Stemborer-induced rice plant volatiles boost direct and indirect resistance in neighboring plants

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Received: *6 August 2022*
Accepted: *4 October 2022*

New Phytologist (2023) **237:** 2375–2387
doi: 10.1111/nph.18548

Key words: *Chilo suppressalis*, *Cotesia chilonis*, defense priming, herbivore-induced plant volatiles (HIPVs), jasmonate (JA), proteinase inhibitor (PI).

Summary

- Herbivore-induced plant volatiles (HIPVs) are known to be perceived by neighboring plants, resulting in induction or priming of chemical defenses. There is little information on the defense responses that are triggered by these plant–plant interactions, and the phenomenon has rarely been studied in rice.
- Using chemical and molecular analyses in combination with insect behavioral and performance experiments, we studied how volatiles emitted by rice plants infested by the striped stemborer (SSB) *Chilo suppressalis* affect defenses against this pest in conspecific plants.
- Compared with rice plants exposed to the volatiles from uninfested plants, plants exposed to SSB-induced volatiles showed enhanced direct and indirect resistance to SSB. When subjected to caterpillar damage, the HIPV-exposed plants showed increased expression of jasmonic acid (JA) signaling genes, resulting in JA accumulation and higher levels of defensive proteinase inhibitors. Moreover, plants exposed to SSB-induced volatiles emitted larger amounts of inducible volatiles and were more attractive to the parasitoid *Cotesia chilonis*.
- By unraveling the factors involved in HIPV-mediated defense priming in rice, we reveal a key defensive role for proteinase inhibitors. These findings pave the way for novel rice management strategies to enhance the plant's resistance to one of its most devastating pests.

Introduction

Plants attacked by herbivores emit complex mixtures of volatile organic compounds (VOCs). These so-called herbivore-induced plant volatiles (HIPVs) play vital roles in the plants' interaction with insect communities at different trophic levels (Schuman & Baldwin, 2018; Turlings & Erb, 2018; He *et al.*, 2019; Hu *et al.*, 2020). For instance, HIPVs can serve as foraging cues for natural enemies to locate their hosts or preys (Dicke & Sabelis, 1988; Turlings *et al.*, 1990; Kessler & Baldwin, 2001; Dicke *et al.*, 2009; Joo *et al.*, 2018; Turlings & Erb, 2018) and can be used by herbivores to find suitable host plants (Landolt, 1993; De Moraes *et al.*, 2001; Knolhoff & Heckel, 2014; Jiao *et al.*, 2018) or escape the attention of their natural enemies (Shiojiri *et al.*, 2002; Desurmont *et al.*, 2018; Hu *et al.*, 2020). In addition to mediating the interactions between plants and insects, HIPVs can be perceived by neighboring plants and thus serve as airborne signals in plant–plant interactions. In various cases, it has been shown that the perceiving neighbors prepare for incoming attack. This is commonly referred to as *defense priming* and results in faster and stronger defense responses when the primed plants are attacked themselves. In other cases, the perceiving plants straightaway launch defense responses even if they are not under attack yet, which is referred to as *defense induction* (Erb, 2018).

The induction or priming effects of HIPVs on plants have been reported numerous times (Baldwin & Schultz, 1983; Bruin *et al.*, 1992; Arimura *et al.*, 2000; Engelberth *et al.*, 2004; Ruther & Fürstenau, 2005; Kost & Heil, 2006; Frost *et al.*, 2007, 2008a; Ton *et al.*, 2007; Rodriguez-Saona *et al.*, 2009; Heil & Karban, 2010; Holopainen & Blande, 2012; Ueda *et al.*, 2012; Das *et al.*, 2013; Karban *et al.*, 2013, 2014; Erb *et al.*, 2015; Delory *et al.*, 2016; Kalske *et al.*, 2019; Zhang *et al.*, 2020; Hu, 2021). Indole, a heterocyclic aromatic compound, released by maize plants is an example of a HIPV that primes conspecific neighboring plants (Erb *et al.*, 2015). An example of a HIPV acting as a true inducer is the ubiquitous homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) released by tea plants after infestation by *Ectropis obliqua* (Prout) (Lepidoptera: Geometridae) caterpillars, which triggers the accumulation of jasmonic acid (JA) in neighboring intact tea plants, making them more resistant to this lepidopteran pest (Jing *et al.*, 2021). Few studies suggest that HIPVs can also act as suppressors of defenses in neighboring plants or trigger the 'wrong' defense (Erb, 2018). For instance, broccoli plants that were exposed to the volatiles of conspecific plants damaged by *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) caterpillars became more susceptible to their oviposition (Li & Blande, 2015). A study on whitefly-induced tomato plant volatiles found that they suppressed JA-dependent defenses in neighbors, thus rendering them more susceptible to whiteflies (Zhang *et al*., 2019). Although the phenomenon of plant–plant interactions mediated by volatiles has been widely reported, the molecular and biochemical processes that are involved are still largely unknown. This is especially true for interactions among rice plants, which have been poorly studied in this context (Ye *et al*., 2019).

Rice (*Oryza sativa*) is one of the world's most important staple crops, and losses due to abiotic and biotic stresses can have tremendous consequences for food security. It has therefore been subject to many investigations, in particular on how it defends itself against insect pest infestations (Liu *et al*., 2016; Dai *et al*., 2019; Wang *et al*., 2020; Xu *et al*., 2021). Protease inhibitors (PIs), vital defense compounds, play an important role in defending rice against lepidopteran larvae (Zhou *et al*., 2009; Q. S. Liu *et al*., 2021). Our recent study on the striped stemborer (SSB), *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae), indicates that SSB caterpillar induces a significantly upregulated expression of PI-related defensive genes, resulting in the accumulation of PIs, thereby causing poorer performance of SSB larvae on infested rice plants (Q. S. Liu *et al*., 2021). Moreover, SSB caterpillar-infested rice plants emit more and larger amounts of volatile compounds than undamaged plants, which attracts the parasitoid *Cotesia chilonis* (Matsumura) (Hymenoptera: Braconidae), an important natural enemy of SSB (Liu *et al*., 2015). These findings prompted us to hypothesize that SSB-induced rice volatiles can prime or induce molecular and chemical changes in neighboring plants, leading to enhanced direct and indirect resistance (Ton *et al*., 2007; Kessler & Heil, 2011; Guo *et al*., 2019; Pearse *et al*., 2020) against SSB. Because rice plants are usually densely planted and neighboring plants are always in very close proximity, volatile-mediated interactions among rice plants can be expected to be particularly important.

To test our hypothesis, we compared the performance of SSB larvae on rice plants that had been exposed to volatiles emitted by either uninfested or SSB-infested rice plants. In addition, we explored the potential molecular responses that could be involved in volatile-mediated priming, with a focus on the induction and expression of PIs in the rice plant. We also studied the effect on indirect resistance by examining the behavioral responses of *C. chilonis* parasitoids to rice plants that had been exposed to volatiles emitted by uninfested or SSB-infested plants either without or with subsequent caterpillar infestation. To identify inducible compounds that might be involved in parasitoid attraction, the volatile emissions of the exposed plants were also investigated. With these experiments, we aimed to provide insight into the physiological responses and ecological implications of HIPV-mediated information exchange between rice plants, and to use this fundamental knowledge for the development of new strategies to exploit the plant's natural chemical defense responses for improved rice pest management.

Materials and Methods

Plants and insects

Rice plants (*Oryza sativa* L., cultivar Minghui63) were grown in a glasshouse at 27 ± 3°C with 75 ± 10% RH (relative humidity) and a photoperiod of 16 h : 8 h, light : dark (L : D). The cultivation of rice plants followed the same procedure as described previously (Hu *et al*., 2020). Potted plants were used in the experiment 30 d after transplanting when they were at the tillering stage with three tillers and 10–12 leaves on the main stem.

