heptanol, and (*E*)-β-farnesene) were measured using a Y-tube olfactometer as previously described[54]. For the three volatile compounds, standards were solved in acetone and transferred into a capillary (100 mm X 0.3 mm i.d., Eppendorf, with volume of 7.065 μL). In the capillary, we measured the emission rate of the three compounds in acetone and found that the emission rate was about 0.2 μL min^{-1}. The emission rates of 2-heptanone, 2-heptanol, and (*E*)-β-farnesene from GF-infested plants and GFN-infested plants were 1.86 ± 0.32, 0.57 ± 0.09, 0.31 ± 0.05, and 0.91 ± 0.26, 0.30 ± 0.06, 0.19 ± 0.01 ng min^{-1} (see above); therefore, the concentrations of the three compounds in acetone were designed as 5, 2, and 2 mg L^{-1}. Two odor sources (treated rice plants vs. control plants or a capillary with a compound in acetone vs. a capillary with acetone) were connected to a Y-shape glass tube through a clean Teflon tube. Each end of the two Teflon tubes was connected with a small glass tube and covered with nylon mesh to separate wasps from odor sources. Before reaching the odor sources, the air was cleaned with activated charcoal and humidified by being passed through a bottle of distilled water. Airflow was 150 ml min^{-1}. Newly (within 24 h) emerged female wasps were released into the base tube of the olfactometer and given 5 min to walk toward the end of one arm; those that did not reach the arm were defined as "no choice." The choice for an odor source was defined as a wasp crossing a line 7 cm after the division of the base tube and staying there for more than 30 sec. The Y-shape tube was changed every 2 wasps, and odor sources were exchanged every 10 wasps. Experiments were conducted from 10 am to 6 pm.

Parasitism quantification

For parasitism quantification in a climate chamber, rice plants treated with 10 GFs or 10 GFs together with 30 3rd-instar nymphs (GFNs) were placed in a plastic cylinder (height, 60 cm; diameter, 16 cm) in pairs. Five females and 3 males of *A. nilaparvatae* were released into the cylinder; 48 hours later, the parasitoids were removed. After 3 days to allow for the development of wasp eggs, the rice leaf sheaths were harvested and dissected under a dissecting microscope. Eggs with an obvious red dot (wasp larvae) were counted as parasitized eggs. This experiment had 10 biological replicates. For the parasitism assay in the paddy field, rice plants were randomly assigned to two groups: GFs and GFNs. The glass cylinder was divided into two parts as described in the section "egg hatch assay", and the lower parts were exposed to GFs or GFNs for 24 h. After the BPHs were removed, the upper part of each plant from the two groups was infested with another 10 gravid females for 24 h. Before these treated plants (with pots) were transferred into paddy fields, the lower parts of plants were sealed with parafilm to prevent these eggs from parasitism by the parasitoid. Seven pots for each of the two groups were placed at seven locations (5 m apart) in a rice paddy at the end of September when the rice was at the heading stage; each location included two pots, GFs, and GFNs. Three days later, these rice plants were transferred into a climate chamber (28 °C, 60–70% relative humidity, and 14 h light phase) for another 3 days to let wasps develop. The parasitized BPH eggs were counted as described above.

Field data analysis

The population density of BPH and the parasitism rate of BPH eggs by *A. nilaparvatae* were mined from a field survey in 2014 that was published by Li et al.[22]. Notably, to avoid the effect of specific genes, only the data from blocks with wild-type (XS11 variety) plants were used for this analysis. For each block on every survey day, the average number of BPH nymphs per plant, the average number of BPH adults per plant, and the average parasitism rate of BPH eggs by the parasitoid were calculated. Then, the ratio of nymphs to adults was calculated using the average number of BPH nymphs per plant in a block divided by the average number of BPH adults per plant in the same block. Paired data, the ratio of nymphs to adults, and the average parasitism rate of BPH eggs by the parasitoid were used for Pearson correlation analysis.

Statistics & reproducibility

All statistical analyses were performed in SPSS (IBM SPSS Statistics 25), except significance for enrichments of DEGs from RNA-seq, which were analyzed using online tools (KEGG: Kyoto Encyclopedia of Genes and Genomes). The DEGs of RNA-seq data were analyzed by the negative binomial distribution of DEseq with the parameter of absolute |FC | > 2 and P_{adj} value < 0.05 as cut-off values, followed by the *P*-value adjustment using the Benjamini-Hochberg method. Data from two groups were analyzed by paired or independent Student's *t* test when data were in a normal distribution. The Wilcoxon's signed rank test and Friedman rank sum test were performed for BPH distribution data. Three or more treatments were analyzed using one-way ANOVA

followed by Tukey's HSD post-hoc tests. A Chi-square goodness of fit test was used for the wasp preference assay data. Pearson correlation was used for the relation between the egg parasitism percentages and the ratios of nymphs to adults, via the bivariate correlation analysis. Statistical values for all the data are represented in the Source Data file. The sample size of each experiment was described in the corresponding method and figure legend. No data were excluded from the analyses.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available within the manuscript and its Supplementary Information files and data. The raw RNA-seq data generated in this study have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in the National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences, under accession code GSA: CRA019145 [https://ngdc.cncb.ac.cn/gsa/s/HdyBBGI5]. Source data are provided in this paper.

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Competing interests

The authors declare no competing interests.

Additional information

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