Research Article

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Differential Levels of Fatty Acid-Amino Acid Conjugates in the Oral Secretions of Lepidopteran Larvae Account for the Different Profiles of Volatiles

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Abstract

BACKGROUND: Plants have evolved sophisticated defense responses to insect herbivore attack, which often involve elicitors in the insects' oral secretions. The major eliciting compounds in insect oral secretions across different species and their potency in inducing volatile emissions have not yet been fully characterized and compared.

RESULTS: Seven lepidopteran insects with variable duration of association with maize were selected, five species known as pests for a long time (*Ostrinia furnacalis*, *Spodoptera exigua*, *Spodoptera litura*, *Mythimna separata*, and *Helicoverpa armigera*) and two newly emerging pests (*Athetis lepigone* and *Athetis dissimilis*). Oral secretions of the newly emerging pests have the highest total contents of Fatty Acid-Amino Acid Conjugates (FACs), and their relative composition was well separated from that of the other five species in principal compound analysis. Redundancy analyses suggested that higher quantity of FACs was mainly responsible for the increases in maize volatiles, of which (*E*)-3,8-dimethyl-1,4,7-nonatriene (DMNT) and (*E*, *E*)-4,-8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) were the most strongly inducible compounds. Adding FACs to the oral secretion of *S. litura* larvae significantly increased the emissions of TMTT and DMNT, confirming the key role of FACs in inducing volatile emissions in maize plants. Additional experiments with artificial diet spiked with linolenic acid suggested that variation in FACs is due to differences in internal FAC degradation and fatty acid excretion.

CONCLUSION: Compared with two newly emerging pests *A. lepigone* and *A. dissimilis*, the long-term pests could diminish the volatile emission by maize through reducing the FAC content in their oral secretions, which may lower the risk of attracting natural enemies.

Supporting information may be found in the online version of this article.

Keywords: maize; lepidopteran larvae; oral secretion; plant defense induction; plant volatiles; fatty acid-amino acid conjugates

1 INTRODUCTION

For effective pest control, it is of great importance to take advantage of the innate defences of crop plants against herbivores. Plants have evolved optimal strategies to fend off herbivorous insects to maximize their fitness.[1] Upon attack, plants can distinguish among pest insects belonging to different feeding guilds and respond by adaptively triggering the appropriate defensive signalling pathway.[2–3] Herbivore-induced plant volatiles (HIPVs) are known to attract natural enemies of the herbivores, to play a role in intra-plant and plant–plant communication and to directly affect the foraging of the herbivores.[4]

When challenged by different herbivorous insects, plants can respond by emitting different blends of HIPVs, which can be recognized by specific natural enemies.[5–9] For example, native and exotic insects, specialist and generalist, sucking and chewing insects triggered different HIPV blends in *Brassica rapa* plants.[10] An increasing number of studies indicate that the diverse volatile patterns induced by different insects depend on the specific recognition of

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oral secretion cues from herbivores.[9, 11–12] For example, oral secretions from *Mythimna loreyi* induce a higher release of volatiles by rice seedlings relative to those of *Parnara guttata*.[13] Also, in the three closely related insect species *Spodoptera frugiperda*, *Spodoptera littoralis* and *Spodoptera exigua*, oral secretions were found to induce different amounts of maize volatiles, which were thought to result from different levels of insect elicitors.[14] While several elicitors have been identified in lepidopteran insects, little is known about the composition of oral secretions in different species and their associations with plant volatile emission.

Patterns of HIPVs induced by different herbivores are closely correlated with herbivore-associated molecular patterns (HAMPs) in the respective oral secretions.[15–16] It has been shown that insect species vary in their oral secretion components, especially some important HAMPs. *Manduca sexta* have far more fatty acid-amino acid conjugates (FACs) than *S. exigua* in their oral secretion when they feed on *Nicotiana attenuata*.[17] And in the extreme case of two rice pests, the secretions of one species, *Mythimna loreyi*, contained large amounts of FACs, while none were detectable in those of the other species, *Parnara guttata*.[18] FACs are typically well-studied HAMPs in lepidopteran insects, which are able to trigger both direct and indirect plant defences through inducing mitogen-activated protein kinase (MAPK) cascade and jasmonic acid (JA) signalling pathway which results in the subsequent synthesis of secondary metabolites.[19–22] Therefore, differences in the abundance of FACs in herbivores may be responsible for the species-specific response by plants. Moreover, FACs with differential fatty acid chains and hydroxylation modification were also reported to have different capacities to induce plant defences.[23] For example, 18:3-Gln and 18:3-Glu are two of the most abundant elicitors in the regurgitant of tobacco hornworm, and 18:3-Gln displays higher activity in inducing plant volatiles than 18:3-Glu.[24–25] Furthermore, while the disulfooxy fatty acid caeliferin A16:0 is only effective in *Arabidopsis*, volicitin can trigger the JA and ethylene (ET) production in maize, soybean and eggplant, suggesting that it is able to induce plant hormone signalling in a wider range of plants.[26] The differential composition of oral secretions and distinct responses triggered by each oral secretion component of lepidopteran insects raises the question of whether the plants' response to herbivorous insects is explained by different proportions of elicitors in the secretions.

Maize is one of the most important crops worldwide and challenged by attack from various herbivores, which can cause great economic losses. Five primary pest insects and two moth species newly emerging as pests that share maize as a host plant were chosen in this study. The five primary insects included the specialist borer *O. furnacalis* (Lepidoptera: Crambidae) and the four generalist *S. exigua*, *S. litura*, *M. separata*, and *H. armigera* (Lepidoptera: Noctuidae), which were reported to damage maize for decades to centuries,[27] the two polyphagous insects *A. epigone* and *A. dissimilis* (Lepidoptera: Noctuidae) are the 'newly emerging' species that were newly discovered as maize pests for no longer than 20 years in China and have outbroken in maize fields recently (Supporting Information, Fig. S1).[28–30] We hypothesize that these seven insects differing in coexistence histories with maize produce distinct oral secretions, which account for different emissions of HIPVs by maize plants. To experimentally test this hypothesis, we aimed to determine: (i) the composition of oral secretions of the seven lepidopteran insects on maize plants; (ii) the effect of FACs on the amounts of maize volatiles released, and (iii) the effect of fatty acid metabolism on FAC contents in the oral secretions.

2 MATERIALS AND METHODS

2.1 Plant and insects

We chose larvae of seven different lepidopterous species, and use the maize inbred line (CML69) for this experiment, because the inbred line shows low degrees of phenotype variations. After germination, maize seedlings were transferred in groups of three into plastic pots, grown in a greenhouse under a 22 ± 2 °C, 14: 10 h (light: dark) regime and watered every 2 days. The maize seedlings were used in the experiment when they were 2 weeks old. Caterpillars of *S. exigua*, *S. litura*, *M. separata*, *O. furnacalis* and *H. armigera* were purchased from Henan keyun biological pesticide co. LTD. *A. lepigone* and *A. dissimilis* were kindly provided by Henan Academy of Agricultural Sciences. The insects were reared on artificial diets in climate chambers and kept under a 14 (26 °C): 10 (24 °C) (light: dark), 70% ± 5% humidity regime.

2.2 Collection of oral secretion

Following the description in Qi *et al*.,[31] we collected the oral secretions from third to fifth instar larvae reared on CML69. Caterpillars were gently squeezed to provoke regurgitation, then oral secretion droplets were collected using a micropipette and transferred to 200 μL ice-cold tubes directly. After having been quickly frozen in liquid nitrogen, the samples were stored at −80 °C until use.