Striped stemborer larvae were obtained from laboratory colonies reared on an artificial diet for many generations with annual introductions of field-collected individuals (Han *et al*., 2012). The larval endoparasitoid *C. chilonis* used in the study was reared on 4th-instar SSB larvae as previously described (Liu *et al*., 2015). Each SSB larva was exposed to a mated female parasitoid for 6 h and then provided with sufficient artificial diet until they yielded parasitoid cocoons. Newly emerged adult parasitoids were supplied with a 20% honey water solution, and female adults mated over 24 h were used in the bioassays. The insect colonies were kept in separated climatic chambers, respectively, at 27 ± 1°C, 70 ± 5% RH, and 16 h : 8 h, light : dark.

Experimental exposure treatment

To expose rice plants to volatiles emitted from SSB larvae-infested or uninfested rice plants, we used a push-pull system as shown and described in Fig. S1. Purified air that was filtered through activated charcoal was pumped into the system at a speed of 600 ml min^{-1}, passing into a first glass bottle (diameter 22 cm; height 75 cm) with four SSB-infested rice plants (emitting plants) and then into the downwind glass bottle with the four healthy rice plants (receiving plants). Each emitting plant was infested with two 3rd-instar SSB larvae that had been starved for 3 h. As control treatments, four uninfested rice plants were used as the emitting plant placed in the upwind glass bottle. All interfaces were connected with Teflon tubes (inner diameter 0.6 cm; outer diameter 0.9 cm). The exposures lasted for 48 h, and the receiving plants were used for the various subsequent experiments.

Performance of caterpillars on exposed plants

In the first bioassay, to obtain emitting herbivore-infested plants, rice plants were infested with two 3rd-instar SSB larvae for 1, 3, 5, or 7 d. Plants from the same cohort but without any insect infestation served as emitting control plants. Healthy rice plants were exposed to volatiles from one of these five types of emitting plants for 48 h as described above. After exposure, each receiving plant was infested with 10 neonate SSB larvae, and the weight of each larva was determined after 7 d on a precision balance (CPA2250; Sartorius AG, Göttingen, Germany; readability = 0.01 mg). The mean weight of caterpillars per plant was considered as one biological replicate. Eight plants (biological replicates) were used for each treatment.

Based on the result from this first bioassay, a second bioassay was conducted in which the receiving plants were exposed for 48 h to volatiles from undamaged plants or plants that had been infested by SSB larvae for 5 d. Insect performance was determined following the same procedure as described above. A total of 12 plants (biological replicates) were used for each treatment. Based on the results of these two series of assays, we used emitting rice plants that had been infested with SSB larvae for 5 d as sources of caterpillar-induced volatiles for the subsequent experiments. All experiments were conducted in climatic chambers at 27 ± 1°C, 70 ± 5% RH, and 16 h : 8 h, light : dark.

RNA extraction and cDNA synthesis

After exposure to volatiles emitted from SSB-infested or uninfested rice plants (control), the receiving plants were subjected to infestation by two 5-d-old SSB larvae for 0, 3, 6, 12, and 24 h, before their stems were harvested. For each treatment, rice stems harvested from four plants of the same treatment were pooled as one sample, and 3–4 samples for each treatment were collected at each time point. All harvested stems were immediately frozen in liquid nitrogen and stored at −80°C for further RNA extraction.

Frozen rice stem samples were ground to a fine powder in liquid nitrogen with a pestle and mortar (Liu *et al.*, 2016; Wang *et al.*, 2020; Q. S. Liu *et al.*, 2021). Total RNA was extracted using TRIzol reagent (Invitrogen), in accordance with the manufacturer's instructions. RNA concentration and purity were determined using a DS-11 spectrophotometer (Denovix, Wilmington, DE, USA), and the integrity of RNA was assessed by 1% agarose gel electrophoresis. One thousand nanograms of total RNA was reverse transcribed using a TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions.

Quantitative real-time PCR

The jasmonate (JA) signaling pathway plays an important role in regulating rice defense against insect herbivores, and PIs have been shown to be the main defense compounds to SSB (Q. S. Liu *et al.*, 2021). Therefore, the expressions of 12 JA pathway genes (*r9-LOX1*, *LOX4*, *AOS1*, *LOX2*, *LOX6*, *LOX8*, *LOX12*, *HI-LOX*, *LOX2.2*, *DOX2*, *WP1*, and *OPR2*) and 9 PIs genes (*APIP4*, *CPI1*, *PEI1*, *CPI8*, *TM1*, *BBTI8*, *PI1*, *PI2*, and *BBTI12*) were monitored in the stems of rice plants that were subjected to the different exposure treatments and subsequent caterpillar infestation. The transcript levels of all genes were quantified by quantitative real-time PCR (qRT-PCR) using Bio-RadCFX96 Touch Real-time PCR Detection System instrument (Bio-Rad, Hercules, CA, USA) with a 96-well plate. The amplification reactions were performed in a final volume of 20 µl that contained 10 µl of 2 × TransStart® Top/Tip Green qPCR Supermix, 1.2 µl of forward primer (10 µM) and reverse primer (10 µM) pairs, 5.8 µl of nuclease-free water, and 3 µl of cDNA first-strand template. The thermocycler setting was as follows: 30 s at 94°C, followed by 40 cycles of 5 s at 94°C, 15 s at 60°C, and 10 s at 72°C. To confirm the formation of single peaks and to exclude the possibility of primerdimer and nonspecific product formation, a melting curve was generated by the end of each PCR. Primer pairs were designed using the PRIMER 6 software (Premier Biosoft International, Palo Alto, CA, USA) and listed in Table S1. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method with *ubiquitin 5* as an endogenous control gene (Zhang *et al.*, 2019). Each gene was tested for each treatment with 3–4 biological replications.

Quantification of endogenous JA and jasmonoyl isoleucine

After exposure to volatiles emitted from SSB-infested or uninfested rice plants, the receiving plants were then subjected to infestation by two 5-d-old SSB larvae for 0, 12, 24, and 48 h, before the stems were harvested. The endogenous JA and jasmonoyl isoleucine (JA-Ile) in the stems were quantified by the plant hormone platform at the Kunming Institute of Botany, Chinese Academy of Sciences, as previously described (Wu *et al.*, 2007; Malook *et al.*, 2019). About 150 mg per sample (precise content was recorded) was ground in liquid nitrogen to a fine powder. Each phytohormone was quantified by comparing its peak area with the peak area of its respective internal standard. Five replicates were conducted for each time point.

Quantification of trypsin protease inhibitors

After exposure to volatiles emitted from SSB-infested or uninfested rice plants, the receiving plants were then subjected to infestation by two 5-d-old SSB larvae for 0, 48, 72, and 96 h, before the stems were harvested. Samples from four individual plants were pooled together as one biological replicate, and four replicates were used for each sampling time point. The analysis of trypsin protease inhibitor (TrypPI) was based on comparison of its content with total protein in samples as previously described (Q. S. Liu *et al.*, 2021). Frozen rice stem samples were ground in liquid nitrogen, and approximately 80 mg was placed in 2-ml Eppendorf tubes. Seven hundred and twenty microlitres of 0.01 M phosphate-buffered saline (PBS) extraction buffer (pH = 7.4) (Sigma-Aldrich, St Louis, MO, USA) was added and homogenized. After centrifugation at 4000 ***g*** for 30 min at 4°C, 400 µl of supernatants was transferred to new 2-ml Eppendorf tubes and stored at 4°C. The content of total protein was determined using a BCA protein quantitation kit (Aidlab Biotechnologies Co. Ltd, Beijing, China), and TrypPIs were detected using an ELISA Kit (J&L Biological, Shanghai, China), in accordance with the manufacturer's instructions. The measurements of absorbance were read at 562 nm for total protein and 450 nm for TrypPI using a microplate spectrophotometer (PowerWave XS2; BioTek, Winooski, VT, USA). Four biological and three technical replicates were conducted for each time point.

