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Intraspecific differences in plant defense induction by fall armyworm strains

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Summary

• The underlying adaptive mechanisms by which insect strains are associated with specific plants are largely unknown. In this study, we investigated the role of herbivore-induced defenses in the host plant association of fall armyworm (*Spodoptera frugiperda*) strains.

• We tested the expression of herbivore-induced defense-related genes and the activity of plant-defensive proteins in maize and Bermuda grass upon feeding by fall armyworm strains.

• The rice strain caterpillars induced greater accumulation of proteinase inhibitors in maize than the corn strain caterpillars. In Bermuda grass, feeding by the corn strain suppressed induction of trypsin inhibitor activity whereas the rice strain induced greater activity levels. Differences in elicitation of these plant defenses by the two strains seems to be due to differences in the activity levels of the salivary enzyme phospholipase C. The levels of plant defense responses were negatively correlated with caterpillar growth, indicating a fitness effect.

• Our results indicate that specific elicitors in the saliva of fall armyworm stains trigger differential levels of plant defense responses that affect caterpillar growth and thus may influence host plant associations in field conditions. The composition and secretion of plant defense elicitors may have a strong influence in the host plant association of insect herbivores.

Introduction

While feeding, herbivores release a variety of cues present in their oral secretions, saliva and frass that come in contact with wounded plant tissues (Acevedo *et al.*, 2015; Kaloshian & Walling, 2015; Schmelz, 2015; Stuart, 2015). In their coevolution with herbivores, plants have evolved mechanisms to recognize these herbivore-derived cues or HAMPs (herbivore-associated molecular patterns) to activate production of defense responses. Both the amount and the type of cues released by insects and the plant's ability to recognize them seem to be species-specific (Acevedo *et al.*, 2015). This phenotypic plasticity of plants and associated herbivores play an important role in survival and have a significant effect in their interactions with one another and their trophic levels (Mooney & Agrawal, 2008). The herbivores' ability to develop physiological, morphological and behavioral adaptations in response to physical and chemical plant barriers directly influences their ability to use a particular host.

Insect host strains are genetically differentiated populations of the same species that exhibit partial reproductive isolation and are adapted to specific host plants (Drès & Mallet, 2002). Host race evolution has been linked to differential host plant association in several insect species (Drès & Mallet, 2002), one of the best documented cases being strain formation in the apple maggot fly (*Rhagoletis pomonella* (Walsh)) associated with a host shift from wild hawthorn to cultivated apple trees (Bush, 1969; Feder *et al.*, 1994). Likewise, the fall armyworm (*Spodoptera frugiperda* (J.E. Smith)) (FAW) is composed of two sympatric strains that exhibit different host preferences under field conditions. The 'corn strain' is primarily found in maize, sorghum and cotton, while the 'rice strain' is mostly associated with rice and forage grasses such as Bermuda grass (Pashley, 1986; Whitford *et al.*, 1988; Machado *et al.*, 2008). These strains exhibit plant-dependent fitness differences in larval and/or pupal weight and developmental time (Meagher *et al.*, 2004; Groot *et al.*, 2010; Meagher & Nagoshi, 2012), suggesting differences in nutrient assimilation and metabolism. Studies aiming to elucidate the factors driving differential host plant association of FAW strains have found greater capacity of the rice strain to metabolize the cyanide present in grasses (Hay-Roe *et al.*, 2011) and lower activity levels of the detoxification enzyme mixed-function oxidase than the corn strain (Veenstra *et al.*, 1995). These studies illustrate the presence of key physiological adaptations of the FAW strains to overcome constitutive defenses of their associated host plants, but how these strains deal with induced plant defenses is largely unknown.

The mechanical damage caused during insect feeding can, by itself, induce some direct and indirect plant defense responses; however, there is evidence that plants recognize herbivore-derived cues to fine-tune the production of defense compounds (Howe & Jander, 2008). During feeding, lepidopteran larvae secrete copious saliva and oral secretions (or regurgitant) onto wounded plant tissues (Peiffer & Felton, 2005; Felton & Tumlinson, 2008). Caterpillar regurgitant is a rich source of HAMPs including β-glucosidase (Mattiacci *et al.*, 1995), fatty acid amino acid conjugates (FACs) (Alborn *et al.*, 1997, 2003; Halitschke *et al.*, 2001; Yoshinaga *et al.*, 2014) and inceptins (Schmelz *et al.*, 2006) that induce defenses in numerous plant species. Likewise, caterpillar saliva, a rich proteinaceous secretion, is known to modulate defense responses in plants (Rivera-Vega *et al.*, 2017). The salivary enzyme glucose oxidase (GOX) is present in more than 80 insect species (Eichenseer *et al.*, 2010) and can act as either an elicitor (inducing defense responses against herbivores) or an effector (suppressing herbivore-induced defenses) depending on the host plant (Musser *et al.*, 2002; Tian *et al.*, 2012). In addition to GOX, several enzymes with ATPase activity, which act as effectors in tomato, were identified in the saliva of the noctuid *Helicoverpa zea* (Boddie) (Wu *et al.*, 2012). Other studies have found that insect-derived lipases can also affect plant defense signaling. Lipases present in the oral secretions of the generalist grasshopper *Schistocerca gregaria* (Forskal) induce the accumulation of oxylipins, especially OPDA (12-oxo-phytodienoic acid) in *Arabidopsis thaliana* (L.) (Schäfer *et al.*, 2011). Moreover, lipase-like proteins with similarity to phospholipases were found in the salivary glands of the Hessian fly larvae (*Mayetiola destructor* (Say)) and may affect wheat immunity by increasing plant cell permeability (Shukle *et al.*, 2009). These studies indicate that insects from different order groups may share some of the identified HAMPs and effectors but their biological relevance is highly dependent upon their host plant association. For instance, components in the saliva of FAW caterpillars are known to induce production of proteinase inhibitors in maize (Chuang *et al.*, 2014), but this defense response is not elicited by the GOX present in their saliva because GOX treatment failed to induce defenses in maize (Louis *et al.*, 2013). The specific FAW salivary elicitors and their potential influence on the strains' host plant associations are unknown.

In a recent study, we identified qualitative and quantitative differences in the salivary proteome of the FAW strains (Acevedo *et al.*, 2017b). Thirteen unique proteins were identified for each strain using label-free LC-MS, and 11 proteins were found to be differentially abundant between the two FAW strains using labeling with isobaric tags (Acevedo *et al.*, 2017b). Changes in salivary protein abundance and concentration were also identified in FAW strains fed on maize and Bermuda grass (Acevedo *et al.*, 2017b). However, the effect of FAW intraspecific salivary changes on induced plant defense responses remains to be tested. In this study, we investigated the role of herbivore-induced defenses in the host plant association of FAW strains. We hypothesize that FAW strains induce different defense responses in their preferred and nonpreferred host plants during their feeding behavior.