2.3 FACs in oral secretion

FACs content were measured as described in Yoshinaga *et al*.[23] with some modifications. Briefly, oral secretions were centrifugated transiently to remove the insoluble parts, and then 5 μL aliquots of oral secretions were added into 95 μL methanol, and FAC content was analyzed by LC–MS (Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS). We injected 2 μL of sample solution into a reverse-phase column (ZORBAX Eclipse Plus C18, 2.1 × 100 mm, Agilent Technologies, USA) eluted with a solvent gradient of 20–90% CH_3CN containing 0.08% acetic acid, in water containing 0.05% acetic acid (0.2 mL min^{-1}, 16 min). The column temperature was maintained at 40 °C. Features were assigned to individual compounds by retention time and peak shape matching, and FACs were identified by comparison with pure compounds and previous MS/MS spectra described in Diezel *et al*.[17] The standard FACs were synthesized by WuXi AppTec. Standard curves were used to quantify FACs content. Five to six replicates were performed for each treatment.

2.4 Glucose oxidase (GOX) activity analysis

GOX catalyzes the production of H_2O_2 from glucose, which is a substrate for peroxidase. The catalytic product of H_2O_2 can be catalyzed by 4-aminoantipyrine coupling phenol, which has an absorption peak at 500 nm. GOX activity in oral secretion was measured using the glucose oxidase assay kit (GOD-2-Y) purchased from Suzhou Comin Biotechnology co. LTD. Boiled samples were set as control. Each treatment had five to six replicates.

2.5 Plant treatments

To induce volatile emission in response to the oral secretions of the different insect species, the third fully expanded leaf was mechanically wounded with a tweezer to produce an area of 0.5 cm^2 damage on both sides of the midvein. Fresh wounds were immediately treated with 5 μL oral secretion of caterpillars or H_2O. Intact plants served as control.

To confirm that the increased HIPVs emission resulted from the high abundance of FACs in oral secretions, four FAC standards

(C18:3-Glu, C18:2-Glu, C18:1-Gln and C16:1-Gln) were dissolved in 0.05% TWEEN 20 at a concentration of 100 ng μL^{-1} and then individually added to samples of oral secretion of *S. litura*, since oral secretions of *S. litura* induced the lowest amounts of maize volatiles. 10 μL mixture solution of each FAC and oral secretion of *S. litura* in a 1:1 ratio was applied on a wounding site of the maize seedling. Control plants were treated with 10 μL mixture solution of 0.05% TWEEN 20 and oral secretion of *S. litura*. The ability in inducing volatile emission was assessed by quantifying the abundance of TMTT and DMNT because these two volatiles exhibited the highest inducibility when the plants were infiltrated with oral secretion regardless of insect species. Each treatment had seven to eight replicates.

2.6 Volatile collection

Volatiles released by maize plants were collected by headspace sampling for a duration of 10 h (n = 6–9), from 9 am to 7 pm. The day before collection, maize seedlings were watered and the plastic pot with the maize seedling was carefully wrapped in aluminium foil. Light intensity was set at a constant level of 18 000 lx during the collection. Maize seedlings were then placed in a sealable glass jar (dm = 7 cm, height = 35 cm). Air purified with an activated charcoal filter was pumped through the inlet port *via* Teflon tube at a rate of 600 mL min^{-1} and headspace volatiles were collected on a trap consisting of a 10 cm glass tube, in which 100 mg 80–100 mesh PoraPakQ adsorbent was kept in place by a small quantity of fiberglass held on each side. Before collection, glass jars used for headspace sample were washed with water and organic solvent and heated to 100 °C for at least 2 h. PoraPakQ traps were rinsed with 3 mL of hexane and methylene chloride alternately. After entrainment, the filters were eluted with 500 μL hexane, 870 ng nonyl acetate was immediately added to the sample as internal standard, and stored at −20 °C temporarily until analysis.

2.7 GC–MS analysis

Headspace samples were analysed by GC–MS (Agilent Technologies 6890 N GC-5973 MSD) as described in Schnee *et al.*[32] with some modifications. The GC was equipped with a HP-5MS column (60 m × 0.25 mm, film thickness 0.25 μm, J&W Scientific, Folsom, CA, USA). After injection of 2 μL samples, the GC temperature program was as follows: temperature was kept at 50 °C for 1 min and then increased to 240 °C at 5 °C min^{-1}. Compounds were identified by retention time and peak shape matching. Volatiles were identified by the use of the mass spectral libraries NIST02 (Rev. D.04.00, Agilent Technologies, Palo Alto, CA, USA) as well as by comparison with retention time and spectra of pure compounds.

2.8 Fatty acid assimilation assay

Diets containing three different concentrations of linolenic acid were used in this experiment. The diets were prepared with 60 g of wheat bran, 12 g of whole wheat flour, 12 g of corn flour, 10 g of soybean flour, 12 g of yeast extract, 600 g of distilled water, 12 g of agar, 2 g of sorbic acid, 2 g ascorbic acid and various amounts of linolenic acid, 0, 2, and 4 mL, respectively. To avoid operational error in the preparation of diets, ultimate linolenic acid concentration was quantified using GC–MS (see below). Third instar caterpillars were fed on these three types of diets for 16 h to eliminate the influence of previous diet before experiment, and frass was discarded. Then the experiment for fatty acid assimilation assay was started, 12 larvae were put individually into Petri dishes to feed on a defined quantity of artificial diet for 24 h. After feeding, oral secretions and frass were collected from caterpillars, and the remaining diet was weighed. FACs in oral secretion were quantified by LC–MS as mentioned above and linolenic acid concentrations were measured as described in the following section.

2.9 Linolenic acid quantification

Fatty acids extracted from artificial diet or frass were analyzed as described by Xia *et al.*[33] Briefly, weighed samples were frozen and ground, and then 2 mL 97% methanol/3% H_2SO_4 and C17:0 were added, and incubated for 1 h at 80 °C. 1 mL hexane containing 0.001% butylated hydroxytoluene (BHT) was added after incubation. Fatty acids were in the organic phase, of which an 1uL aliquot was injected into GC–MS for further analysis.

Fatty acid identification and quantification were carried out by coupled gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890 N GC-5973 N MSD. The GC was equipped with an HP-5MS column as described above, helium was used as carrier gas with a constant flow rate 1 mL min^{-1}. A 1 μL sample was injected in spiltless mode. Following injection, the column temperature was held at 50 °C for 1 min, increased from 50 to 120 °C at 4 °C min^{-1}, increased from 120 to 280 °C at 20 °C min^{-1}, and hold for 8 min. Fatty acids were identified by comparing their retention times with those of authentic compounds and the spectra with those of mass spectral libraries NIST02 (Rev. D.04.00, Agilent Technologies, Palo Alto, CA, USA). Fatty acids were quantified by their areas of Selected ion monitoring (SIM) ions relative to that of the internal standard C17:0 as described by Mandal *et al.*[34]

2.10 Statistical analysis

Data analyses were performed with the statistical analysis software SPSS 20 and R 3.5.1. The effect of moth species on composition of oral secretions and on induced volatile blends were tested using one-way analyses of variance (ANOVA) with SPSS 20. Two-way analyses were made to verify the influence of linolenic acid content and herbivore species on FACs content. Student's *t*-tests were used for two-group comparisons. Multiple comparisons were carried out with Turkey's test with α set at 0.05. To examine the overall differences in the profiles of oral secretion components among different herbivores, the abundance of the detected features was subjected to principal component analysis (PCA). To examine the overall relation between profiles of oral secretion component and HIPV profiles, the relative abundance of the detected features was subjected to redundancy analysis (RDA) using different treatments as a unique explanatory variable. RDA analysis was performed using 'vegan' package by R version 3.5.1.

