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THE WEAR AND TEAR OF FEEDING: INDUCED PLANT DEFENSES BY FALL ARMYWORM HERBIVORY AND COUNTER INSECT ADAPTATION MECHANISMS

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ABSTRACT

Feeding by insect herbivores induces defense responses in plants. Numerous studies have shown that plants recognize the mechanical damage as well as a variety of herbivore-derived cues present in insect oral secretions, saliva and frass to activate the production of specific defenses responses. Plant defenses negatively affect herbivore fitness; therefore to be able to feed on plants, insects need to develop specific adaptations to overcome defenses of their hosts. This can be challenging for herbivores that feed on a wide range of plants with diverse structural and biochemical defenses. In this dissertation, I studied defense responses induced by a polyphagous herbivore in some of its host plants as well as some of its adaptation mechanisms to counter the effect of these defenses. I used the lepidopteran fall armyworm (FAW) *Spodoptera frugiperda*, and its host plants maize, Bermuda grass, rice and tomato as a model system. The FAW comprises two host strains that are associated with different host plants in field conditions; the corn strain is mainly associated with maize, while the rice strain is mostly associated with forage grasses and rice. I specifically tested the presence of intraspecific differences in plant defense induction by the FAW strains and the composition of their caterpillar saliva. I also investigated the effect of caterpillar-associated gut bacteria on the modulation of defense responses in different hosts. In addition, I documented morphological and developmental adaptations of the FAW larvae to cope with their host structural defenses. The results of this study show that feeding by the FAW strains induced different defense responses in maize and Bermuda grass; this plant defense induction was associated with differences in the caterpillar saliva. The saliva of the FAW strains also has divergent protein profiles and differing expression of several salivary proteins. In addition, the saliva of the FAW contains non-protein compounds that actively regulate defenses in different hosts; these include several phytohormones and other presumptive small molecules. During feeding, the FAW larvae also secrete small amounts of regurgitant, I identified two bacteria isolates in the caterpillar oral secretions that modulated defense responses in tomato and maize plants. In addition, feeding by this insect induced the production of glandular trichomes in tomato and the deposition of silica in maize and rice

plants. As adaptive strategies to feed on Bermuda grass and rice, the FAW larvae increased their head size to house larger mandibular muscles and potentially increase the biting force needed to feed on tough leaves. I conclude that FAW feeding modulates the induction of plant biochemical and physical defenses, which in turn induced plastic physiological and morphological changes in this insect species. The results of this dissertation highlight the importance of insect physiological and morphological plastic adaptations as means to feed on different host plants.

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Chapter 1

Introduction

Insects are the most diverse group of organisms on earth (Kim 1993); among them, herbivores are exceptionally species-rich, accounting for about one quarter of all described species (Janz *et al.* 2006). Therefore, herbivory appears to promote diversification (Mitter *et al.* 1988) in some insect orders (Wiens *et al.* 2015). For example, Lepidoptera with 99% of its species being herbivores (Grimaldi & Engel 2005), appears to have the fastest diversification rates of any insect order (Wiens *et al.* 2015). However, plant feeding is challenging for insects due to their low nutrient content and the presence of structural and chemical defenses (War *et al.* 2012). Consequently, to feed on plants, insects need to develop specific adaptations to overcome the defenses of their hosts and acquire the necessary nutrients to grow and reproduce (Simon *et al.* 2015). Most phytophagous insects are specialists, meaning that they specialize to feed on a particular group of plants, usually within the same genus, while a smaller portion (< 10 %) of them are generalists, or insects that feed on more than three different plant families (Ali & Agrawal 2012). Specialist herbivores adapt their physiology, morphology and behavior to feed on plants with similar chemistry and therefore are less affected by the defenses of their hosts than generalists. Conversely, generalist insects need to have a broader range of mechanisms to tolerate plant defenses (Ali & Agrawal 2012). However, within a given range of suitable hosts, generalists usually perform better on some of them. The effect of herbivore-induced plant defenses on the particular "host preference" in polyphagous insects and their adaptations to feed on these plants is unknown.

Mechanical damage caused by chewing herbivores induces plant defenses, but, through their coevolution with insects, plants have evolved the capability to recognize herbivore-derived cues to induce specific defense responses (Fürstenberg-Hägg *et al.*

2013). Several of these cues have been identified in insect secretions including, their saliva, regurgitant, frass and oviposition fluids (reviewed in Acevedo *et al.* 2015; Schmelz 2015). Some of the herbivore-derived cues are plant defense elicitors and have been named HAMPS (Herbivore–associated Molecular Patterns) while others are effectors, or molecules that suppress anti-herbivore defenses. Defenses against chewing herbivores are usually regulated by the activation of jasmonic acid (JA) within the plant octadecanoid pathway (Turner *et al.* 2002) while defenses against pathogens and sucking insects are usually activated by salicylic acid (SA) within the shikimate pathway (Heil & Bostock 2002). These two pathways also interact with ethylene and other plant hormones to regulate the synthesis of specific defense compounds (Adie *et al.* 2007). In many plant species the JA and SA pathways are antagonistic so that the activation of one suppresses the other (Thaler *et al.* 2012). Insects have evolved the ability to use this plant hormone antagonism, or crosstalk, to their benefit by either directly activating the SA pathway or by using associated symbionts that upon recognition by plants down regulate the synthesis of anti-herbivore defenses (Chung *et al.* 2013). Since induced plant defenses usually affect herbivore's fitness, is it conceivable that polyphagous herbivores would prefer to feed on plants in which they trigger less anti-herbivore defenses. The main questions that this research aimed to answer were: 1) Do polyphagous insects trigger different defense responses in different host plants? 2) Are herbivore-induced defenses correlated with insect host preference? 3) Does the composition of insect secretions changes with the host plant type they feed on? and 4) How do polyphagous herbivores cope with plant defenses from different hosts?

To answer these questions I used the polyphagous lepidopteran fall armyworm (FAW) *Spodoptera frugiperda,* and its host plants maize, Bermuda grass, rice and tomato as a model system. FAW has been reported to feed on more than 80 different plant species, both monocots and dicots and is an important agricultural pest of some of them (Luttrell & Mink 1999). Additionally, this species comprises two different host strains or populations of the same species that are genetically differentiated and exhibit partial reproductive isolation due in part to different host-plant adaptations (Drès & Mallet

2002). These strains are morphologically indistinct and exhibit different host preferences. In field conditions, the corn strain is mainly associated with corn, sorghum, and cotton while the rice strain is mostly associated with forage grasses and rice, Bermuda grass being one of its preferred hosts (Pashley 1986; Whitford *et al.* 1988; Machado *et al.* 2008). These strains also differ in their genetic makeup, sex pheromone blends, and mating time (Pashley 1986; Groot *et al.* 2008; Schöfl *et al.* 2009). Studies aiming to understand their differential host–plant association have found greater capability 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). Furthermore, positive fitness effects on larval and/or pupal weight and developmental time have been found when the FAW strains are associated with their preferred hosts compared with alternative plants (Pashley 1986; Whitford *et al.* 1988; Pashley *et al.* 1995; Veenstra *et al.* 1995). Together these studies suggest that the FAW strains may have evolved specific adaptive mechanisms to use their host plants species. Host race evolution has been linked to the development of differential adaptation to host plants in different insects species (Drès & Mallet 2002), one of the best documented cases being the strain formation in the apple maggot fly (*Rhagoletis pomonela)* associated with a host shift from wild hawthorn to cultivated apple trees (Bush 1969; Feder *et al.* 1994). Numerous studies have reported differences in fitness when insect strains are associated with different hosts (Katakura *et al.* 1989; Via 1991; Pashley *et al.* 1995; Horner *et al.* 1999; Caillaud & Via 2000); however, the underlying mechanisms driving these adaptations are yet to be determined.

This dissertation covers some fundamental aspects of the interaction of the polyphagous insect FAW with some of its host plants. In the first chapter I present a brief overview on induced plant defenses. The second chapter focuses on intraspecific differences in plant defense induction by the FAW strains, the identification of differences in the composition of insect-derived plant defense elicitors and the effect of induced plant defenses in caterpillar fitness. In the third chapter I present a proteomic analysis of the FAW saliva along with the identification of proteins differentially

expressed in the saliva of the two FAW strains associated with different host plants. The fourth chapter focuses on the identification of non-protein plant defense elicitors present in the saliva of the FAW strains with emphasis in phytohormones and small molecules. In the fifth chapter I present the effect of FAW-associated gut bacteria in the modulation of defense responses in different host plants. The sixth chapter focuses on the identification of physical plant defenses induced by FAW herbivory and counter insect adaptations to overcome these defenses. Lastly, the seventh chapter presents a short discussion of the main findings of this dissertation.

Induced plant defenses

Plants have evolved a variety of defense mechanisms against environmental and biotic stressors that can be classified as physical or biochemical. Physical defenses are morphological structures that include thorns, spines, hairs (trichomes), lignification of cell walls, deposition of waxes and minerals into plant tissues (War *et al.* 2012). Biochemical defenses include the production of secondary metabolites (compounds that are not needed for plant growth, development or reproduction), plant defensive proteins and enzymes (War *et al.* 2012). The production of defensive compounds is metabolically costly and in some cases can be cytotoxic for plants; thus, some of these defensive traits are constitutive while others are induced only in the presence of threat (Steppuhn & Baldwin 2008). Herbivory triggers a cascade of biochemical reactions that often leads to the biosynthesis or direct and indirect plant defenses (Bonaventure 2014). Direct defenses are deterrents, anti-nutritional or toxic compounds that negatively affect insects themselves, while indirect defenses are plant-produced molecules that attract natural enemies of insect herbivores (War *et al.* 2012). Plant defensive mechanisms against phytophagous insects and their perception have been nicely reviewed by War *et al.* (2012), Fürstenberg-Hägg *et al.* (2013), and Bonaventure (2014); while the effect of insect derived secretions on plant defense induction have been recently reviewed by Acevedo *et al.* (2015) and Schmelz (2015). This brief overview only aims to present a few plant defensive strategies considered in this dissertation.

