

Silencing the alarm: an insect salivary enzyme closes plant stomata and inhibits volatile release

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Summary

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- Herbivore-induced plant volatiles (HIPVs) are widely recognized as an ecologically important defensive response of plants against herbivory. Although the induction of this 'cry for help' has been well documented, only a few studies have investigated the inhibition of HIPVs by herbivores and little is known about whether herbivores have evolved mechanisms to inhibit the release of HIPVs.
- To examine the role of herbivore effectors in modulating HIPVs and stomatal dynamics, we conducted series of experiments combining pharmacological, surgical, genetic (CRISPR-Cas9) and chemical (GC-MS analysis) approaches.
- We show that the salivary enzyme, glucose oxidase (GOX), secreted by the caterpillar *Helicoverpa zea* on leaves, causes stomatal closure in tomato (*Solanum lycopersicum*) within 5 min, and in both tomato and soybean (*Glycine max*) for at least 48 h. GOX also inhibits the emission of several HIPVs during feeding by *H. zea*, including (Z)-3-hexenol, (Z)-jasmones and (Z)-3-hexenyl acetate, which are important airborne signals in plant defenses.
- Our findings highlight a potential adaptive strategy where an insect herbivore inhibits plant airborne defenses during feeding by exploiting the association between stomatal dynamics and HIPV emission.

Introduction

Since the discovery that airborne signals are released by plants during herbivore attack (Baldwin & Schultz, 1983; Dicke & Sabelis, 1987; Turlings *et al.*, 1990), herbivore-induced plant volatiles (HIPVs) have become a vigorous area of research with thousands of studies describing how herbivory elicits the release of these airborne cues (Heil, 2014). Although many questions have yet to be answered (e.g. the adaptive value of HIPVs), several functions have been identified (Heil, 2014). Notably, HIPVs have been proposed as a strategy by which plants 'cry for help' and have been shown to help protect plants from insect herbivores by attracting natural enemies of those herbivores, priming defenses in neighboring plants, and mediating systemic defense responses via within-plant signaling (Dicke & Baldwin, 2010). While these studies highlight the function of HIPVs in mediating

community-wide interactions, the question of whether insect herbivores can modulate the release of HIPVs during feeding has received almost no attention (Delphia *et al.*, 2006; Heil, 2014; Turlings & Erb, 2018), and the mechanisms by which insect herbivores might interfere with HIPV release and/or production remain unclear (but see Jones *et al.*, 2019).

The release of plant volatile organic compounds (VOCs), including HIPVs, was believed to be controlled by their rates of synthesis (Niinemets *et al.*, 2004). However, growing evidence has shown that stomata, the gates that link internal plant tissues to the atmosphere, exert some control over the release of VOCs (Niinemets *et al.*, 2004; Seidl-Adams *et al.*, 2015). The openness of stomata not only directly affects the release of VOCs, but also controls the inflow of CO₂, which is essential for VOC synthesis (Niinemets *et al.*, 2004). Plant stomata might therefore provide a point of manipulation for insect herbivores. Previous work has shown that caterpillars alter HIPVs; however, the specific components that lead to these modulations remain mostly unclear and

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speculative (Jones *et al.*, 2019). Salivary glucose oxidase (GOX) of the polyphagous caterpillar *Helicoverpa zea* (feeds on >100 species of plants; Supporting Information Fig. S1) is a well-studied insect enzyme known to modulate plant defenses (Acevedo *et al.*, 2015). Although the adaptive function of GOX was first hypothesized to reduce defenses in plants and facilitate the consumption of various host plants, it was later discovered that the influence of GOX on plant defenses is species-specific, and that it can in fact induce defenses in many plant species (Lin *et al.*, 2020). Given the context-dependent nature of GOX on plant defense (not only the species of host plant but also the type of plant defenses tested), it is likely that its adaptive value is linked to the modulation of other plant physiological processes. In the presence of D-glucose, GOX catalyzes the production of hydrogen peroxide (H₂O₂), which is an important signaling molecule involved in plant physiological processes (Mittler *et al.*, 2004), including stomatal closure (Wang & Song, 2008). As mentioned earlier, stomatal closure not only inhibits the direct release of VOCs, but also reduces overall gas-exchange efficiency, slowing VOC production. Cytoplasmic glucose concentrations are also linked to the capacity for stomatal opening, and GOX might prevent stomatal opening by depleting glucose concentration (Flutsch *et al.*, 2020). We therefore hypothesized that *H. zea* larvae use GOX to modulate stomatal dynamics in plants to prevent them from releasing HIPVs or ‘crying for help’. This function could be one of the reasons why high concentrations of GOX are typically detected in the saliva of *H. zea* larvae, and can potentially affect plant processes beyond ‘crying for help’.

Materials and Methods

Plants and insects

Host plants of *H. zea*, including tomato (*Solanum lycopersicum* cv Better Boy), soybean (*Glycine max* var. FS Hisoy HS33A14-98SB132B) and cotton (*Gossypium hirsutum* cv UA222) were grown in a glasshouse at Pennsylvania State University (PA, USA) at ambient temperature 25°C, 16 h : 8 h, light : dark, and 60% relative humidity. Plants were fertilized with Osmocote Plus 15-9-12 Fertilizer (ICL Specialty Fertilizers, Summerville, SC, USA) 1 wk after seedlings were transplanted to 76 mm (3 inch) square pots (volume = 450 cm³) with Metromix 400 potting mix (Griffin Greenhouse & Nursery Supplies, Tewksbury, MA, USA). Plants with four mature leaves were used for all experiments. Wild-type tomato fruitworm, *Helicoverpa zea*, were purchased from Benzon Research. The colony was kept with ambient temperature 25°C, 6 h : 8 h, light : dark and reared on artificial diet as previously described (Peiffer & Felton, 2005). Mutant *H. zea* with impaired GOX were kept under the same conditions. Feeding damage to plants by *H. zea* larvae is shown in Fig. S1.

Impact of GOX on stomatal conductance

Plants with four mature leaves were used to test if GOX affected leaf stomatal conductance. A puncture was created in

the center of the first mature leaf of each plant using 200 µl pipette tips cut in the middle (diameter = 4 mm). The wounded area was then treated with different solutions, including phosphate-buffered saline (PBS) buffer (1X, pH = 7.4), or 20 µl of 0.01 mg ml⁻¹ GOX (Sigma-Aldrich) in PBS buffer. Conductance data were then measured using SC-1 Leaf Porometer (Meter; Pullman, WA, USA) at different time points, including 1, 3, 5, 7, 24 and 48 h after treatment. The diameter of the measuring chamber was 6.35 mm (area = 42.43 mm²). The measurements took place parallel to the wound on both sides of the leaf, and an average was calculated for each leaf as the final leaf stomatal conductance. Plants damaged by thrips during the experiment were excluded from the experiment (Figs 1c,d, S2c).