3 RESULTS

3.1 FACs content and GOX activity in Oral secretion of insects

To determine the differences in the composition of oral secretions among the seven species, the contents of nine FACs in the oral secretion and GOX activity were quantified. C18:2-Glu and C18:3-Glu were only detected in *A. lepigone* and *A. dissimilis* when fed on maize plants, while they were not detected in the other species (C18:3-Glu: $F_{6,31}$ = 71.903, $P < 0.001$; C18:2-Glu: $F_{6,31}$ = 242.864, $P < 0.001$). The highest contents of C18:3-Gln ($F_{6,31}$ = 84.1, $P < 0.001$), C18:2-Gln ($F_{6,31}$ = 292.2, $P < 0.001$), C18:1-Gln ($F_{6,31}$ = 133.4, $P < 0.001$), C16:1-Gln ($F_{6,31}$ = 274.9, $P < 0.001$) and C16:0-Gln ($F_{6,31}$ = 559.8, $P < 0.001$) were found in

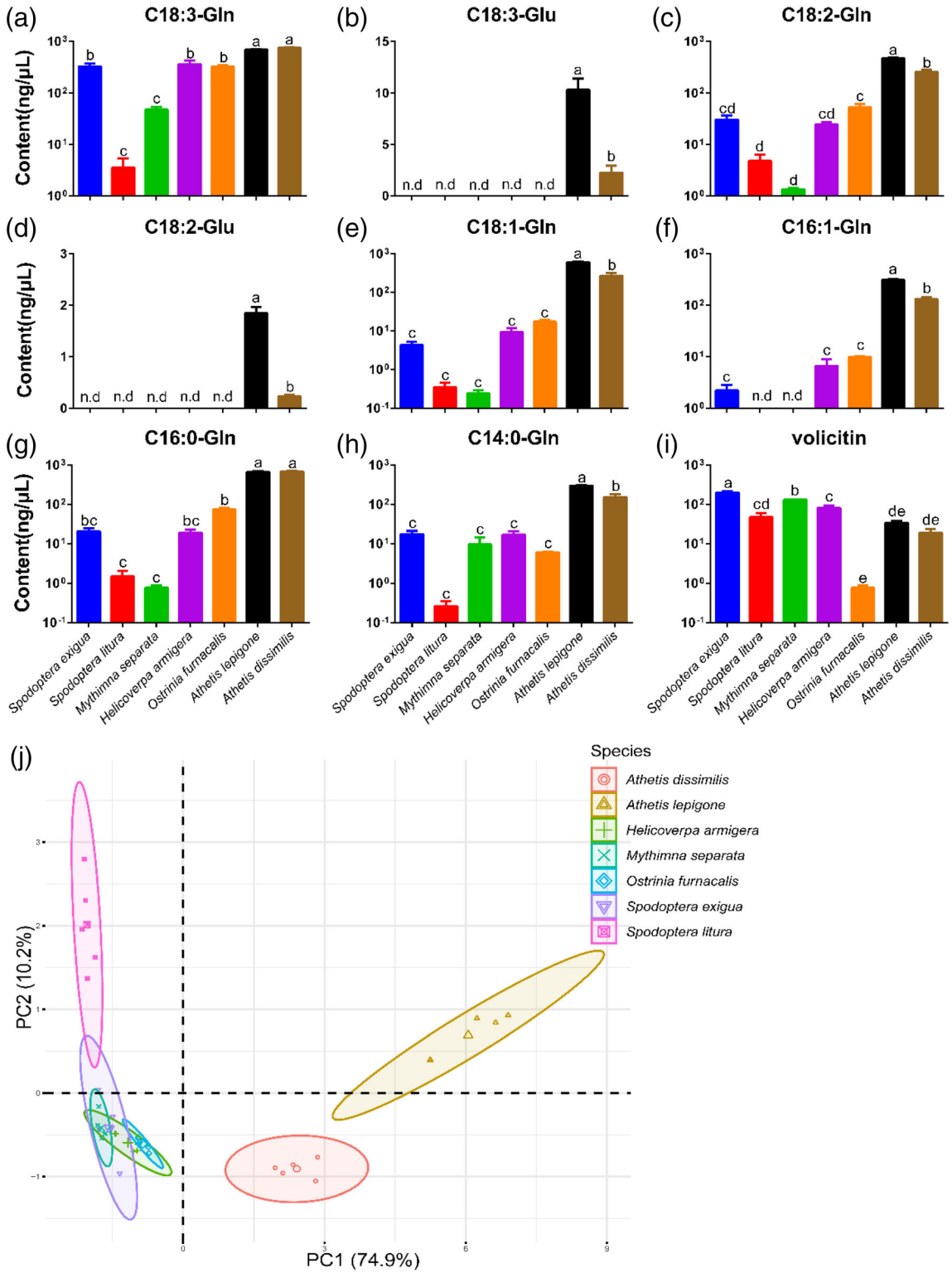

Figure 1. FACs content in the oral secretion of different lepidopteran larvae. (a–i) Concentration of nine major FAC components in the oral secretions of insects. Mean (± SE) for concentration of FACs are shown, the scale of the Y-axes are logarithmic except (b) and (d), different letters indicate significant differences between the insect species ($P < 0.05$; n = 5–6). (j) Principal component analysis (PCA) of the oral secretion components from seven insect species. The first two axes (PC1 and PC2) explained 74.9% and 10.2% of the total variation, respectively. Ellipses delimit the 95% statistical confidence areas for each biological group in the score plot.

oral secretions of *A. lepigone* and *A. dissimilis*, the lowest in those of *S. litura* and *M. separata* (Fig. 1(a)–(h)). Furthermore, oral secretion of *S. exigua* contained the highest concentration of volicitin, while *O. furnacalis* had the lowest content among the seven insect species ($F_{6,31} = 65.310$, $P < 0.001$) (Fig. 1(i)). For GOX activity, *S. litura* and *S. exigua* had higher GOX activity than other species

