



Phytohormones in Fall Armyworm Saliva Modulate Defense Responses in Plants

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Abstract

Insect herbivory induces plant defense responses that are often modulated by components in insect saliva, oral secretions or regurgitant, frass, or oviposition fluids. These secretions contain proteins and small molecules that act as elicitors or effectors of plant defenses. Several non-protein elicitors have been identified from insect oral secretions, whereas studies of insect saliva have focused mainly on protein identification. Yet, insect saliva may also contain non-protein molecules that could activate defense responses in plants. The goal of this study was to identify non-protein plant defense elicitors present in insect saliva. We used the fall armyworm (FAW), *Spodoptera frugiperda* and its host plants tomato, maize, and rice as a model system. We tested the effect of protein-digested saliva or non-protein components on herbivore-induced defense responses in maize, rice and tomato. We identified phytohormones in FAW saliva using high performance liquid chromatography coupled with mass spectrometry. The results of this study show that non-protein components in FAW saliva modulated defense responses in different plant species. The saliva of this insect contains benzoic acid, and the phytohormones jasmonic acid, salicylic acid, and abscisic acid at concentrations of <5 ng per µl of saliva. Plant treatment with similar phytohormone quantities detected in FAW saliva upregulated the expression of a maize proteinase inhibitor gene in maize, and down-regulated late herbivore-induced defenses in tomato plants. We conclude that FAW saliva is a complex fluid that, in addition to known enzymatic plant defense elicitors, contains phytohormones and other small molecules.

Keywords Oral secretions · Insect saliva · Phytohormones · Plant defenses · Induced defenses · Salicylic acid · Jasmonic acid · Benzoic acid · Abscisic acid

Introduction

Plant defense responses to insect herbivores are mediated by the action of hormones. The jasmonic acid (JA) and ethylene (ET) pathways are frequently activated in response to insect herbivory whereas salicylic acid (SA) tends to be activated in response

to pathogens (Zarate et al. 2007). Evidence suggests that plants are able to integrate a variety of signals to regulate a repertoire of defenses in a specific manner, through the interaction of JA/ET/SA and other hormones, including auxin, abscisic acid (ABA), cytokinins, gibberellins and brassinosteroids (Erb et al. 2012). For example, there is extensive support for the antagonistic activation of JA and SA signaling pathways in a variety of plant species (Thaler et al. 2012). Crosstalk among pathways may be hijacked by herbivores and their symbionts to control induction of defenses and better exploit their hosts (Chung et al. 2013). Insects are also able to activate the SA pathway directly by the excretion of plant hormones (Schwartzberg and Tumlinson 2014). Phytohormones have been identified in insect secretions, including honeydew, regurgitant and frass (Dafoe et al. 2013; Schwartzberg and Tumlinson 2014; Tooker and De Moraes 2006). Some plant hormones have also been found in insect salivary glands (Suzuki et al. 2014; Tooker and De Moraes 2006), but it is unknown if these hormones are secreted in insect saliva and their potential role in plant defense induction.

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Some insect species appear to use phytohormones to regulate plant defenses. For example, feeding by the pea aphid, *Acyrtosiphon pisum*, downregulates JA-defense responses in *Vicia faba*. This plant defense suppression appears to be mediated by SA present in the aphid's honeydew via induction of the SA pathway (Schwartzberg and Tumlinson 2014). Similarly, SA in the mucus of the slug, *Deroceras reticulatum*, induces SA-related gene expression in *Arabidopsis thaliana* (Kästner et al. 2014). JA has been detected in different tissues of *Heliothis virescens* larvae, including their gut, regurgitant, salivary glands, frass and remaining body (Tooker and De Moraes 2006). Likewise, indole-3-acetic-acid (IAA) and its precursors were found in the regurgitant and salivary glands of silkworms (Suzuki et al. 2014). Because some of these insect secretions, such as regurgitant, saliva and frass, come in contact with plants during insect feeding, it is possible that their phytohormone content could modulate plant defense responses.

In addition to phytohormones, insect secretions also contain fatty acids and proteins that induce plant defenses. The oral secretions of several caterpillar species contain fatty acid-amino acid conjugates (FACs), some of which are strong plant defense elicitors (Tumlinson and Engelberth 2008). N-(17-hydroxylinolenoyl)-L-glutamine or “volicitin” was the first FAC identified from the regurgitant of the beet armyworm, *Spodoptera exigua*; this FAC elicits production of the same volatile organic compounds from maize seedlings as caterpillar feeding (Alborn et al. 1997). In addition to volicitin, the oral secretions of *S. exigua* contain other fatty acids as well as free linoleic and linolenic acid (Alborn et al. 2000). FACs seem to be common constituents of insect oral secretions as they have been found in several species (Alborn et al. 2000, 2007; Halitschke et al. 2001; Mori et al. 2003; Pohnert et al. 1999; Yoshinaga et al. 2007, 2014). Insect regurgitant also contains enzymes and plant-derived peptides that induce plant defenses (Mattiacci et al. 1995; Schmelz et al. 2006). Although insect saliva may also contain FACs and other unknown plant defense elicitors, most studies thus far have focused on protein composition. For example, the salivary enzymes glucose oxidase (GOX), adenosine triphosphatases (ATPases) and a recently identified phospholipase C (PLC), regulate defense responses in several host plants. However, insect-induced plant defenses are not exclusively triggered by the action of phytohormones, FACs or enzymes (Acevedo et al. 2015). For example, oral secretions from *Spodoptera littoralis* and *Pieris brassicae* downregulated expression of several wound-inducible genes in *Arabidopsis thaliana*; evidence suggests that this suppression was caused by a small molecule (< 3 kD) rather than by the action of GOX or FACs (Consales et al. 2012). These studies suggest that insect oral secretions and saliva are complex mixtures of compounds from which only a few have been identified.

Saliva of the polyphagous lepidopteran fall armyworm (FAW), *Spodoptera frugiperda*, induces defense responses in maize (Acevedo et al. 2018; Chuang et al. 2014). We have recently shown that the “corn” and “rice” strains of this insect induce differential plant defense responses due to differences in the proteomic composition of their caterpillar saliva (Acevedo et al. 2018). The saliva of FAW strains contains GOX, ATPases, and PLC, which are known plant defense elicitors (Acevedo et al. 2017b); but their saliva also contains heat-resistant molecules that induce plant defenses (Acevedo et al. 2018). Therefore, the main goal of this study was to identify non-protein plant defense elicitors in the saliva of FAW strains feeding on different diets. We specifically quantified the presence of phytohormones using high performance liquid chromatography/Mass Spectrometry (HPLC/MS). This study found benzoic acid (BA), SA, JA and ABA in the saliva of FAW caterpillars; application of these commercially available hormones in similar amounts found in caterpillar saliva regulated defense responses in some plant species.