Materials and Methods

Insects

The FAW strains were obtained from a laboratory colony maintained at the USDA-ARS in Gainesville, FL, USA. 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 Hendry and Palm Beach Counties (South Florida). For each strain, the field-collected insects were pair-mated to select the F_1 individuals containing the corresponding mitochondrial marker that identifies each strain (Nagoshi & Meagher, 2003).

Plants

Seeds of the maize cultivar (*Zea mays*) inbred line B73 were kindly provided by W. P. Williams from Mississippi State University and the USDA-ARS (Mississippi State, MS, USA). Maize seeds were germinated in Promix potting soil (Premier Horticulture Inc., Quakertown, PA, USA). The seedlings were transplanted 10 d after germination into 3.78-litre 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. Bermuda grass (*Cynodon dactylon*) hulled seeds were purchased from Seed World USA (Tampa, FL, USA) and directly grown in 2.8-litre pots (C300; Nursery Supplies Inc.) containing Hagerstown loam soil. The seedlings were fertilized once with 5 g of Osmocote plus and used for experiments 4 wk after germination. All plants were grown under glasshouse conditions (14 : 10 h, light : dark) at Pennsylvania State University, University Park, PA, USA.

Plant defense responses

Plant defense responses to different treatments were evaluated by measuring the expression of jasmonic acid (JA) defense-related genes and the activity of defense-related proteins using quantitative real-time PCR (qPCR) and biochemical assays, respectively. In maize plants, we measured the relative expression of the genes for maize proteinase inhibitor (*mpi*), allene oxide synthase (*aos*) and ribosome-inactivating protein 2 (*rip 2*). In Bermuda grass, we measured the activity of trypsin protease inhibitor (trypsin PI), which inhibits the activity of digestive serine proteases in insects impairing their growth and development (Dorrah, 2004).

Plant mechanical wounding

In maize plants, the third youngest leaf was mechanically wounded once using the tool described by Bosak (2011) and Ray *et al.* (2015). The five younger leaves of Bermuda grass plants were wounded (one wound per leaf) using a cork borer (Harris unicore -2.0 (Ted Pella Inc., Redding, CA, USA)).

Plant defense responses to feeding of FAW strains

To evaluate the effect of feeding of FAW strains on induced plant defenses, maize and Bermuda grass plants were challenged with actively feeding last-instar caterpillars of both strains. These caterpillars were grown from egg hatch on detached leaves of maize and Bermuda grass before placing them onto their respective plants. In maize, caterpillars were either placed directly in the whorl of the plants for 24 h or enclosed in clip cages (polypropylene with metallic micromesh screen, 23 mm diameter and 18 mm height) to control for the amount of injury (Supporting Information Fig. S1a). Caterpillars were removed after they ate the 415.48 mm^2 of leaf tissue contained in the cage. The leaf tissue around the feeding sites was harvested 24 h later for gene expression analysis. Bermuda grass plants were treated by exposing 6–10 leaves to caterpillars enclosed in cages (5.5 cm diameter, 1.5 cm high, 23.76 cm^2 area) built with two plastic Petri dish bottoms (60 × 15 mm, VWR, West Chester, PA, USA) with air holes punched through for airflow. With the lip of the Petri dish padded with felt, the leaf and caterpillars were sandwiched between two dishes held together with aluminum hair clips. The cage was supported by a wooden stick to prevent leaf breakage (Fig. S1b). Insect-fed leaf samples were harvested 24 h later for further analyses. For maize and Bermuda grass experiments, each plant (n = 6–7) was treated with one caterpillar in a complete randomized design.

Plant defense response to caterpillar saliva

We studied the effect of caterpillar saliva from the FAW strains on induced defense responses of maize and Bermuda grass plants using two different methods: (1) by heat cauterizing the caterpillar's spinneret, which is the structure that secretes saliva from the labial glands; and (2) by dissecting and applying caterpillar salivary gland homogenates or saliva onto mechanically wounded plants. For the first method, caterpillars were cooled on ice for 15 min and ablated by cauterizing their spinneret with a hot pin. Ablated caterpillars were allowed to recover and eat for 12 h before placing them onto the plants. Each plant (n = 5–6) was treated with one caterpillar in a complete randomized design. For the second method, saliva or salivary glands were obtained from last-instar actively feeding caterpillars grown from egg hatch on maize or Bermuda grass leaves, and were used to treat these plants, respectively. Labial salivary glands were dissected from caterpillars chilled on ice for *c.* 15 min and immobilized in wax-dissecting dishes (VWR) using pins; the outer caterpillar cuticle was cut longwise in the ventral side and the salivary glands – that freely floated in the hemolymph – were picked up with dissecting forceps, quickly rinsed in milliQ water and placed into 1.5 ml tubes kept on ice. The salivary glands were then homogenized in 100 µl of 1× PBS (137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH. 7.2) using polypropylene pellet pestles (VWR), centrifuged for 3 min at 6810 ***g*** and the supernatant collected into a new tube. The amount of protein was quantified using a Bradford assay. Each plant (n = 6–10) was wounded and treated with 10 µg of homogenate obtained from three to five pairs of salivary glands within 1 h of their dissection. Saliva was collected following a previously described procedure (Acevedo *et al.*, 2017b), and stored at −80°C until use. Plants were wounded and treated with a 12 µl mixture of saliva (3–5 µg of protein) diluted in 1× PBS. To investigate if protein components in the FAW caterpillar's saliva would trigger plant defense responses, we boiled saliva or salivary gland homogenates (30 min at 98°C) to heat-inactivate the proteins and used it to treat the plants (n = 6–8). The levels of defense responses of wounded plants treated with saliva or salivary glands were compared against those from wounded plants treated with 1× PBS buffer, and unwounded controls in a complete randomized design.

Effect of induced plant defenses on caterpillar weight

We investigated if induced plant defenses by FAW saliva would affect the performance of naïve FAW larvae. Maize and Bermuda grass plants (n = 5–7) were challenged with ablated and intact caterpillars from the two FAW strains. Caterpillars were enclosed in clip cages (23 mm diameter and 18 mm height) to standardize the amount of damage and were removed after eating the provided leaf area (23.76 cm^2). Twenty-four hours later, the damaged leaves were detached from plants and used to feed neonates of both strains for 1 wk. The damaged tissue of each plant was used to grow three caterpillar neonates, and their average weight was used as one independent biological replicate for statistical analysis. The effect of the *strain* and *treatment* factors on weight gain was tested using a two-factor factorial design.