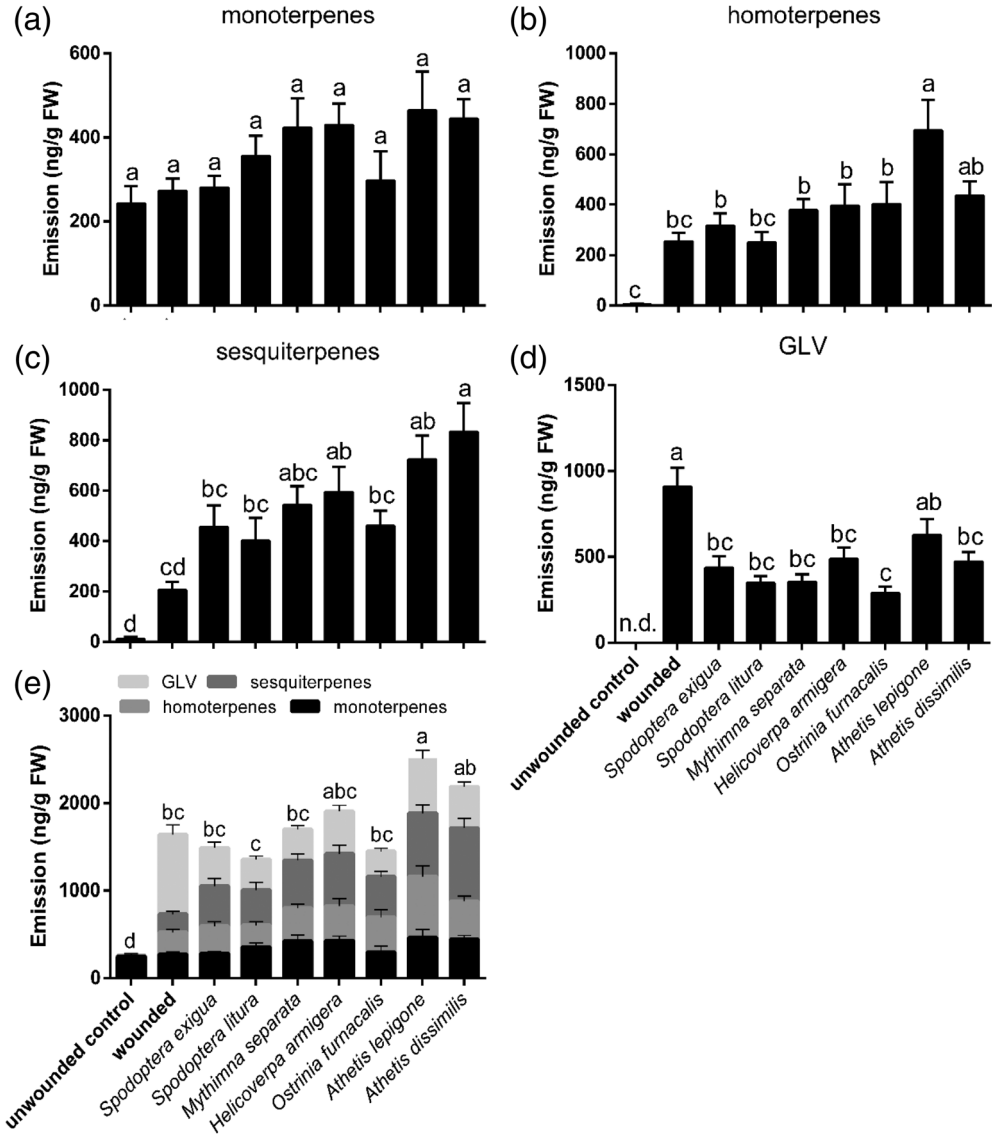

Figure 2. HIPV emissions by maize seedlings in response to treatments with oral secretions from seven herbivorous insects. (a)–(d) Total emissions of four major groups of HIPVs after elicitation: monoterpenes (α-pinene, limonene, linalool); homoterpenes (DMNT, TMTT); sesquiterpenes ((e)-α-bergamotene, (e)-β-farnesene); green leaf volatiles ((z)-3-hexenal, (Z)-3-hexenol, (z)-3-hexenyl acetate), respectively. (e) Total amount of HIPVs, all four major categories combined. Mean (±SE) for amount of HIPVs are shown. Different letters indicate significant differences among insect species ($P < 0.05$; n = 5–6). For individual volatiles, see Supporting Information, Table S2.

(Supporting Information, Fig. S2). PCA analysis revealed that *S. exigua*, *S. litura*, *M. separata*, *O. furnacalis* and *H. armigera* were clustered together, suggesting these five insect species shared higher similarity for their oral FAC components than *A. lepigone* and *A. dissimilis* (Fig. 1(j), Supporting Information, Table S1).

3.2 Plant volatiles induced by Oral secretions

To determine the effect of oral secretions on the induction of plant volatiles, the volatiles released by maize treated with oral secretion of different insect species were collected and quantified (Supporting Information, Table S2). The ten most abundant plant volatiles were then selected for further analysis and divided into four groups: the monoterpenes α-pinene, linalool, limonene, the sesquiterpenes (E)-α-Bergamotene and (E)-β-Farnesene, the homoterpenes DMNT and TMTT, and the green leaf volatiles (GLVs) (Z)-3-Hexenal, (Z)-3-Hexenol, (Z)-3-Hexenyl acetate (Fig. 2). Mechanical wounding and oral secretion treatment from seven insects significantly increased sesquiterpenes, homoterpenes and GLVs when compared with healthy plant control (monoterpenes: $F_{8,60} = 2.584$, $P = 0.017$; sesquiterpenes: $F_{8,60} = 10.378$, $P < 0.001$; homoterpenes: $F_{8,60} = 8.67$, $P < 0.001$; GLVs: $F_{8,60} = 8.517$, $P < 0.001$) (Fig. 2(a)–(d)). Treatment with oral secretion from *A. lepigone* increased the emission of homoterpenes and sesquiterpenes, treatment with oral secretion from *H. armigera* and *A. dissimilis* significantly increased the release of sesquiterpenes when compared with mechanical wounding (Fig. 2(b), (c)). By contrast, except for *A. lepigone*, treatment with oral secretion from all other insect species suppressed emission of GLVs released from maize when compared to the wounding treatment (Fig. 2(d)). Among all the seven insect species, only *A. lepigone* oral secretion significantly increased total volatile emission when compared with wounding treatment (Fig. 2(e)).

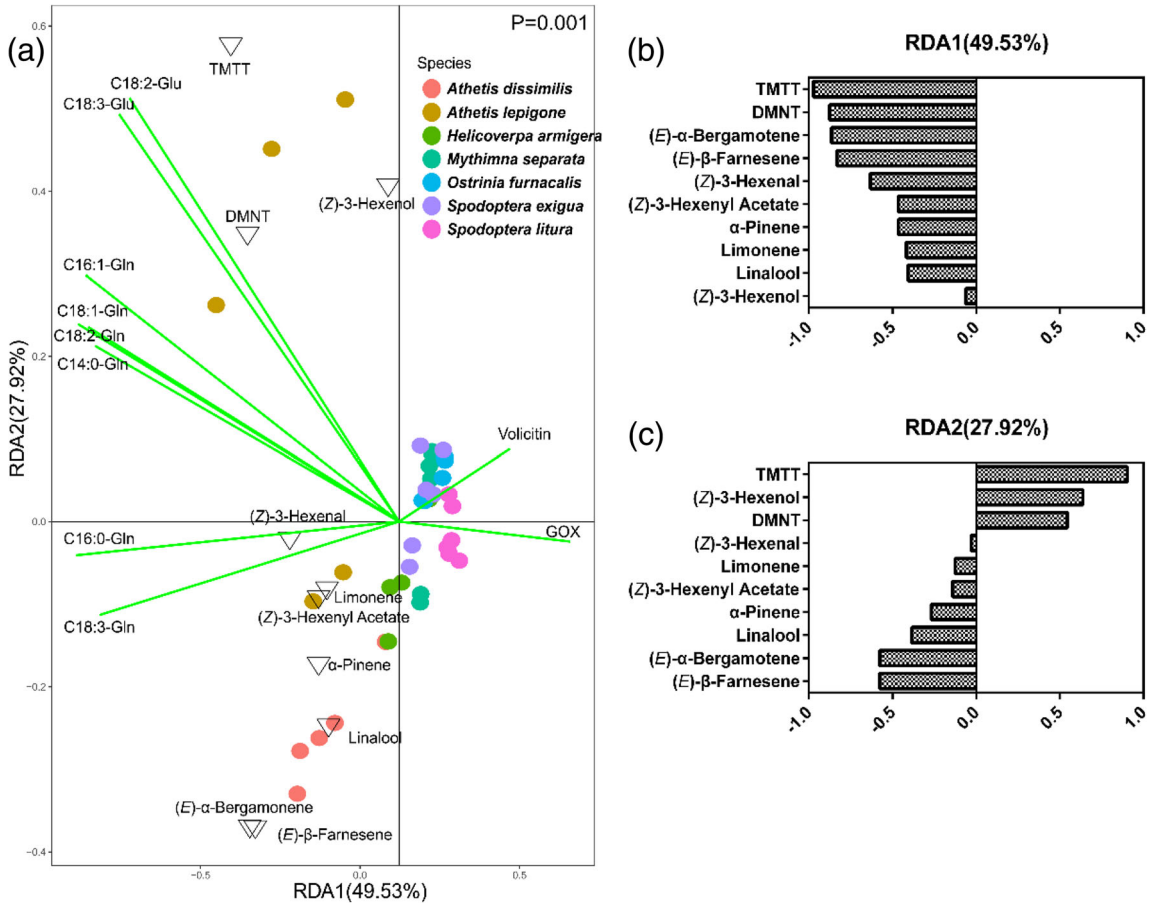

Figure 3. Composition of the oral secretion of the seven moth species shape the volatile response of maize. (a) Redundancy analysis (RDA) of the maize volatile response to different compounds in the oral secretions. Model significance is indicated above the plots. The first two axes explain 49.53% and 27.92% of the total variation, respectively. Axes report the proportions of constrained variation explained by the constrained axes. Data points represent individual replicates. (b, c) The ordination of volatiles explained for the total variance in the first two axes. The ordination obtained revealed that HIPVs of maize were differently induced by FACs.