To identify whether the same stomatal conductance responses were observed under herbivory, fifth-instar *H. zea* were used for damage treatments. Caterpillar spinnerets were ablated to prevent salivation using the method previously described (Musser *et al.*, 2006). Caterpillars were placed in clip cages on the center of the first mature leaf of the plant and allowed to feed for 3 h to ensure complete removal of leaf area within the clip cages, which created equal sized holes (3.14 cm²) in the center of leaves, as previously described (Chung *et al.*, 2013). Leaf stomatal conductance was then measured as described earlier. Caterpillars that did not consume the entire area within the clip cage were removed from the experiment (Fig. 1d). In addition, the effect of GOX on photosynthesis activity was also measured using the LI-6800 portable photosynthesis system (Li-Cor, Lincoln, NV, USA) (Fig. S3; Methods S1; Notes S1).

GOX mutant of *H. zea*: target sequence selection

The mRNA sequence of glucose oxidase gene *H_z-gox* was obtained from NCBI (GenBank FJ460711.1). In order to identify exons and introns of this sequence, we tried to align several portions of the mRNA to locations within the whole genome shotgun sequences of *H. zea* (GenBank NFMG0000000.1) without success. Therefore, we blasted the sequence against the *gox* gene mRNA sequence of *Helicoverpa armigera* (*Ha-gox* 97% identity, GenBank EU629216.1) and used the *Ha-gox* genomic nucleotide sequence to annotate potential exons in *H_z-gox* mRNA. We designed primers (Table S1) to amplify potential contiguous exons and obtained PCR amplicons and the sequences for candidate exons and introns on the *H_z-gox* gene. We then designed primers within the introns to be able to sequence mutants.

For targeted mutagenesis, we used the ALT-R Cas9-HF and crRNA system from Integrated DNA Technologies (IDT, Coralville, IA, USA). We designed crRNA targeting several exons that matched the best on-target effects using the IDT designing platform; however, we focused our experiments on targeting exon 4 for embryonic microinjections. We sequenced PCR fragments from individuals of our *H. zea* colony to verify that the target sites were present and matched the designed sequence exactly (Table S2).

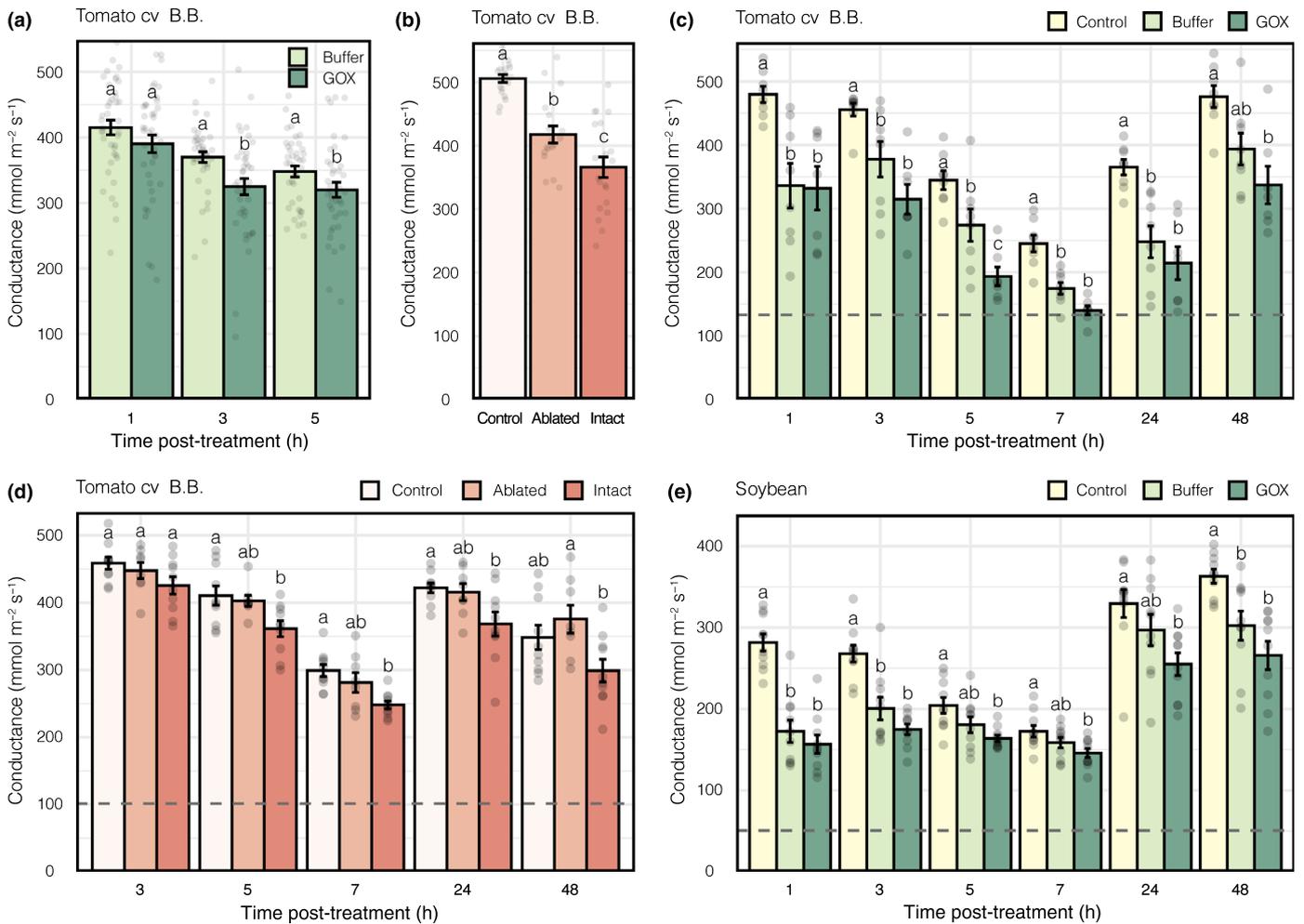


Fig. 1 Impact of glucose oxidase (GOX) and *Helicoverpa zea* saliva on stomatal conductance of leaf. (a) Stomatal conductance of tomato cv Better Boy (cv B.B.; all treatments, $n = 40$). (b) Stomatal conductance of tomato 5 h after damage by intact caterpillars ($n = 20$) or ablated caterpillars ($n = 18$), and undamaged control ($n = 20$). (c) Stomatal conductance of tomato (GOX, $n = 7$; control and buffer, $n = 8$). (d) Stomatal conductance of tomato damaged by caterpillars (control, $n = 10$; ablated, $n = 8$; intact, $n = 10$). (e) Stomatal conductance of soybean (all treatments, $n = 10$). Bars indicate means \pm SE. Dots indicate individual observations. Different letters indicate significant differences between means within each time point ($P < 0.05$, Tukey's honestly significant difference, ANOVA). Dashed lines indicate night-time conductance of plant.

GOX mutant of *H. zea*: embryo injection

We prepared embryo microinjection mixes using the protocol for Cas9-HF from IDT. Briefly, each crRNA at 2 nmol was diluted in 20 μ l of TE buffer to obtain 100 μ M, then we mixed 1 μ l of the crRNA with 1 μ l of tracrRNA 100 μ M and 0.5 μ l of nuclease-free duplex buffer and incubated at 95°C for 5 min obtaining sgRNA at 40 μ M. Then, 2.5 μ l of this sgRNA was used to prepare a master mix containing 3 μ l of Cas9 protein (1 mg ml⁻¹), 1 μ l of 10 \times injection buffer (0.1 M sodium phosphate buffer, pH 6.8, and 1 M KCl, prepared following the manufacturer's instructions) and 3.5 μ l of water. Newly oviposited eggs were collected from cages at 15 min intervals, dried in a desiccation chamber for 5 min, attached to a coverslip taped to a mounting slide using double-sided tape and injected using a Femtojet microinjector (Eppendorf, Enfield, CT, USA). After injection, eggs were kept in a growth chamber at 25°C, under 16 h : 8 h, light : dark photoperiod, until hatching. Individual larvae were

placed into a rearing cup (30 ml) immediately after hatching. Larvae were reared on wheat germ-based artificial diet as previously described (Peiffer & Felton, 2005) until adults.