Behavioral bioassays with the parasitoid *C. chilonis*

To test whether caterpillar-infested rice volatiles induce/prime the release of such volatiles in neighboring plants, we tested the attraction of *C. chilonis* to rice plants that had been exposed to the volatiles of differently treated plants. Two groups of behavioral bioassays were conducted: rice plants exposed to volatiles from uninfested rice plants (Control) vs rice plants exposed to volatiles from SSB-infested rice plants (HIPVs); and, rice plants exposed to volatiles from uninfested rice plants followed by infestation with two 4th-instar SSB larvae infestation for 6 h (Control + SSB) vs rice plants exposed to volatiles from SSB-infested rice plants followed by the same caterpillar infestation (HIPVs + SSB). The aim of bioassay was to determine whether rice plants exposed to HIPVs show increased attractiveness to females of the parasitoid (induction), and to determine whether rice plants exposed to HIPVs followed by caterpillar damage show faster and stronger attractiveness to the parasitoids than similarly infested control plants (priming). The olfactory behavior assays were conducted using Y-tube olfactometers (15-cm stem, 15-cm arms at 75° angle, and 1.5 cm internal diameter) as previously described (Liu *et al.*, 2015; Hu *et al.*, 2020). A total of 80–100 female parasitoids were tested for each treatment pair. All tests were conducted between 10:00 and 16:00 h in a climate-controlled laboratory room at 27 ± 1°C and 40% RH.

Collection and analysis of headspace rice volatiles

As in the previous experiment, four types of rice plants were prepared as follows: rice plants exposed to volatiles from uninfested rice plants (Control); rice plants exposed to volatiles from SSB-infested rice plants (HIPVs); rice plants exposed to volatiles from uninfested rice plants followed by caterpillar infestation for 6 h (Control + SSB); and, rice plants exposed to volatiles from SSB-infested rice plants followed by caterpillar infestation for 6 h (HIPVs + SSB).

We used a dynamic headspace device to collect rice volatiles and analyzed them by gas chromatography (GC) coupled with a mass spectrometry (MS) system (Agilent 8890/5977B GC-MS; Agilent Technologies Inc., Beijing, China) as previously described (Hu *et al.*, 2020). Each volatile collection, in accordance with the timing and conditions of behavioral bioassays with the parasitoids, lasted for 3 h (12:00–15:00 h) under continuous light in a climate chamber. After collection, trapping filters filled with 20 mg Super Q traps (80/100 mesh; ANPEL Laboratory Technologies (Shanghai) Inc., China) were rinsed with 200 μl dichloromethane, and 500 ng nonyl acetate was added as internal standard. Volatile compounds were identified by mass spectral matches to library spectra and by matching observed retention times with those of available authentic standards. If standards were unavailable, tentative identifications were made based on referenced mass-spectra available from NIST (Scientific Instrument Services Inc., Ringoes, NJ, USA) or based on previous studies (Jiao *et al.*, 2018; Hu *et al.*, 2020). Relative quantification of identified compounds was first based on comparison of their peak areas with the internal standard. Although not allowing for precise quantification, these relative amounts provide accurate information for comparisons among volatile collections. We also used these values as rough estimates of quantity to select the dosages of chemical standards that we used in the following olfactometer assay. For each treatment, rice volatiles were collected from four plants as one biological sample and repeated 7–8 times in parallel.

Identification of volatiles regulating behavior of *C. chilonis* parasitoids

Based on the results of GC-MS analyses, four consistently induced volatile compounds (linalool, DMNT, methyl salicylate (MeSA), and hexadecane; see Table S2) were selected to test their effects on the behavior of *C. chilonis* wasps. For that, the absolute concentrations of the four compounds were first calculated based on their response factors relative to the internal standard as described in our previous study (Hu *et al.*, 2020). The synthetic volatile compounds were individually dissolved in hexane, and each compound was tested at three doses, including a low dose that was equal to their concentrations in the volatile blend emitted by rice plants that had been exposed to volatiles from SSB-infested rice plants and were consequently subjected to caterpillar damage (HIPVs + SSB), a medium dose that was equal to a tenfold of the low dose and a high dose that was equal to a 100-fold of the low dose. Filter papers (1 × 2 cm) loaded with either 10 μl of the solution with one of the four volatiles or with 10 μl pure hexane were placed into separate Teflon tubes connecting to two ports of the Y-tube (Guo *et al.*, 2019; Hu *et al.*, 2020). The procedure for the Y-tube behavioral assays was the same as described above. A total of 60–90 individuals were tested for each treatment pair.

Statistical analysis

The data obtained for gene expression, phytohormone, and TrypPI levels were analyzed by two-way analysis of variance (ANOVA), with exposure treatment and caterpillar infestation time after exposure as independent variable, followed by pairwise comparisons through Bonferroni adjustment. The normality of the data was verified by inspecting residuals through Shapiro–Wilk tests, and the variance homogeneity was tested using Levene's test. Datasets that did not fit these assumptions were either log-transformed or quartic-root-transformed to meet the requirements of equal variance and normality. The data for larval weights were analyzed using two-sided Student's *t*-test. Behavioral responses of *C. chilonis* in Y-tube assays were analyzed using a binomial test with an expected response of 50% for either olfactometer arm; parasitoids that did not make a choice were excluded from the analysis. Quantities of specific volatile compounds released upon different treatments were compared using the one-way ANOVA followed by Tukey's honest significant difference (HSD) test. The data on volatile emissions were further investigated by discriminant analysis. These data were normalized by medians, log-transformed, and then auto-scaled (mean-centered and divided by the standard deviation of each variable) using the METABOANALYST 4.0 software before being analyzed using orthogonal partial least squares-discriminant analysis (OPLS-DA). Statistical analyses were conducted with SPSS 25.0 (IBM SPSS, Somers, NY, USA), except that the OPLS-DA was performed using the SIMCA 14.1 software (Umetrics, Umea, Sweden).

Results

Pre-exposure to caterpillar-induced volatiles increases SSB resistance in rice plants

The results of our first bioassay showed that the mean weight of SSB caterpillars was not significantly affected when fed on plants that had been exposed to volatiles emitted from plants that had been infested by caterpillars for 1 or 3 d ($t = 0.828$, df = 13, $P = 0.423$; $t = 0.255$, df = 13, $P = 0.803$, respectively). Yet, their weight gain was significantly lower when feeding on plants that had been exposed to volatiles from plants infested for 5 or 7 d ($t = 2.592$, df = 14, $P = 0.021$; $t = 2.943$, df = 14, $P = 0.011$, respectively), compared with those fed on plants that had been exposed to volatiles emitted from uninfested plants (Fig. 1a). The enhanced resistance was further confirmed in the second bioassay, showing significantly lower weight of SSB caterpillars fed on plants exposed to volatiles emitted from rice plants infested by caterpillars for 5 d than caterpillars on plants that received volatiles from the uninfested control plants ($t = 4.919$, df = 19, $P < 0.001$) (Fig. 1b).

Pre-exposure to caterpillar-induced volatiles increases the expression of JA signaling genes and JA accumulation in rice plants

Among the 12 tested JA-signaling genes, two genes (*DOX2* and *LOX8*) were directly induced after exposure to caterpillar-induced volatiles. Upon subsequent infestation by caterpillars, 11 genes showed an earlier and/or stronger transcriptional priming in receiving plants that had been exposed to volatiles emitted from SSB-infested rice plants. By contrast, two genes (*WPI* and *OPR2*) showed a lower expression in the HIPVs-exposed plants damaged by caterpillars for 6 h, than in similarly infested control plants (Fig. 2).