Methods and Materials

Insects FAW strains were obtained from a laboratory colony maintained at the USDA - ARS in Gainesville, Florida. The Rice strain was collected from a Tifton 85 Bermuda grass field in Chiefland (Levy County) and from pasture fields at Jacksonville, FL, whereas the corn strain was obtained from sweet corn fields at Hendy and Palm Beach County (South Florida). For each strain, the field-collected insects were paired in order to select the F1 individuals containing the corresponding mitochondrial marker that identify each strain (Nagoshi and Meagher 2003).

Plants Maize plants (*Zea mays*, inbred line B73) were kindly provided by W. P. Williams from Mississippi State University and the USDA-ARS, (Mississippi State, MS). Maize seeds were germinated in Promix potting soil (Premier Horticulture Quakertown, PA, USA). The seedlings were transplanted 10 days after germination into 3.78-l pots (C400 Nursery Supplies Inc. Chambersburg, PA, USA) containing Hagerstown loam soil and fertilized once with 10 g of the slow release fertilizer Osmocote plus (15–9–12, Scotts, Marysville, OH, USA). Plants in the V8–V9 physiological stage were used for the experiments. Rice plants (*Oryza sativa*, cv Nipponbare) were obtained from the USDA-ARS Dale Bumpers National Rice Research Center in Arkansas. The seeds were germinated in moist towels at 25 °C (16 h light/8 h dark) and further transplanted to 10 cm square pots (Dillen, Griffin Greenhouse Supplies, Morgantown PA, USA) containing the potting soil Metro-mix 360 (SunGro). One week after emergence, the seedlings were fertilized with a

solution containing 4 g of the slow release iron chelate Sprint 330 (Becker underwood, INC) and 20 g of ammonium sulfate Sulf-N Pro (Lawn & Landscape) diluted in one gallon of water. Weekly thereafter the plants were watered with a solution of 20 g of ammonium sulfate diluted in 3.8 l of water. Rice plants in the V6 physiological stage were used for the experiments. Tomato plants (*Solanum lycopersicum*, cv Better Boy) were grown in Promix potting soil (Premier Horticulture) in 10 cm square pots (Dillen, Griffin Greenhouse Supplies, Morgantown PA, USA), and used when their 5th leaf was fully extended. All plants were grown under glasshouse conditions (14 h light: 10 h dark) at the Pennsylvania State University, University Park, PA.

Plant Defense Responses Plant defense responses to different treatments were evaluated by measuring expression of JA defense-related genes and activity of defense-related proteins using quantitative real-time PCR (qPCR) and biochemical assays, respectively. We measured relative expression of the *Maize proteinase inhibitor (mpi)* gene in maize, activity of trypsin protease inhibitors (Trypsin PI) and relative expression of the *Bowman-Birk proteinase inhibitor (rpi)* gene in rice, and activity of polyphenol oxidase (PPO) and peroxidase (POX) in tomato plants. These genes and proteins have previously been used as markers to evaluate herbivore-induced defense responses in plants (Mahanil et al. 2008; Stout et al. 1994; Tamayo et al. 2000; Xu et al. 2003; Zavala et al. 2004). Activity of plant defensive enzymes was standardized by the total amount of protein in each sample. RNA extraction, cDNA synthesis, real time PCR, PPO and Trypsin PI activity were performed as previously described (Acevedo et al. 2017a, 2018). The primers used in qPCR for the target genes *mpi* and *rpi*, and the endogenous *actin* genes were the ones reported in Ray et al. (2016).

Plant Mechanical Wounding In maize plants, the third youngest leaf was mechanically wounded once using the wounding tool described in Bosak (2011). The two youngest leaves in rice plants were wounded (two wounds per leaf) using a cork borer (Unicore –2.0 Harris, USA). In tomato, the leaflet of the 5th leaf was wounded using the tool described in Bosak (2011).

Effect of Non-protein Molecules from FAW Saliva on Induced Plant Defenses We have previously shown that boiled saliva from FAW induces defense responses in maize (Acevedo et al. 2018); therefore, we hypothesized that FAW saliva contains other non-protein molecules that elicit plant defenses. To test this hypothesis, we evaluated the effect of boiled salivary gland homogenates on defense responses of maize, rice and tomato plants. We further explored the influence of protease-treated salivary gland extracts, as well as protein-precipitated saliva on maize defense responses.

Plant Treatment with Boiled Salivary Gland Homogenates

Salivary glands were dissected from last instar corn strain caterpillars (two days after molting) fed on detached plant leaves and homogenized in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH. 7.2). The homogenates were centrifuged at 8000 rpm for three minutes; the supernatant was recovered and kept on ice. Subsequently, protein concentration was quantified using the Bradford assay with a bovine serum albumin standard curve (Bradford 1976) in a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale CA). Each plant was mechanically wounded and treated with 15–20 µl of 1X PBS solution containing 20 µg of either boiled (95 °C for 1–4 h) or non-boiled salivary gland homogenates obtained from five caterpillars. The effect of these treatments on plant defense responses was compared with wounding plus PBS and untreated controls under a completely randomized design.

Maize Treatment with Protease-Treated Salivary Gland Extract

Labial salivary glands were dissected from last instar corn strain FAW caterpillars (2 d after molting) fed on corn from egg hatch. Immediately after being dissected, the salivary glands were quickly rinsed by immersion in deionized water, and then transferred to 1.5 µl tubes containing Ringer's solution (136 mM NaCl, 1.8 mM CaCl₂, 2.7 mM KCl, 2.4 mM NaHCO₃). The tubes were placed on an open-air platform shaker Lab-Line MaxQ2000 (Barnstead International/Lab-Line Melrose Park, IL 60,160) at 150 rpm for 15 min at room temperature to allow the content of the salivary glands to be released into the solution (Rivera-Vega et al. 2018). The supernatant was recovered and kept on ice for protein quantification using the Bradford assay described above. Proteins were digested by incubating salivary gland extracts with pronase from *Streptomyces griseus* (Calbiochem cat # 53702) at 37 °C for 30 min, followed by a denaturation step at 95 °C for 45 min to inactivate the proteases. The concentration of salivary protein to protease was 10:1 µg. Each plant was mechanically wounded and treated with 15 µl (20 µg) of either untreated or protease-treated salivary gland extract obtained from five caterpillars, and diluted in 1X PBS. The effect of these treatments on plant defense responses was compared with the effect of wounding plus treatment with Ringer's solution and PBS, and with the effect from untreated control plants under a completely randomized design.