3.3 Impact of the composition of oral secretions on volatile release

Redundancy analysis (RDA) was conducted to evaluate the effect of oral secretion on maize volatile composition. All the applied variables account for 49.53% and 27.92% of the total variance in volatile emission in the first two axes, respectively. Overall, FACs displayed strong positive correlation with volatile emission of maize. The strongest positive correlation was observed between the oral secretion components C18:3-Glu, C18:2-Glu and TMTT, DMNT and (Z)-3-Hexenol. Moreover, C18:3-Gln and C16:0-Gln mostly induced the emission of the other seven volatiles (Fig. 3(a)).

The RDA ordination of volatiles in the first two axes is presented in Fig. 3(b), (c), highlighting that TMTT and DMNT ranked among the top three compounds in both axes. This suggests that TMTT and DMNT were most strongly released in response to oral secretions compared with other volatiles.

3.4 Effect of exogenous application of individual FAC components on plant volatiles

Previous studies found that some individual FACs significantly increased plant volatiles, but it was unclear that the changes of individual FAC could affect the elicitor role of the whole oral secretion. Individual pure FAC compounds were added into oral secretion of *S. litura* to examine their activity in inducing TMTT and DMNT. Four individual FACs were selected (C18:3-Glu, C18:2-Glu, C18:1-Gln and C16:1-Gln) as they showed a highly positive correlation with DMNT and TMTT according to RDA analysis. Plants that were treated with oral secretion of *S. litura* combined with individual FACs showed higher volatile emissions, which were similar to those of plants infiltrated with oral secretion of insects with high FAC content (Fig. 4(a)). The exogenous application of C18:3-Glu ($F_{1,13}$ = 1.737, P = 0.001), C18:2-Glu ($F_{1,13}$ = 0.137, P = 0.006), C18:1-Gln ($F_{1,12}$ = 5.183, P = 0.009) and C16:1-Gln ($F_{1,12}$ = 2.573, P = 0.002) enhanced the amount of TMTT emitted (Fig. 4(a)), while application of C18:2-Glu ($F_{1,13}$ = 0.495, P = 0.002), C18:1-Gln ($F_{1,12}$ = 8.395, P = 0.025) and C16:1-Gln ($F_{1,12}$ = 6.117, P = 0.01) increased the amount of DMNT released by maize plants, respectively (Fig. 4(b)). These results suggested that increased FACs triggered higher content releases of DMNT and TMTT in maize plants.

3.5 The relationship between fatty acid ingestion and FAC content

Since the fatty acid chain of FACs is derived from the diet,[35] we speculated that exogenously supplementing fatty acids could increase FAC content in oral secretion of insects. To verify this hypothesis, *S. exigua* and *A. dissimilis* that had extremely distinct

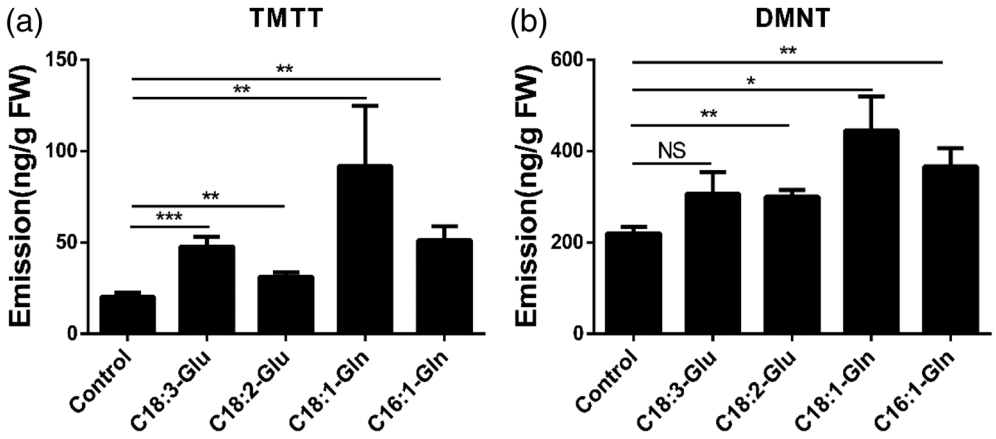

Figure 4. Effect of exogenous application of FACs on volatile release of maize. Maize seedlings were treated with *S. litura* oral secretions combined with individual FACs: C18:3-Glu, C18:2-Glu, C18:1-Gln and C16:1-Gln, *S. litura* oral secretion combined with solvent serving as control. The amounts of TMTT (a) and DMNT (b) (mean ± SE) induced by oral secretion combined with exogenous FACs are presented. Asterisks indicate statistical differences in the amounts released between the control and FAC-treated plants. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS: not significant; n = 7–8).

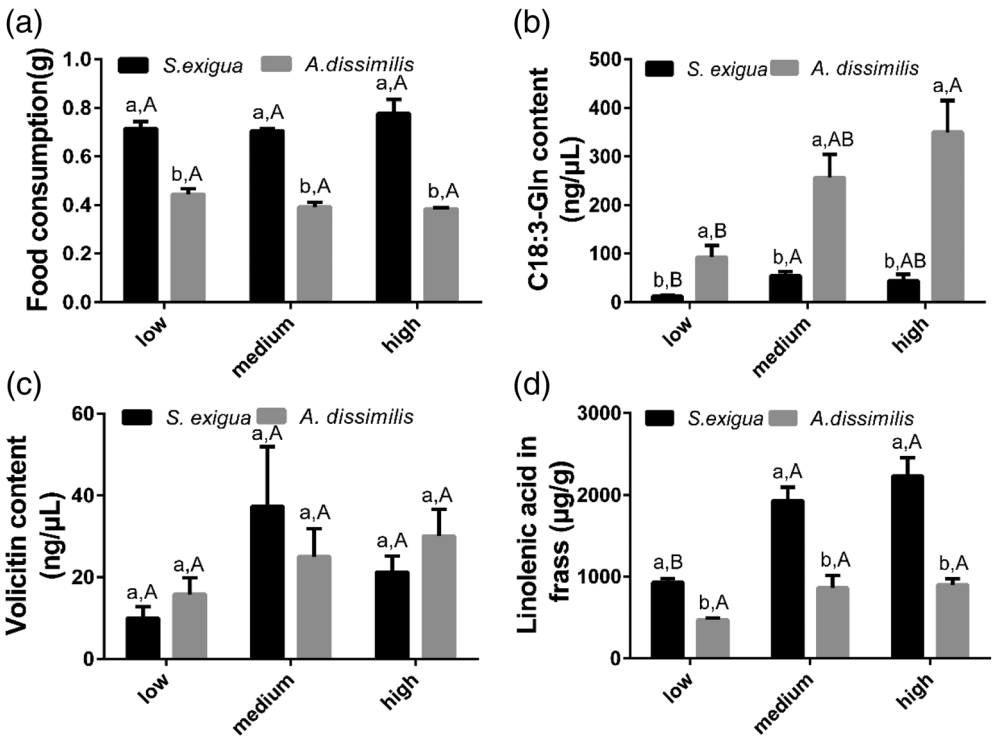

Figure 5. The effect of supplemented linolenic acid on FAC contents of oral secretions. *S. exigua* and *A. dissimilis* were fed on artificial diets enriched with three concentrations of linolenic acid, which defined as low, medium and high. Quantified linolenic acid concentrations are indicated in the Supporting Information, Fig. S3. (a) Food consumption over 24 h. (b, c) The content of C18:3-Gln and volicitin in oral secretions of the two insect species after feeding on artificial diet for 24 h. (d) Linolenic acid excreted in the frass. Different lowercase letters indicate significant differences between the two insects within the same concentrations of linolenic acid, and different uppercase letters indicate significant differences within three concentrations of linolenic acid in the same insect ($P < 0.05$, n = 3–4). Means and standard errors are shown.