Consistent with the differences observed for the expression of most of the JA signaling genes, exposure to SSB-induced volatiles did not directly change the accumulation of JA ($F_{1,32} = 1.907$, $P = 0.177$), but significantly higher amounts of JA were detected in plants that had been exposed to caterpillar-induced volatiles and then were infested for 12, 24, and 48 h ($F_{1,32} = 32.179$, $P < 0.001$; $F_{1,32} = 39.783$, $P < 0.001$; $F_{1,32} = 6.246$, $P = 0.018$, respectively), than in plants that had first been exposed to volatiles from uninfested plants (Fig. 3a). The content of JA-Ile showed a very similar treatment effect, but not anymore after 48 h of infestation (Fig. 3b).

Pre-exposure to caterpillar-induced volatiles increases the expression of PI genes and PI accumulation in rice plants

In general, all nine tested PI genes showed inducible expressions after SSB caterpillar infestation, independent of pre-exposure. Among the PI genes, only *CPI8* was directly induced in receiving plants that had been exposed to volatiles emitted from caterpillar-infested plants, compared with receiving plants that had been exposed to volatiles emitted from uninfested plants. Upon subsequent SSB infestation, the four genes, *APIP4*, *CPI1*, *PEI1*, and *CPI8*, showed an earlier and stronger transcriptional induction in receiving plants that had been exposed to volatiles emitted from SSB-infested plants (Fig. 4). As in our earlier study (Q. S. Liu *et al.*, 2021), *APIP4* was found to positively regulate TrypPI production. Other tested PI genes also showed an increased expression trend in the treatment of pre-exposure to SSB-infested rice volatiles, although the differences were not significant. Unexpectedly, *CPI8* showed a significantly lower transcriptional expression in the HIPVs-exposed plants followed by 6 h of caterpillar damage, as compared to control plants (Fig. 4).

To investigate whether the activation of PI genes is associated with higher accumulation of PIs, the contents of TrypPI in HIPVs-exposed and control plants were measured. Pre-exposure to SSB-induced rice volatiles did not increase the contents of TrypPI before caterpillar infestation ($F_{1,24} = 0.135$, $P = 0.717$) but significantly increased the levels of TrypPI after infestation by caterpillar for 72 h or 96 h ($F_{1,24} = 48.819$, $P < 0.001$; $F_{1,24} = 22.473$, $P < 0.001$, respectively) (Fig. 5).

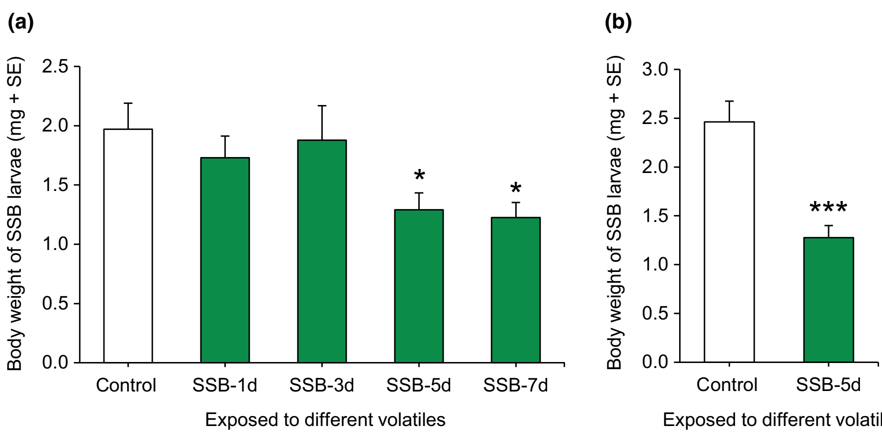

Fig. 1 Weight of striped stemborer (SSB) caterpillars fed on rice plants for 7 d that had been pre-exposed to volatiles emitted from differently treated rice plants. (a) Rice plants were exposed for 48 h to volatiles emitted from uninfested plants (control) or plants that had been infested with SSB caterpillars for 1, 3, 5, or 7 d, respectively ($n = 7$–8). (b) Rice plants were exposed for 48 h to volatiles emitted from uninfested plants (control) or plants that had been infested with SSB caterpillars for 5 d ($n = 10$–11). Neonates of SSB were allowed to feed on exposed plants for 7 d, then the body weight of SSB larvae was determined. Bars represent mean + SE. Asterisks indicate statistically significant differences between treatments relative to control (Student's t-test: *, $P < 0.05$; ***, $P < 0.001$).