Maize Treatment with Protein-Precipitated Saliva Saliva was collected from last instar corn strain FAW caterpillars (2 d after molting) fed on corn from egg hatch following a previously described procedure (Acevedo et al. 2017b). The saliva collected from 43 caterpillars was diluted in water to a volume of 15 µl. Salivary proteins were then precipitated with nine volumes (135 µl) of cold 100% ethanol (200 proof) at –20 °C

overnight. Samples were centrifuged at 14,000 rpm for 15 min at 4 °C; the supernatant was recovered, transferred to a new tube and allowed to dry at room temperature. Lastly, the samples were resuspended in 45 µl of MQ water and used to treat plants. Each plant was mechanically wounded and treated with 10 µl of either saliva, precipitated saliva or water under a completely randomized design.

SDS Polyacrylamide Gels (PAGE) Proteins from saliva and salivary gland homogenates from the experiments above were visualized in 0.75 mm PAGE gels to verify protein denaturation. Protein separation was carried out by loading ~0.5 µg of protein into 12% SDS PAGE gels run at 75 V for ~3 h in a vertical electrophoresis camera (Biorad Mini-Protean #165800FC). The protein bands were then visualized by staining with silver nitrate.

Quantification of Phytohormones in Saliva of the FAW Strains

The quantification of JA, SA, ABA, cinnamic acid (CA), and BA (SA precursor) in caterpillar saliva was carried out using HPLC/MS with isotope-labeled standards at the Penn State University Metabolomics Facility. Saliva was collected from last-instar caterpillars fed on either artificial diet (wheat germ) or on detached leaves of maize, rice, and tomato plants following a previously described procedure (Acevedo et al. 2017b). The saliva samples were collected in a micropipette tip (catalog no. 53550–178 VWR, West Chester, PA) preloaded with 1 µl of MQ water and stored at –80 °C until use. Each saliva sample comprised the pooled saliva quantities (1.2–4.5 µl) collected from 30 to 50 caterpillars. The volume of saliva from each sample was obtained using a mechanical Eppendorf micropipette (0.5–10 µl) (Fisher Scientific Ottawa, ON, Canada). There were 3 biological replications for each FAW strain and diet combination (except for tomato in which only the corn strain was tested). The saliva samples were diluted in MQ water to a final volume of 10 µl and their proteins precipitated overnight at –20 °C by the addition of 3.5 volumes (35 µl) of 100% methanol to which internal isotope-labeled standards had been added. The concentration of each isotope standard [α - ^{13}C SA (Campro Scientific #CS01-183_473), D₅ JA (CDN isotopes #D-6936), d₆ ABA (ICON #1001), D₅ CA (CDN isotopes #D-5284), and BA ring $^{13}\text{C}_6$ (ICON #IC3089)] in the 35 µl solution was 1.4 µM. The samples were then centrifuged at 20,000 rcf for 15 min at 4 °C. The supernatant was removed and transferred to polypropylene tubes sealed with aluminum crimp top caps. Five microliters of each sample were separated by reverse phase HPLC using a Prominence 20 UFLCXR system (Shimadzu, Columbia MD) with a Waters (Milford, MA) BEH C18 column (100 mm × 2.1 mm 1.7 µm particle size) maintained at 55 °C and a 20-min aqueous acetonitrile gradient, at a flow rate of 250 µl/min. Solvent A was HPLC grade water with 0.1% formic acid and Solvent B was HPLC grade acetonitrile

with 0.1% formic acid. The initial conditions were 97% A and 3% B, increasing to 45% B at 10 min, 75% B at 12 min where it was held at 75% B until 17.5 min before returning to the initial conditions. The eluate was delivered into a TripleTOF® 5600 system using a Duospray™ ion source (AB Sciex, Framingham, MA). The capillary voltage was set at 4.5 kV in negative ion mode, with a declustering potential of 80 V. The MS was operated in IDA (Information Dependent Acquisition) mode with a 100 ms survey scan from 100 to 1200 *m/z*, and up to 20 MS/MS product ion scans (100 ms) per duty cycle using collision energy of 50 V with a 20 V spread. Data were acquired and analyzed using the Analyst software (Applied Biosystems). Phytohormone quantities were determined by the analysis of the Gaussian smoothed peak areas of each compound with respect to their corresponding isotopic standards. We constructed individual standard curves for each phytohormone using different concentrations of unlabeled standards containing a fixed concentration of the corresponding isotope. We calculated the concentration of phytohormones in each saliva sample using the regression equation obtained from the standard curve of each compound by replacing the dependent variable “Y” for the ratio of the sample peak area over the isotope peak area, and multiplying this result by the concentration of the isotope. Finally, we divided the concentration of each phytohormone by the volume of insect saliva in each sample to report results as ng/µl of saliva. The *m/z* values of corresponding [M-H] ions, and the retention times for each labeled compound are in Table 1.

Effect of Phytohormones Present in Insect Saliva on Plant Defense Induction

It has been shown that exogenous applications of phytohormones modulate defense responses in plants (Bari and Jones 2009); therefore, we hypothesized that the phytohormones present in FAW saliva could induce plant defense responses. To test this, we treated tomato, maize and rice plants with a mixture of phytohormones estimated to be in 1 µl of caterpillar saliva when feeding on each respective plant type. Although, it is experimentally challenging to calculate accurately the amount of saliva deposited by a caterpillar during its feeding activity, we conservatively estimated that FAW secretes at least 1 µl

Table 1 Retention time and *m/z* values of isotope-labeled phytohormone standards

Compound	Formula	[M-H] <i>m/z</i>	Retention time (min)
Jasmonic acid	C ₁₂ H ₁₃ D ₅ O ₃	214.1511	10.9
Salicylic acid	¹³ C ₆ H ₆ O ₃	138.0288	8.92
Abscisic acid	C ₁₅ H ₁₄ D ₆ O ₄	269.1679	8.56
Benzoic acid	¹³ C ₆ H ₅ O ₂	127.0455	7.36
Cinnamic acid	C ₉ H ₇ D ₅ O ₂	152.0725	9.01

of saliva based on the specific activity of the salivary enzyme glucose oxidase (GOX) (Eichenseer et al. 2010), tissue immunoblot assays with anti-GOX antibody (Chuang et al. 2014), and the amount of protein in the saliva (Acevedo et al. 2017b). For plant treatment, commercial phytohormones were diluted in 100% methanol to a concentration of 10 μ M, and then further diluted with MQ water. Each plant was mechanically wounded, as described above, and treated with 15–20 μ l of the corresponding aqueous phytohormone mixture. Plant defense responses elicited by mechanical wounding plus phytohormones were compared against those elicited by wounding and the application of water and methanol (at the same concentrations used in the hormone treatment) as well as untreated controls. Plant defense responses to the application of phytohormones and corresponding controls were measured in time course experiments as follows: 24, 48, 72 and 96 h for tomato plants; 12, 24, 48, 72 and 96 h for rice; and 24, 48, and 72 h for maize. In all cases, each time point had a separate group of plants randomly assigned to each treatment under a completely randomized design.