Screening and generation of *gox* knockout mutant lines

Injected individuals (G0 generation) were reared on artificial diets as described, and 1 mm of tissue from the tip of one leg was collected for further genotyping when larvae reached the fourth instar. PCR was performed using Phusion (New England Biolabs, Beverly, MA, USA) polymerase with primers (forward, 5'-GAAGAGCTCGTTTAATGAGGG-3'; reverse, 5'-CGCATCCTAAGATCATTCG-3'; product size, 1028 bp) that target the full span of exon 2 to exon 4 on each individual. Gel electrophoresis was conducted to identify indels based on the presence of two or more bands within each individual. PCR products were then purified using ExoSAP-IT (ThermoFisher) and sent to the Genomic Core Facility at the Pennsylvania State

University for sequencing. Sequences from individual G0 were aligned to the wild-type sequence to identify indels. G0 individuals that showed the same indels were mated to produce families. The screening processes (PCR and sequencing) were repeated with the offspring of each family at generations G1–G4. Once we identified that 100% of the offspring within a family showed the same band pattern and indels, we assumed that the line was homozygous, and we proceeded to validate the phenotype of the lines using salivary gland GOX activity assay and GOX gene expression in salivary glands.

Activity, expression and detection of salivary GOX

To screen for individuals with low GOX activity, fifth-instar larvae were fed overnight on a disk of filter paper (diameter = 6 mm) soaked in 10% sucrose solution. The remaining portion of the disk was then placed in a 50 μ l droplet of GOX reaction mixture containing 0.17 mM *o*-dianisidine-HCl in 0.1 M potassium phosphate (pH 7.0), 92.7 mM D-glucose, 2.0 mg ml⁻¹ horseradish peroxidase (Eichenseer *et al.*, 1999). After 20 min, the disks were photographed. The presence of functional GOX was determined by the presence of brown precipitate.

To quantify GOX activity in mutant larvae, labial salivary glands were collected from fifth-instar larvae and frozen with liquid nitrogen, homogenized in 30 μ l 0.1 M potassium phosphate buffer (pH 7.0), and assayed in the GOX reaction mixture described previously. We combined 5 μ l of homogenate with 200 μ l of the reaction mixture and the change in absorbance was recorded at 460 nm. Specific activities were calculated using an extinction coefficient of 8.3 cm mM⁻¹. Protein concentration was determined by the Bradford Assay (Bradford, 1976).

To quantify the abundance of GOX transcript in salivary glands, gland tissues were collected 48 h after the larvae molted into the fifth instar and frozen with liquid nitrogen. Gland tissue was homogenized, and RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using the extracted RNA and used as templates for quantitative real-time PCR. Detailed methods and primer sequences were as previously described (Tan *et al.*, 2018).

The GOX proteins were visualized by polyacrylamide gel electrophoresis (PAGE) and Western blotting. Briefly, denatured labial gland proteins were separated in 0.75 mm sodium dodecyl sulfate (SDS). Gels were stained with SimplyBlue (cat. no. LC6060; Invitrogen). A duplicate gel was transferred to 0.2 μ m nitrocellulose and Western blotted as previously described (Peifer & Felton, 2005). Native gels were run as previously described (Dussourd *et al.*, 2016).

Impact of GOX on stomatal aperture

To examine the stomatal response to GOX at the cellular level, we conducted a series of experiments that observed stomata microscopically. The same artificial damage regime was applied to tomato (*S. lycopersicum* cv Better Boy) with specific treatments that aimed to understand: the timing of stomatal closure after initial GOX and damage treatment; (ii) the impact of GOX

concentration on stomatal conductance; and (iii) the impact of distance of GOX application on stomata. Approximately 1 cm² of leaf tissue was excised from the treated leaf next to the wound site using a razor blade. Each leaf section was then mounted on a slide and observed immediately using a Zeiss Axio Observer Microscope equipped with a Nikon D5100 DSLR camera under a $\times 63$ objective. Five different pictures of each sample were taken randomly. Aperture of all stomata from the five pictures were averaged to get the mean stomatal aperture of the leaf. To isolate the impact of GOX from other oral secretions of *H. zea*, the impacts of damage by mutant and wild-type *H. zea* on stomatal aperture was compared. Fifth-instar caterpillars were confined in clip cages for 3 h to remove all leaf tissue in the cage. The stomatal aperture of tomato leaves was then observed at 3 h after the end of clip cage treatment. Caterpillars that did not consume the entire area were excluded from the analysis (Figs 2d, S4). In addition, the effects of active and denatured GOX on stomatal aperture were also compared (Fig. S5; Notes S2).

Volatile organic compound collections

Volatile organic compound (VOC) collections were performed in a growth chamber under 16 h : 8 h, 25°C : 23°C, light : dark conditions. Plants were placed in individual 4 l glass chambers, each with two small openings serving as the inlet and outlet of the system which were connected to two Teflon tubes. A push-pull system (Analytical Research Systems, Micanopy, FL, USA) was used to collect VOCs (Paudel *et al.*, 2019). A constant pressure (4 l min⁻¹) of charcoal-purified air was provided to the inlet, and air was pulled with a constant pressure of 1 l min⁻¹ through a trap containing 50 mg of HayesepQ adsorbent, 80/100 mesh (Alltech Assoc., New York, NY, USA). VOCs were collected from 08:00 to 20:00 h for 3 d with a caterpillar feeding freely on each plant. For experiments using *gox* mutants, a single third-instar larva (ecologically relevant stage that is susceptible to predation and parasitism) was placed in the chamber before the collection. We conducted five independent trials to obtain a total of 20 replicates for tomato and soybean. Tomato plants that experienced stem boring, which led to wilting of shoots, were removed from further analysis. After each collection, the volatile filter traps were eluted with 150 μ l of dichloromethane into 2.0 ml glass vials with a 300 μ l glass insert. Standard solution (5 μ l) containing nonyl acetate (80 ng μ l⁻¹) was added to all samples for quantification and stored at -80°C until further analysis. VOCs were analyzed using methods previously described (Helms *et al.*, 2019). Briefly, 1 μ l of sample was injected into a GC system (Agilent 7890A, Santa Clara, CA, USA) coupled with MS (Agilent 5975C). The splitless injector was held at 250°C and the column (Rxi[®] - 1 ms, 30 m, 0.25 mm ID, 0.25 μ m film thickness) at 40°C for 2 min, and then ramped to 280°C at a speed of 10 min °C⁻¹. VOCs were identified using the recorded mass spectra and retention times. Tentative VOC identifications were made by comparison with mass spectral libraries (NIST17, Adams2 (Allured Publishing Corp., Carol Stream, IL, USA), and the University of Göteborg Library). Compounds with scores < 86 were removed from further analysis