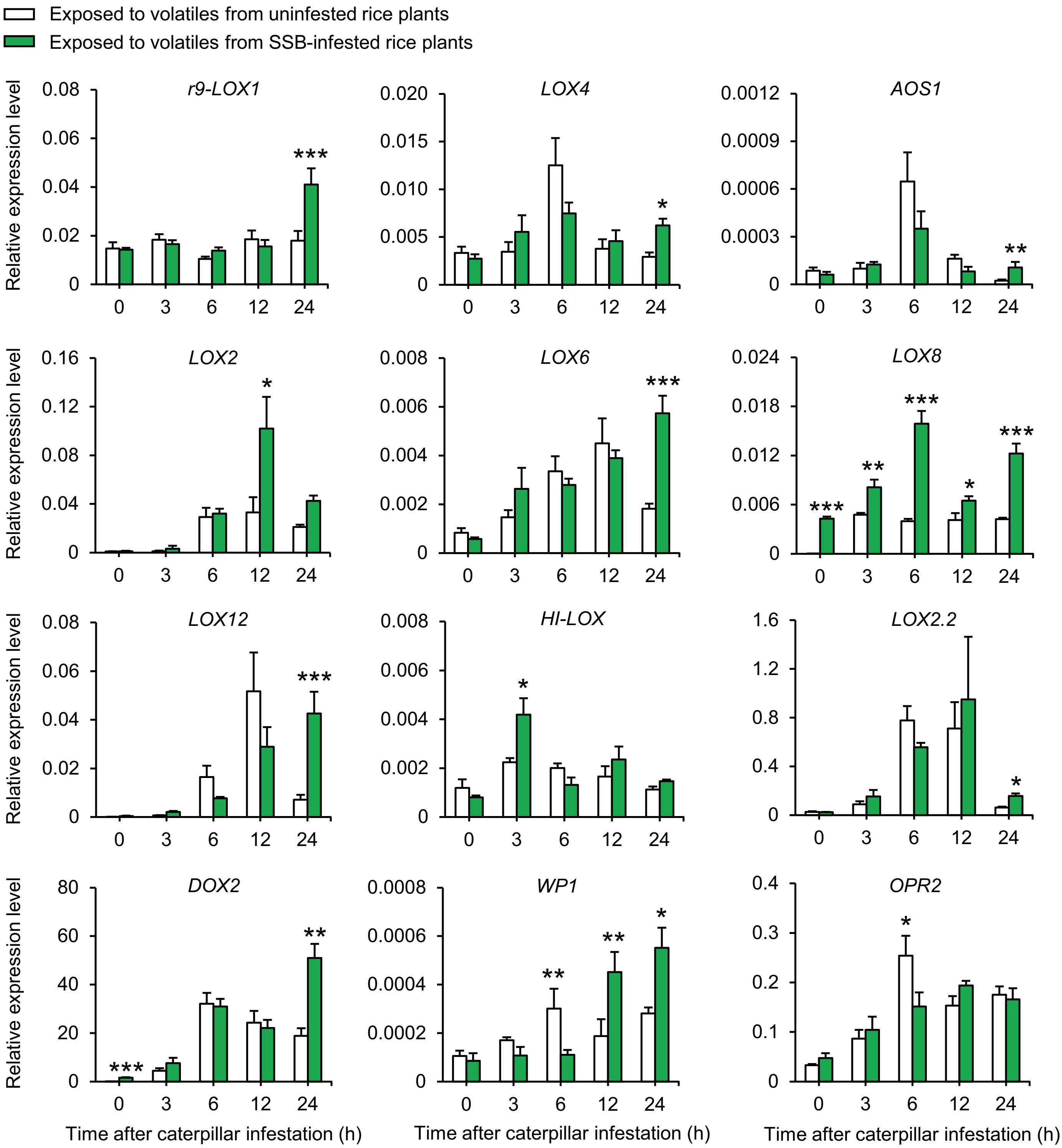

Fig. 2 Expression levels of jasmonic acid-related defensive genes in receiving plants that had been exposed to volatiles emitted from differently treated rice plants either without or with subsequent striped stemborer (SSB) caterpillar infestation at different time points. *AOS1*, allene oxide synthase 1; *DOX2*, alpha-dioxygenase 2; *HI-LOX*, lipoxygenase; *LOX12*, lipoxygenase 12; *LOX2*, lipoxygenase 2; *LOX2.2*, lipoxygenase 2.2; *LOX4*, lipoxygenase 4; *LOX6*, lipoxygenase 6; *LOX8*, lipoxygenase 8; *OPR2*, oxophytodienoate reductase 2; *r9-LOX1*, lipoxygenase 1; *WP1*, wound/stress protein 1. Bars represent mean + SE. Asterisks indicate statistically significant differences between volatile exposure treatments at different time points (n = 3–4; two-way ANOVA followed by pairwise comparisons through Bonferroni adjustment: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

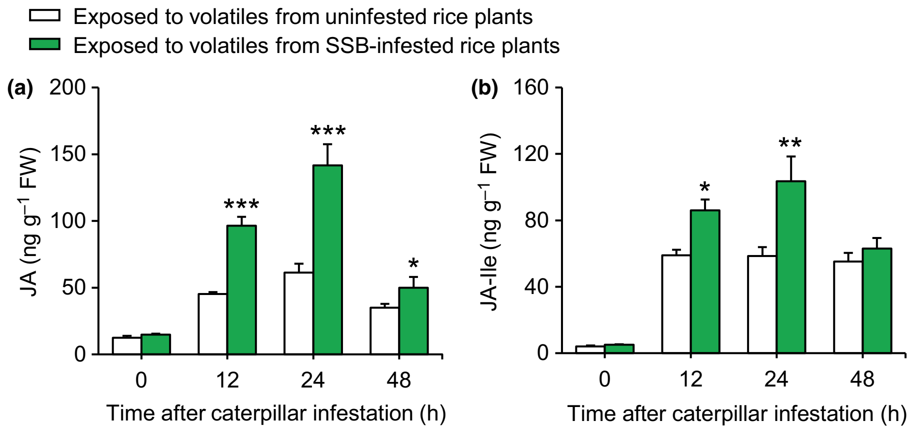

Fig. 3 The concentrations of (a) jasmonic acid (JA) and (b) jasmonoyl isoleucine (JA-Ile) in receiving plants that had been exposed to volatiles emitted from differently treated rice plants either without or with subsequent striped stemborer (SSB) caterpillar infestation at different time points. Bars represent mean + SE. Asterisks indicate statistically significant differences between volatile exposure treatments at different time points ($n = 5$; two-way ANOVA followed by pairwise comparisons through Bonferroni adjustment: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Pre-exposure to caterpillar-induced volatiles increases the attractiveness of caterpillar-infested rice plants to parasitic wasps

The results of the olfactometer assay showed no significant differences in the preference of parasitoids when given the choice between rice plants exposed to uninfested (Control) or caterpillars-infested plant volatiles (HIPVs) ($P = 0.598$). However, the wasps exhibited a stronger preference for rice plants pre-exposed to HIPVs that were then subjected to SSB-infestation (HIPVs + SSB) than those pre-exposed to uninfested plant volatiles followed by SSB-infestation (Control + SSB) ($P < 0.001$) (Fig. 6).

Pre-exposure to caterpillar-induced volatiles increases volatile emissions in rice plants

A total of 19 compounds were detected in the headspace of rice plants pre-exposed to volatiles from uninfested rice plants (Control), whereas 20 compounds were detected for rice plants pre-exposed to volatiles from SSB-infested rice plants (HIPVs). Compared with control plants, plants pre-exposed to HIPVs emitted larger amounts of several volatile compounds, such as acetophenone, dodecane, tetradecane, and hexadecane (Table S2). Pre-exposure to HIPVs changed the composition and contents of rice volatiles after subsequent SSB infestation. A total of 27 compounds were detected in the headspace of plants that were pre-exposed to volatiles from uninfested rice plants and then infested with SSB larvae (Control + SSB) and 29 compounds in the headspace of plants that were first exposed to SSB-induced rice plant volatiles and then infested with SSB larvae (HIPVs + SSB). The emitted relative quantities of five compounds: linalool, DMNT, ethyl benzoate (in trace amounts), methyl salicylate (MeSA), and hexadecane, were significantly higher for HIPVs pre-exposed rice plants (Table S2).

A projection to orthogonal partial least squares-discriminant analysis (OPLS-DA) using the relative amounts of all detected volatiles showed a clear separation between SSB-infested plants and uninfested plants independent of pre-exposure, as well as between plants pre-exposed to caterpillar-induced volatiles followed by SSB infestation (HIPVs + SSB) and plants pre-exposed to volatiles from uninfested plants followed by the same infestation (Control + SSB) (Fig. 7). The first two significant OPLS components explained 26.4% and 6.9% of the total variance, respectively. The first component showed a clear separation between volatile profiles of plants with SSB infestation vs plants without SSB infestation independent of pre-exposure, while the second component separated volatile profiles released by plants pre-exposed to caterpillar-induced volatiles followed by SSB infestation vs plants pre-exposed to volatiles from uninfested plants followed by the same infestation. However, the first two components do not separate the plants pre-exposed to caterpillar-induced volatiles vs control plants (Fig. 7).

Responses of female *C. chilonis* wasps to single inducible volatile compounds

In the olfactometer, female parasitoids were attracted to linalool and DMNT whether at a low, medium, or high dose (all $P < 0.01$) (Fig. 8a,b). MeSA was attractive at a low dose ($P = 0.040$) but was repellent at a high dose ($P = 0.029$) (Fig. 8c). Hexadecane had no effects on parasitoids attraction at any dose (Fig. 8d).

Discussion

Plants have the ability to eavesdrop on the volatile signals emitted by the neighboring conspecific plants (Baldwin *et al.*, 2002; Dudareva *et al.*, 2006; Heil & Ton, 2008; Heil & Karban, 2010; Karban *et al.*, 2014). Upon perceiving volatiles from plants attacked by herbivores or pathogens, plants have been found to prepare themselves for the incoming threat and show enhanced activation of defensive responses once being harmed by the same attacker (Engelberth *et al.*, 2004; Ton *et al.*, 2007; Delory *et al.*, 2016; Turlings & Erb, 2018; Hu, 2021). Here, we provide another example whereby pre-exposure to SSB caterpillar-induced rice volatiles boosts direct (increased plant toxicity to caterpillars) and indirect (enhanced attractiveness to parasitoids) resistance in neighboring rice plants upon subsequent caterpillar infestation. In virtually all cases, the differences in plant defense traits between plants exposed