Effect of Exogenous Application of BA on Tomato Defense Responses We tested the effect of commercial non-isotope labeled BA on PPO activity of tomato plants. Wounded plants were treated with the estimated amount of BA contained in 1 μ l of caterpillar saliva. Commercial BA (Sigma) was diluted in 100% methanol to a concentration of 10 μ M, and then further diluted with MQ water to treat the plants. The PPO activity elicited by BA was compared with the activity elicited by a phytohormone mixture (prepared as indicated above), a blank (aqueous solution of 18% methanol), and untreated control plants. PPO activity of tomato plants was measured 96 h after treatment. All plants were assigned to the treatments in a completely randomized design.

Experimental Design and Statistical Analysis Plant defense responses (gene expression) to the treatments (wounding plus boiled salivary gland homogenates, protease-treated saliva extract, precipitated saliva, PBS and untreated controls) were analyzed with one-way ANOVA following post hoc tests of Tukey and Fisher at $\alpha = 0.05$. Likewise, the effect of the treatments (phytohormone mixture, BA, blank, and untreated controls) on tomato PPO activity was analyzed with one-way ANOVA following the Tukey post hoc test at $\alpha = 0.05$. Differences in phytohormone quantities found in FAW caterpillar saliva were analyzed using a two-factor factorial design; the factors were *strain* (corn or rice) and *diet type* (artificial diet, maize, rice, and tomato). The effects of *time* (specific for each plant type) and *treatment* (wounding plus phytohormone mixture, blank or untreated controls) on plant defense responses (PPO, POX, trypsin PI activity, and *mpi* relative expression) in time course experiments were analyzed using a two-factor factorial design. All the statistical analyses were

performed using the software Minitab 18 (Minitab Inc., State College, PA, USA), and all graphs were generated in R version 3.2.4 (Foundation for Statistical Computing, Vienna, Austria).

Results

Non-protein Components in the FAW Saliva Induce Defense Responses in Plants To verify that FAW saliva contained non-protein plant defense elicitors, we measured plant defense responses after wounding and treatment with saliva whose proteins were heat-inactivated, digested or precipitated. Both non-boiled and boiled salivary gland homogenates applied to wounded plants, induced higher expression of the *mpi* and *rpi* genes in maize and rice, respectively, than their corresponding PBS treatment (Fig. 1 a,b). Conversely, in tomato plants, the application of salivary gland homogenates suppressed PPO activity compared to buffer-treated plants (Fig. 1c). In the plant species tested, the defense responses elicited by non-boiled and boiled salivary gland homogenates were not different from each other ($P > 0.05$), indicating that some of the elicitors contained in the saliva of this insect are not heat sensitive (Fig. 1). Likewise, when pronase-treated salivary gland extracts were applied to wounded maize plants, the *mpi* gene expression was higher than the PBS treatment but not different from the expression levels triggered by untreated salivary extracts (Fig. 2a). Similar results were obtained with saliva in which the salivary proteins were precipitated with ethanol (Fig. 2c).

FAW Saliva Contains Phytohormones As an attempt to identify non-protein salivary elicitors in FAW saliva, this study identified and quantified the plant hormones JA, SA, ABA, CA, and the SA precursor BA using HPLC/MS. The most abundant plant hormone in FAW saliva was SA, and its precursor BA; JA and ABA were also present at lower amounts (Fig. 3), but CA was not found. There were no differences in the quantities of these hormones for the two FAW strains, but there was a strong effect of the type of diet on the quantities of SA and JA (Table 2). The saliva of rice-fed caterpillars contained greater amounts of SA compared with the saliva of caterpillars fed on maize, and artificial diet. Rice and tomato-fed caterpillars had greater content of JA in their saliva compared with the ones fed on maize. No differences among diets were observed for either BA or ABA (Fig. 3).

Phytohormones Present in FAW Saliva Modulate Defense Responses in some Plants We tested the effect of phytohormones found in FAW saliva on defense responses of tomato, rice and maize plants. Each wounded tomato plant was treated with either a mixture of hormones [0.1426 ng of JA, 0.3281 ng of SA, 3.67 ng of BA, and 0.004 ng of ABA

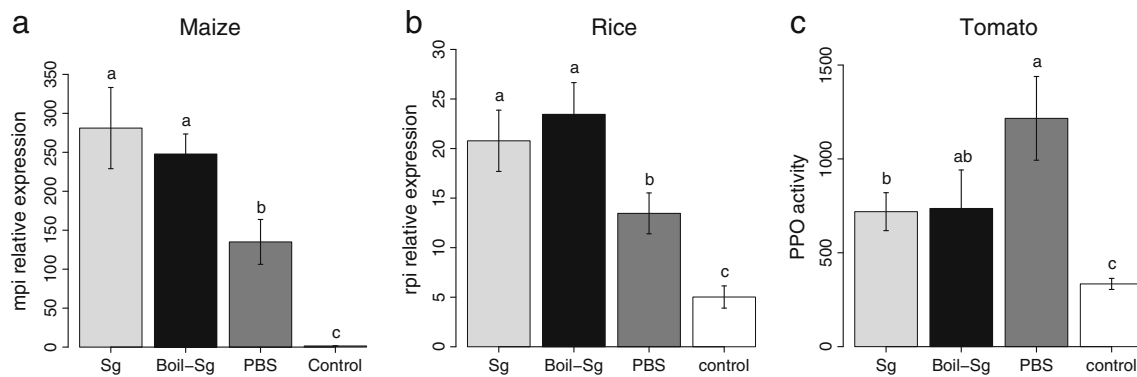


Fig. 1 Plant defense response to wounding plus boiled (Boil-Sg) or non-boiled salivary gland homogenates (Sg) from fall armyworm caterpillars. **a** Maize proteinase inhibitor (*mpi*) gene expression 24 h after treatment ($F_{3,17} = 214.97$, $P < 0.001$; Tukey test; $n = 4-5$; log transformed data). **b** Rice Bowman-Birk proteinase inhibitor (*rpi*) gene expression 24 h after treatment ($F_{3,23} = 12.28$, $P < 0.001$; Fisher test; $n = 5-6$; log transformed

data). **c** Polyphenol oxidase (PPO) activity 48 h after treatment ($F_{3,20} = 4.07$, $P = 0.021$; Fisher test; $n = 6$; untransformed data). Bar values are untransformed means $\pm$ SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at $\alpha = 0.05$. PBS are buffer-treated plants. Controls are undamaged plants