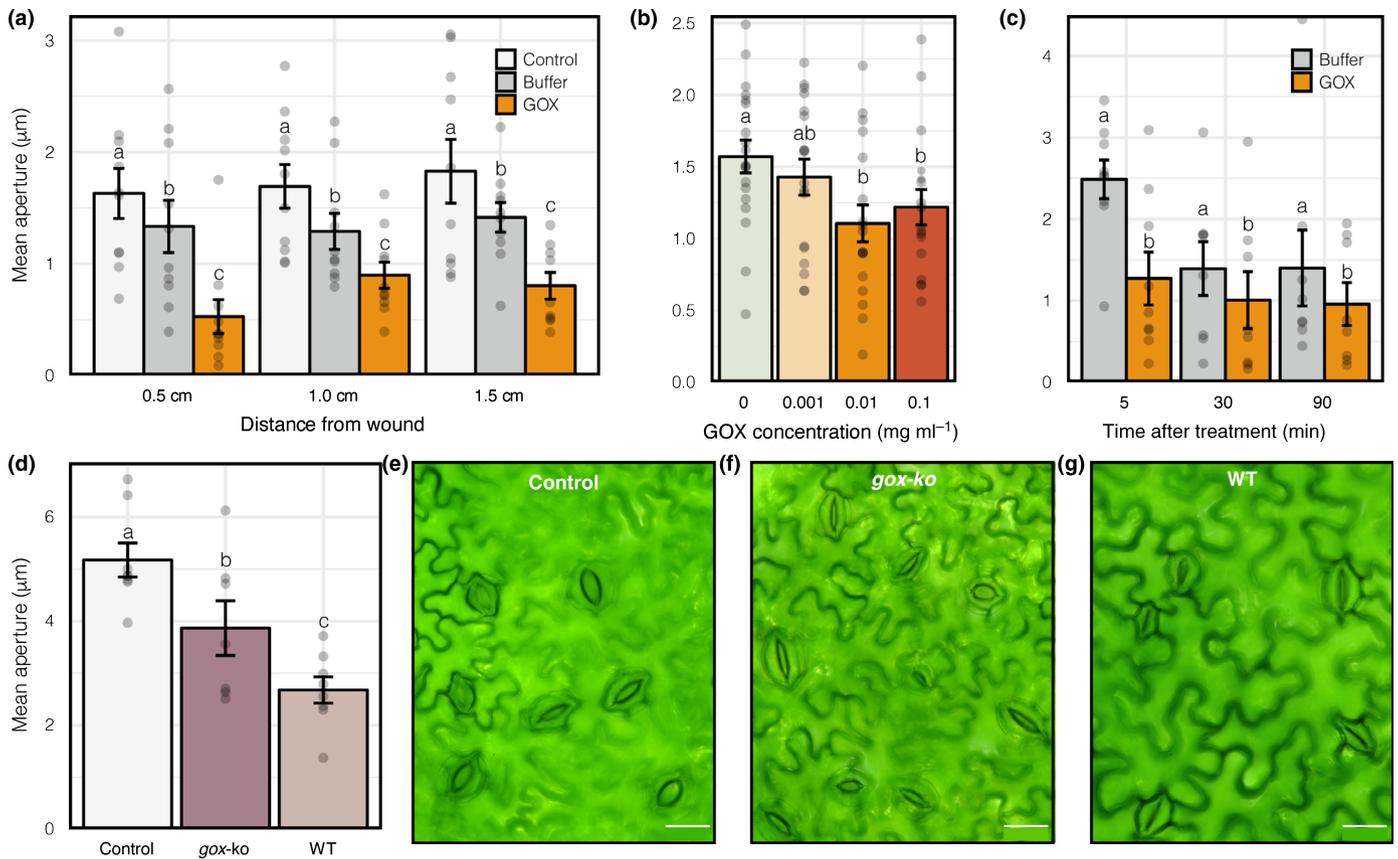


Fig. 2 Impact of glucose oxidase (GOX) on stomatal aperture of tomato leaves (cv Better Boy). (a) Stomatal aperture at 3 h after treatment at different distances from wounds (all treatments, $n = 10$). (b) Stomatal aperture at 3 h after treatment with varying concentrations of GOX (all treatments, $n = 6$). (c) Stomatal aperture within 90 min after the treatments (all treatments, $n = 9$ for 5 min, $n = 10$ for 30 and 90 min). (d) Stomatal aperture at 3 h after damage by fifth-instar larvae of *Helicoverpa zea* with different GOX activity (*gox* = inbred line: M1-33/02 (*gox*, $n = 7$); wild-type *H. zea* (WT) and control, $n = 8$). (e–g) Representative images of leaf stomata in (d); bars, 20 μm . Bars indicate means \pm SE. Dots indicate individual observations. Different letters indicate significant differences among means ($P < 0.05$, Tukey's honestly significant difference, ANOVA). Control, undamaged; *gox*, *gox* mutant *H. zea*. Unit of n is plant.

to ensure quality, and structure assignments were confirmed where possible by comparison of mass spectra and retention times with authentic standards. Compounds were quantified relative to standard concentrations.

Statistical analysis

All data were analyzed using R (R Core Team, 2017). For continuous response variables, including conductance, aperture and total VOC emission, data were analyzed using general linear models (ANOVA, package: *car* (Fox & Weisberg, 2018)) to test for treatment effects. Independent variables in experiments involving conductance were treatment and trial (blocking factor, two levels, in Fig. 1a,b). Measurements at different time points were repeated on the same individual. Data from each time point were fitted to separate models (Fig. 1a,c–e). For experiments focusing on stomatal aperture, independent variables included treatment, position (three levels in Fig. 2a,b, as blocking factor in Fig. 2b), trial (blocking factor, three levels in Fig. 2a,c,d; two levels in Fig. 2b), and time (three levels in Fig. 2c, not repeated measures). For total VOC emission, data from each day were

fitted to separate models. Diagnostic plots were performed to test for violation of model assumptions (Zuur *et al.*, 2010). Data that needed transformation (e.g. total VOC emission) were transformed using the package *BESTNORMALIZE* (Ryan and Peterson, 2019). Full models were initially fitted to the data to determine and remove nonsignificant terms. *Post hoc* comparisons (Tukey's honestly significant difference (HSD)) were performed with the package *EMMEANS* (Lenth, 2018). GOX activity and expression data were analyzed using a nonparametric test (Kruskal–Wallis test) followed by Bonferroni *post hoc* comparison.

For VOC data, random forest analysis was performed on full sets of VOCs to identify a subset that was important in separating the predictors (package *RANDOMFOREST* (Liaw & Wiener, 2002)) as described by Mann *et al.* (2012). Predictors consisted of a combination of independent variables (treatment + day). VOCs with mean decrease accuracy (MDA) > 2 were selected for further analysis (Mann *et al.*, 2012). Principal component analysis was performed to visualize associations between responses and explanatory variables (package *FACTOEXTRA* (Kassambara & Mundt, 2016)). For quantity analysis of individual VOCs, as a result of many dropout events after the quality control step based

on identification scores, the data were overdispersed and zero-inflated. To cope with this problem, the data were analyzed using zero-inflated Gaussian mixed model (ZIGMM; package `NBZIMM` (Zhang *et al.*, 2018)), setting the quantity of VOCs as response variable, treatment and day as fixed factors and individual plant as a random factor. *Post hoc* comparisons (Tukey's HSD) were performed to determine the difference between different treatments.