Plant defense responses to caterpillar regurgitant

It is well known that oral secretions from caterpillars induce defenses in plants; however, caterpillars do not always secrete regurgitant during feeding (Peiffer & Felton, 2009). Therefore, we first quantified the amount of regurgitant secreted by the FAW strains on their host plants following a previously described procedure (Peiffer & Felton, 2009; Acevedo *et al.*, 2017a), and then tested the plant defense response to the application of those regurgitant quantities. Regurgitant was collected from the oral cavity of plant-fed caterpillars (by gently tapping their heads) and immediately placed on ice. The regurgitant was further diluted in 1× PBS and 10 µl of the dilution was applied to wounded plants within 1 h of its collection. The tissue surrounding the wounds was further collected for gene expression and biochemical analyses. Each plant (n = 5–10) was treated with regurgitant obtained from at least three caterpillars. Regurgitant-treated plants were compared against wounded plants treated with PBS and unwounded controls in a complete randomized design. A two-factor factorial design was used to analyze the effect of the factors *strain* and *plant* on the amount of regurgitant secreted.

Maize defense responses to caterpillar frass

Recent studies have demonstrated that components in the frass of FAW caterpillars trigger defense responses in maize plants (Ray *et al.*, 2015); we therefore tested for differences in induced plant

defenses by frass from FAW strains. Fresh frass from last instar caterpillars, reared from egg hatch on detached maize leaves, was collected and used to treat maize plants ($n = 7$–8). Plants were mechanically wounded and fresh frass pellets were pressed by hand against the wounds. After 24 h, the tissue surrounding the wounded sites was collected for gene expression analyses. The effect of the treatments was tested in a complete randomized design.

FAW salivary elicitors

To identify potential plant defense elicitors in the saliva of FAW strains, we selected a few salivary enzymes previously identified in the FAW salivary proteome (Acevedo *et al.*, 2017b), and previously reported as plant defense elicitors in other insect species. We specifically tested for differences in the activity of GOX, ATPases and phospholipase C (PLC) in both strains feeding on an artificial diet (wheat germ), maize and Bermuda grass. GOX activity in saliva and salivary glands was measured following the protocol developed by Eichenseer *et al.* (1999) and adjusted for a microplate reader. ATPase hydrolysis activity was measured using the ENLITEN ATP Assay System Bioluminescence Detection Kit (Promega) following the manufacturer's procedures. The PLC enzymatic assays were carried out following a published protocol (Kurioka & Matsuda, 1976; Le Chevalier *et al.*, 2015), and adapted for a microplate reader. Each sample ($n = 5$) contained 3–5 pairs of salivary glands extracted from last-instar caterpillars (second day after molting). The effect of the *strain* and *plant* factors on GOX, ATPase and PLC activity was tested in a two-factor factorial design.

Plant response to PLC and GOX treatment

To evaluate the effect of PLC and GOX on plant defense responses, plants ($n = 4$–10) were wounded and treated with 40 μg of commercial PLC from *Clostridium perfringens* and GOX from *Aspergillus niger* (P7633 and G2133, respectively, Sigma) diluted in 1× PBS. After 24 h, the wounded tissue was harvested for gene expression and biochemical analyses. The amount of commercial enzyme applied to the plants had activity levels within the range of those found in the FAW caterpillars' saliva. Wounded plants treated with PLC or GOX were compared against wounded plants treated with PBS and unwounded controls in a complete randomized design.

Effect of PLC-induced plant defenses on caterpillar weight

PLC from labial glands of a noctuid caterpillar, *Helicoverpa zea*, was cloned and expressed in *Escherichia coli* (M. Peiffer *et al.*, unpublished). The purified recombinant protein was diluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) containing 1 mM $CaCl_2$ for activity assays and plant treatment (M. Peiffer *et al.*, unpublished). The third youngest leaf of B73 plants was wounded (as indicated above) twice, and each wound was treated with 15 μl buffer containing 2.7 μg of recombinant PLC. After 24 h, the damaged leaves were detached and used to feed FAW caterpillar neonates following the same procedure as already described.

RNA extraction, cDNA synthesis and qPCR

Leaf tissue (60–90 mg) frozen in liquid nitrogen was homogenized in a GenoGrinder 2000 (OPS Diagnostics, Lebanon, NJ, USA) and total RNA extracted using a modified Trizol protocol previously described (Acevedo *et al.*, 2017a). Complementary DNA (cDNA) was synthesized from 1 μg of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) using Oligo-dT following the manufacturer's protocol. qPCR was conducted using the 7500 Fast Real-Time PCR System (Applied Biosystems) with SYBR green (Roche Applied Science). Specific primers for each of the genes (Table S1) were designed with Primer Express 3.0 (Life Technologies).

Trypsin protease inhibitor activity

We measured the activity of trypsin PI following the procedure described by Chung & Felton (2011). Trypsin PI activity was calculated as PI (%) = (1 − (slope of sample/slope of noninhibitor)) × 100, and the resulting activity values were normalized by the amount of protein (mg) contained in the sample.

Statistical analysis

Plant defense responses (gene expression and trypsin PI activity) to the treatments (caterpillar feeding, application of salivary glands, regurgitant, frass, commercial PLC and GOX) were analyzed with one-way ANOVA following the post-hoc tests of Tukey and Fisher at $\alpha = 0.05$. The significance of the factors *strain* (corn or rice) and *host plant/diet type* (maize, Bermuda grass, artificial diet) as well as their interaction on the variables enzymatic activity of PLC and GOX in the salivary glands of the FAW strains, amount of secreted regurgitant, and larva weight gain was analyzed using a two-way ANOVA following post-hoc tests. The association between plant defense responses and weight gain by caterpillar neonates of the FAW strains was tested using linear regression analysis. When needed, the response variables were transformed to meet the assumptions of normality and equal variances. All the statistical analyses were performed using the Statistical Software Minitab 16 (Minitab Inc., State College, PA, USA) and R v.3.2.2 (Foundation for Statistical Computing, Vienna, Austria). All graphs were generated in R.