(Sigma-Aldrich) diluted in an aqueous solution of 18% methanol] or a solution of 18% methanol. The activity of PPO and POX was affected by the treatments and time points at which the samples were harvested (PPO time effect: $F_{3,97} = 11.51$, $P < 0.001$, PPO treatment effect: $F_{2,97} = 29.41$, $P < 0.001$; POX time effect: $F_{3,97} = 39.93$, $P < 0.001$, POX treatment effect: $F_{2,97} = 179.64$, $P < 0.001$). Therefore, differences among treatments were analyzed for each time point using one-way ANOVA. The activity of PPO was similar in wounded plants treated with the phytohormone mixture, and the ones treated with water + methanol solution at 24, 48 and 72 h after treatment. However, at 96 h, the activity of PPO was suppressed by application of the phytohormone mixture (Fig. 4). The activity of POX was similar in wounded plants treated with the phytohormone mixture and water + methanol, but both treatments had higher POX activity when compared to untreated controls at the time points tested (Fig. S1).

Wounded rice plants were treated with a phytohormone mixture containing 0.126 ng of JA, 0.55 ng of SA, 3.26 ng of BA, and 0.0045 ng of ABA diluted in an aqueous solution of 8% methanol. There was a significant effect of both, treatment ($F_{3,126} = 22.65$, $P < 0.001$) and time ($F_{4,126} = 16.37$, $P < 0.001$) on the activity of Trypsin PI. Individual ANOVAs for each time point followed by the Tukey multiple comparison test, showed similar effects of the phytohormone mixture, and the treatment of water + methanol on Trypsin PI activity, but both treatments were significantly higher than untreated controls at 24 and 48 h post treatment (Fig. 5).

Wounded maize plants were treated with a mixture of phytohormones containing 0.055 ng of JA, 0.186 ng of SA, 1.99 ng of BA, and 0.0024 ng of ABA diluted in an aqueous solution of 13.3% methanol. There was a significant effect of both treatment ($F_{2,61} = 159.66$, $P < 0.001$) and time ($F_{2,61} = 11.27$, $P < 0.001$) on *mpi* relative expression. Individual ANOVAs for each time point followed by the Tukey multiple

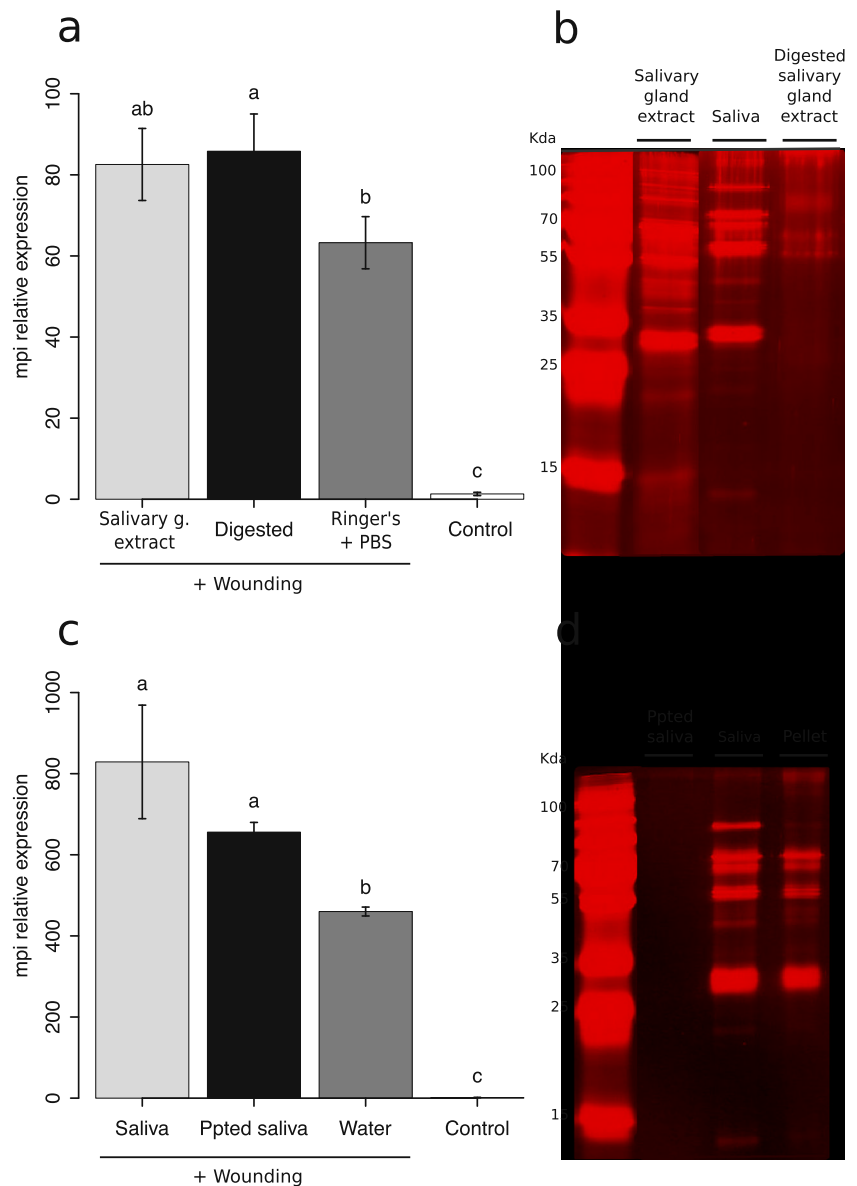
comparison test, found higher transcript accumulation of *mpi* in maize plants treated with the phytohormone mixture compared with the ones treated with water + methanol at 48 and 72 h after treatment (Fig. 6). No differences were found between the phytohormones and water + methanol treatments at 24 h (Fig. 6).

Exogenous Application of BA Suppresses PPO Activity on Tomato Plants Due to the abundance of BA in secreted FAW saliva, we tested its effect on tomato PPO activity. Wounded tomato plants were treated with one of the following treatments: a) 3.67 ng of BA diluted in an aqueous solution of 18% methanol, b) an aqueous solution of 18% methanol (blank), and c) a mixture of hormones (0.1426 ng of JA, 0.3281 ng of SA, 3.67 ng of BA, and 0.004 ng of ABA; all of these compounds were diluted in an aqueous solution of 18% methanol). The activity of PPO was significantly lower in plants treated with BA when compared with the blank treatment. The PPO activity induced by the phytohormone mixture was lower than the blank, but not different from it or the BA treatment (Fig. 7).