Feeding by insect herbivores triggers a cascade of events that lead to the production of plant defensive compounds. The first steps involve changes in the potential of cell membranes followed by electrical signals from wounded sites to distant parts of the plant. Soon after, the cells experience fluctuation in intracellular calcium ions that act as second messengers of signal pathways. Calcium signals activate calcium sensing proteins and calcium binding protein kinases that promote downstream reactions like protein phosphorylation and gene expression (Fürstenberg-Hägg *et al.* 2013). The biosynthesis of JA, the main hormone activated in response to chewing insects, starts from the release of linolenic acid from membrane phospholipids by the action of phospholipases (Fürstenberg-Hägg *et al.* 2013). Linolenic acid passes through a series of reactions mediated by lipoxygenases (LOX), allene oxide synthase (AOS), and 12-oxo-phytodecanoic acid reductase (OPR) resulting in the synthesis of JA (Shivaji *et al.* 2010). JA is a long-distance trafficking molecule that induces the biosynthesis of other defense responses; it also interacts with other plant hormones like ethylene, SA, and abscisic acid to regulate the expression of defenses (Fürstenberg-Hägg *et al.* 2013). Some of these defenses include production of anti-nutritional proteins and volatile organic compounds.

Reactive oxygen species (ROS)

These are partially reduced forms of oxygen like superoxide (O^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-) collectively referred as ROS (War *et al.* 2012). Their synthesis is induced by abiotic and biotic stressors including pathogens and herbivores (Tripathy & Oelmüller 2012). Insect feeding promotes an increase in ROS, which are directly toxic, but ROS also act as second messengers to regulate other defense responses including anti nutritional proteins (Fürstenberg-Hägg *et al.* 2013).

Proteinase inhibitors (PIs)

These are plant-produced proteins that bind to insect digestive enzymes and inhibit their catalytic activity (War *et al.* 2012). The ingestion of PIs compromises

insects' ability to obtain nutrients from its food reducing growth and development. Most PIs target insect serine proteases like trypsin and chymotrypsin, which are the main digestive proteases in lepidopterans (Jongsma & Beekwilder 2008). However, the protective effect of PIs is usually transient because insects are able to inactivate ingested PIs and produce PI-insensitive proteases (War *et al.* 2012).

Polyphenol oxidases (PPO)

They are copper-containing enzymes that catalize the oxidation of phenols to quinones, which are able to cross link proteins reducing food nutritional quality (Constabel & Barbehenn 2008). PPO are important defensive enzymes that affect insect growth and development by reducing nutrient availability and inducing oxidative stress in the insect gut (War *et al.* 2012).

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Chapter 2

Intraspecific differences in plant defense induction by the fall armyworm strains

Abstract

Insect host strains are genetically differentiated populations of the same species that exhibit partial reproductive isolation and are adapted to specific host plants. The fall armyworm (FAW) *Spodoptera frugiperda*, comprises two strains with different host preferences. The corn strain is mainly associated with maize, while the rice strain is mostly associated with forage grasses and rice. The association of insect strains to different host plants has been claimed to influence reproductive isolation. However, the underlying mechanisms by which these strains adapt to different host plants are mostly unknown. In this chapter I investigated the role of induced plant defenses in the host plant association of the fall armyworm strains. I tested the expression of defense-related genes and the activity of plant-defensive proteins in maize, rice and Bermuda grass upon feeding by the FAW strains. The FAW strains induced different defense responses in their host plants; feeding by rice strain caterpillars induced greater accumulation of proteinase inhibitors in maize than feeding by the corn strain. In Bermuda grass, feeding by the corn strain down regulated the activity of trypsin protease inhibitors while the rice strain induced greater activity levels. These differences in plant defense induction were associated with variances in the caterpillar saliva, specifically these strains exhibited differences in activity of the salivary enzyme phospholipase C (PLC), which triggered different defense responses in maize and Bermuda grass. I conclude that specific elicitors in the saliva of the FAW strains triggered differential levels of plant defense responses that subsequently affected caterpillar fitness and thus may influence host–plant preference in field conditions.

Introduction

During 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 co-evolution with herbivores, plants have evolved mechanisms to recognize these herbivore-derived cues or HAMPs (herbivore-associated molecular patterns) to activate the production of defense responses. Both the amount and type of cues released by the insect and the plant's ability to recognize them seem to be species specific (Acevedo *et al.* 2015). This phenotypic plasticity of both plants and their 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.

Host-race evolution has been linked to differential host-plant associations in several insects species (Drès & Mallet 2002), one of the best documented cases being the strain formation in the apple maggot fly, *Rhagoletis pomonella,* associated with a host shift from wild hawthorn to cultivated apple trees (Bush 1969; Feder *et al.* 1994). Likewise, the fall armyworm–*Spodoptera frugiperda,* (FAW) comprises two sympatric strains that exhibit different host preferences under field conditions. The "corn strain" is mainly associated with maize, sorghum, and cotton, while the "rice strain" is mostly associated with forage grasses and rice (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 (Groot *et al.* 2010; Meagher & Nagoshi 2012), indicating differences in nutrient assimilation and metabolism. Studies aiming to elucidate the factors driving the differential host plant association of the 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 widely 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 is a rich proteinaceous secretion known to modulate defense responses in plants. 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 as 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* (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*, induce the accumulation of oxylipins, especially OPDA (12-oxo-phytodienoic acid) in *Arabidopsis thaliana* (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*, 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 the 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 association are unknown.

In this study, I investigated the role of herbivore-induced defenses in the host plant association of the FAW strains. I specifically addressed the following questions: (1) Do the FAW strains induce different defense responses in their host plants during their feeding behavior? (2) Do induced plant defenses affect the fitness of the FAW strains? (3) Do the FAW strains exhibit differences in the composition of known insect-derived elicitors?

The results of this study show that feeding by the FAW strains induced different defense responses in their host plants. From the potential cocktail of elicitors, I identified one enzyme, phospholipase C, present in the caterpillars' saliva that may drive different levels of defense induction. Furthermore, plant defense responses affected caterpillar fitness, and therefore may strongly influence the strains' host plant association in field conditions.

Methods

Insects

The 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, while 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

F1 individuals containing the corresponding mitochondrial marker that identify 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, U.S.A.). The seeds were germinated in Promix potting soil (Premier Horticulture Quakertown, PA, USA) and transplanted 10 days after germination into 1-gallon 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 (*Oryza sativa*) seeds of the cultivar Nipponbare were obtained from the USDA-ARS Dale Bumpers National Rice Research Center in Arkansas. The seeds were germinated in moisted towels at 25 ˚C (16:8 hours light: dark) and further transplanted to four-inch 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) diluted in one gallon of water. Weekly there after the plants were watered with 20 g of ammonium sulfate diluted in 1 gallon of water. Plants in the V6 physiological stage were used for the experiments.

Bermuda grass (*Cynodon dactylon*) hulled seeds were purchased from Seed World USA (Tampa, Fl) and directly grown on 0.734 gallon pots (C300 Nursery Supplies Inc. Chambersburg, PA, USA) containing Hagerstown loam soil and fertilized with five g of Osmocote plus. 4-week old plants were used for the experiments.

All plants were grown under glasshouse conditions (14 hours light: 10 hours dark) at the Pennsylvania State University, University Park, PA.

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 *Maize proteinase inhibitor (MPI), Allene Oxide Synthase (AOS)* and *Ribosome-inactivating protein 2 (RIP2).* In Bermuda grass, we measured the activity of trypsin protease inhibitor (PI), which inhibits the activity of digestive serine proteases in insects impairing their growth and development (Dorrah 2004). In rice plants we measured the expression of the JA-related genes *Lipoxygenase (LOX)* and *the Bowman-Birk proteinase inhibitor (RPI).*

Plant mechanical wounding

In maize plants, the third youngest leaf was mechanically wounded once using the wounding tool described in Bosak (2011). The five youngest leaves of Bermuda grass plants (one wound per leaf) and the second youngest leaf in rice plants (4 wounds) were wounded using a cork borer (Unicore -2.0 Harris, USA).

Plant defense response to the FAW strains feeding

To evaluate the effect of the FAW strains feeding on induced defenses, plants were challenged with actively feeding last-instar caterpillars of both strains. These caterpillars were grown from egg hatch on detached leaves of maize, Bermuda grass and rice before placing them onto their respective plants. In maize, caterpillars were either placed directly in the whorl of the plants for 24 hours or enclosed in clip cages (metallic micromesh screen diameter 23 mm, height 18 mm. Polypropylene) to control for the amount of injury. 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 hours later for gene expression analysis. Bermuda grass and rice plants were treated by exposing 6-10 leaves to caterpillars enclosed in Petri dish cages (5.5 cm diameter, 1.5 cm high, 23.76 cm^2 area) built with two bottoms of plastic petri dishes (60 *15 mm, VWR, West Chester, PA) with air wholes pocked through for air flow. The petri dishes are hinged with an aluminum hair clip bended to fit and gasketed with felt attached to a wood stick 12 inches long. Leaf samples were harvested 24 hours later. Each plant (n = 6 - 7) was treated with one caterpillar in a complete randomized design.

Plant defense response to caterpillar saliva

I 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 minutes and ablated by cauterizing their spinneret with a hot pin. Ablated caterpillars were allowed to recover and eat for 12 hours 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, rice or Bermuda grass leaves, and were used to treat these plants, respectively. Saliva was manually collected from caterpillars chilled on ice for 45 minutes and immobilized into a metallic hairclip, as they warmed up, their saliva was collected using a micropipette tip (VWR cat No. 53509-015) under a light microscope (Olympus SZ30). On average, each caterpillar secreted ~0.2 µl of saliva. The saliva was stored at -80 $^{\circ}$C until use. Each plant was wounded and treated with 2 µl of saliva (~5 µg of protein) diluted in 10 µl of 1X PBS (137 mM NaCl, 2.7 mM KCl, 10.14 mM Na2HPO4, 1.76 mM KH2PO4, pH. 7.2). Labial salivary glands were dissected from caterpillars chilled on ice for ~ 15 minutes 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 MQ water and placed into 1.5 ml tubes kept on ice. The salivary glands were homogenized in 100 µl of 1X PBS using polypropylene pellet pestles (VWR), centrifuged for three minutes at 8000 rpm and the supernatant collected into a new tube. The amount of protein was determined using a Bradford assay.

Each plant (n= 6-10) was wounded and treated with 10 µg of the homogenates obtained from 3-5 pairs of salivary glands within one hour of their dissection. To investigate if protein components in the FAW caterpillar's saliva would trigger plant defense responses, I boiled the 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 the ones from wounded plants treated with PBS buffer and unwounded controls in a complete randomized design.