Results

To test whether GOX affects stomatal dynamics, we applied GOX (0.01 mg ml⁻¹, biologically relevant) (Peiffer & Felton, 2005) to artificially wounded leaves of tomato (*Solanum lycopersicum* cv Better Boy). Treatment of wounds with GOX reduced stomatal conductance of tomato leaves at 3 h ($F_{1,77} = 9.58$, $P = 0.003$; Fig. 1a) and 5 h ($F_{1,77} = 5.51$, $P = 0.022$; Fig. 1a) after application compared with buffer-treated plants. To determine whether GOX-containing saliva of *H. zea* causes the same response, we repeated this experiment by wounding plants with intact larvae and larvae with ablated spinnerets to prevent saliva deposition (Musser *et al.*, 2006). Salivating caterpillars (i.e. intact spinnerets) significantly reduced leaf stomatal conductance compared with ablated larvae at 5 h after the start of feeding ($F_{2,54} = 32.33$, $P < 0.001$; Fig. 1b). The significant reduction in conductance in ablated caterpillars was a result of physical damage itself, which can be observed in other experiments comparing between undamaged controls and damage treatments (Fig. 1c–e). The further reduction of stomatal conductance by GOX applications and salivating caterpillars suggests that the salivary enzyme GOX modulates stomatal conductance in tomato plants, but it was not known whether this effect persists longer than 5 h or whether these effects are observed in other plant species. Similar sets of experiments were performed to evaluate the timing and generality of this response in distantly related plants, including soybean (*Glycine max*) and cotton (*Gossypium hirsutum*). The effect of GOX on stomatal conductance of tomato cv Better Boy continued for at least 48 h, with GOX application reducing conductance at 5 h ($F_{2,20} = 14.75$, $P < 0.001$; Fig. 1c) after application compared with buffer-treated plants, and 48 h ($F_{2,20} = 8.4$, $P = 0.002$; Fig. 1c) after application compared with undamaged controls. At 48 h, buffer-treated plants had an intermediate stomatal conductance between undamaged controls and GOX-treated plants (no significant differences between either), while stomatal conductance remained suppressed in GOX-treated plants compared with undamaged controls. A similar pattern was observed for treatment with salivating caterpillars, which again significantly reduced leaf stomatal conductance at 5 h ($F_{2,25} = 4.85$, $P = 0.016$; Fig. 1d), 7 h ($F_{2,25} = 7.51$, $P = 0.003$; Fig. 1d), and 24 h ($F_{2,25} = 4.91$, $P = 0.016$; Fig. 1d) after treatment compared with undamaged controls, and 48 h ($F_{2,25} = 4.38$, $P = 0.023$; Fig. 1d) after treatment compared with plants treated by ablated caterpillars. GOX treatment also reduced stomatal conductance in soybean plants at 5 h ($F_{2,27} = 5.99$, $P = 0.007$; Fig. 1e), 7 h ($F_{2,27} = 4.33$,

$P = 0.023$; Fig. 1e) and 24 h ($F_{2,27} = 4.86$, $P = 0.016$; Fig. 1e) after treatment compared with undamaged controls, while the conductance level of buffer-treated plants was between undamaged controls and GOX-treated plants (no significant differences between either). A similar effect of GOX on stomatal conductance was not observed in cotton (Fig. S2).

To examine plant stomatal responses to GOX at a cellular level, we conducted a series of experiments to observe stomata microscopically. The stomatal aperture of tomato leaves was significantly decreased by artificial damage with buffer application and was further decreased by application of GOX ($F_{2,82} = 24.36$, $P < 0.001$; Fig. 2a). The effect of GOX was similar at distances of 0.5, 1.0 and 1.5 cm from the artificial wound ($F_{2,82} = 0.91$, $P = 0.408$; Fig. 2a). GOX applied at biologically relevant concentrations (0.1 and 0.01 mg ml⁻¹) (Peiffer & Felton, 2005) significantly reduced stomatal aperture ($F_{3,65} = 5.19$, $P = 0.003$; Fig. 2b). The effect of GOX was rapid, with a reduction in stomatal aperture within 5 min of application compared with the buffer-treated plants ($F_{1,44} = 8.65$, $P = 0.005$; Fig. 2c). Despite these observations, the effect of GOX during natural herbivory was unclear. Insect saliva is complex and comprises many molecules with variable effects on plant defenses. Spinneret ablation not only prevents secretion of salivary GOX, but also prevents secretion of all other salivary components, which may also affect stomatal dynamics. To circumvent this limitation and provide direct evidence for the function of GOX in *H. zea*, we created several *gox* knockout mutant lines using CRISPR/Cas9-mediated mutagenesis (Figs 3, S6; Notes S3). Performance experiments revealed no observable differences in growth or feeding behavior between caterpillars from all mutant lines and wild-type. As predicted, damage by wild-type caterpillars (controlled for damage level and time using clip cage; see section in the Materials and Methods for more detail) led to smaller stomatal apertures than mutant caterpillars ($F_{2,73} = 26.12$, $P < 0.001$; Fig. 2d). Similar results were found in two additional trials using different mutant lines (Fig. S4), further confirming that the effect of *H. zea* saliva on stomatal conductance was mainly linked to salivary GOX activity.

To further investigate the effects of GOX-associated stomatal closure on VOC emission, we compared the HIPVs emitted by host plants during feeding by wild-type or *gox* mutant *H. zea* caterpillars (Figs 4, S7; Table S3; Notes S4). For experiments using *gox* mutants, total VOCs emitted from tomato (cv Better Boy) were significantly affected by caterpillars only in the first day ($F_{2,52} = 18.06$, $P < 0.001$; Fig. 4a), whereas total VOCs from soybean were not affected by treatments (Fig. 4e). Within the first 24 h, damage by wild-type caterpillars inhibited emissions of several VOCs in tomato and soybean plants that are known to attract a parasitoid wasp, *Microplitis croceipes*, specialized on *H. zea* (Whitman & Eller, 1992) compared with the *gox* knockout lines. These compounds included (*Z*)-3-hexenol in tomato and soybean (Fig. 4b,f), (*Z*)-jasmone in soybean (Fig. 4g) and (*Z*)-3-hexenyl acetate in soybean (Fig. 4h). By contrast, the emission of β -phellandrene was higher in tomato damaged by wild-type caterpillars than in mutants, indicating induction of certain VOCs by GOX (Fig. 4d).