Discussion

During feeding, Lepidoptera larvae secrete saliva that comes in contact with plant wounds (Peiffer and Felton 2005); cues present in insect saliva and oral secretions are recognized by plants to trigger antiherbivore defense responses (Acevedo et al. 2015; Peiffer and Felton 2005). Insect saliva contains an abundance of proteins, some of which modulate plant defense responses (Acevedo et al. 2018; Musser et al. 2002; Rivera-Vega et al. 2017; Wu et al. 2012). Although, insect saliva is likely also to contain small molecules, their identity and their role in plant defense induction are unknown.

Fig. 2 Maize defense response to wounding plus the application of salivary gland extracts, and protein-inactivated saliva from fall armyworm caterpillars. **a** *Maize proteinase inhibitor (mpi)* gene expression 24 h after treatment with untreated salivary gland extract, protein-digested salivary gland extract (pronase-treated), and dilution buffers (Ringer's solution + PBS) ($F_{3,19} = 181.93$, $P < 0.001$; Fisher test; $n = 5$ –6; log transformed data). **b** SDS PAGE gel showing protein degradation after pronase treatment, and protein profiles of salivary gland extract and saliva from fall armyworm caterpillars. **c** *Maize proteinase inhibitor (mpi)* gene expression 24 h after treatment with saliva and protein-precipitated saliva (Pptd saliva) ($F_{3,15} = 420.32$, $P < 0.001$; Fisher test; $n = 4$ –5; log transformed data). **d** SDS PAGE gel showing the absence of salivary protein bands after ethanol precipitation. Bar values are untransformed means $\pm$ SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at $\alpha = 0.05$. Controls are undamaged plants

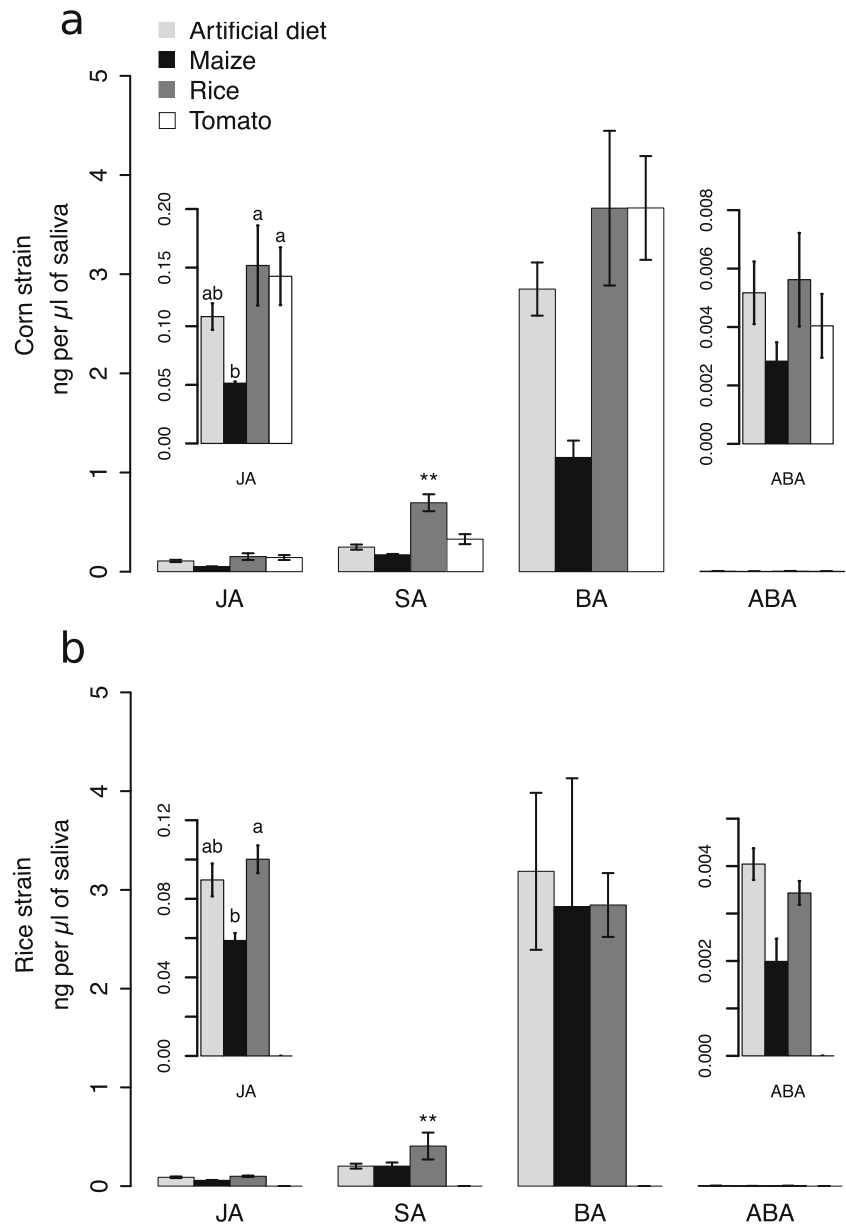


Our results demonstrate that saliva of fall armyworm caterpillars contain non-protein molecules that regulate defense responses in plants. Treatment with saliva or salivary gland homogenates with heat-inactivated enzymes, and digested and precipitated proteins, induced defense responses in maize at similar levels found with untreated saliva, but higher than buffer-treated controls. In tomato, application of salivary gland homogenates from FAW caterpillars had decreased PPO activity compared to buffer-treated plants (Fig. 1).

These results suggest that FAW saliva contains non-protein molecules that trigger defense responses in some plant species. However, FAW saliva also contains the enzymatic elicitors GOX and PLC (Acevedo et al. 2018); Therefore, we expected different levels of defense responses in plants treated with non-boiled, and boiled salivary gland homogenates in which these enzymatic elicitors were inactivated. A possible

explanation for this is that the activity of GOX and PLC is affected by the freshness of the samples and the type of diet (Acevedo et al. 2018). Except for the experiments in tomato, we did not use saliva or salivary gland samples that were collected the same day, possibly affecting the activity of PLC. Moreover, GOX activity levels in tomato-fed FAW caterpillars are very low compared to caterpillars reared on other diets (Acevedo et al. 2017b), possibly helping explain why untreated salivary gland homogenates did not induce greater PPO activity than boiled samples or buffer-treated controls (Fig. 1). Furthermore, enzymatic elicitors may also be degraded by other proteases that may be present in FAW saliva, possibly influencing our results. The differential plant defense responses in maize and tomato to the application of phytohormones may be due to differences in signal transduction pathways between these plant species (Acevedo et al. 2017a).