FAC contents were selected to determine the effect of application of variable concentrations of linolenic acid in the diet on FAC content (Supporting Information, Fig. S3). With increased linolenic acid in artificial diets, the food consumption and volicitin content in oral secretions were not influenced ($F_{2,5} = 0.763$, $P = 0.488$) (Fig. 5(a), (c)), while C18:3-Gln content was significantly increased ($F_{2,5} = 7.502$, $P = 0.007$) (Fig. 5(b), Supporting Information, Table S3), indicating that the fatty acid ingested was an important factor to affect C18:3-Gln content. The food consumption of *S. exigua* was greater than that of *A. dissimilis* regardless of linolenic acid concentration in artificial diets ($F_{1,5} = 185.534$, $P < 0.001$) (Fig. 5(a)), but its oral C18:3-Gln content was much lower than that of *A. dissimilis* ($F_{1,5} = 40.851$, $P < 0.001$) (Fig. 5(b), Supporting Information, Table S3). Furthermore, *S. exigua* showed a considerably higher linolenic acid concentration in frass than *A. dissimilis* ($F_{1,5} = 70.701$, $P < 0.001$) (Fig. 5(d)), indicating that decreased FAC content in oral secretion of *S. exigua* was resulting from its faster rate of surplus fatty acid excretion.

4 DISCUSSION

Herbivore-induced plant volatiles can vary considerably depending on the identity of the attacking herbivore.[9, 36] Here, we demonstrated that seven lepidopteran pest species exhibit different FACs profiles in their oral secretions when fed on maize plants, which in turn induces distinct HIPVs emissions. Among seven lepidopteran insects, the newly emerging maize pests *A. lepigone* and *A. dissimilis* exhibited much higher FAC secretion and triggered higher amounts of HIPVs than the tested primary maize pest species. This result suggests that, compared with two newly emerging pests, the primary pest avoids secreting FACs and minimizes HIPV emission by the host plant as a consequence of their long coexistence history with the host plant.

The interaction between plants and herbivores originated 350 million years ago. The variable duration of the evolutionary association between herbivore and its host plant could affect plant defence and herbivore counteradaptation.[37–39] According to the phytochemical coevolution hypothesis, herbivores with long coexistence time with host plants have greater tolerance or better detoxification of the host toxins.[40–42] *Plutella xylostella*, a pest on cruciferous plants, encodes three genes with glucosinolate sulfatase activity which are responsible for the detoxification of different types of glucosinolates, and such gene duplication in the *P. xylostella* genome has evolved under positive selection during the long-time interaction with host plants.[43] However, for those associations with a short coexistence time, herbivory may provoke a stronger plant defence response. For example, *Spodoptera frugiperda* has been divided into a rice strain and a corn strain, and these two genetic subgroups were separated according to their coexistence history with certain host plants. The corn strain larvae could induce more cyanogenic compounds in *Cynodon* spp., a grass species closely related to rice, than the rice strain larvae.[44]

Here, seven insects from primary insects to emerging pests of maize were selected in our research. On the basis of phytochemical coevolution hypothesis, due to the relatively shorter coexistence time between the two newly emerging pests *Athetis* species and maize, we speculated that the oral secretions of *Athetis* species would trigger stronger volatile emission by maize plants than the five primary insects. In accordance with our hypothesis, maize leaves treated with the oral secretion of *A. lepigone* and *A. dissimilis* released significantly higher amounts of sesquiterpenes and homoterpenes, especially DMNT and TMTT, which have been well documented to function both in attracting natural enemies and in strengthening plant–plant communication.[9, 45–47] Therefore, the oral secretion of two newly emerging herbivorous insects could be more easily detected by host plants, which in turn release more HIPVs to attract natural enemies, which would prevent the newly emerging insects from forming a stable feeding niche on the new host plants. Because of the limited insect species chosen in our research, more newly emerging insects should be tested, as such information will help to better confirm this conclusion.

It is increasingly acknowledged that herbivores could suppress host plant direct defence by secreting their oral saliva into plant.[25, 48–49] For example, phloem-feeding insects like the whitefly *Bemisia tabaci* possess a Bt56 protein in their saliva that can suppress JA defence by eliciting the salicylic-acid signalling pathway in tobacco plants, which can promote the phloem feeding of *B. tabaci*.[50] Recently, an effector named HARP1 in the oral secretion of *H. armigera* was found to block the JA signalling pathway.[51] As a widespread oral secretion component, the proportion of FACs in lepidopteran insects can affect the induction of plant resistance.[52] For example, *Manduca sexta* produces more FACs than *S. exigua* when fed on *Nicotiana attenuata*, and consequently more strongly induces the JA and ET signalling pathways that confer effective resistance against chewing insects.[17] In our study, all seven insects fed on maize had FACs but differed in their abundance and relative proportion, which may trigger distinct HIPV emissions.[53] Newly emerging pests *A. lepigone* and *A. dissimilis* had significantly higher FACs content which were separated from the other five insects according to PCA analysis. Among nine FACs we analyzed, the one first discovered, volicitin, is the most important compound in oral secretion of many caterpillars and exhibits elicitor activity among a wide range of plant species.[54] It was also found that volicitin was one of the main components among FACs that we detected in all seven species. Additional FACs have also been identified in lepidopteran larvae with some of the most abundant forms consisting of linoleic (18:2) and linolenic (18:3) acids conjugated to glutamate (Glu) or glutamine (Gln).[23] Interestingly, *A. lepigone* and *A. dissimilis* had much higher total FACs, especially C18:3-Glu and C18:2-Glu than the five primary insects. Furthermore, the RDA analysis showed that the total FAC content is positively correlated with emission of homoterpenes. Addition of individual C18:3-Glu and C18:2-Glu to the oral secretion of *S. litura* induced an increase of DMNT and TMTT emission. Thus, the total FAC amount in oral secretion was shown to determine the induction of plant HIPV. In addition, *S. litura* had the highest GOX activity in oral secretion among seven insects but induced the lowest volatile emission. In contrast, two *Athetis* insects with low GOX activity can induce the highest amounts of volatiles. These results were consistent with other studies that GOX activity was negatively correlated with plant defence.[55,56]

Lepidopteran insects can change the FACs content of their oral secretion by behavioural or physiological regulation. To avoid parasitoid attack, *Heliothis subflexa* preferably feeds on fruit that are free of linolenic acid rather than on leaves. The lack of linoleic acids in fruits prevents *H. subflexa* from forming FACs.[57] Our results showed that with increasing concentration of linolenic acid in the diet, the food consumption of these two insects were not influenced. However, the food consumption of *S. exigua* was higher than *A. dissimilis*, suggesting that *S. exigua* uptake more linolenic acid. A previous study found that the most ingested linolenic acid can incorporate into FACs within 6 h.[58] We therefore suppose that most ingested linolenic acid has been incorporated into FACs under our sampling time (after feeding for 24 h). Interestingly, *S. exigua* ingested more linolenic acid but has lower FAC content. We then speculated that the biosynthesized FACs in *S. exigua* has higher FACs degradation rate to diminish the FAC content and then excrete excess fatty acids relative to *A. dissimilis*. Notably, several enzymes are involved in FAC biosynthesis and hydrolysis. Aminoacylase-like protein (L-ACY-1) levels, responsible for FAC hydrolyzation, were found to be different in *Heliothis virescens*, *Helicoverpa zea*, and *Heliothis subflexa*.[59–60] Highly specialized *H. subflexa* on *Physalis* plants has the highest L-ACY-1 levels, which may in turn result in lower FAC content and higher fitness on *Physalis* plants. Thus, further research needs to determine how fatty acid metabolism of insects affect the composition of FAC.