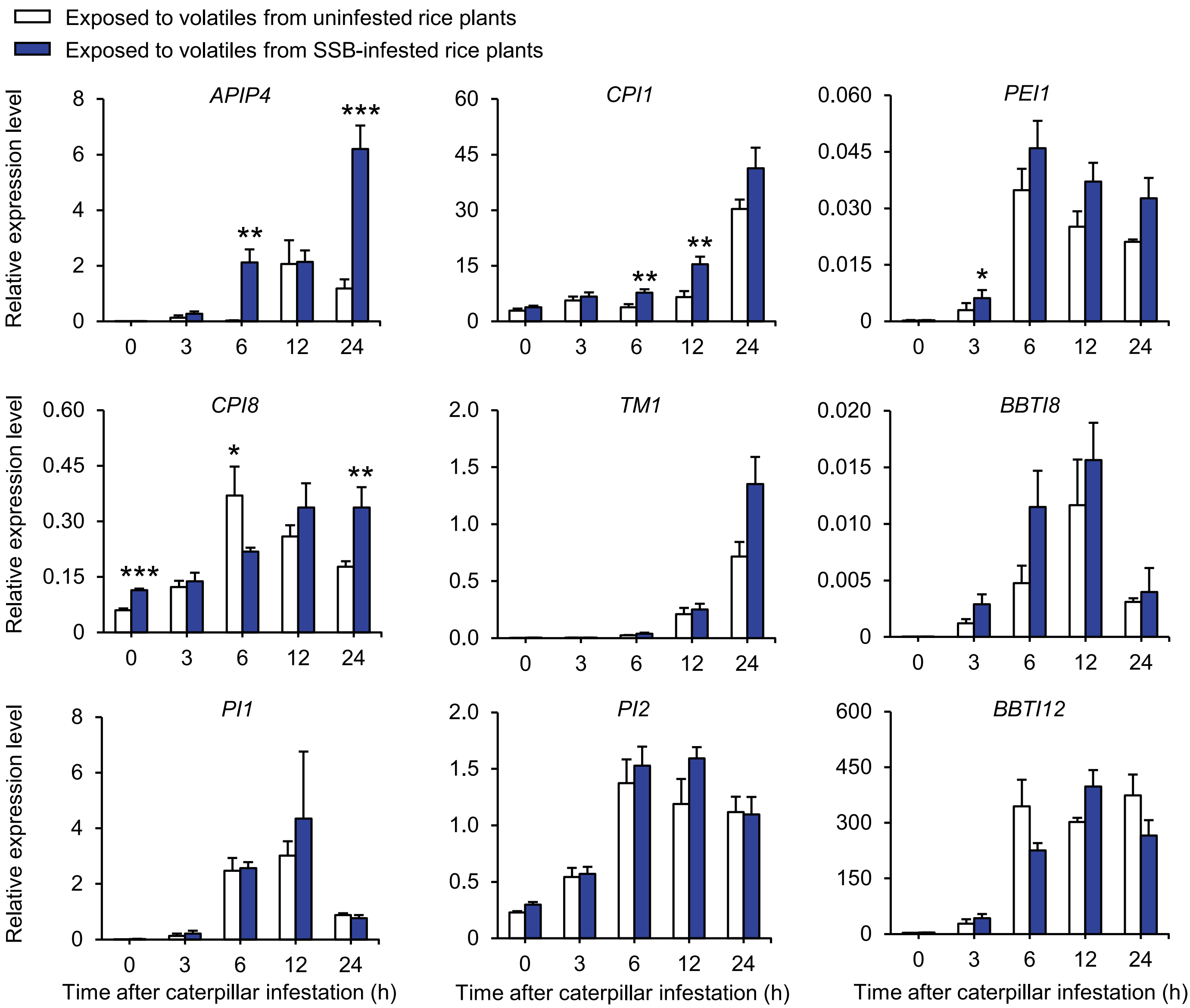

Fig. 4 Expression levels of protease inhibitor (PI)-related defensive genes in receiving plants that had been exposed to volatiles emitted from differently treated rice plants either without or with subsequent striped stemborer (SSB) caterpillar infestation at different time points. *APIP4*, Bowman–Birk inhibitor AvrPiz-t interacting protein 4; *BBTI12*, Bowman–Birk trypsin inhibitor 12; *BBTI8*, Bowman–Birk trypsin inhibitor 8; *CPI1*, cysteine proteinase inhibitor 1; *CPI8*, cysteine proteinase inhibitor 8; *PEI1*, pectinesterase inhibitor 1; *PI1*, protease inhibitor 1; *PI2*, protease inhibitor 2; *TM1*, thaumatin 1. Bars represent mean + SE. Asterisks indicate statistically significant differences between volatile exposure treatments at different time points (n = 3–4; two-way ANOVA followed by pairwise comparisons through Bonferroni adjustment: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

to HIPVs and control plants were measurable only after insect attack, implying the HIPVs triggered priming, rather than induction. The only exception was a slight upregulation of three defense-related genes (*LOX8*, *DOX2*, and *CPI8*) also before the exposed plants were subjected to insect attack (Figs 2, 4). Importantly, with chemical and molecular analyses, this study also reveals a key part of the underlying processes of the volatile-mediated priming in rice plants.

In previous work, we showed that the growth of SSB larvae is considerably impaired when they feed on rice plant that has already been attacked by conspecifics (Q. S. Liu *et al.*, 2021). In the current study, this negative effect on SSB caterpillar performance was further enhanced when caterpillars fed on rice plants that had been pre-exposed to volatiles emitted by SSB-infested conspecific plants (Fig. 1). We therefore speculated that SSB-induced volatiles may trigger the similar defense-related pathways in the receiving rice plants as in the plants infested by the caterpillars. Our subsequent experiments indeed showed that pre-exposure to SSB-induced rice volatiles led to an earlier and/or stronger expression of several JA signaling genes, and consequently a higher accumulation of JA and JA-Ile in the receiving plants when they themselves were infested with SSB larvae (Figs 2, 3). These results support our hypothesis and are consistent with results obtained for other plants (Erb, 2018). A

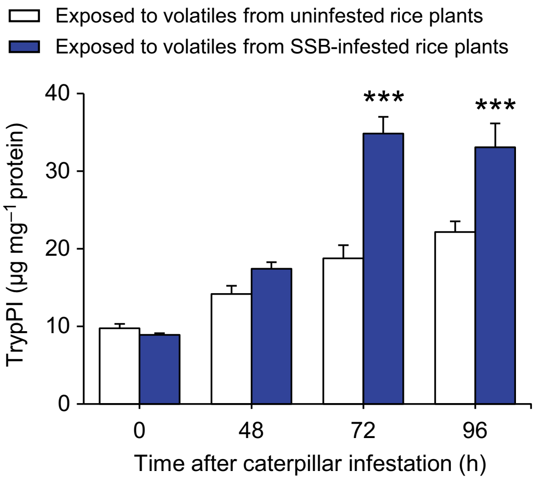

Fig. 5 The content of TrypPI in receiving plants that had been exposed to volatiles emitted from differently treated rice plants either with or without subsequent striped stemborer (SSB) caterpillar infestation at different time points. Bars represent mean + SE. Asterisks indicate statistically significant differences between volatile exposure treatments at different time points ($n = 4$; two-way ANOVA followed by pairwise comparisons through Bonferroni adjustment: ***, $P < 0.001$).