Effect of induced plant defenses on caterpillar weight gain

I investigated if induced plant defenses by the 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; 24 hours later, the caterpillars were removed and the damaged leaves were used to feed neonates of both strains for a week. The damaged tissue of each plant was used to grow three caterpillar neonates, and their average weight 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, I first quantified the amount of regurgitant secreted by the FAW

strains on their host plants following the procedure described by Peiffer & Felton (2009) 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 1X PBS and 10 µl of the dilution were applied to wounded plants within an hour 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 the 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 response to caterpillar frass

Recent studies have demonstrated that components in the frass of the FAW caterpillars trigger defense responses in maize plants (Ray *et al.* 2015); therefore I tested for differences in induced plant defenses by frass from the 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 pressed by hand against the wounds. After 24 hours, the tissue around 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 the FAW strains, I searched for salivary enzymes that have previously been identified in other insect species. I specifically tested for differences in the activity of GOX, ATPases and PLC in both strains feeding on 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 procedures. The PLC enzymatic assays were carried out following the protocol developed by (Kurioka & Matsuda 1976), and adapted for a microplate reader, briefly, 5 µl of salivary gland homogenates (2 - 3 pairs diluted in 50 µl of 0.25 M Tris-HCl pH. 7.2) were mixed with 100 µl of the assay solution containing 20 mM of NPPC (O-4 nitrophenylphosphoryl-choline) (Sigma N5879) and 60% sorbitol (Sigma S7547) in 0.25 M Tris-HCl pH. 7.2. Phospholipase C hydrolyzes NPPC to phosphorylcholine and p-nitrophenol. The change in absorbance of p-nitrophenol was measured at 410 nm for 5 min. The PLC specific activity was calculated using an extinction coefficient of 1.51×10^4. Each sample (n = 5) contained 3 - 5 pairs of salivary glands from 2-day old last-instar caterpillars. The effect of the *strain* and *plant* factors on the 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, St Louis, MO, USA) diluted in 1X PBS. After 24 hours, 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.

RNA extraction, cDNA synthesis and qPCR

Leaf tissue (60 - 90 mg) frozen in liquid nitrogen was homogenized in a GenoGrinder 2000 (OPS Diagnostics, USA) and their total RNA extracted using a modified Trizol protocol [**See Appendix A].** Complementary DNA (cDNA) was synthesized from 1µg of RNA using the High Capacity cDNA Reverse Transcription kit

(Applied Biosystems, USA) using Oligo-dT. Quantitative real-time PCR (qRT-PCR) was carried out in a 10 µl volume containing 0.05 µM of each forward and reverse primer (Table 2-1), 5 µl of the Fast Start Universal 2X SYBR Green Master Mix (Roche Applied Science, USA), 2.9 µl of ultrapure water, and 2 µl of cDNA template. The PCR had a an initial denaturation step of 10 min at 95 ^{o}C, followed by 40 cycles of 15 sec at 95 ^{o}C, 60 sec at 60 ^{o}C. The C(T) values of each sample were normalized using the reference gene *Actin* and the relative quantification was calculated using the $2^{-\Delta\Delta C(T)}$ method (Livak & Schmittgen 2001) using undamaged control plants as reference. Specific primers for each of the genes were designed with Primer Express 3.0 (Life technologies, USA) and their efficiency was tested by comparing the normalized C(T) values of standard curves built using five serial dilutions of cDNA. Only primer pairs with similar efficiencies for the target and endogenous genes were used to quantify gene expression.

Table 2-1. Primers used in real-time PCR.

Plant	**Gene name**	**NCBI Accession No.**	**Primer sequences**	**Reference**
Maize	MPI	X78988	Forward 5'-GCGGATTATCGCCCTAACC-3' Reverse 5'-CGTCTGGGCGACGATGTC-3'	Chuang *et al.* 2014b
	AOS	NM_001111774	Forward 5'-CAAACCGACGAATTTGAGCAA-3' Reverse 5'-GGAGGCTCGCAACAAGTTG-3'	Chuang *et al.* 2014b
	RIP2	L26305	Forward 5'-GAGATCCCCGACATGAAGGA-3' Reverse 5'-CTGCGCTGCTGCGTTTT-3'	Chuang *et al.* 2014a
	Actin	U60511.1	Forward 5'-GGAGCTCGAGAATGCCAAGAGCAG-3' Reverse 5'-GACCTCAGGGCATCTGAACCTCTC-3'	Chuang *et al.* 2014b
Rice	LOX	Os12g37260 *	Forward 5'-TATCCCATCCCCATCCACTTAT-3' Reverse 5'-GTGTGAATGATTTGCAGCTGAAC-3'	Designed by Flor E. Acevedo
	RPI	AB098712.1	Forward 5'-CGTTCGATCATTCAGAGTTGGTATA-3' Reverse 5'-AAGCATGCAAGATGCACAAAA-3'	Designed by Flor E. Acevedo
	Actin	NM_001057621.1	Forward 5'-ATCCTGACGGAGCGTGGTTA-3' Reverse 5'-TAGTCCAGGGCGATG11TAGGAA-3'	Designed by Flor E. Acevedo

Trypsin protease inhibitor activity

I measured the activity of trypsin PI following the procedure described in Chung & Felton (2011). The trypsin PI activity was calculated as PI (%) = (1-(slope of sample/slope of Non inhibitor))*100 and the resulting activity values normalized by the amount of protein (mg) contained in the sample.

Development of the FAW strains on different plants

Caterpillars of both FAW strains were grown from egg hatch on artificial diet (wheat germ) as a control, and detached leaves of maize and Bermuda grass. The caterpillars were kept at 25 ºC with a photoperiod of 14 hours light: 10 hours dark, in plastic 1 oz cups (DART, Mason Mi, USA, Ref. 100PC) containing 1ml of 3% agar. The cups were kept clean and fresh leaves were provided as needed. Each treatment contained 30 randomly selected caterpillars from at least 10 different egg masses. We measured four fitness parameters for each larva: time of development from egg hatch to pupation, pupa weight, time from egg hatch to moth emergence, and the percentage of survival (individuals that completed their cycle from neonate to moth). The effect of the *strain* and *diet* factors on pupa weigh was analyzed using a two-factor factorial design.

Statistical analysis

The plant defense response (gene expression and trypsin PI activity) to the treatments (caterpillar feeding, application of salivary glands, regurgitant, frass, commercial PLC and GOX) was 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, larva weight gain, and pupa weight was analyzed using 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. The association between strain (corn and rice) and either larva developmental time or time to moth emergence was tested for each plant/diet using Chi Square. All the statistical analyses were performed using the Statistical Software Minitab 16 (Minitab Inc., State College, PA, USA) and R version 3.2.2 (Foundation for Statistical Computing, Vienna, Austria). All graphs were generated in R.

Results

The FAW strains triggered different levels of induced defenses on maize and Bermuda grass but not in rice.

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. 2-1 a-b); five out of six independent experiments showed the same results. Two independent experiments in Bermuda grass, showed that feeding by the corn strain suppressed induction of trypsin PI activity to similar levels found in undamaged controls; feeding by the rice strain, on the other hand, induced significantly greater activity of trypsin PI compared with the corn strain ($P = 0.023$) and untreated controls ($P = 0.002$) (Fig. 2-1 c). When feeding on rice plants, caterpillars from both strains induced similar transcript accumulation of the *LOX* and *RPI* genes (Fig. 2-2 a-b). This was observed in two independent experiments.

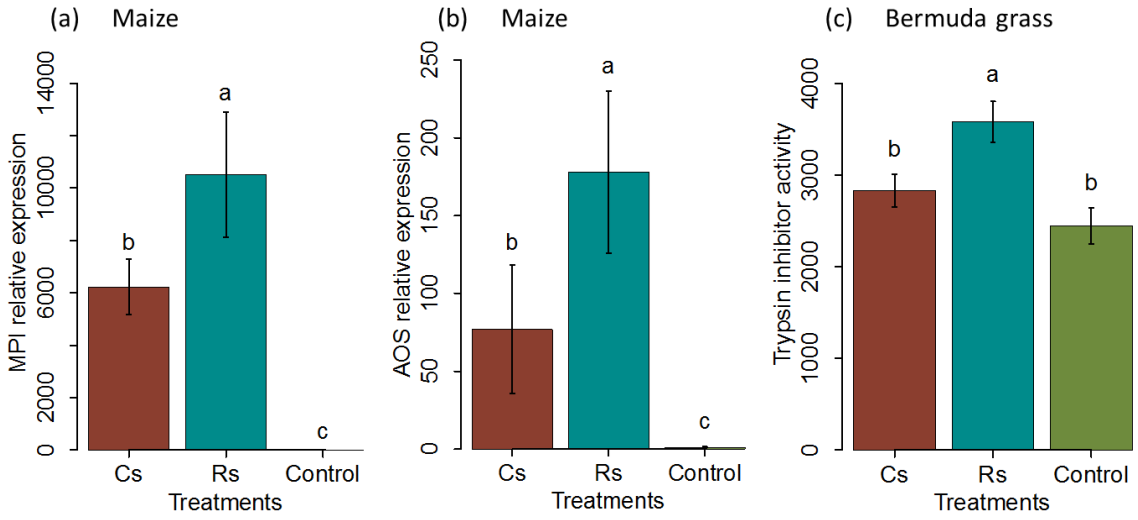

Figure 2-1. Plant defense response to the FAW strains caterpillar feeding. Cs = Corn strain, Rs = Rice strain, controls are undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α = 0.05. (a) *Maize Proteinase Inhibitor (MPI)* gene expression 24 hours after caterpillar treatment ($F_{2,15}$ = 497.05, P = 0.000; Tukey test; n = 6; log transformed data). (b) Maize *Allene Oxide Synthase (AOS)* gene expression 24 hours after caterpillar treatment ($F_{2,13}$ = 6.3, P = 0.046; Fisher test; n = 6; log transformed data). (c) Bermuda grass *trypsin inhibitor activity* 24 hours after caterpillar damage ($F_{2,18}$ = 8.23, P = 0.003; Tukey test; n = 7).