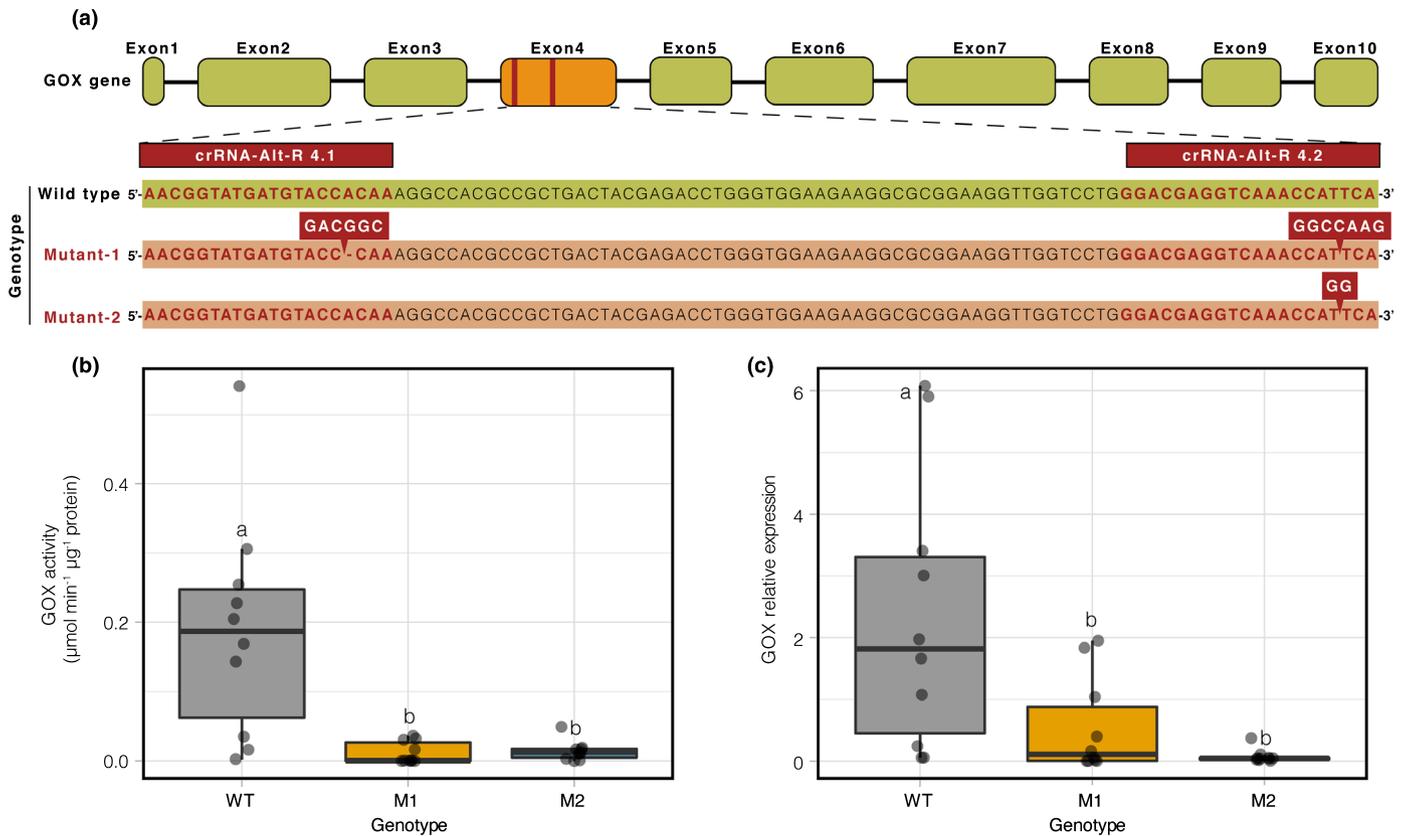


Fig. 3 Genotypes and phenotypes of *gox* mutants of *Helicoverpa zea*. (a) Genotypes of *H. zea*. Insertions are indicated in red boxes. Deletion is indicated by '-'. (b) Salivary glucose oxidase (GOX) activity ($n = 10$). (c) Salivary GOX expression ($n = 10$). Individuals of *gox* mutants were the third generation (embryo injection took place at generation zero). Points indicate individual observations. Box plot represents the median, 25th and 75th quantiles of the response variables. Different letters indicate significant differences among means ($P < 0.05$, Bonferroni comparison, Kruskal–Wallis test).

Discussion

Leaf stomata of plants are structures that mediate growth, defenses and responses of plants to the environment (Niinemets *et al.*, 2004; Melotto *et al.*, 2008; Zhu *et al.*, 2012), which are strictly regulated by plants and closely linked to plant survival. While it has been shown that certain pathogens have evolved intricate mechanisms to manipulate stomata for their benefits (Melotto *et al.*, 2008), whether herbivores have evolved a similar ability and the underlying mechanisms associated with modulating stomatal behavior are unknown. Through a series of experiments, we show that salivary GOX of *H. zea* larvae causes stomatal closure of plants. Although physical damage to the leaf tissue can itself cause significant reduction in stomatal conductance, probably caused in part by the production of H_2O_2 (Orozco-Cardenas & Ryan, 1999), the presence of GOX further reduced conductance and maintained the reduction for a longer period of time. The lack of effect of GOX in cotton suggests that the impacts of GOX on stomatal conductance is species-dependent, given that reactive oxygen species (ROS, e.g. H_2O_2) are widely involved in plant stress responses, and the varying ability to maintain ROS homeostasis among physiological status (whether they have been subjected to stresses; see Nayyar & Gupta, 2006), ontogeny (Apel & Hirt, 2004; Mittler *et al.*,

2004) and plant genotypes (Shalata & Tal, 1998). Shalata & Tal (1998) have shown that different cultivars of tomato varied in the amounts of antioxidants, which probably affect their ability to eliminate ROS. It is therefore expected that the degree of impact from GOX on stomata will vary among plants. We also show that the observed reduction in stomatal conductance by GOX was linked to stomatal closure. These data reveal a newly discovered mechanism where insects can influence stomatal dynamics of their host plants, not unlike certain pathogenic bacteria (Melotto *et al.*, 2008).

The contrasting effects of GOX on different types of VOCs follows the predicted control of stomata over VOCs with different physiochemical characteristics (Harley, 2013). VOCs with high volatility (high Henry's law constant), such as monoterpenes (e.g. β -phellandrene (Harley, 2013), $5670 \text{ Pa m}^3 \text{ mol}^{-1}$), cannot be controlled effectively by stomata (Niinemets *et al.*, 2004), whereas VOCs that are more soluble in water (low Henry's law constant), such as alcohols (e.g. (*Z*)-3-hexenol (Richards-Henderson *et al.*, 2014), $1.6 \text{ Pa m}^3 \text{ mol}^{-1}$), carbonyls (e.g. (*Z*)-3-hexenyl acetate (Richards-Henderson *et al.*, 2014), $0.036 \text{ Pa m}^3 \text{ mol}^{-1}$; methyl salicylate (Richards-Henderson *et al.*, 2014), $0.38 \text{ Pa m}^3 \text{ mol}^{-1}$), aldehydes and oxygenated monoterpenes are more easily regulated by stomata (Harley, 2013). Although green leaf volatiles (GLVs, e.g. (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate) are