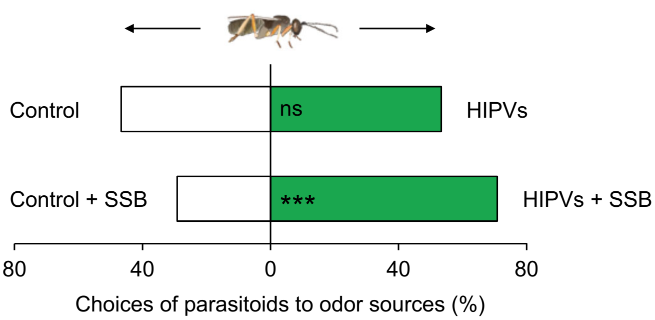

Fig. 6 Behavioral responses of females *Cotesia chilonis* to rice plants that had been exposed to volatiles from differently treated conspecific plants. Rice plants exposed to volatiles from uninfested rice plants (Control) vs rice plants exposed to volatiles from striped stemborer (SSB)-infested rice plants (herbivore-induced plant volatiles (HIPVs)). Rice plants exposed to volatiles from uninfested rice plants followed by infestation with two 4th-instar SSB larvae for 6 h (Control + SSB) vs rice plants exposed to volatiles from SSB-infested rice plants followed by infestation with two 4th-instar SSB larvae for 6 h (HIPVs + SSB). The bars are average percentages of parasitoids choosing the Y-tube olfactometer arm. Asterisks indicate significant differences between the treatment pairs for olfactometer assays ($n = 80$–100; binomial test: ***, $P < 0.001$; ns, not significant).

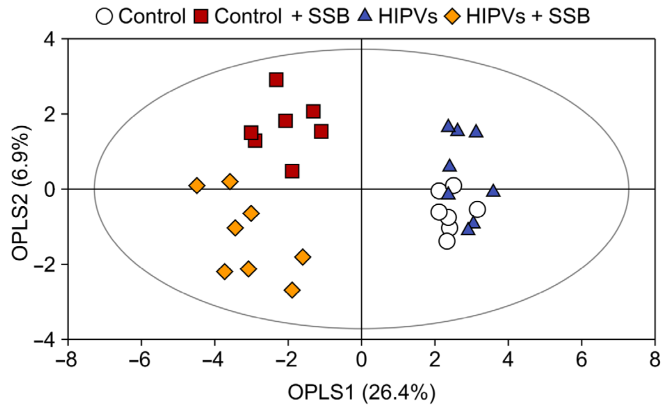

Fig. 7 Orthogonal partial least squares-discriminant analysis (OPLS-DA) of the relative quantities of volatiles emitted by rice plants. The plants were either exposed to volatiles from uninfested rice plants (Control), from striped stemborer (SSB)-infested rice plants (herbivore-induced plant volatiles (HIPVs)), from uninfested rice plants and then infested with SSB larvae (Control + SSB), or from SSB-infested rice plants and then infested with SSB larvae (HIPVs + SSB). The score plot display the grouping pattern according to the first two components and the ellipse defines the Hotelling's T2 confidence interval (95%) for the observations.

study by Ye *et al.* (2019) had already shown that exposure of rice plants to the common aromatic HIPV indole can directly enhance the expression of the leucine-rich repeat-receptor-like kinase *OsLRR-RLK1*. This response leads to priming of various defense-related genes and results in JA accumulation after herbivore attack (Ye *et al.*, 2019). Here, we show a key role of TrypPI downstream of this priming effect, similar to the priming effect of HIPVs on a TrypPI in poplar (Frost *et al.* 2008b). It was already known that TrypPIs are key rice defense compounds that negatively affect the performance of SSB caterpillars and that the JA signal transduction pathway is responsible for the production of TrypPI in rice plants (Q. S. Liu *et al.*, 2021). We therefore tested and confirmed that the upregulated expression of TrypPI genes and a higher accumulation of TrypPI in rice plants explained the increased resistance after exposure to SSB-induced rice volatiles compared with plants exposed to volatiles from uninfested plants following SSB attack (Figs 4, 5).

The phenomenon that HIPVs emitted from a plant attacked by an herbivore can enhance the direct resistance of its neighboring plants against the same herbivore has been found for multiple plant species (Karban *et al.*, 2014). For instance, exposure to volatiles emitted from maize plants infested by *Mythimna separata* (Walker) (Lepidotera: Noctuidae) caterpillars results in increased resistance in neighboring plants (Ali *et al.*, 2013). The development of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) caterpillars is negatively affected when they feed on maize plants that have been exposed to volatiles emitted from *S. littoralis* caterpillar-infested maize plants (Ton *et al.*, 2007). Exposure to the volatile compound DMNT emitted from *E. obliqua*-infested tea plants induces the accumulation of JA and thus promotes resistance in neighboring plants to the same herbivore (Jing *et al.*, 2021). Although rice is one of the world's most important staple crops, and the interactions among individual plants should be particularly important due to dense planting practices, HIPVs-mediated rice–rice interactions have rarely been studied (see Ye *et al.*, 2019).

Herbivore-induced plant volatiles not only prime direct defense responses but also trigger indirect resistance, for instance in the form of volatiles that attract natural enemies of the herbivorous insects (Turlings & Erb, 2018). Our previous study showed that rice plants attacked by SSB caterpillars emit a large number and quantity of volatiles and are more attractive to the parasitoid *C. chilonis* (Liu *et al.*, 2015). Because pre-exposure to SSB-induced volatiles enhanced the expression of JA signaling