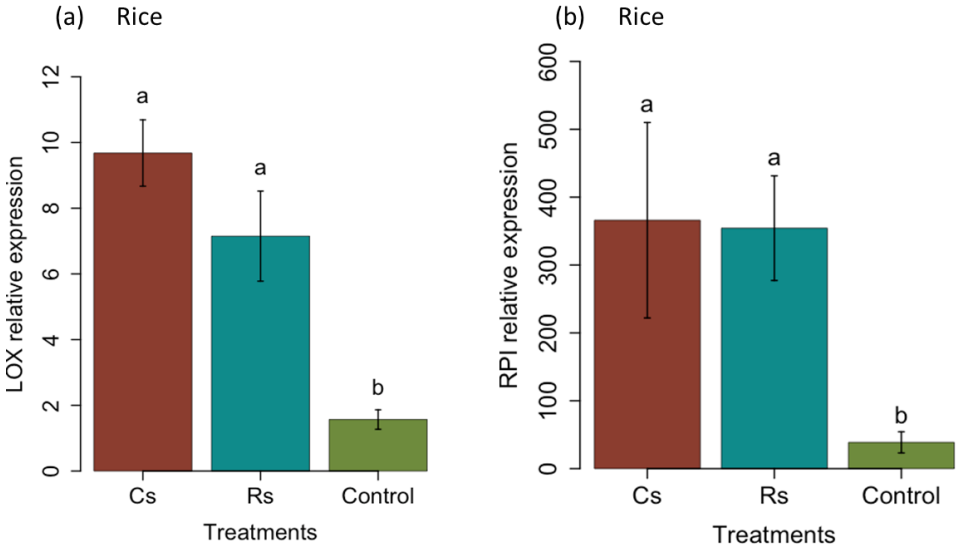

Figure 2-2. Rice defense response to the FAW strains caterpillar feeding. Cs = Corn strain, Rs = Rice strain, controls are undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α = 0.05.

(a) *Lipoxygenase (LOX)* gene expression 24 hours after caterpillar treatment ($F_{2,14}$ = 14.65, P = 0.000; Tukey test; n = 5 - 6; log transformed data). (b) *Bowman-Birk proteinase inhibitor (RPI)* gene expression 24 hours after caterpillar treatment ($F_{2,14}$ = 5.73, P = 0.015; Fisher test; n = 5 - 6; log transformed data).

Induction of defenses in maize and Bermuda grass negatively affected 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. The neonates gained greater weight when fed on undamaged plants (controls) and plants previously damaged by ablated rice-strain caterpillars (Fig. 2-3 a). There was a significantly negative correlation between the transcript accumulation of *MPI* and the weight gained by young FAW larvae ($F_{1,18}$ = 44, P = 0.000; Fig. 2-3 c). In Bermuda grass, neonates grew faster when fed on leaves previously damaged by intact corn strain caterpillars and untreated controls compared with the ones grown on leaves previously damaged by ablated corn strain and intact rice strain caterpillars (Fig. 2-3 b). There was a significant but not strong negative correlation between trypsin PI activity and caterpillar weight gain ($F_{1,31}$ = 6.67, P = 0.015; Fig. 2-3 d).

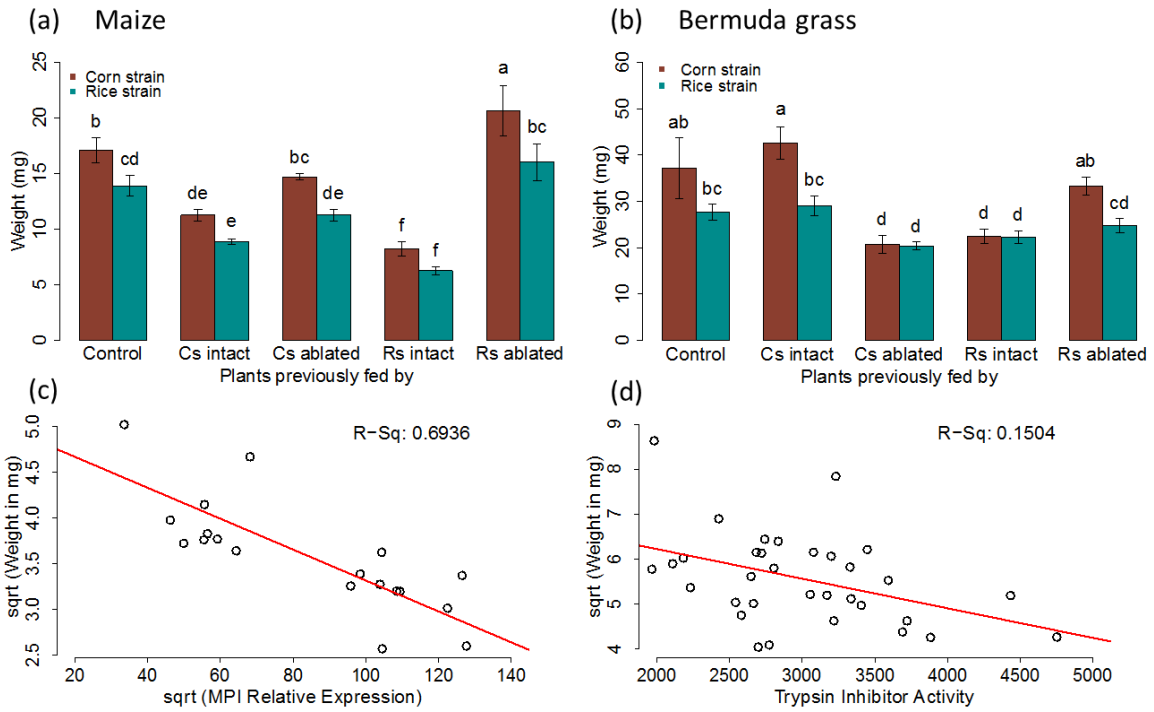

Figure 2-3. Larvae weight gain reared on detached leaves previously damaged by FAW strain caterpillars able (intact) and impaired to salivate (ablated). Cs = Corn strain, Rs = Rice strain, controls are undamaged plants**.** Bars are untransformed means ± SEM. **(a)** Larvae grown on damaged maize leaves; strain effect: $F_{1,40} = 22.10$, P = 0.000; treatment effect: $F_{4,40} = 34.47$, P = 0.000; strain*treatment effect: $F_{4,40} = 0.48$, P = 0.088; Fisher test; n = 5. **(b)** Larvae grown on damaged Bermuda grass leaves; strain effect: $F_{1,55} = 11.21$, P = 0.000; treatment effect: $F_{4,55} = 15.22$, P = 0.000; strain*treatment effect: $F_{4,55} = 2.14$, P = 0.065; Tukey test; n = 5-7; (1/sqrt 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 inhibitor activity [sqrt (mg) = 7.54 – 0.00066 trypsin PI].

Caterpillar saliva of the FAW strains triggered 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 = 0.000) (Fig. 2-4 a). In Bermuda grass, intact caterpillars from the corn strain suppressed the induction of trypsin PI activity to similar levels found in undamaged

controls, while ablated caterpillars induced the production of these inhibitors. On the contrary, intact rice strain caterpillars induced greater production of trypsin PI than the corresponding ablated ones (P = 0.045; Fig. 2-4 b); two independent experiments showed the same 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. 2-4 c-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. 2-4 d).

Protein components of the caterpillar's saliva from the FAW strains elicited different plant defense responses.

When boiled saliva or salivary gland homogenates were applied to wounded plants, the levels of plant defense responses were not different between the strains (Fig. 2-5). However, boiled salivary glands still induced greater defenses 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 (not shown).

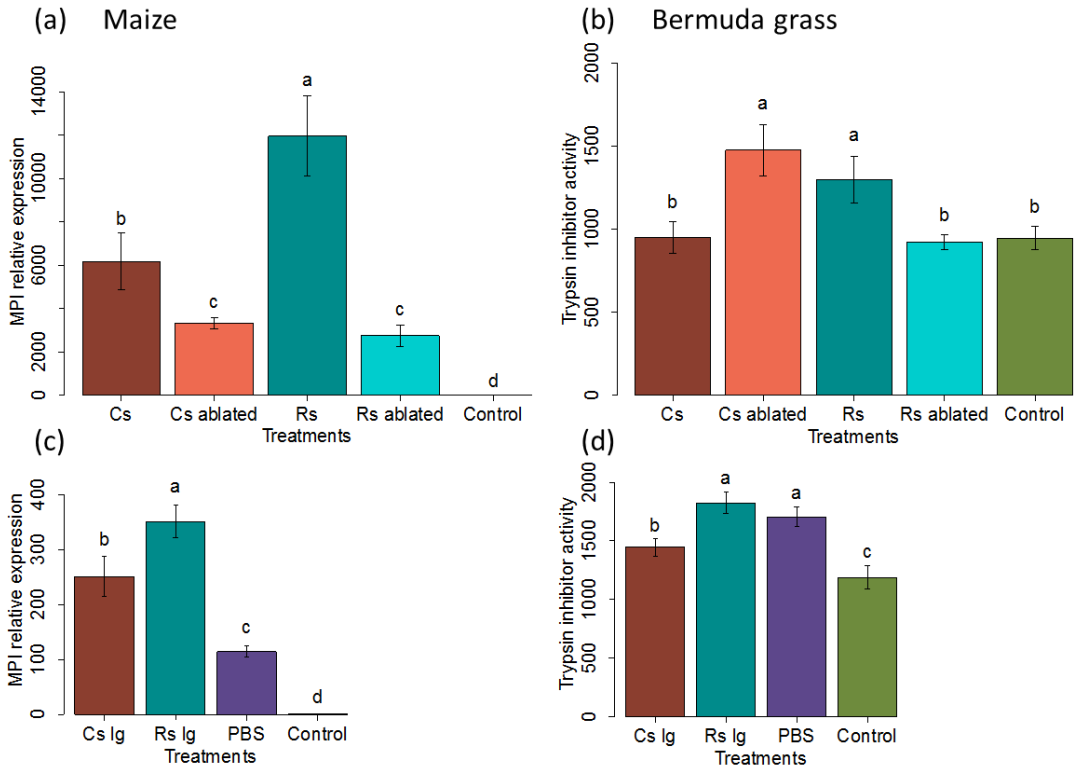

Figure 2-4. Plant response to caterpillar saliva from the FAW strains. Cs = Corn strain, Rs = Rice strain, ablated = caterpillars impaired to salivate, lg = labial salivary glands, PBS are buffer treated controls, controls are undamaged plants. Values are untransformed means ± SEM. (**a**) *Maize Proteinase Inhibitor (MPI)* gene expression 24 hours after caterpillar treatment ($F_{4,20}$ = 394.78, P = 0.00; Fisher test; n = 5; log transformed data). (**b**) Bermuda grass *trypsin inhibitor activity* 24 hours after caterpillar damage ($F_{4,25}$ = 5.38, P = 0.045; Fisher test; n = 6). (**c**) *Maize Proteinase Inhibitor (MPI)* gene expression 24 hours after treatment with salivary glands from the FAW strains ($F_{3,20}$ = 493.9, P = 0.000; Fisher test; n = 6; log transformed data). (**d)** Bermuda grass *trypsin inhibitor activity* 24 hours after treatment with salivary glands ($F_{3,36}$ = 10.44, P = 0.000; Tukey test; n = 10).