Results

FAW strains trigger different levels of induced defenses on maize and Bermuda grass

In maize, feeding by the rice strain induced greater expression of the *mpi* ($P = 0.019$) and *aos* ($P = 0.046$) genes than the corn strain (Fig. 1a,b); five out of six independent experiments showed similar results. In Bermuda grass, two independent experiments showed that feeding by the corn strain suppressed the induction

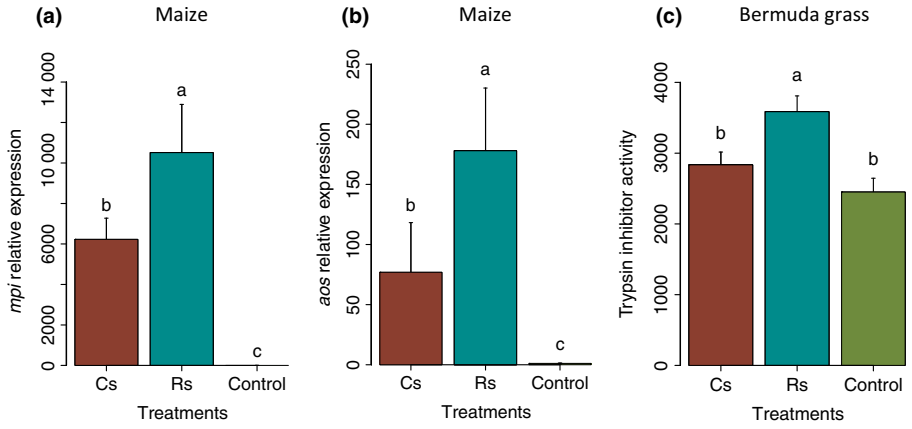

Fig. 1 Induced plant defense responses by feeding from *Spodoptera frugiperda* strains: Cs, corn strain; Rs, rice strain; controls, undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post-hoc tests at $\alpha = 0.05$. (a) *Maize proteinase inhibitor* (*mpi*) gene expression 24 h after caterpillar treatment ($F_{2,15} = 497.05$, $P < 0.001$; Tukey test; $n = 6$; log-transformed data). (b) *Maize allene oxide synthase* (*aos*) gene expression 24 h after caterpillar treatment ($F_{2,13} = 6.3$, $P < 0.05$; Fisher test; $n = 6$; log-transformed data). (c) Bermuda grass trypsin protease inhibitor activity 24 h after caterpillar damage ($F_{2,18} = 8.23$, $P < 0.05$; Tukey test; $n = 7$).

of trypsin PI activity to levels similar to those found in undamaged controls; feeding by the rice strain, by contrast, induced significantly greater activity of trypsin PI compared with the corn strain ($P = 0.023$) and untreated controls ($P = 0.002$) (Fig. 1c).

Induction of defenses in maize and Bermuda grass negatively affect FAW caterpillar growth

In maize, neonates gained less weight when grown on leaves previously damaged by intact (able to salivate) rice strain caterpillars than when grown on leaves damaged by intact and ablated (impaired to salivate) caterpillars of the corn strain. FAW neonates gained greater weight when fed on undamaged plants (controls) and plants previously damaged by ablated rice strain caterpillars (Fig. 2a). There was a significant negative correlation between the transcript accumulation of *mpi* and the weight gained by young FAW larvae ($F_{1,18} = 44$, $P < 0.001$) (Fig. 2c). In Bermuda grass, neonates grew faster when fed on leaves previously damaged by intact corn strain caterpillars and untreated controls compared with those grown on leaves previously damaged by ablated corn strain and intact rice strain caterpillars (Fig. 2b). There was a significant negative correlation between trypsin PI activity and caterpillar weight gain ($F_{1,31} = 6.67$, $P < 0.05$) (Fig. 2d).

Caterpillar saliva of the FAW strains triggers different levels of induced defenses on maize and Bermuda grass

The expression of plant defense-related genes was significantly different when plants were challenged with ablated and intact caterpillars of both strains. In maize, two independent experiments showed that intact caterpillars from the rice strain induced the highest expression of *mpi* compared with intact corn strain and ablated caterpillars of both strains ($P < 0.001$) (Fig. 3a). In Bermuda grass, intact caterpillars from the corn strain suppressed induction of trypsin PI activity to similar levels found in undamaged controls, while ablated caterpillars induced production of these inhibitors. By contrast, intact rice strain caterpillars induced greater production of trypsin PI than the corresponding ablated caterpillars ($P = 0.045$) (Fig. 3b); two independent experiments showed similar results. The effect of caterpillar saliva on plant defense induction was confirmed by the application of fresh salivary gland homogenates from both strains onto wounded plants. In maize and Bermuda grass, salivary glands from the rice strain induced greater expression of *mpi* ($P = 0.04$) and trypsin PI than the corn strain ($P = 0.017$), respectively (Fig. 3c,d). Salivary glands from the corn strain suppressed the activity of trypsin PI in Bermuda grass compared with buffer-treated plants ($P = 0.025$), while the rice strain induced the same response as the buffer treatment (Fig. 3d).

Protein components of caterpillar saliva from the FAW strains elicit different plant defense responses

When boiled saliva or salivary gland homogenates were applied to wounded plants, the levels of induced plant defense responses were not different between the strains (Fig. 4). However, boiled salivary glands still induced greater defense responses than the PBS-treated plants in maize but not in Bermuda grass. In maize these experiments were also performed using boiled saliva with similar results (data not shown).

FAW strains exhibit differential activities of the salivary enzymes PLC and GOX

PLC activity varied with the type of diet for each of the strains. ANOVA showed a significant interaction between strain and diet type ($F_{2,24} = 21.6$, $P < 0.0001$), so significant differences were obtained using a two-sample *t*-test for the two strains on each diet type followed by the Bonferroni correction to account for multiple tests. When feeding on maize the rice strain had significantly higher activity than the corn strain; conversely, when feeding on Bermuda grass the corn strain had higher activity than the rice