Fig. 3 Phytohormone quantities in the saliva of fall armyworm caterpillars reared on different diet types. Values are untransformed means $\pm$ SEM. Asterisks and different letters indicate significant differences ($\alpha = 0.05$) among diet types obtained with ANOVA and Tukey test for (salicylic acid (SA) ($F_{3, 16} = 9.56$, $P < 0.001$), and jasmonic acid (JA) ($F_{3, 16} = 6.74$, $P = 0.004$), respectively. ABA = abscisic acid and BA = benzoic acid



Together, these results indicate that saliva of FAW caterpillars contain non-protein elicitors of plant defenses that interact with plants in a host-dependent manner.

Attempts to identify some of the non-protein elicitors in FAW saliva, led us to screen for phytohormones. Saliva of this insect contained JA, SA, BA, and ABA; BA and SA were found in the greatest amounts (Fig. 3). BA and SA occur naturally in plants and are precursors to several primary and secondary metabolites as well as plant defense responses against biotrophic pathogens (Widhalm and Dudareva 2015; Zarate et al. 2007). Previous studies have shown that benzoic acid or its conjugates influence SA accumulation in *Arabidopsis*, cucumber and tobacco plants (Chong et al. 2001; Doherty et al. 1988; Dorey et al. 1997; Mauch-Mani and Slusarenko 1996; Meuwly et al. 1995). Therefore, we

hypothesized that exogenous application of these molecules through caterpillar saliva could induce defense responses in plants. The results of this study showed that wounded tomato plants treated with a mixture of commercial phytohormones, at similar quantities detected in FAW saliva, had lower activity of the anti-nutritional protein PPO four days after treatment compared with their respective controls (Fig. 4). Similar results were found when tomato plants were treated with only BA, the main constituent of the phytohormone mixture identified in FAW saliva (Fig. 7). Even though BA has been reported as a known inhibitor of PPO (Doğan et al. 2013; Janovitz-Klapp et al. 1990), the concentrations of BA needed to reduce enzyme activity are much higher than the concentrations applied to plants in this study (Doğan et al. 2013). Wounded maize plants treated with a mixture of

Table 2 Effect of strain and diet on the phytohormone quantities detected in FAW saliva. Asterisks (*) indicate significant differences at $\alpha = 0.05$

Phytohormone	Factor	<i>F</i> (treatment, error df)	ANOVA <i>P</i> value
JA	strain	$F_{1,16} = 2.13$	0.163
	diet type	$F_{3,16} = 6.74$	0.004*
SA	strain	$F_{1,16} = 2.72$	0.119
	diet type	$F_{3,16} = 9.56$	0.001*
BA	strain	$F_{1,16} = 0.46$	0.508
	diet type	$F_{3,16} = 1.74$	0.2
ABA	strain	$F_{1,16} = 3.87$	0.067
	diet type	$F_{3,16} = 2.86$	0.069

phytohormones detected in FAW saliva, had a higher expression of the *maize proteinase inhibitor (mpi)* gene 48 and 72 h after treatment compared with their respective controls (Fig. 6). In rice plants, application of these hormones did not affect activity of trypsin PI during the four days tested, perhaps because rice shoots contain very high levels of free SA ($>10 \mu\text{g/g}$ fresh weight; Silverman et al. 1995) and, therefore, exogenous application of SA and BA at these small quantities ($< 5 \text{ ng}$) were not enough to trigger defense responses. Alternatively, these hormones may influence plant defenses in concert with other constituents of FAW saliva.

The amounts of phytohormones detected in FAW saliva were similar in the two FAW strains. These results suggest that the phytohormone content in the saliva of these insects does not appear to influence intraspecific differences in plant

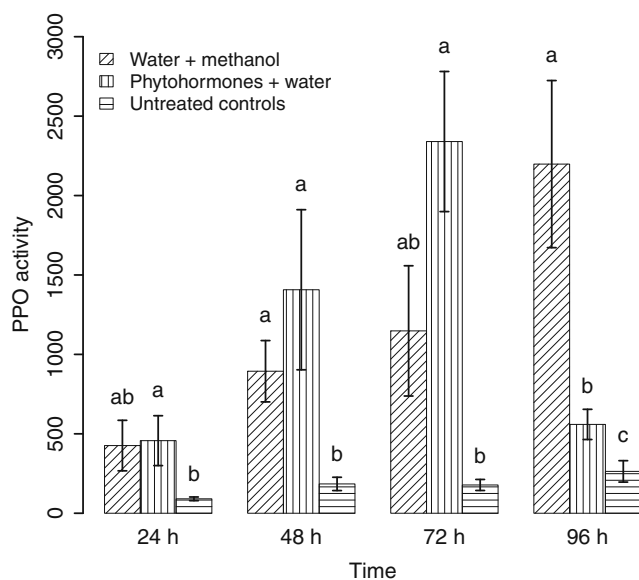


Fig. 4 Polyphenol oxidase (PPO) activity in tomato plants treated with either a mixture of phytohormones or water plus methanol at different time points. Bar values are untransformed means $\pm$ SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$ [24 h ($F_{2,23} = 3.57$, $P = 0.046$), 48 h ($F_{2,22} = 76$, $P = 0.010$), 72 h ($F_{2,22} = 5.7$, $P = 0.010$), 96 h ($F_{2,26} = 30.26$, $P < 0.001$)]. Controls are undamaged plants

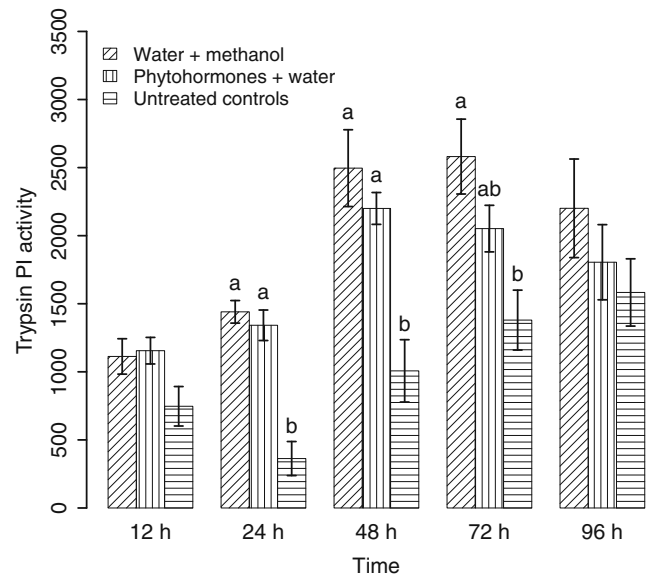


Fig. 5 Trypsin proteinase inhibitor (Trypsin PI) activity in rice plants treated with either a mixture of phytohormones or water plus methanol at different time points. Bar values are untransformed means $\pm$ SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$ [12 h ($F_{2,24} = 2.99$, $P = 0.069$), 24 h ($F_{2,21} = 16.18$, $P < 0.001$), 48 h ($F_{2,21} = 11.10$, $P = 0.001$), 72 h ($F_{2,27} = 7.08$, $P = 0.003$), 96 h ($F_{2,21} = 1.06$, $P = 0.366$)]. Controls are undamaged plants

defense induction previously reported for FAW strains in maize plants (Acevedo et al. 2018). Nevertheless, the phytohormone quantities in FAW saliva were strongly influenced