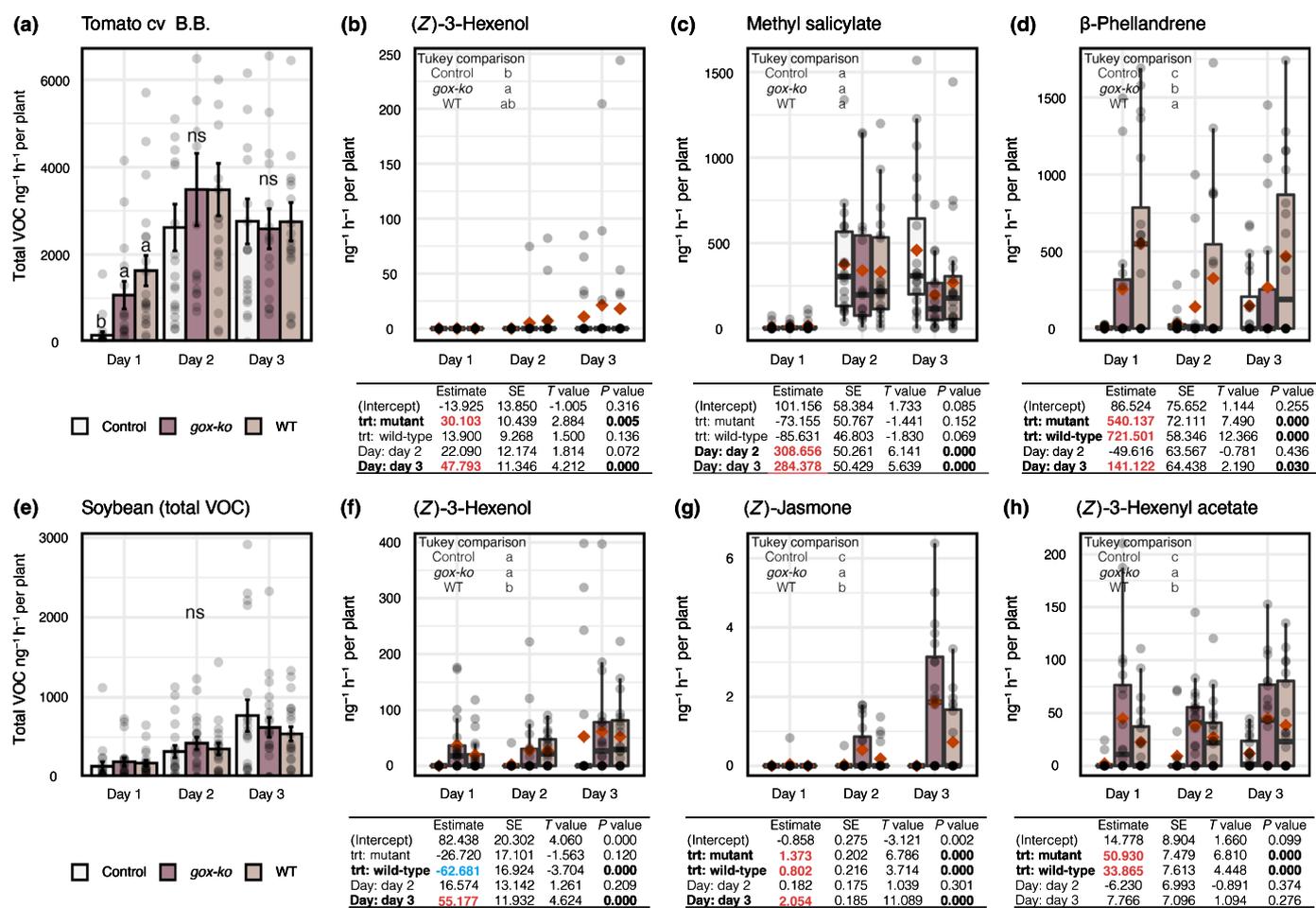


Fig. 4 Volatile organic compound (VOC) emissions during feeding by *Helicoverpa zea* larvae with different salivary glucose oxidase (GOX) activities. (a) Total VOC emission of tomato cv Better Boy (*gox*, $n = 15$; wild-type (WT) and control, $n = 20$). Bars indicate means \pm SE. Different letters indicate significant differences between means ($P < 0.05$, Tukey's honestly significant difference (HSD), ANOVA). (b–d) Box plots of individual VOCs emitted by tomato. (e) Total VOC emission of soybean (*gox*, WT and control, $n = 20$). (f–h) Box plots of individual VOCs emitted by soybean. Red diamonds indicate means. Dots indicate individual observations. Estimates indicate the direction and magnitude of influence each factor has on VOC emission. trt, treatment, including: *gox*, WT and control. P -value indicates whether the estimate is statistically significant ($P < 0.05$). Different letters indicate significant differences between averaged means of treatments across 3 d ($P < 0.05$, Tukey's HSD, zero-inflated Gaussian mixed model). ns, not significant. Text in bold in the table indicates significant factors; red text indicates positive estimates; blue text indicates negative estimates.

presumed to be released directly from wound sites, they are also released systemically through other pathways. For example, infection by plant pathogens that do not create open wounds has generally been reported to emit much higher amounts of GLVs compared with herbivore damage (Ameye *et al.*, 2018). We speculate that in addition to enzymatic modulation of chemical property of GLVs by insect herbivore (Jones *et al.*, 2019), stomata may also play an important role in this pattern. The induction of terpene release is a common characteristic of plants under abiotic stress, such as drought (Loreto & Schnitzler, 2010), and terpenes can serve as antioxidants that protect tissues from oxidative damage during stress (Loreto & Schnitzler, 2010). As such, the induction of monoterpenes (i.e. β -phellandrene) in tomato is potentially a response to increased H_2O_2 generated by GOX. In terms of the implication of these HIPVs for plant defenses, the two groups of VOCs, categorized by the degree of control by stomata, are functionally divergent. HIPVs that can be controlled by stomata (e.g. (Z)-3-hexenol and (Z)-3-hexenyl acetate) have

been implicated in the attraction of natural enemies of herbivores and plant–plant communication (Engelberth *et al.*, 2004). In addition, (Z)-3-hexenol is a compound that can be absorbed by many plants, including tomato, and converted into a toxic glycoside (Sugimoto *et al.*, 2014). The suppression of (Z)-3-hexenol through stomatal closure would not only suppress a potential indirect defense, but also prevent both the release and uptake of an important defensive precursor molecule. By contrast, VOCs that are less affected by stomatal dynamics (e.g. terpenes) or those stored in trichomes often deter herbivores directly (Loivamaki *et al.*, 2008).