This study showed that the coexistence time between herbivore and host plant could influence the content of FACs in oral secretion and in turn affect the emission of HIPV. The newly emerging maize pests *A. lepigone* and *A. dissimilis* can secrete much higher

FACs and triggered higher amounts of HIPVs than the primary maize pest species. With longer coexistence time, the primary herbivores tend to excrete more linolenic acid in their frass to reduce concentration of linolenic acid and in turn suppress FAC formation. Our findings support the hypothesis that decreased FAC contents in the oral secretions can be considered an adaptation meant to suppress release HIPVs by the plants, which lowers the risk that the caterpillars are exposed to natural enemy attack. Based on the crucial role of FACs in regulating plant HIPV emission, a RNAi- or CRISPER-Cas9-based genetic approach, in which the expression of candidate regulatory genes in FAC metabolism are systematically edited, might be one candidate way to control crop pest.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Agrawal AA, Current trends in the evolutionary ecology of plant defence. *Funct Ecol* **25**:420–432 (2011).
2 Machado RA, Ferrieri AP, Robert CA, Glauser G, Kallenbach M, Baldwin IT *et al.*, Leaf-herbivore attack reduces carbon reserves and regrowth from the roots via jasmonate and auxin signaling. *New Phytol* **200**:1234–1246 (2013).
3 Mithofer A and Boland W, Plant defense against herbivores: chemical aspects. *Annu Rev Plant Biol* **63**:431–450 (2012).
4 Pierik R, Ballare CL and Dicke M, Ecology of plant volatiles: taking a plant community perspective. *Plant Cell Environ* **37**:1845–1853 (2014).
5 Aljbory Z and Chen M-S, Indirect plant defense against insect herbivores: a review. *Insect Sci* **25**:2–23 (2018).
6 Christensen SA, Nemchenko A, Borrego E, Murray I, Sobhy IS, Bosak L *et al.*, The maize lipoxygenase, ZmLOX10, mediates green leaf volatile, jasmonate and herbivore-induced plant volatile production for defense against insect attack. *Plant J* **74**:59–73 (2013).
7 Maag D, K€ohler A, Robert CAM, Frey M, Wolfender J-L, TCJ T *et al.*, Highly localized and persistent induction of Bx1-dependent herbivore resistance factors in maize. *Plant J* **88**:976–991 (2016).
8 Vincent TR, Avramova M, Canham J, Higgins P, Bilkey N, Mugford ST *et al.*, Interplay of plasma membrane and vacuolar ion channels, together with BAK1, elicits rapid cytosolic calcium elevations in Arabidopsis during aphid feeding. *Plant Cell* **29**:1460–1479 (2017).
9 Clavijo McCormick A, Unsicker SB and Gershenzon J, The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. *Trends Plant Sci* **17**:303–310 (2012).
10 Danner H, Desurmont GA, Cristescu SM and van Dam NM, Herbivore-induced plant volatiles accurately predict history of coexistence, diet breadth, and feeding mode of herbivores. *New Phytol* **220**:726–738 (2018).
11 McCormick AC, Boeckler GA, Köllner TG, Gershenzon J and Unsicker SB, The timing of herbivore-induced volatile emission in black poplar (*Populus nigra*) and the influence of herbivore age and identity affect the value of individual volatiles as cues for herbivore enemies. *BMC Plant Biol* **14**:304 (2014). https://doi.org/10.1186/s12870-014-0304-5
12 Verheggen FJ, Haubruge E, De Moraes CM and Mescher MC, Aphid responses to volatile cues from turnip plants (*Brassica rapa*) infested with phloem-feeding and chewing herbivores. *Arthropod Plant Interact* **7**:567–577 (2013).
13 Sobhy IS, Miyake A, Shinya T and Galis I, Oral secretions affect HIPVs induced by generalist (Mythimna loreyi) and specialist (Parnara guttata) herbivores in Rice. *J Chem Ecol* **43**:929–943 (2017).
14 De Lange ES, Laplanche D, Guo H, Xu W, Vlimant M, Erb M *et al.*, *Spodoptera frugiperda* caterpillars suppress herbivore-induced volatile emissions in maize. *J Chem Ecol* **46**:344–360 (2020).
15 Hilker M and Meiners T, How do plants "notice" attack by herbivorous arthropods? *Biol Rev* **85**:267–280 (2010).
16 Wu JQ and Baldwin IT, New insights into plant responses to the attack from insect herbivores. *Annu Rev Genet* **44**:1–24 (2010).
17 Diezel C, von Dahl CC, Gaquerel E and Baldwin IT, Different lepidopteran elicitors account for cross-talk in herbivory-induced Phytohormone signaling. *Plant Physiol* **150**:1576–1586 (2009).
18 Shinya T, Hojo Y, Desaki Y, Christeller JT, Okada K, Shibuya N *et al.*, Modulation of plant defense responses to herbivores by simultaneous recognition of different herbivore-associated elicitors in rice. *Sci Rep* **6**:32537 (2016).
19 Bonaventure G, VanDoorn A and Baldwin IT, Herbivore-associated elicitors: FAC signaling and metabolism. *Trends Plant Sci* **16**:294–299 (2011).
20 Truitt CL, Wei H-X and Pare´ PW, A plasma membrane protein from Zeamays binds with the herbivore elicitor Volicitin. *Plant Cell* **16**: 523–532 (2004).
21 Yang LH, Wang XY, Bai SF, Li X, Gu SH, Wang CZ *et al.*, Expressional divergence of insect GOX genes: from specialist to generalist glucose oxidase. *J Insect Physiol* **100**:21–27 (2017).
22 Yoshinaga N, Physiological function and ecological aspects of fatty acid-amino acid conjugates in insects. *Biosci, Biotechnol, Biochem* **80**:1274–1282 (2016).
23 Yoshinaga N, Alborn HT, Nakanishi T, Suckling DM, Nishida R, Tumlinson JH *et al.*, Fatty acid-amino acid conjugates diversification in lepidopteran caterpillars. *J Chem Ecol* **36**:319–325 (2010).
24 Alborn HT, Brennan MM and Tumlinson JH, Differential activity and degradation of plant volatile elicitors in regurgitant of tobacco hornworm (Manduca sexta) larvae. *J Chem Ecol* **29**:1357–1372 (2003).
25 Mori N and Yoshinaga N, Function and evolutionary diversity of fatty acid amino acid conjugates in insects. *J Plant Interact* **6**:103–107 (2011).
26 Schmelz EA, Engelberth J, Alborn HT, III JHT and Teal PEA, Phytohormone-based activity mapping of insect herbivore-produced elicitors. *Proc Natl Acad Sci U S A* **106**:653–657 (2009).
27 Wang YZ, Kim KS, Guo WC, Li QY, Zhang YY, Wang ZY *et al.*, Introgression between divergent corn borer species in a region of sympatry: implications on the evolution and adaptation of pest arthropods. *Mol Ecol* **26**:6892–6907 (2017).
28 Li LT, Zhu YB, Ma JF, Li ZY and Dong ZP, An analysis of the Athetis lepigone transcriptome from four developmental stages. *PLoS One* **8**: e73911 (2013).
29 Liu XL, Sun SJ, Khuhro SA, Elzaki MEA, Yan Q and Dong SL, Functional characterization of pheromone receptors in the moth Athetis dissimilis (Lepidoptera: Noctuidae). *Pestic Biochem Physiol* **158**:69–76 (2019).
30 Sun H, Song Y, Du J, Wang X and Cheng Z, Identification and tissue distribution of chemosensory protein and odorant binding protein genes in Athetis dissimilis (Lepidoptera: Noctuidae). *Appl Entomol Zool* **51**:409–420 (2016).
31 Qi J, Sun G, Wang L, Zhao C, Hettenhausen C, Schuman MC *et al.*, Oral secretions from Mythimna separata insects specifically induce defence responses in maize as revealed by highdimensional biological data. *Plant, Cell Environ* **39**:1749–1766 (2016).
32 Schnee C, Kollner TG, Held M, Turlings TCJ, Gershenzon J and Degenhardt J, The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. *Proc Natl Acad Sci U S A* **103**:1129–1134 (2006).
33 Xia Y, Gao QM, Yu KS, Lapchyk L, Navarre D, Hildebrand D *et al.*, An intact cuticle in distal tissues is essential for the induction of systemic acquired resistance in plants. *Cell Host Microbe* **5**:151–165 (2009).
34 Mandal MK, Chandra-Shekara AC, Jeong RD, Yu KS, Zhu SF, Chanda B *et al.*, Oleic acid-dependent modulation of NITRIC OXIDE ASSOCIATED1 protein levels regulates Nitric Oxide-mediated defense signaling in Arabidopsis. *Plant Cell* **24**:1654–1674 (2012).
35 Mithofer A and Boland W, Recognition of herbivory-associated molecular patterns. *Plant Physiol* **146**:825–831 (2008).