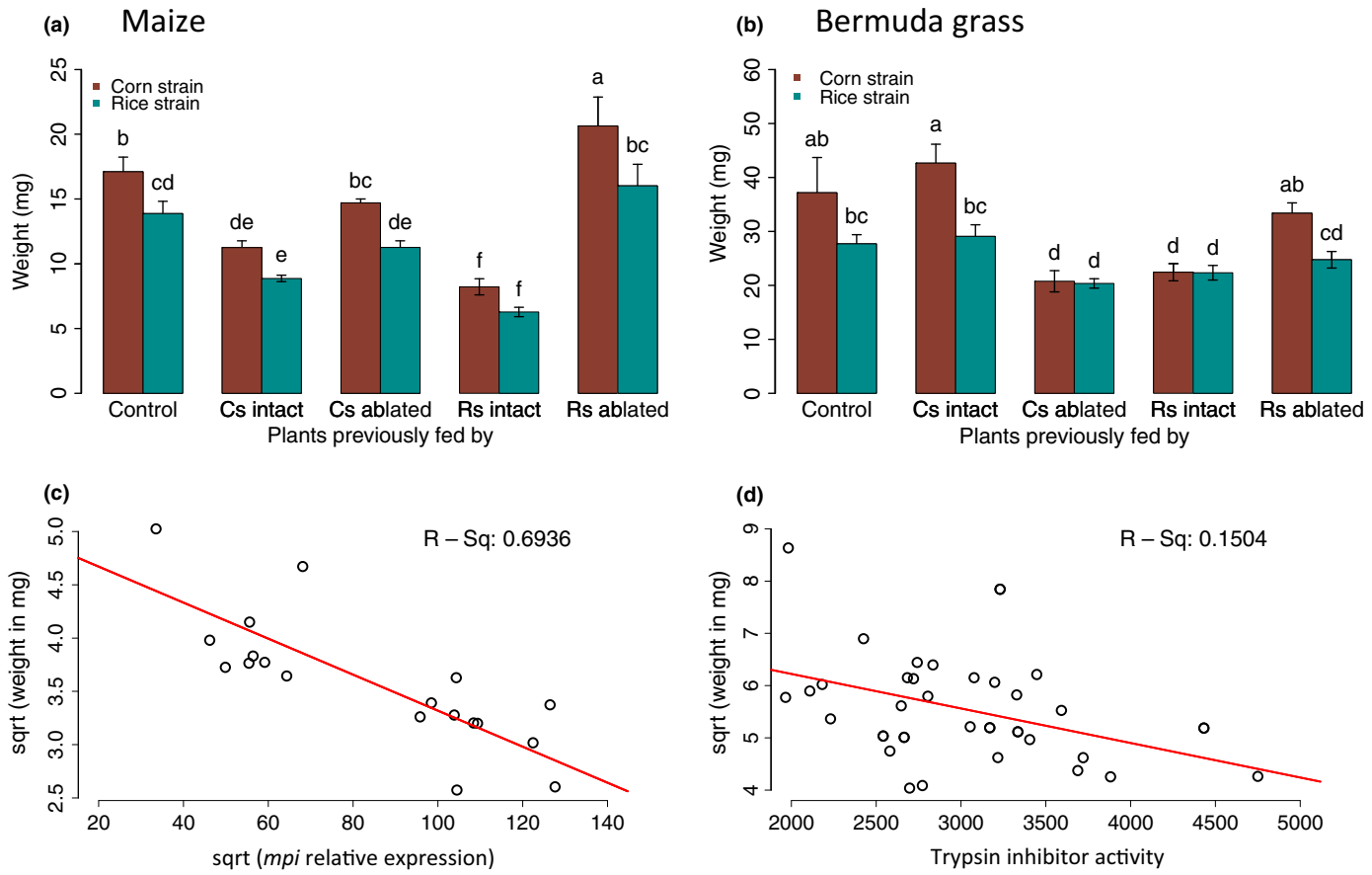

Fig. 2 Weight gain of larvae reared on detached leaves previously damaged by caterpillars able (intact) or impaired to salivate (ablated) from *Spodoptera frugiperda* strains: Cs, corn strain; Rs, rice strain; controls, undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post-hoc tests at $\alpha = 0.05$. (a) Larvae grown on damaged maize leaves; strain effect: $F_{1,40} = 22.10$, $P < 0.001$; treatment effect: $F_{4,40} = 34.47$, $P < 0.001$; strain × treatment effect: $F_{4,40} = 0.48$, $P > 0.05$; Fisher test, $n = 5$. (b) Larvae grown on damaged Bermuda grass leaves; strain effect: $F_{1,55} = 11.21$, $P < 0.001$; treatment effect: $F_{4,55} = 15.22$, $P < 0.001$; strain × treatment effect: $F_{4,55} = 2.14$, $P > 0.05$; Tukey test, $n = 5$–7 (1/square root-transformed data). (c) Regression analysis of caterpillar weight gain and *maize proteinase inhibitor* (*mpi*) relative expression ($\sqrt{}$(mg) = 5.008–0.01689 $\sqrt{}$(*mpi*)). (d) Regression analysis of caterpillar weight gain and Bermuda grass trypsin protease inhibitor activity ($\sqrt{}$(mg) = 7.54–0.00066 trypsin PI).

strain; lastly, when feeding on artificial diet, the two strains had similar PLC activities (Fig. 5a). GOX activity levels for the two FAW strains were also diet-dependent. There was a significant effect of both strain ($F_{1,20} = 25.2$, $P < 0.0001$) and type of diet ($F_{2,20} = 11.1$, $P < 0.0001$), but no significant interaction between the two ($F_{2,20} = 2.9$, $P > 0.05$). For all diets tested the corn strain had significantly higher GOX activity than the rice strain. GOX activity was higher in diet-fed caterpillars of both strains followed by the maize and Bermuda grass-fed caterpillars (Fig. 5b). No activity of ATPases was detected for either of the strains (data not shown).

PLC modulates defense responses in maize and Bermuda grass and reduces caterpillar weight gain

In maize, commercial PLC from *C. perfringens* induced higher expression of the herbivore-responsive genes *mpi* ($P = 0.015$) and *rip 2* ($P = 0.028$) compared with buffer-treated plants (Fig. 6a,b). Conversely, in Bermuda grass, PLC suppressed production of trypsin PI to similar levels found in untreated controls (Fig. 6c). These defense responses affect insect growth as FAW neonates gained less weight when fed on maize leaves previously treated with recombinant PLC (Fig. 7).

Effect of GOX on plant defense responses

In Bermuda grass, application of commercial GOX and PBS to wounded plants induced similar levels of trypsin PI activity ($F_{2,27} = 1.1$, $P > 0.05$) (Fig. S2). In maize, previous studies have shown no effect of GOX on induced-defense responses (Louis *et al.*, 2013), so its effect on this plant was not tested here again.

Role of oral secretions in plant defense induction by FAW strains

All the caterpillars tested ($n = 30$) released regurgitant into the plants during feeding. The amount of regurgitant released varied from 2 to 6 nl. There was a significant effect of strain ($F_{1,16} = 5.8$, $P < 0.05$) and plant × strain interaction ($F_{1,16} = 5.3$, $P < 0.05$), but not a significant effect of plant alone ($F_{1,16} = 3.5$, $P > 0.05$)