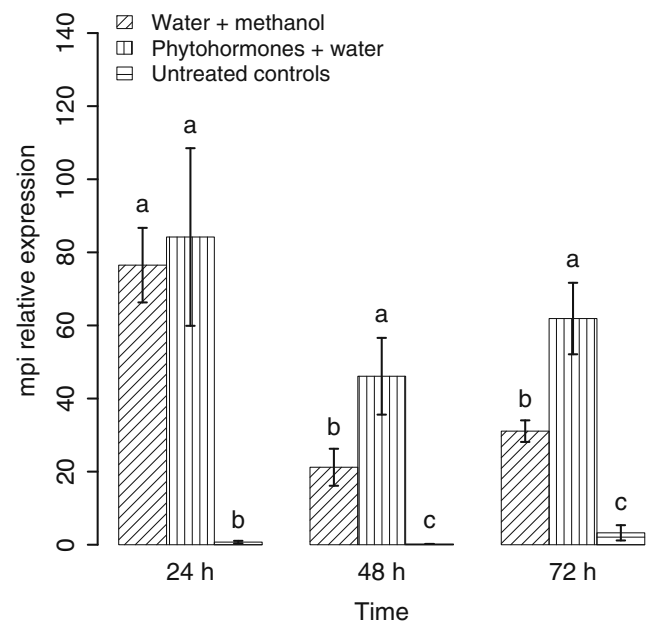


Fig. 6 Maize proteinase inhibitor (*mpi*) gene expression treated with either a mixture of phytohormones or water plus methanol at different time points. Bar values are untransformed means $\pm$ SEM; different letters indicate significant differences obtained with ANOVA following a Tukey test at $\alpha = 0.05$ [24 h ($F_{2,19} = 73.35$, $P < 0.001$), 48 h ($F_{2,19} = 75.48$, $P = 0.001$), 72 h ($F_{2,19} = 46.69$, $P < 0.001$), log transformed data, $n = 9$ for treatments, and 4 for controls]. Controls are undamaged plants

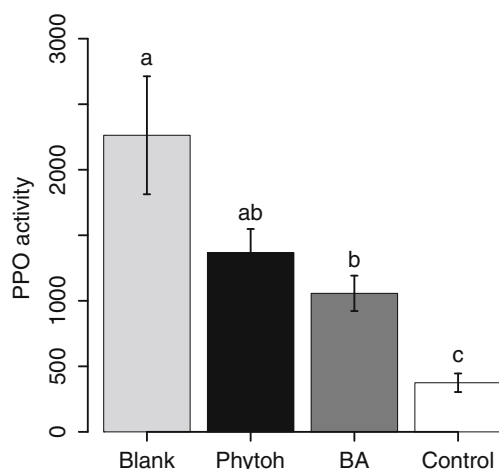


Fig. 7 Polyphenol oxidase (PPO) activity in tomato plants treated with either benzoic acid (BA), a mixture of phytohormones (Phytoh) or water plus methanol (Blank) four days after treatment. Bar values are untransformed means $\pm$ SEM; different letters indicate significant differences obtained with ANOVA following a Tukey test at $\alpha = 0.05$ ($F_{3,36} = 21.09$, $P < 0.001$, $n = 10$, log transformed data). Controls are undamaged plants

by the type of diet on which caterpillars were grown. SA amounts were higher in saliva of rice-fed caterpillars, probably because of the higher quantity of this hormone in rice leaves (Silverman et al. 1995) compared with amounts in tomato ($0.27 \mu\text{g/g}$ fresh weight), and maize ($<0.01 \mu\text{g/g}$ fresh weight; Raskin et al. 1990). However, greater amounts of JA were also found in rice and tomato-fed caterpillars compared with other diet types. In contrast, the type of diet had no effect on quantities of BA, and ABA. Previous studies have shown that amounts of JA, BA and SA in diets are not associated with quantities of these compounds detected in insect eggs (Tooker and De Moraes 2007). The amounts of JA were higher in insects than those found in their diets (Tooker and De Moraes 2005), suggesting that insects may be able to sequester these compounds selectively for re-delivery to the plants. Alternatively, insects or their associated symbionts may be able to synthesize them. For example, several galling insects contain indole-3-acetic-acid (IAA) and cytokinins, hormones that regulate plant cell growth and division (Dorchin et al. 2009; Straka et al. 2010; Tanaka et al. 2013; Tooker and Moraes 2011; Werner et al. 2001; Yamaguchi et al. 2012; Zhao 2010). These hormones are either synthesized by insects or by their symbionts; the galling sawfly *Pontania* sp. is able to produce IAA de novo from tryptophan (Yamaguchi et al. 2012), but the leaf-mining moth *Phyllonorycter blancardella* seems to rely on endosymbiotic bacteria for the production of cytokinins (Kaiser et al. 2010). The associated bacteria of *P. blancardella* induce formation of photosynthetically active green areas in senescent leaves (green-island phenotype; Kaiser et al. 2010); because these green islands are rich in cytokinins (Giron et al. 2007), it is likely that symbiotic bacteria in the caterpillars are producing them (Giron et al. 2013).

In conclusion, our study has shown that, in addition to enzymatic elicitors, FAW saliva contains non-protein compounds that modulate defense responses in different plants. We successfully identified and quantified BA, SA, JA, and ABA in secreted FAW saliva and tested their role in plant defense induction. Our experiments indicate that a mixture of these hormones suppresses herbivore-induced defenses in tomato, but induces them in maize, which correlates with the effect elicited by saliva treatment on these plants. It is unknown if these hormones are sequestered by these insects from their diet, or are synthesized by them or by their associated symbionts. Future experiments aiming to elucidate these mechanisms are needed.

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