36 Heil M, Herbivore-induced plant volatiles: targets, perception and unanswered questions. *New Phytol* **204**:297–306 (2014).
37 Lopez-Carretero A, Boege K, Diaz-Castelazo C, Dominguez Z and Rico-Gray V, Influence of plant resistance traits in selectiveness and species strength in a tropical plant-herbivore network. *Am J Bot* **103**: 1436–1448 (2016).
38 Qi J, Malook SU, Shen G, Gao L, Zhang C, Li J *et al*., Current understanding of maize and rice defense against insect herbivores. *Plant Diversity* **40**:189–195 (2018).
39 Steinbrenner AD, Munoz-Amatriain M, Chaparro AF, Aguilar-Venegas JM, Lo S, Okuda S *et al*., A receptor-like protein mediates plant immune responses to herbivore-associated molecular patterns. *Proc Natl Acad Sci U S A* **117**:31510–31518 (2020).
40 Cornell HV and Hawkins BA, Herbivore responses to plant secondary compounds: a test of phytochemical coevolution theory. *Am Nat* **161**:507–522 (2003).
41 Rotter MC, Couture JJ, Rothwell EM, Garcia J and Holeski LM, Evolutionary ecology of plant resistance traits across the herbivore diet spectrum: a test in the model plant *Mimulus guttatus*. *Evol Ecol Res* **19**: 423–440 (2018).
42 Sarmento RA, Lemos F, Bleeker PM, Schuurink RC, Pallini A, Oliveira MGA *et al*., A herbivore that manipulates plant defence. *Ecol Lett* **14**:229–236 (2011).
43 Heidel-Fischer HM, Kirsch R, Reichelt M, Ahn SJ, Wielsch N, Baxter SW *et al*., An insect Counteradaptation against host plant defenses evolved through concerted Neofunctionalization. *Mol Biol Evol* **36**: 930–941 (2019).
44 Hay-Roe MM, Meagher RL and Nagoshi RN, Effects of cyanogenic plants on fitness in two host strains of the fall armyworm (*Spodoptera frugiperda*). *J Chem Ecol* **37**:1314–1322 (2011).
45 Brillada C, Nishihara M, Shimoda T, Garms S, Boland W, Maffei ME *et al*., Metabolic engineering of the C-16 homoterpene TMTT in Lotus japonicus through overexpression of (E,E)-geranyllinalool synthase attracts generalist and specialist predators in different manners. *New Phytol* **200**:1200–1211 (2013).
46 Liu DF, Huang XZ, Jing WX, An XK, Zhang Q, Zhang H *et al*., Identification and functional analysis of two P450 enzymes of *Gossypium hirsutum* involved in DMNT and TMTT biosynthesis. *Plant Biotechnol J* **16**:581–590 (2018).
47 Meents AK, Chen SP, Reichelt M, Lu HH, Bartram S, Yeh KW *et al*., Volatile DMNT systemically induces jasmonate-independent direct antiherbivore defense in leaves of sweet potato (*Ipomoea batatas*) plants. *Sci Rep* **9**:17431 (2019).
48 Acevedo FE, Peiffer M, Ray S, Meagher R, Luthe DS and Felton GW, Intraspecific differences in plant defense induction by fall armyworm strains. *New Phytol* **218**:310–321 (2018).
49 Bruce TJ, Interplay between insects and plants: dynamic and complex interactions that have coevolved over millions of years but act in milliseconds. *J Exp Bot* **66**:455–465 (2015).
50 Xu HX, Qian LX, Wang XW, Shao RX, Hong Y, Liu SS *et al*., A salivary effector enables whitefly to feed on host plants by eliciting salicylic acid-signaling pathway. *Proc Natl Acad Sci U S A* **116**:490–495 (2019).
51 Chen CY, Liu YQ, Song WM, Chen DY, Chen FY, Chen XY *et al*., An effector from cotton bollworm oral secretion impairs host plant defense signaling. *Proc Natl Acad Sci U S A* **116**:14331–14338 (2019).
52 Yoshinaga N, Ishikawa C, Seidl-Adams I, Bosak E, Aboshi T, Tumlinson JH *et al*., N-(18-Hydroxylinolenoyl)-L-glutamine: a newly discovered analog of Volicitin in Manduca sexta and its elicitor activity in plants. *J Chem Ecol* **40**:484–490 (2014).
53 Voelckel C and Baldwin IT, Generalist and specialist lepidopteran larvae elicit different transcriptional responses in *Nicotiana attenuata*, which correlate with larval FAC profiles. *Ecol Lett* **7**:770–775 (2004).
54 Alborn HT, Turlings TCJ, Jones TH, Stenhagen G, Loughrin JH and Tumlinson JH, An elicitor of plant volatiles from beet armyworm oral secretion. *Science* **276**:945–949 (1997).
55 Musser RO and Hum-Musser SM, Caterpillar saliva beats plant defences. *Nature* **416**:599–600 (2002).
56 Lin PA, Chen YT, Chaverra-Rodriguez D, Heu CC, Bin Zainuddin N, Sidhu JS *et al*., Silencing the alarm: an insect salivary enzyme closes plant stomata and inhibits volatile release. *New Phytol* **230**:793–803 (2021).
57 De Moraes CM and Mescher MC, Biochemical crypsis in the avoidance of natural enemies by an insect herbivore. *Proc Natl Acad Sci U S A* **101**:8993–8997 (2004).
58 Yoshinaga N, Aboshi T and Abe H, Active role of fatty acid amino acid conjugates in nitrogen metabolism in Spodoptera litura larvae. *Proc Natl Acad Sci U S A* **105**:18058–18063 (2008).
59 Kuhns EH, Seidl-Adams I and Tumlinson JH, Heliothine caterpillars differ in abundance of a gut lumen aminoacylase (L-ACY-1)-suggesting a relationship between host preference and fatty acid amino acid conjugate metabolism. *J Insect Physiol* **58**:408–412 (2012).
60 Kuhns EH, Seidl-Adams I and Tumlinson JH, A lepidopteran aminoacylase (L-ACY-1) in Heliothis virescens (Lepidoptera: Noctuidae) gut lumen hydrolyzes fatty acideamino acid conjugates, elicitors of plant defense. *Insect Biochem Mol Biol* **42**:32–40 (2012).