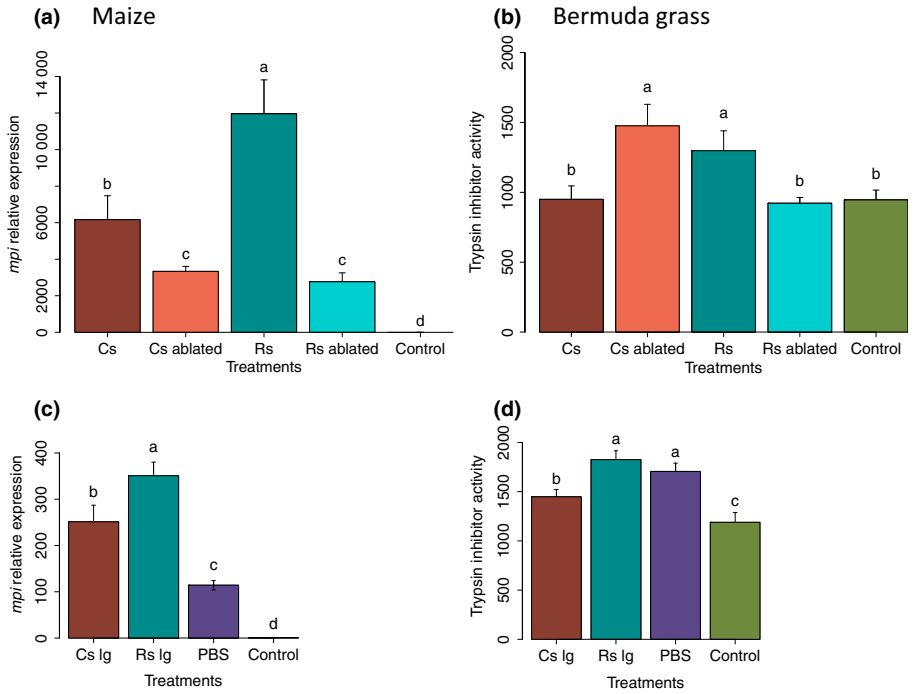

Fig. 3 Induced plant defense responses by caterpillar saliva from *Spodoptera frugiperda* strains: Cs, corn strain; Rs, rice strain; ablated, caterpillars impaired to salivate; lg, labial salivary glands; PBS, buffer-treated controls; controls, undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post-hoc tests at $\alpha = 0.05$. (a) *Maize proteinase inhibitor* (*mpi*) gene expression 24 h after caterpillar treatment ($F_{4,20} = 394.78$, $P < 0.001$; Fisher test; $n = 5$; log-transformed data). (b) Bermuda grass trypsin protease inhibitor activity 24 h after caterpillar damage ($F_{4,25} = 5.38$, $P < 0.05$; Fisher test; $n = 6$). (c) *Maize proteinase inhibitor* (*mpi*) gene expression 24 h after wounding and treatment with salivary glands from fall armyworm strains ($F_{3,20} = 493.9$, $P < 0.001$; Fisher test; $n = 6$; log-transformed data). (d) Bermuda grass trypsin inhibitor activity 24 h after wounding and treatment with fall armyworm salivary glands ($F_{3,36} = 10.44$, $P < 0.001$; Tukey test; $n = 10$).

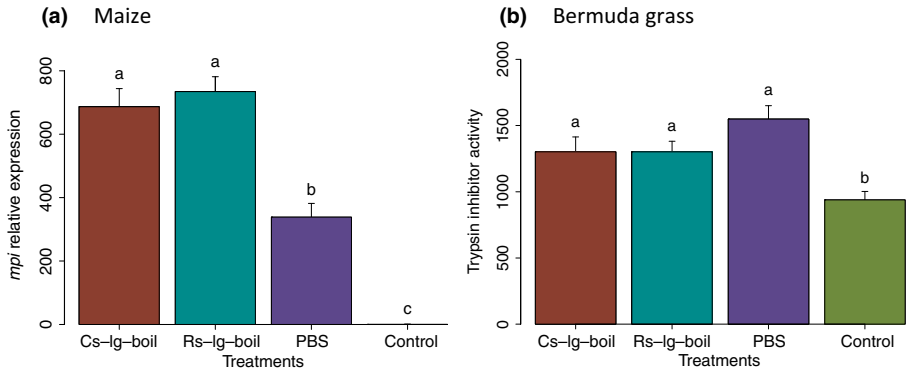

Fig. 4 Plant defense response to wounding plus the application of boiled salivary gland homogenates from *Spodoptera frugiperda* strain caterpillars: Cs, corn strain; Rs, rice strain; lg, labial salivary glands; boil, boiled; PBS, buffer-treated controls; controls, undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post-hoc tests at $\alpha = 0.05$. (a) *Maize proteinase inhibitor* (*mpi*) gene expression 24 h after treatment ($F_{3,21} = 714.06$, $P < 0.001$; Tukey test; $n = 6$; log-transformed data). (b) Bermuda grass trypsin protease inhibitor activity 24 h after treatment ($F_{3,30} = 8.61$, $P < 0.001$; Fisher test; $n = 8$).

in the amount of regurgitant secreted. Both strains released similar amounts of regurgitant when feeding on Bermuda grass ($t = -0.11$, $P = 0.917$), but when feeding on maize, the rice strain released four times more regurgitant than the corn strain ($t = -2.75$, $P = 0.025$) (Fig. S3). In maize, the application of meaningful quantities of regurgitant induced higher transcript accumulation of the *mpi* gene compared with wounded + PBS-treated plants ($F_{2,15} = 7.7$, $P < 0.005$), but there were no differences in induction for the strains despite the different amounts applied ($P = 0.802$) (Fig. S4a). In Bermuda grass there were no differences observed among regurgitant or PBS-treated plants compared with controls ($F_{3,36} = 1.0$, $P = 0.387$) (Fig. S4b).

Effect of caterpillar frass

Caterpillar frass induced higher levels of *mpi* transcript accumulation compared to wounding alone ($F_{2,20} = 73.9$, $P < 0.001$), but the levels of defense induction were not different for the two strains (Fig. S5).

Discussion

Our results show that caterpillars of the two FAW strains induce different defense responses in their host plants during feeding, which have a fitness effect on young larvae and may affect their host plant association. The rice strain induced greater defense responses than the corn strain in maize and Bermuda grass, whereas the corn strain suppressed induction of trypsin PI in Bermuda grass to similar levels found in undamaged controls. Neonate larvae gained more weight when fed on

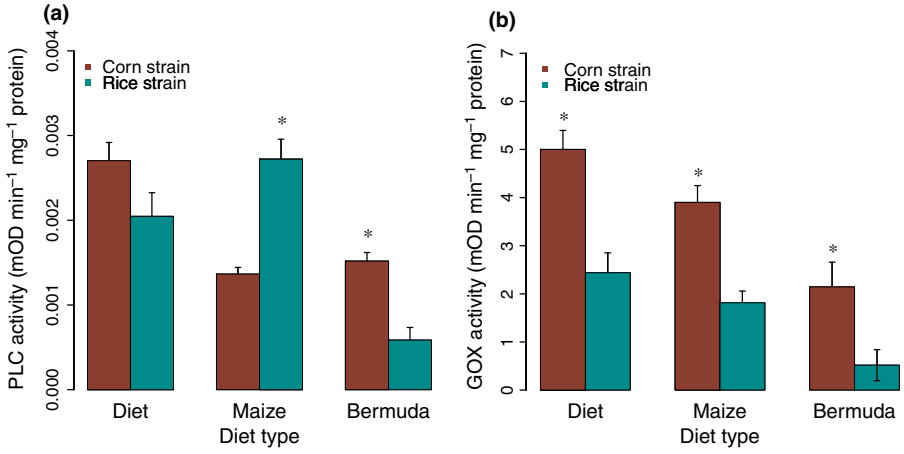

Fig. 5 Phospholipase C (PLC) and glucose oxidase (GOX) activity in labial salivary glands of *Spodoptera frugiperda* strains fed on different diets. Values are untransformed means ± SEM, and asterisks indicate significant differences ($\alpha = 0.05$) between strains per diet type. (a) PLC activity per mg of protein; artificial diet: $t = 1.87$, $P > 0.01667$; maize: $t = -5.48$, $P < 0.01667$; Bermuda grass: $t = 5.17$, $P < 0.01667$; $n = 5$; Bonferroni 0.05/3 = 0.01667. (b) GOX activity per mg of protein; significant differences between strains for each diet type were determined by the Bonferroni method at 95% confidence.