These findings beg the question as to why certain categories of plant VOCs can be controlled by stomata, and more studies are needed to reveal the evolutionary and ecological importance of stomatal control over VOCs. GOX activity is present in many caterpillars in addition to *H. zea* (Eichenseer *et al.*, 2010). A survey by Eichenseer *et al.* (2010) showed that higher GOX activity is associated with broader host range in caterpillars across

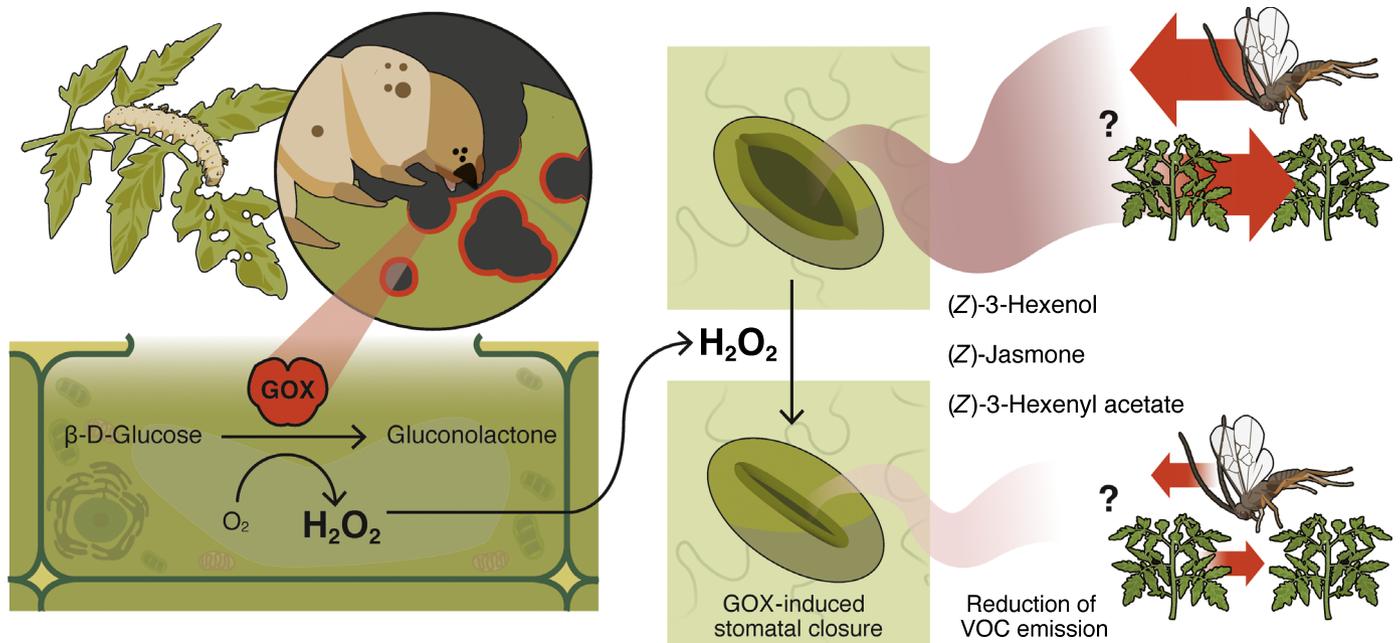


Fig. 5 Graphical summary. Salivary glucose oxidase (GOX) of *Helicoverpa zea* larvae induces stomatal closure and inhibits emission of herbivore-induced plant volatiles (HIPVs) involved in plant communication and defenses. The direct involvement of H_2O_2 is speculative. VOC, volatile organic compound.

Lepidoptera lineages. A common characteristic of such generalist caterpillars (e.g. *H. zea*) is their mobility and tendency to move among host plants, which is risky and associated with higher predation rates (Bernays, 1997). The links among GOX, host range and HIPVs led us to speculate that using GOX to prevent defenses associated with HIPVs, including attraction of natural enemies, may be an important adaptive feeding strategy in some lepidopteran taxa. Together with the finding that certain generalist caterpillars were able to modulate GLVs (Jones *et al.*, 2019), it appears that modulation of HIPVs is a more common trait of generalist herbivores than was previously recognized. The higher HIPV induction observed by specialist herbivores than by generalist herbivores (Rowen & Kaplan, 2016) supports this hypothesis. It was predicted by Ali & Agrawal (2012) that generalist herbivores may evolve ways to modulate fundamental plant traits (e.g. stomata physiology) to facilitate effective use of diverse host plants. However, the lack of stomatal responses to GOX in some tested plants also reveals the context dependency of whether GOX can influence HIPV release (Mittler *et al.*, 2004), which could influence the patterns of host use by generalist caterpillars such as *H. zea*.

In summary, our findings suggest a novel mechanism by which an insect herbivore inhibits emission of HIPVs during feeding (Fig. 5). By inducing stomatal closure, *H. zea* inhibits the release of HIPVs, which can have cascading effects on the synthesis of VOCs and perception of HIPVs by plants or other members in the community (Dicke & Baldwin, 2010). It is known that herbivory can lead to changes in plant gas-exchange properties and primary metabolism, both of which are closely associated with stomatal dynamics (Nabity *et al.*, 2009). Our results suggest that stomata play a more important role in plant–insect interactions than previously recognized. Changes in

stomatal dynamics can have downstream impacts on plant development and defenses that affect insect herbivores. Although the ecological and evolutionary importance of stomatal dynamics to herbivory and plant defense responses remains unknown at present, we believe future research could reveal the important roles for stomata in multiple aspects of plant–insect interactions. In addition, GOX might serve as an example where the costs of HIPVs to insect herbivores have led to the evolution of traits that are observed across lineages in generalist caterpillars (Eichenseer *et al.*, 2010), underscoring the potential importance of HIPVs and natural enemies in shaping herbivore traits. Given the ubiquity of HIPVs in plants, it is likely that traits that influence HIPVs have evolved broadly among insect herbivores.

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Author contributions

P-AL and GWF conceived the project. P-AL, GWF, YC, DC-R, JLR, CTA, JL, AH, JA and NBZ designed experiments. P-AL, YC, DC-R, JSS, CCH, C-WT, MP, DK and NBZ performed experiments. P-AL, YC, DC-R, CCH, NBZ and AH analyzed the data. P-AL and GWF wrote the manuscript in consultation with YC, DC-R, JLR, CTA, JL and JA. P-AL, YC and DC-R contributed equally to this work.

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Data availability

The datasets and R codes generated during the study will be available in the Dryad repository: Lin *et al.* (2020), Impact of *Helicoverpa zea* salivary GOX on stomatal conductance and volatile emission of host plants, Dryad dataset <https://doi.org/10.5061/dryad.q573n5tgj>.

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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 Feeding damage of *Helicoverpa zea* larvae on host plants.

Fig. S2 Impact of GOX on leaf stomatal conductance of cotton within 2 d.

Fig. S3 Impact of GOX on photosynthetic activity and leaf stomatal conductance at 3 h after treatment.

Fig. S4 Leaf stomatal aperture of tomato at 3 h after damage by fifth-instar larvae of *Helicoverpa zea* with different GOX activity in generation G3 compared with undamaged controls.

Fig. S5 Impact of active and denatured GOX on leaf stomatal aperture.

Fig. S6 Mating process of *gox* mutant *Helicoverpa zea*.

Fig. S7 Impact of *Helicoverpa zea* salivary GOX activity on volatile organic compound (VOC) emission of plants.

Methods S1 Measurements of photosynthetic activity and stomatal conductance (LI-6800 portable photosynthesis system).

Notes S1 Impact of GOX on photosynthetic activity of plants.

Notes S2 Impact of active and denatured GOX on stomatal aperture.

Notes S3 CRISPR/Cas9 mutagenesis and *gox* mutant screening.

Notes S4 Volatiles emitted by plants damaged by *gox-ko* or wild-type *H. zea*.

Table S1 Primers for glucose oxidase gene in *Helicoverpa zea*.

Table S2 crRNA sequences for targeted mutagenesis.

Table S3 Model summaries of volatile analysis.

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