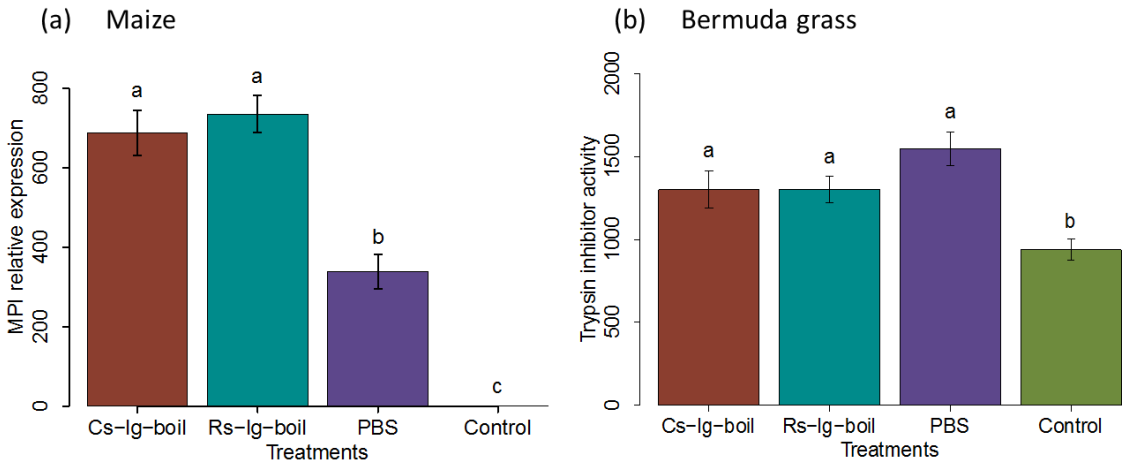

Figure 2-5. Plant response to the application of boiled salivary gland homogenates from caterpillars of the FAW strains. Cs = Corn strain, Rs = Rice strain, lg = labial salivary glands, boil = boiled, PBS are buffer treated controls, controls are undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at $\alpha = 0.05$. (**a**) *Maize Proteinase Inhibitor (MPI)* gene expression 24 hours after treatment ($F_{3,21} = 714.06$, P = 0.000; Tukey test; n = 6; log transformed data). **(b)** Bermuda grass *trypsin inhibitor activity* 24 hours after treatment ($F_{3,30} = 8.61$, P = 0.000; Fisher test; n = 8).

FAW strains exhibited differential activities of the salivary enzymes phospholipase C and glucose oxidase.

PLC activity varied with the type of diet for each of the strains. The analysis of variance showed a significant interaction between strain and diet type ($F_{2,24} = 21.6$, P = 0.000), therefore significant differences were obtained using a 2-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 that the rice strain; lastly, when feeding on artificial diet, the corn strain had similar PLC activity that the rice strain (Fig. 2-6 a). The GOX activity levels for the two FAW strains were also diet-dependent. There was a significant effect of both the strain ($F_{1,20} = 25.2$, P = 0.000), and the type of diet ($F_{2,20} = 11.1$, P = 0.000), but not a significant interaction between them ($F_{2,20} = 2.9$, P = 0.077). For all diets tested the corn strain had significantly higher GOX activity than the rice strain. The GOX activity was higher in diet-fed

caterpillars of both strains followed by the maize and Bermuda grass-fed ones (Fig. 2-6 b). No activity of ATPases was detected for either of the strains (not shown).

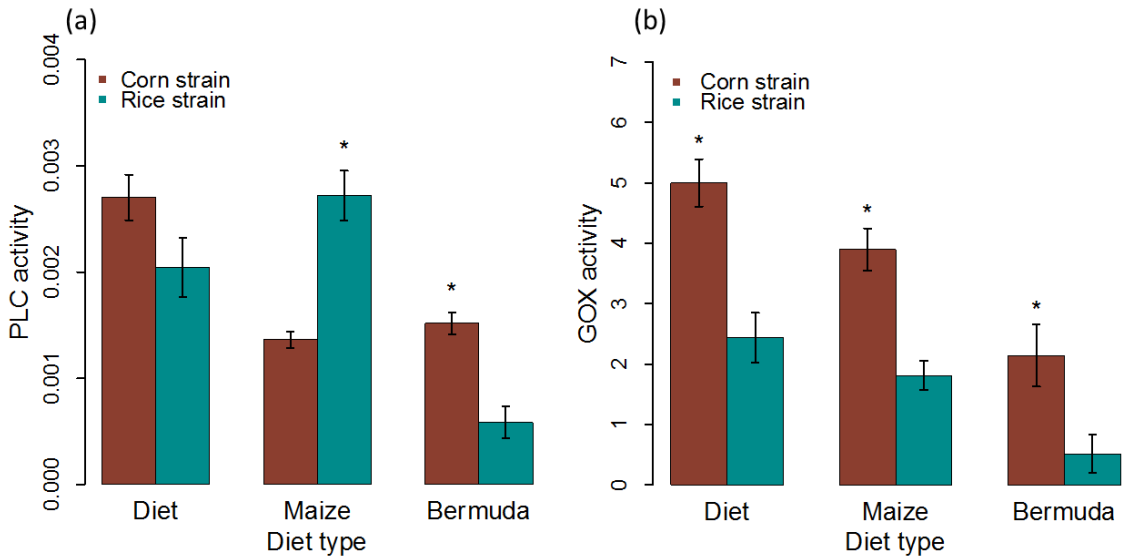

Figure 2-6. Phospholipase C (PLC) and glucose oxidase (GOX) activity in labial salivary glands of the FAW strains fed on different diets. Values are untransformed means ± SEM, asterisks indicate significant differences (α = 0.05) between the strains per diet type. (**a**) PLC activity per mg of protein: artificial diet: t = 1.87, P = 0.104; maize: t = -5.48, P = 0.005; Bermuda grass: t = 5.17, P = 0.001; n = 5; Bonferroni 0.05/3 = 0.01667. **(b)** GOX activity per mg of protein, significant differences between the strains for each diet type were determined by the Bonferroni method at 95% confidence.

Phospholipase C modulated defense responses in maize and Bermuda grass.

In maize, commercial PLC from *Clostridium perfringens* induced higher expression of the herbivore-responsive genes *MPI* (P = 0.015) and *RIP2* (P = 0.028) compared with buffer treated plants (Fig. 2-7 a-b). Conversely, in Bermuda grass, PLC suppressed the production of trypsin PI to similar levels found in untreated controls (Fig. 2-7 c).

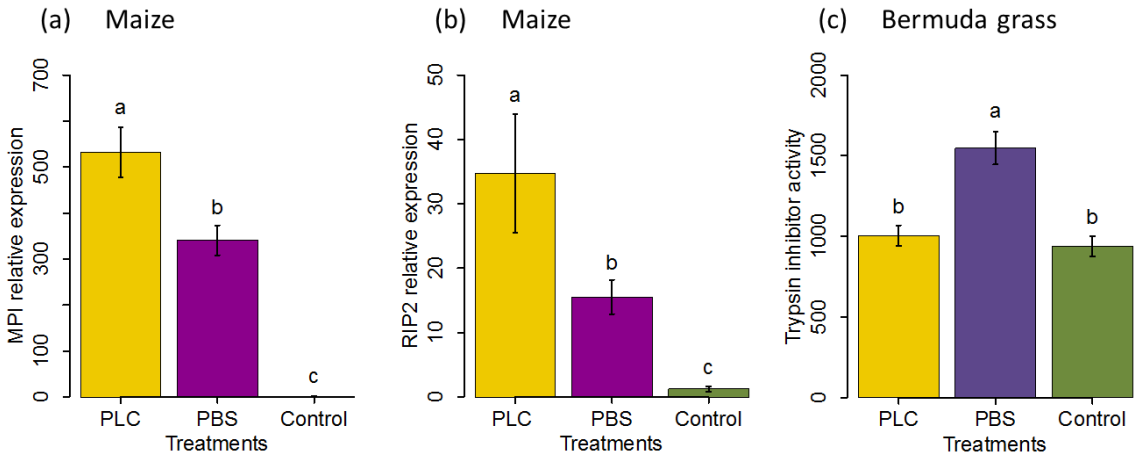

Figure 2-7. Plant response to the application of commercial phospholipase C (PLC). PBS are buffer treated controls, controls are undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at $\alpha = 0.05$. (**a**) *Maize proteinase inhibitor (MPI)* gene expression 24 hours after treatment ($F_{2,12} = 298.58$, P = 0.000; Tukey test; n = 4 - 7; ¼ root transformed data) **(b)** *Ribosome-inactivating protein 2 (RIP2)* gene expression 24 hours after treatment ($F_{2,12} = 37.16$, P = 0.000; Tukey test; n = 4-7; log transformed data) (**c**) *Trypsin inhibitor activity* per mg of protein 24 hours after treatment with PLC ($F_{2,27} = 18.17$, P = 0.000; Tukey test; n = 10).

Effect of glucose oxidase on plant defense responses.

The application of commercial GOX in Bermuda grass, induced similar trypsin PI activity levels than the PBS-treated plants ($F_{2,27} = 1.1$, P = 0.336) (Fig. 2-8). In maize, previous studies have shown no effect of GOX on induced-defense responses (Louis *et al.* 2013), therefore, its effect on this plant was not tested here again.

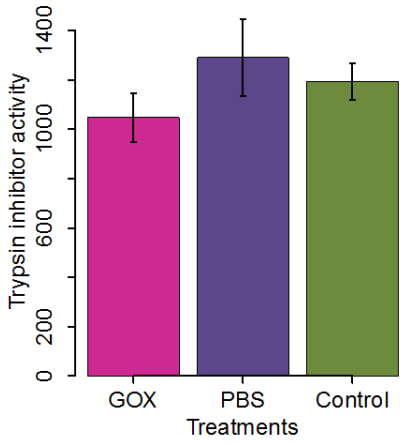

Figure 2-8. Trypsin protease inhibitor activity in Bermuda grass 24 hours after treatment with glucose oxidase (GOX). ($F_{2,27}$ = 1.13, P = 0.336; n = 10). Values are untransformed means ± SEM.