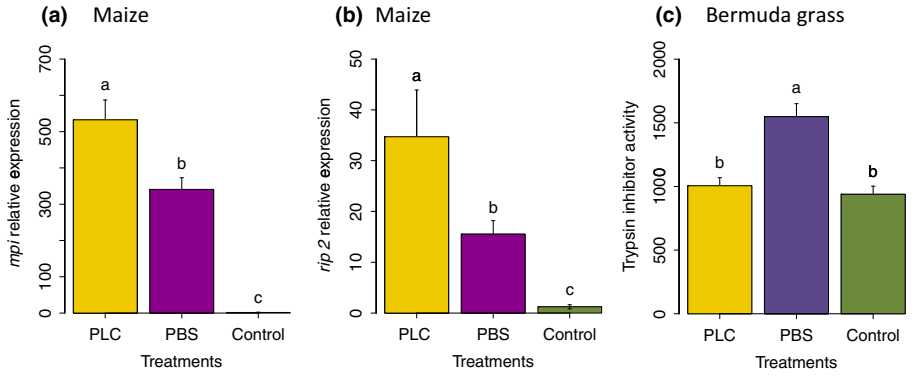

Fig. 6 Plant defense response to wounding plus application of commercial phospholipase C (PLC) from *Clostridium perfringens*. PBS, buffer-treated controls; controls, undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post-hoc tests at $\alpha = 0.05$. (a) *Maize proteinase inhibitor* (*mpi*) gene expression 24 h after treatment ($F_{2,12} = 298.58$, $P < 0.001$; Tukey test; $n = 4$–7; ¼ root-transformed data). (b) *Ribosome-inactivating protein 2* (*rip 2*) gene expression 24 h after treatment ($F_{2,12} = 37.16$, $P < 0.001$; Tukey test; $n = 4$–7; log-transformed data). (c) Trypsin protease inhibitor activity per mg of protein 24 h after treatment with PLC ($F_{2,27} = 18.17$, $P < 0.001$; Tukey test; $n = 10$).

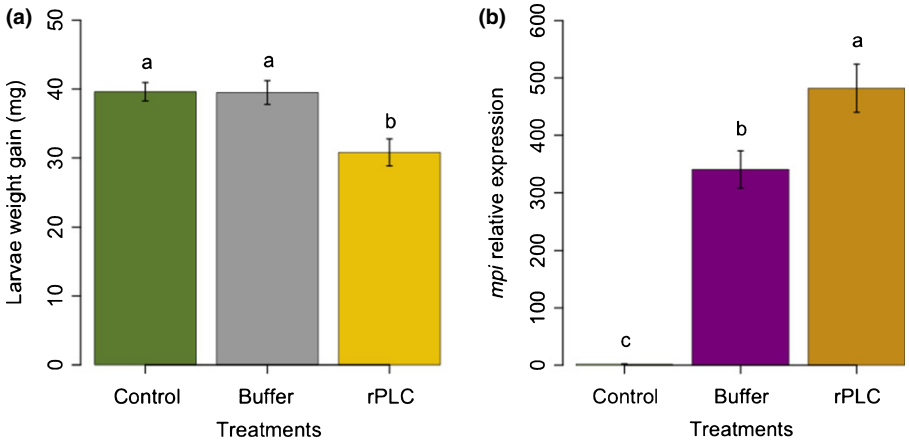

Fig. 7 (a) Weight gain of *Spodoptera frugiperda* larvae reared on detached leaves previously wounded and treated with recombinant phospholipase C (rPLC) and buffer. Bars are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA ($F_{2,27} = 8.9018$, $P = 0.0011$; $n = 10$) following Tukey tests at $\alpha = 0.05$. (b) *Maize proteinase inhibitor* (*mpi*) gene expression 24 h after wounding plus the application of recombinant PLC from *Helicoverpa zea* ($F_{2,18} = 365.69$, $P < 0.001$; Tukey test; $n = 7$; ¼ root-transformed data). Controls are undamaged plants.

leaf tissue previously damaged by the corn strain than when fed on tissue previously exposed to the rice strain; caterpillar weight gain was negatively correlated with the levels of induced plant defenses in both hosts (Fig. 2). Our results suggest that components in caterpillar saliva, specifically differences in activity of the enzyme PLC, elicit these differential plant defense responses by the FAW strains. (1) A similar trend of *mpi* expression and trypsin PI activity induced by intact caterpillars was observed when plants were treated with salivary gland homogenates of the two strains. (2) When plants were treated with boiled salivary gland homogenates the plant defense responses were no longer different for the strains, indicating that the associated salivary component triggering different defense responses was inactivated by heat. (3) Application of commercial PLC induced production of protease inhibitors in maize but suppressed activity of trypsin PI in Bermuda grass (Fig. 6). Likewise, treatment with either FAW caterpillars or their salivary glands induced similar responses in these plants. (4) Saliva of the rice strain had higher PLC activity when feeding on maize where it elicited greater expression of *mpi* than the corn strain, while the corn strain had higher PLC activity in Bermuda grass where it suppresses the induction of trypsin PI activity (Fig. 5a). (5) FAW neonates gained less weight when fed on maize leaves previously treated with recombinant PLC (Fig. 7). (6) Neither the application of regurgitant nor the application of frass from the two strains induced different defense responses in maize or Bermuda grass. Therefore, differences in the salivary PLC activity seem to explain the different plant defense responses triggered by the FAW strains. Although specific PLC inhibitors such as the aminosteroid U73122 and the PLC ether lipid analog edelfosin (ET-18-OCH3) may have been useful to confirm these results, we did not use them because their cytotoxicity and secondary effects resulting from alkylation of various proteins (Horowitz *et al.*, 2005) could have affected plant defense responses.