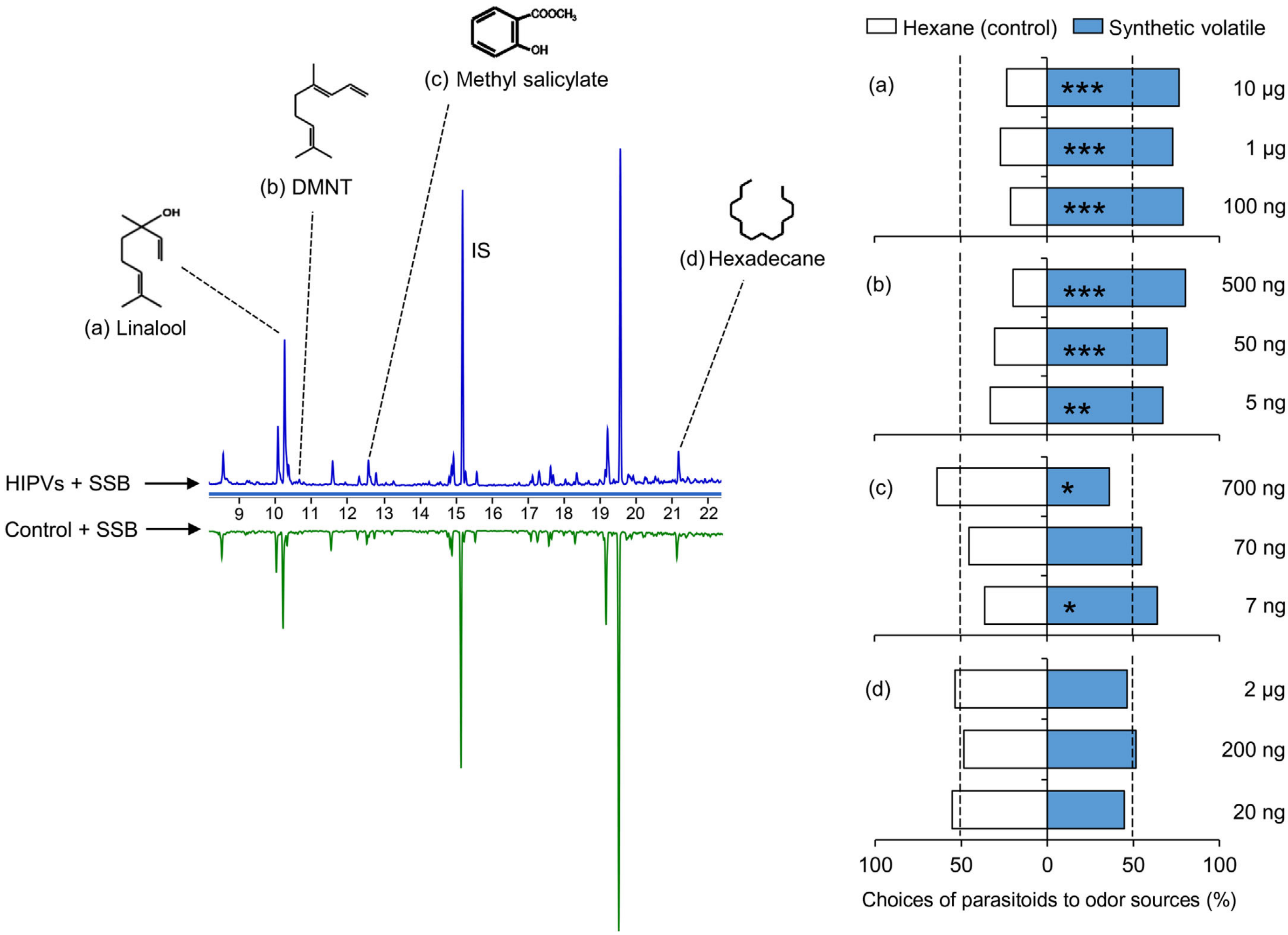

Fig. 8 Chromatograms of GC-MS headspace analyses of rice plants that had first been exposed to caterpillar-infested volatiles and were then infested with striped stemborer (SSB) larvae (herbivore-induced plant volatiles (HIPVs) + SSB) as compared to rice plants that had first been exposed to uninfested rice volatiles and were then infested with SSB larvae (Control + SSB). IS, internal standard. In the right of diagram, behavioral responses of female *Cotesia chilonis* wasps to individual inducible volatile compound (a–d: right hand panel). The synthetic volatile compounds were individually dissolved in hexane (control), and each compound was tested at three doses, including a low dose equivalent to their emissions from rice plants exposed to volatiles from SSB-infested rice plants that were subsequently infested with SSB caterpillars, a medium dose equivalent to a tenfold of the low dose and a high dose equivalent to a 100-fold of the low dose. The bars are average percentages of parasitoids choosing the Y-tube olfactometer arm. Asterisks indicate significant differences between the treatment pairs for olfactometer assays (n = 60–90; binomial test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

genes and JA content in rice plants, especially when the exposed plants were then themselves infested with the caterpillars (Figs 2, 3), we hypothesized that pre-exposure to SSB caterpillar-induced rice volatiles would also enhance the attractiveness of receiving rice plants following caterpillar infestation to female *C. chilonis*. Indeed, rice plants pre-exposed to SSB-induced rice volatiles were more attractive to the parasitoid, than nonprimed plants if they were subsequently subjected to caterpillar infestation, but not if they were left unharmed (Fig. 6). This was consistent with results from the volatile collections; after HIPV-exposure and subsequent infestation, rice plants emitted larger amounts of linalool and MeSA, and they released two new compounds, DMNT and ethyl benzoate, which were not detected in the headspace of uninfested plants (Table S2). Linalool, MeSA (at a low dose), and DMNT were confirmed to be attractive to *C. chilonis* females (Fig. 8). In fact, linalool and DMNT have been widely reported to be attractants to different parasitoid species. For example, linalool attracts *Anagrus nilaparvatae* (Pang et Wang) (Hymenoptera: Mymaridae), an egg parasitoid of the brown planthopper of *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae) (Hu *et al.*, 2020), *Campoletis chlorideae* (Uchida) (Hymenoptera: Ichneunmonidae), a larval endoparasitoid of many noctuid species (Yan & Wang, 2006), and *Aphidius ervi* (Haliday) (Hymenoptera: Aphidiidae), a parasitoid of the pea aphid *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae) (Du *et al.*, 1998). (*E*)-4,8-dimethyl-1,3,7-nonatriene has been linked to the attraction of *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), a generalist parasitoid of caterpillars (Turlings & Tumlinson, 1992), *A. nilaparvatae* (Hu *et al.*, 2020), and *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae), an endoparasitoid of multiple lepidopteran larvae (D. F. Liu *et al.*, 2021). A previous study also found that exposure to

S. littoralis-induced maize volatiles enhanced levels of aromatic and terpenoid volatiles after defense elicitation, which led to enhanced attraction of *C. marginiventris* wasps, but the specific compounds responsible for the attraction were not further identified (Ton *et al.*, 2007).

Several plant hormone-associated signaling pathways are known to be involved in the production of herbivore-induced volatiles and are therefore key to plant–insect and plant–plant interactions (Ozawa *et al.*, 2000; Van Poecke & Dicke, 2002; Hu, 2021). For example, MeJA-treated transgenic rice plants that overexpress *OsTPS3* produce more (*E*)-*β*-caryophyllene, which results in enhanced attraction of the parasitoid wasp *A. nilaparvatae* (Cheng *et al.*, 2007). When transgenic *Arabidopsis* plants that only constitutively emitted (3*S*)-*E*-nerolidol were sprayed with JA, (*E*)-DMNT was detected in the headspace and attracted carnivorous predatory mites *Phytoseiulus persimilis* (Athias-Henriot) (Acarina: Phytoseiidae) (Kappers *et al.*, 2005). It is also known that HIPVs can prime defense hormone production in exposed plants, causing an enhanced hormone production and volatile emission in the exposed plant, but only when it itself is attacked (Frost *et al.*, 2008b). For instance, exposure to indole from *S. littoralis*-infested plants significantly increased JA and ABA levels in early stages and then enhanced terpenoids volatile emissions in neighboring maize after elicitation (Erb *et al.*, 2015). Similarly, exposure to volatiles emitted by tobacco plants infested by *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) or *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) enhances JA accumulation and subsequent volatile emissions after simulated herbivory (Timilsena *et al.*, 2019). Amongst the different plant defense hormones, JA has been widely considered to be the major plant hormone involved in plant defense signaling and mediating emission of plant volatiles (Dicke *et al.*, 1999; Lou *et al.*, 2005). Our current results suggest that the indirect resistance in rice plants primed by SSB-induced volatiles is also controlled by the JA pathway. This is in line with previous findings that exogenous application of JA induces volatile emissions in rice and enhances the attraction of the natural enemies of the herbivores (Lou *et al.*, 2005).

Despite the considerable evidence that HIPVs-mediated plant–plant interactions are of significant ecological importance (Kost & Heil, 2006; Frost *et al.*, 2007; Karban *et al.*, 2013; Meents *et al.*, 2019; Zhang *et al.*, 2020; Jing *et al.*, 2021; Yao *et al.*, 2021), little is known about the exact signals or the biochemical and molecular responses that are involved. We here show that in rice, exposure to HIPVs strongly primes the JA pathway, resulting in enhanced production of TrypPI and specific volatiles upon insect attack. This renders these primed plants more resistant to caterpillars and more attractive to their natural enemies when they themselves are under attack by SSB caterpillar. As rice is a highly densely planted crop that suffers attacks by multiple insect pest species, priming is expected to be particularly important in this plant that feeds billions. Understanding the details of the underlying processes should facilitate studies to elucidate similar interactions in other systems and may lead to strategies that exploit the odorous alert signals to manage SSB and other destructive pests.

Acknowledgements

The study was supported by the National Natural Science Foundation of China (32120103009 and 31972984 to YL). The contribution by TCJT was supported by European Research Council Advanced Grant 788949.

Author contributions

YL conceived and directed the project. YL, CY and TCJT designed the study. CY and LD performed the experiments. CY, QL, XH and YL analyzed the data. CY, QL, XH, YL, WY and TCJT wrote the manuscript. All authors have read and approved the manuscript for publication.

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Data availability

The data that support the findings of the study are available in the Supporting Information of this article.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Schematic diagram depicting exposure of rice plants to volatiles emitted from caterpillar-infested or uninfested rice plants.

Table S1 Genes and primer pairs used for quantitative real-time polymerase chain reaction.

Table S2 Volatile compounds collected from the headspace of rice plants.

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