The role of oral secretions in plant defense induction by the FAW strains

All the caterpillars tested (N = 30) released regurgitant into the plants during feeding. The amount of regurgitant released varied from 2- 6 nl. There was a significant effect of the strain ($F_{1,16}$ = 5.8, P = 0.028) and the plant*strain interaction ($F_{1,16}$ = 5.3, P = 0.035) on the amount of regurgitant secreted. Both strains released the same amount of regurgitant when feeding on Bermuda grass (t = -0.11, P = 0.917), but when feeding on maize, the rice strain released 4 times more regurgitant than the corn strain (t = -2.75, P = 0.025) (Fig. 2-9). In maize, the application of meaningful quantities of regurgitant induced higher transcript accumulation of the *MPI* gene compared with wounded + PBS treated plants ($F_{3,20}$ = 509.55, P = 0.000), but there were no differences in induction for the strains despite the different amounts applied (P = 0.802) (Fig. 2-10 a). 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. 2-10 b).

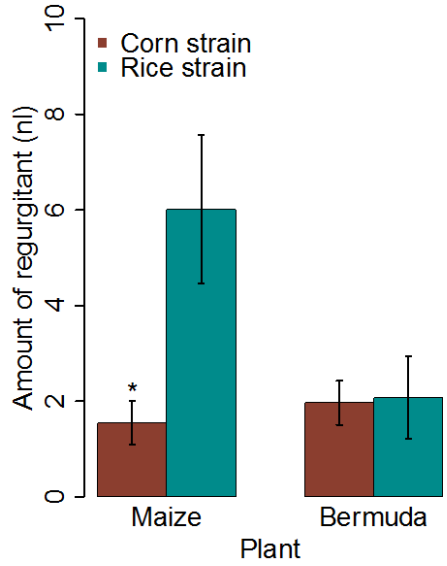

Figure 2-9. Amount of secreted regurgitant in nanoliters (nl) by the FAW strain caterpillars feeding on different host plants. Values are untransformed means ± SEM. Asterisks indicate significant differences (α = 0.05) between the strains per plant type. Maize: t = -2.75, P = 0.025; Bermuda grass: t = -0.11, P = 0.917.

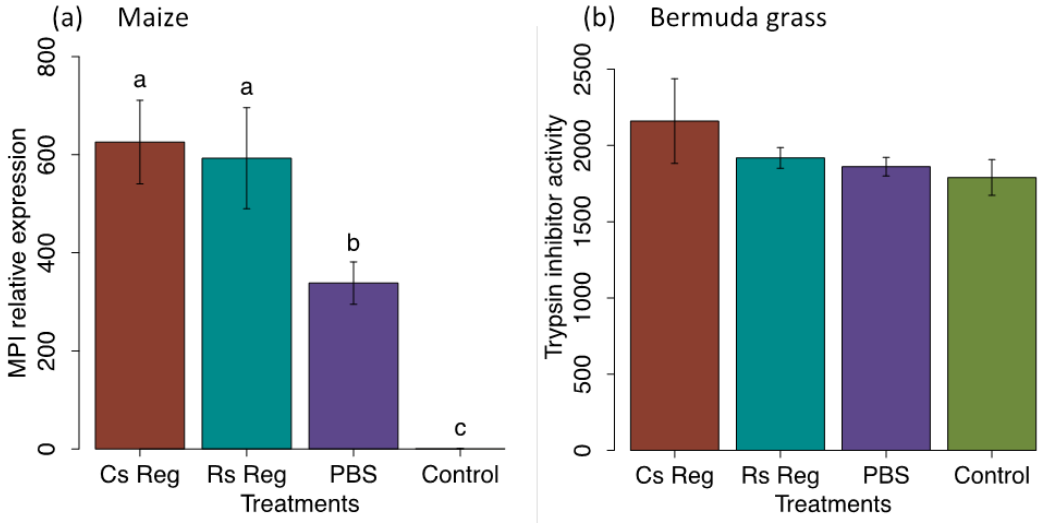

Figure 2-10. Plant response to the application of regurgitant from the FAW strain caterpillars. Cs = Corn strain, Rs = Rice strain, Reg = regurgitant, controls are undamaged plants. Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α = 0.05. (**a**) *Maize Proteinase Inhibitor (MPI)* gene expression 24 hours after treatment ($F_{3,20}$ = 509.55, P = 0.000; Tukey test; n = 5-7; log transformed data). **(b)** Bermuda grass Trypsin inhibitor activity 24 hours after treatment ($F_{3,36}$ = 1.04, P = 0.387; n = 10).

The effect of caterpillar frass

Caterpillar frass induced higher levels of *MPI* transcript accumulation compared to wounding alone ($F_{2,20} = 73.9$, P = 0.000), but the levels of defense induction were not different for the two strains (Fig. 2-11).

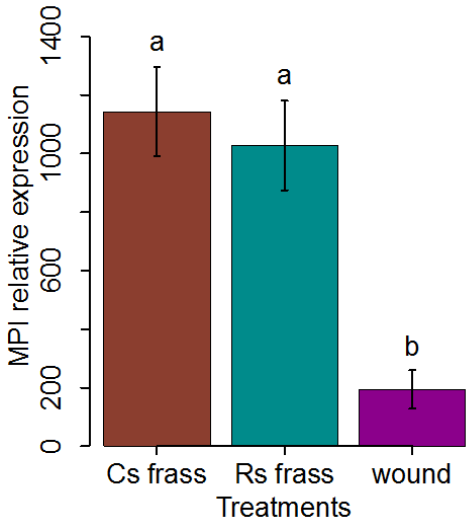

Figure 2-11. Relative expression of the Maize Proteinase inhibitor (MPI) gene 24 hours after frass treatment from the FAW strains. Cs = Corn strain, Rs= Rice strain; values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at $\alpha = 0.05$. ($F_{2,20} = 73.96$, P = 0.000; Tukey test; n = 7-8; log transformed data).

The caterpillars from the FAW strains had different developmental time in maize but not in Bermuda grass.

The FAW strains developed faster and had greater survival when reared on maize and Bermuda grass than when reread on artificial diet. When feeding on maize and artificial diet, the corn strain larvae developed faster than the rice strain, but in Bermuda grass the larvae of the two strains developed similarly (Table 2-2). However, when measuring the days taken from neonate to moth emergence, the corn strain developed faster than the rice strain in all diets tested. When comparing the development of each strain on different diets separately, there were not differences in the time from neonate to

moth on maize or Bermuda grass for either strain (P > 0.05 Chi square test); but when grown on artificial diet, the development was significantly longer (P < 0.05 Chi square test). Similar results were obtained for the larvae developmental time with the exception that the corn strain larvae did develop significantly faster in maize than in Bermuda grass (Chi-squared = 12.784, df = 3, P = 0.0051). The corn strain had higher pupa weight than the rice strain in all diets tested except for Bermuda grass where both strains had the same weight (Fig. 2-12). Both strains had higher pupa weight when fed on maize and artificial diet than when fed on Bermuda grass. However mortality levels where quite different, the rice strain had higher percentage of mortality when feeding on maize than when feeding on Bermuda grass, while the corn strain had similar levels of mortality on both hosts. Differences in the above fitness parameters reflect the differential ability of the strains to utilize nutrients and cope with chemical/physical defenses of their host plants.

Table 2-2. Fitness parameters of the FAW strains reared on non-induced host plants

Strain	**Diet type**	**Larvae time of development from egg hatch to pupation (days)**					**Time from neonate to moth emergence (days)**					**% survival to moth**
		Days (95% CI)	**n**	**Chi square**	**df**	**p**	**Days (95% CI)**	**n**	**Chi square**	**df**	**p**	
Cs	Maize	13.4 ± 0.29	27	29.135	3	2.09e-06	25.8 ± 0.34	27	11.671	4	0.0199	90
Rs	Maize	14.7 ± 0.31	25				26.6 ± 0.49	22				73.3
Cs	Bermuda	13.9 ± 0.27	28	5.6225	3	0.1315	25.6 ± 0.29	27	19.184	5	0.0017	90
Rs	Bermuda	14.3 ± 0.30	29				26.5 ± 0.39	29				96.67
Cs	Diet	14.4 ± 0.45	30	30.082	7	9.17e-05	26.6 ± 0.46	26	24.754	5	0.00015	86.67
Rs	Diet	16.9 ± 0.88	24				28.7 ± 0.42	19				63.33

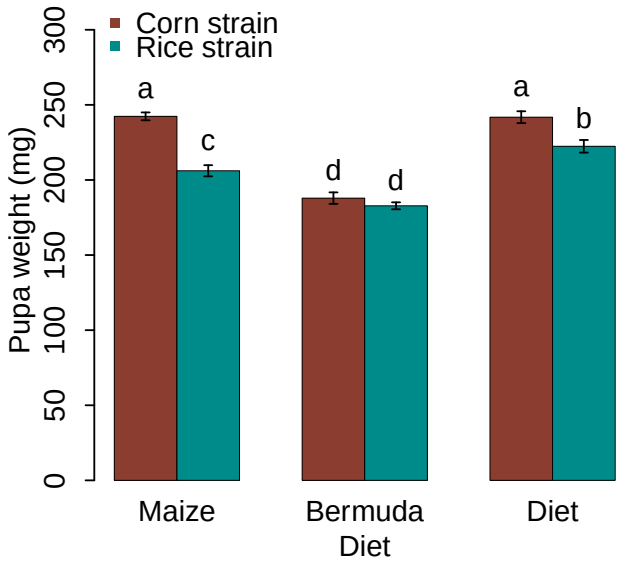

Figure 2-12. Pupa weight of the FAW strains reared on different diet types. Values are untransformed means ± SEM, different letters indicate significant differences obtained with ANOVA following post hoc tests at $\alpha = 0.05$. Strain effect: $F_{1,148} = 51.05$, $P = 0.000$; diet effect: $F_{2,148} = 109.35$, $P = 0.000$; Strain* diet effect: $F_{2,148} = 10.56$, $P = 0.000$. n = 21 – 29.

Discussion

The results of this study show that caterpillars of the two FAW strains induced different defense responses in maize and Bermuda grass plants during feeding, resulting in cascading fitness effects on young larvae that may affect the strains' host-plant association. The rice strain induced greater defense responses than the corn strain in maize and Bermuda grass, while 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 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. 2-3). Several lines of evidence suggest that components in the caterpillar saliva, specifically, differences in the activity of the enzyme PLC elicited these differential plant defense responses by the

strains. *First*, the same 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. *Second*, 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. *Third*, application of commercial PLC induced production of protease inhibitors in maize but suppressed the activity of trypsin PI in Bermuda grass (Fig. 2-7). Likewise, treatment with either FAW caterpillars or their salivary glands induced similar responses in these plants. *Fourth*, 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 suppressed the induction of trypsin PI activity (Fig. 2-6 a). *Lastly*, neither application of regurgitant nor frass from the two strains induced different defense responses in maize or Bermuda grass. Therefore, differences in the salivary PLC activity are likely to explain the different plant defense responses triggered by the FAW strains. Although specific PLC inhibitors like the aminosteroid U73122 and the PLC ether lipid analogue edelfosin (ET-18-OCH3) may have been useful to confirm these results, we did not use them because of their cytotoxicity and several secondary effects resulting from alkylation of various proteins (Horowitz *et al.* 2005).