PLC hydrolyzes the phospholipids phosphatidylinositol 4,5-biphosphate (PIP2) and phosphatidylinositol-4-phosphate (PI_4P) in the plasma membrane by breaking the bond between head and tail before the phosphate group. Hydrolysis of PI_4P and PIP2 produces inositol 1,4,5-triphosphate (IP3) and diacylyglycerol (DAG), which act as second messengers for downstream signal transduction (Canonne *et al.*, 2011). PLC suppressed activity of trypsin PI in Bermuda grass but induced production of proteinase inhibitors in maize. This discrepancy in response to the same compound could be explained by possible differences in the mechanisms of receptor-mediated recognition in different plant species (Schmelz *et al.*, 2009), differences in the availability of enzyme substrates and/or differences in hormonal crosstalk between plant defense pathways.

Our results also show differences in activity of salivary proteins within the strains. The corn strain had greater salivary PLC activity when feeding on artificial diet than when feeding on Bermuda grass or maize. Conversely, the rice strain had greater PLC activity when feeding on maize compared with artificial diet or Bermuda grass (Fig. 5a). Changes were also observed in the salivary enzyme GOX, where the corn strain had significantly higher GOX activity than the rice strain regardless of the host plant (Fig. 5b); this agrees with our previous results of salivary protein abundance in the FAW strains (Acevedo *et al.*, 2017b). However, GOX alone did not trigger defense responses in maize (Louis *et al.*, 2013; Chuang *et al.*, 2014) or Bermuda grass, so its variation in activity may not affect the interaction of the FAW strains with these plants, but because FAW is a polyphagous insect it may play an important role in their interaction with other hosts. These results suggest that FAW strains plastically modify activity levels of their salivary elicitors when feeding on different hosts. Because induction of plant defenses has a fitness effect on FAW caterpillars, plastic differences in the salivary composition that modulate these defenses are likely to be adaptive (Mooney & Agrawal, 2008). Besides PLC and GOX, the FAW saliva has other components affecting defense responses in plants. Boiled salivary gland homogenates from both strains induced significantly greater *mpi* gene expression than buffer-treated plants; however, it is beyond the scope of this paper to identify those salivary molecules.

This work supports the hypothesis that a controlled production and secretion of herbivore elicitors/effectors is critical in insect host associations and may influence host shifts. Intraspecific differences in the protein composition of insect saliva have been identified in the FAW strains (Acevedo *et al.*, 2017b) as well as in other insect species. For example, biotypes of the Russian wheat aphid *Diuraphis noxia* (Mordvilko) that exhibit different virulence to wheat have different salivary protein profiles that may interfere with their host defense signaling and phytotoxicity (Nicholson *et al.*, 2012). Also, the host races of the pea aphid *Acyrthosiphon pisum* (Harris) differ in several genes encoding salivary proteins (Jaquiéry *et al.*, 2012). Furthermore, some insects are also able to modify the composition of their regurgitant to avoid plant defenses. For instance, caterpillars of the legume specialist *Anticarsia gemmatalis* (Hübner) release an antagonistic form of the plant elicitor inceptin that suppresses the induction of indirect defenses in cowpea (Schmelz *et al.*, 2012). The composition of a herbivore's oral secretions and saliva are important factors in the modulation of host immunity and may have a direct influence in the insects' ability to exploit a particular host.

It has been under debate whether the host plant association influences the separation of the FAW strains. In a phylogenetic study of the genus *Spodoptera*, morphological and molecular data suggest that the ancestral members of this genus were probably dicot-feeders, while the use of crop grasses as host plants is a more recent event influenced by human agricultural practices (Kergoat *et al.*, 2012). A molecular dating analysis suggests that the FAW strains have diverged more than 2 Myr ago, long before the domestication of maize (*c.* 9000 yr ago) (Matsuoka *et al.*, 2002). Therefore, separation of the strains is unlikely to have arisen due to the current host plant association. Other factors including differences in sex pheromone blends and mate calling times (Groot *et al.*, 2008; Schöfl *et al.*, 2009) may have influenced the partial reproductive isolation of the strains. However, the host bias distribution of the FAW

strains in field conditions, the presence of host-associated specific detoxification enzymes, along with the differential induction of plant defenses and the associated variances in the salivary composition of the strains, suggest a strong adaptation to different host plants. The separation of these host races may not have originated with a host plant shift itself, but their subsequent adaptation to different hosts may help to re-enforce the strain's separation and may have the potential to affect their level of genetic divergence.

Based on our results, we draw three main conclusions from this study. (1) The FAW strains induce different defense responses in maize and Bermuda grass via specific differences in their saliva composition. Differences in activity of the salivary enzyme PLC appear to be responsible for elicitation of differential plant defense responses by the strains. (2) The differential plant defense induction affects caterpillar growth; therefore, the composition of insect saliva as a plant defense modulator may be under strong selective pressure. (3) The FAW strains plastically modify the composition of their salivary elicitors when feeding on different hosts. Intrastrain-specific differences in PLC and GOX activity may influence the strain's ability to exploit a particular host. Saliva of insect herbivores may represent the first line of protection against plant defenses (Felton, 2008). Salivary glands have evolved rapidly compared to other organ systems and thus saliva could represent one of the primary mechanisms that species use to adapt to new food sources (Tabak & Kuska, 2004).

This study gives important contributions to the fields of insect evolutionary biology, insect–plant interactions and insect pest management. The composition and secretion of herbivore-derived plant defense elicitors may have a strong influence in the host range expansion of insect herbivores, which in turn may influence population dynamics and ecosystem communities of ecological and agricultural importance.

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Author contributions

F.E.A., G.W.F. and D.S.L. designed the study, F.E.A., M.P. and S.R. performed the experiments, and M.P. standardized the PLC assays, expressed the PLC protein and did the confocal fluorescence experiments. S.R. contributed to the frass experiments, bioassays and protein assays. R.M. collected the FAW strains from the field, did the genotyping and maintained the colonies. F.E.A. analyzed the data and wrote the first draft of the paper from which all authors contributed to revisions.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Cages used to enclose *Spodoptera frugiperda* caterpillars in maize and Bermuda grass experiments.

Fig. S2 Trypsin protease inhibitor activity in Bermuda grass 24 h after treatment with glucose oxidase (GOX).

Fig. S3 Amount of secreted regurgitant by *Spodoptera frugiperda* caterpillars feeding on different host plants.

Fig. S4 Induced plant defense responses by the application of regurgitant from *Spodoptera frugiperda* strain caterpillars.

Fig. S5 Relative expression of the *maize proteinase inhibitor* (*mpi*) gene 24 h after frass treatment from *Spodoptera frugiperda* strains.

Table S1 Maize primers used for quantitative PCR

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