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 differences in the mechanisms of receptor-mediated recognition in different plant species (Schmelz *et al.* 2009), differences in availability of enzyme substrates and/or differences in hormonal crosstalk between plant defense pathways.

The results also show differences in the 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. 2-6 a). 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. 2-6 b). GOX alone did not trigger defense responses in maize (Louis *et al.* 2013) or Bermuda grass, therefore, its variation in activity may not affect the interaction of the FAW strains with these plants, but because the FAW is a polyphagous species it may play an important role in their interaction with other hosts. These results suggest that the FAW strains adaptively modify the composition of their salivary elicitors when feeding on different hosts. Since the induction of plant defenses had a fitness effect on the caterpillars, the plastic differences in the salivary composition that modulate these defenses are likely to be adaptive (Mooney & Agrawal 2008). Besides PLC and GOX, FAW saliva has other components affecting defense responses in plants. Boiled salivary gland homogenates from both strains induced significantly greater *MPI* gene expression that the buffer treated plants; however, it is beyond the scope of this study to identify these salivary molecules.

This work supports the hypothesis that a controlled production and secretion of herbivore elicitors/effectors is critical in insect host adaptations and may influence host shifts. Intra-specific differences in the protein composition of insect saliva have been identified in other insect species. For example, biotypes of the Russian wheat aphid, *Diuraphis noxia,* 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*, 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*, 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 the herbivore's oral secretions and saliva are important factors modulating host defenses and have a direct influence in the insects' ability to exploit a particular host.

Constitutive plant defenses also have a differential effect on the fitness of the FAW strains. When feeding on plants that were never exposed to herbivory, the strains exhibited differences in the time of larvae development; pupa weight and time spend from hatch to moth emergence. The corn strain seems to be better adapted to feed on maize where it reached greater pupa weight and shorter larvae developmental time. The rice strain larvae had the same developmental time in maize and Bermuda grass, but higher pupa weight when feeding on maize. Overall, the rice strain had less pupa weight and longer developmental time than the corn strain for all diets tested indicating either that none of the host tested were suitable, or that this strain is in general smaller and takes longer time to develop that the corn strain regardless of the host. It is also likely that the rice strain may be better adapted to feed on a different grass species; For instance, the rice strain develops faster on stargrass (*Cynodon nlemfuensis*) than on Bermuda grass (Meagher *et al.* 2007). Differences in development and pupa weight reflect the differential ability of the FAW strains to uptake and utilize nutrients from their specific hosts. A greater ability to convert ingested food into body mass reflects a greater metabolic efficiency and need suitable physiological adaptations to break down plant tissues and avoid intoxication. The ability to absorb in a short time nutrients to reach a specific weight at pupation has a direct effect on fitness. It will reduce developmental time which in turn increases the number of generations over time and decreases the larval exposure to natural enemies. Also, well-nourished larvae will have greater weight at pupation, which is directly correlated with higher egg loads at the adult stage (Barah & Sengupta 1991). The FAW strains also exhibited differences in survival when feeding on different plants. Survival of the rice strain was 23.4% higher in Bermuda grass compared with maize, and it is therefore the only fitness parameter tested that favors the association of this strain with Bermuda grass in not-induced hosts. These differences may be

explained by different detoxification efficiencies, for example, the rice strain appears to have greater efficiency to detoxify cyanide in Bermuda grass than the corn strain (Hay-Roe *et al.* 2011). Both constitutive and herbivore-induced defenses of maize and Bermuda grass affected the fitness of the FAW strains. Altogether, the corn strain seems to be better adapted to feed on maize where it had greater fitness and induced less direct defenses than the rice strain. Conversely, the rice strain had greater survival in Bermuda grass and induced higher defense responses in maize than the corn strain.

It has been debated whether or not host-plant associations are influencing the separation of the FAW strains. In a recent phylogeny of the genus *Spodoptera*, morphological and molecular data suggest that the ancestral members of this genus were likely dicot feeders, while use of crop grasses as hosts 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, much before the domestication of maize (about 9,000 years ago) (Matsuoka *et al.* 2002). Therefore, the 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 biased distribution of these 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, are strong adaptations to different host plants. This adaptation to different hosts helps re-enforce the strain separation and has the potential to differentially affect levels of genetic divergence.

I draw three main conclusions from this study. *First*, the FAW strains induce different defense responses in maize and Bermuda grass via specific differences in the activity of the caterpillar salivary enzyme PLC. To my knowledge this is the first study to identify differences in induction of plant defenses by insect strains along with the associated elicitor. *Second*, differential plant defense induction affects caterpillar fitness;

therefore, the composition of insect saliva as plant defense modulator may be under strong selective pressure. *Third*, the FAW strains plastically modify the composition of their salivary elicitors when feeding on different hosts. Intra-strain-specific differences in PLC and GOX are likely to strongly influence the strains' ability to exploit a particular host species. 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 have a strong influence in the host range expansion of insect herbivores, which in turn, influence population dynamics and ecosystem communities of ecological and agricultural importance.

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Chapter 3

Quantitative proteomic analysis of the fall armyworm saliva

Abstract

Lepidopteran larvae secrete saliva into plant-wounded sites during feeding. Components in this saliva help in food digestion but some are recognized by plants as cues to initiate production of anti-herbivore defense responses. Despite the ecological and economical importance of these insects, knowledge of their salivary composition is limited to only a few species. The goals of this study were: first, to identify the salivary protein composition of the fall armyworm (FAW) *Spodoptera frugiperda*; second, to identify qualitative and quantitative differences in the salivary protein expression of the two host races (corn and rice strains) of this insect; and third, to identify changes in the salivary protein expression associated with different diets. Saliva for these studies was manually collected from caterpillars reared on maize, Bermuda grass and tomato and the quantitative proteomic analyses of this saliva were performed using isobaric tags for relative and absolute quantification (iTRAQ). A total of 85 proteins were confidentially identified (> 99%) in the FAW saliva. These proteins were further categorized into five different functional groups: proteins involved in plant defense regulation, herbivore offense, insect immunity, detoxification, hydrolysis and other functions. Moreover, this study identified qualitative and quantitative differences in the salivary proteome of the two FAW strains. Eleven proteins were differentially expressed between the two strains and several others differentially expressed for each strain and diet combination. There were also differences in the total salivary protein content and protein expression associated with the host plant in which caterpillars were reared. Based on these results, it was concluded that the FAW saliva is a complex mixture of proteins involved in different functions that can change plastically in response to diet type.

Introduction

The composition and secretion of saliva is of paramount importance to the nutrition and health of higher animals (Lamy & Mau 2012). For arthropod parasites, their saliva is essential not only for digestion but also as a mechanism to overcome host immunity. This is better understood for blood feeding insects whose saliva contains anticoagulants, vasodilators and antiplatelet compounds to facilitate the ingestion of blood meals (Ribeiro & Francischetti 2003). Similar to hematophagous insects, insect herbivores need to overcome immune responses of their hosts to successfully grow and develop. Insect feeding triggers production of a wide array of plant physical and chemical defenses that can be poisonous, reduce food digestibility, or recruit insect natural enemies (Howe & Jander 2008). Both mechanical injury and insect-derived cues are recognized by plants to activate production of specific defense responses (Howe & Jander 2008). Insect saliva, oral secretions and frass are known sources of both HAMPS (Herbivore Associated Molecules Patterns) and effectors; HAMPS are molecules that trigger plant defenses while effectors are compounds that suppress them (Kaloshian & Walling 2015; Schmelz 2015; Stuart 2015). Furthermore, the effect of insect-derived compounds on plant immunity is highly specific; that is, an individual compound can elicit defense responses in some plants but suppress them in others (Acevedo *et al.* 2015). Plant defenses usually affect herbivore fitness; therefore, it is plausible to suggest that oral secretions of herbivores are adapted to trigger less defense responses in plants to which they are preferentially associated (Chapter 2 this thesis).

Although, the salivary proteome appears to be species specific, a few studies have found variation in the protein composition among populations of the same species. In insect biotypes, the salivary proteome appears to rapidly change in response to host-plant chemistry. For instance, quantitative changes in 14 different salivary proteins were identified when comparing the salivary proteome of four biotypes of the Russian wheat aphid, *Diuraphis noxia* (Nicholson *et al.* 2012). Likewise, six proteins were quantitatively different among four biotypes of the greenbug, *Schizaphis graminum*

(Nicholson & Puterka 2014). Because these biotypes have variable virulence to resistant wheat varieties it is possible that changes in their salivary composition are part of the mechanisms to overcome host resistance (Cui *et al.* 2012; Nicholson *et al.* 2012; Nicholson & Puterka 2014). Similarly, populations of the brown planthopper *Nilaparvata lugens,* with different virulence to rice, had differences in the transcript accumulation of 67 genes encoding secretory proteins in their salivary glands (Ji *et al.* 2013). These studies suggest that the saliva composition of these sucking insects is likely to play an important role in plant colonization and therefore is under strong selection pressure.

Furthermore, quantitative differences in protein activity or gene expression have been found within a given insect genotype feeding on different host plants. Larvae of the tomato fruitworm, *Helicoverpa zea,* have glucose oxidase (GOX) in their saliva; this enzyme catalyzes the reaction of glucose into gluconic acid and hydrogen peroxide, which regulates defense responses in a variety of plants (Eichenseer *et al.* 1999). Both the activity and amount of GOX secreted by this insect changes when feeding on different host plants (Peiffer & Felton 2005). Likewise, in fourth-instar larvae of the beet armyworm, *Spodoptera exigua,* the activity of GOX in the salivary glands was higher in caterpillars fed on artificial diet compared with those fed on *Medicago truncatula* (Merkx-Jacques & Bede 2005). Later experiments found that the activity of glucose oxidase in insect salivary glands was positively associated with the amount of glucose and protein present in their diet (Babic *et al.* 2008; Hu *et al.* 2008). Similar variation has been found in other salivary enzymes. For instance, the transcript accumulation of a lysozyme-encoding gene in the salivary glands of *H. zea,* was higher when caterpillars fed on tomato and cotton compared with tobacco plants (Liu *et al.* 2004). Lastly, salivary protein-secretion pathways in *S. exigua* caterpillars were influenced by the nutritional quality of their diet (Afshar *et al.* 2013). Together these studies suggest plastic variations in the biochemical composition and secretion of insect saliva associated with diet.

In this study, we examined differences in the salivary composition of insect host races using the fall armyworm (FAW) (*Spodoptera frugiperda*) strains as a model system.

The FAW is a polyphagous insect comprising two host strains with different plant preferences; in field conditions the "corn strain" is mainly associated with maize, sorghum and cotton, while the "rice strain" is mainly associated with forage grasses and rice (Pashley 1986; Whitford *et al.* 1988; Machado *et al.* 2008). Studies aiming to understand their differential host plant adaptation have found differences in detoxification enzymes, oviposition preference, and host-associated differences in larvae growth and development (Veenstra *et al.* 1995; Groot *et al.* 2010; Hay-Roe *et al.* 2011; Meagher *et al.* 2011; Meagher & Nagoshi 2012), but the effect of induced plant defenses is only beginning to be explored (Chapter 2 this thesis). We have shown that feeding by the FAW strains induce different levels of defense responses in different host plants, which affected insect performance. The differential plant defense induction appears to be elicited by different components in the caterpillar saliva of these strains, especially differences in the activity of the salivary enzyme phospholipase C (PLC) (Chapter 2 this thesis). In this work we further investigated the proteomic composition of the caterpillar saliva from the FAW strains associated with different host plants. Our hypotheses were that 1) the saliva of the two strains was qualitative and quantitatively different and 2) within each strain, there were quantitative changes in the protein abundance associated with the host plant they fed on.

We identified a total of 85 proteins with > 99 % confidence, 11 proteins were differentially expressed between the two strains and several others differentially expressed for each strain and diet combination. The biological function of these proteins is discussed.

Methods

Insects

The 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, while 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 pair-mated in order to select the F1 individuals containing the corresponding mitochondrial marker that identify each strain (Nagoshi & Meagher 2003).

Plants

Maize plants (*Zea mays*, inbred line B73) were grown in Hagerstown loam soil until they reached the V8-V9 physiological stage. Bermuda grass (*Cynodon dactylon*) were grown in Hagerstown loam soil and used four weeks after germination. Tomato plants (*Lycopersicon esculentum*, cultivar Better Boy) were grown in Promix potting soil (Premier Horticulture Quakertown, PA, USA) in four-inch 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 hours light: 10 hours dark) at the Pennsylvania State University, University Park.

Saliva collection

Caterpillars for saliva collection were grown from egg hatch on detached leaves of maize, Bermuda grass, rice and tomato plants. Two day-old last-instar caterpillars were chilled on ice for 45 minutes and immobilized into a metallic hairclip, as they warmed up, their saliva was collected using a micropipette tip (VWR cat No. 53509-015) under a dissecting microscope (Olympus SZ30). Saliva for proteomic analysis was collected in 1X protease inhibitor (Sigma P2714, diluted in MQ water), kept on ice during its collection and stored at – 80 $^{\circ}$C until use. Three independent saliva samples were collected for each strain (corn and rice) per diet type (maize and Bermuda grass), while only one saliva sample was collected from corn strain caterpillars fed on tomato. Each sample was composed of the pooled saliva amounts collected from 40 – 50 caterpillars.

Protein gel electrophoresis

To identify differences in the salivary protein profiles of the FAW strains, denatured proteins were visualized in 0.75 mm SDS polyacrylamide gels **(**PAGE). Protein separation was carried out by loading ~ 0.5 µg of protein per sample into 12% SDS PAGE gels run at 75 V for ~3 hours in a vertical electrophoresis camera (Biorad Mini-Protean #165800FC). The protein bands were then visualized by staining with silver nitrate.

Proteomic analyses

Quantitative proteomic analyses of the FAW saliva were performed using isobaric tags for relative and absolute quantification (iTRAQ). Two different experiments were carried out: one aimed to identify differences in the salivary protein composition of the FAW strains when feeding on maize and Bermuda grass; the other experiment quantified differences in the saliva of the corn strain only when feeding on maize, Bermuda grass and tomato plants. The procedures and analyses for these experiments are described below.

LC-MS-MS

The saliva samples were thawed, combined and their protein quantified using a Bradford assay (Bradford 1976) in a SpectraMax 190 microplate reader (Molecular Devices) The amount of saliva was quantified using a Bovine Serum Albumin (BSA) standard curve. Sixteen saliva samples each containing 10 µg of saliva and 0.3978 µg of protease inhibitor (Sigma P2714) diluted in 10 µl of MQ water were used for proteomic analysis using iTRAQ. These samples were run in two different iTRAQ 8-plex plates, one same sample was run in the two plates for normalization between the two different runs. The protein samples were prepared following the Pennsylvania State University College of Medicine Mass Spectrometry and Proteomics Core Facility standard protocol [**See Appendix B]** adapted from the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). After trypsin digestion and incubation with specific iTRAQ tags, equal

protein amounts from each sample were combined into a single tube. The iTraq-labeled peptides were then separated by strong cation-exchange chromatography (SCX) at a flow rate of 1 ml/min through a passivated Waters 600E HPLC system, using a 4.6 X 250 mm PolySULFOETHYL Aspartamide column (PolyLC, Columbia, MD). For the SCX separation, Buffer A was 10 mM ammonium formate, pH 3.6, in 20% acetonitrile. Buffer B was 666 mM ammonium formate, pH 3.6, in 20% acetonitrile, with a separation gradient following injection of 22 minutes isocratic Buffer A, increasing linearly to 40% buffer B at 48 minutes post-injection, then a steeper increase to 100% buffer B over 1 minute, which remained isocratic at 100% buffer B until 56 minutes, at which point the gradient was switched back to 100% buffer A to re-equilibrate the column for 10 minutes prior to any subsequent injection. The MS spectra were taken from each of the SCX fractions of the iTRAQ-labeled peptides in a 5600 TripleTOF mass spectrometer (ABSciex), after separation of each fraction with a 120 minute gradient from an Eksigent NanoLC-Ultra-2D Plus and Eksigent cHiPLC Nanoflex through a 200 µm x 0.5 mm Chrom XP C18-CL 3 µm 120 Å trap Column and elution through a 75 µm x 15 cm Chrom XP C18-CL 3 µm 120 Å Nano cHIPLC Column. The parent scan was acquired for 250 msec, then up to 50 MS/MS spectra were acquired over 2.5 seconds for a total cycle time of 2.8 seconds.

Protein identification and quantification

Identification and relative quantification of peptides and proteins using iTraq label intensities was done with the Paragon algorithm implemented in Protein Pilot 5.01 software (Shilov *et al.* 2007). Peptide and protein identification was carried out by searching the observed MS/MS spectra against the Lepidoptera Uniprot database (SwissProt /TrEMBL) containing 277,761 proteins (5/24/2016) plus the sequences of 536 common lab contaminants, and a concatenated decoy database consisting of all the protein sequences from the forward databases in reversed order. At any Paragon algorithm Unused Score for a protein, the Local False Discovery Rate, FDR (also called Posterior Error Probability) was estimated from the number of accumulated forward and

decoy database IDs from proteins with Unused Scores higher than the current protein, using the Proteomics System Performance Evaluation Pipeline (PSPEP) algorithm (Tang *et al.* 2008). Only proteins with a global FDR < 1 % (> 99% confidence by the Paragon algorithm's internal scoring metrics), which corresponded to Protein Pilot Unused Scores > 2.39 were considered to be confidently identified. Proteins identified by shared peptides only were not listed as individual IDs, but instead were listed as a single group of proteins to satisfy the principles of parsimony.

Statistical analysis

The statistical analyses of changes in protein levels among sample groups in iTRAQ experiments were done using PSUTraq, an in-house modification of the MatLab program WHATraq developed by Zhou *et al.* (2014). In PSUTraq, we added a Local False Discovery Rate calculation (qLFDR) based on Storey & Tibshirani (2003) to correct for multiple testing. This qLFDR was calculated using the significant p-values (< 0.05) obtained by Protein Pilot for the ratios of two samples with similar peak intensity values. iTraq labels from peptides shared by multiple proteins were not included in quantitative analyses. Quantitative differences in the protein composition of saliva from caterpillars for each FAW strain and diet combination were analyzed using pairwise comparisons of the log2 ratios of their peptide abundances. Proteins were considered differentially expressed if they had a qLFDR $p < 0.05$. The putative biological function to each protein was assigned with QuickGo, the UniProt browser for gene ontology terms and annotations (http://www.ebi.ac.uk/QuickGO. Lastly, the effect of each strain (corn and rice) and plant type (maize and Bermuda grass) on the total amount of protein secreted per microliter of saliva was determined using a two-factor factorial design. In the case of interactions between factors, differences between treatment means were analyzed using t-test at $\alpha = 0.05$.

Additional proteomic studies

Because peptide identification with isobaric tags is affected by the multiplexing of tags used (Pichler *et al.* 2010), we did additional label-free proteomic analysis of two saliva samples for each FAW strain feeding on maize. These supplementary proteomic analyses were carried out by Applied Biomics (Hayward, CA, USA). Saliva samples from 50 caterpillars (for each strain) were collected in 5 mM EDTA, 50 mM Tris-HCl, pH 8.0 and stored at – 80 ^{o}C until use. Proteins were exchanged into a 50 mM ammonium bicarbonate buffer. DTT was added to a final concentration of 10 mM and incubated at 60 ^{o}C for 30 min, followed by cooling down to room temperature. Iodoacetamide was then added to a final concentration of 10 mM and incubated in the dark for 30 min at room temperature. The proteins were then digested by Trypsin (Promega) overnight at 37 ^{o}C. NanoLC-MS/MS was carried out using a Dionex Ultimate 3000 (Milford, MA). Mobile phase solvents A and B were 0.1% TFA (v/v) in water and 0.1% TFA (v/v) in 80% acetonitrile, respectively. Tryptic peptides were loaded into a μ-Precolumn Cartridge (5 μm, 100A, 300 μm i.d., Dionex) and separated on an acetonitrile gradient (ranging from 5% to 60%) on a Nano LC column (3 μm, 100A, 75μm i.d., Dionex). Fractions were collected at 20-second intervals followed by Mass Spectrometry analysis on AB SCIEX TOF/TOF™ 5800 System (AB SCIEX). Both of the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix Science, London, UK) to search the Lepidoptera database of Swiss-Prot. Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters.

Results

The FAW strains have different protein profiles

Although the saliva of the FAW strains has multiple shared proteins with the same molecular weight, there are some proteins that appear to be specific for each strain (Fig. 3-1)

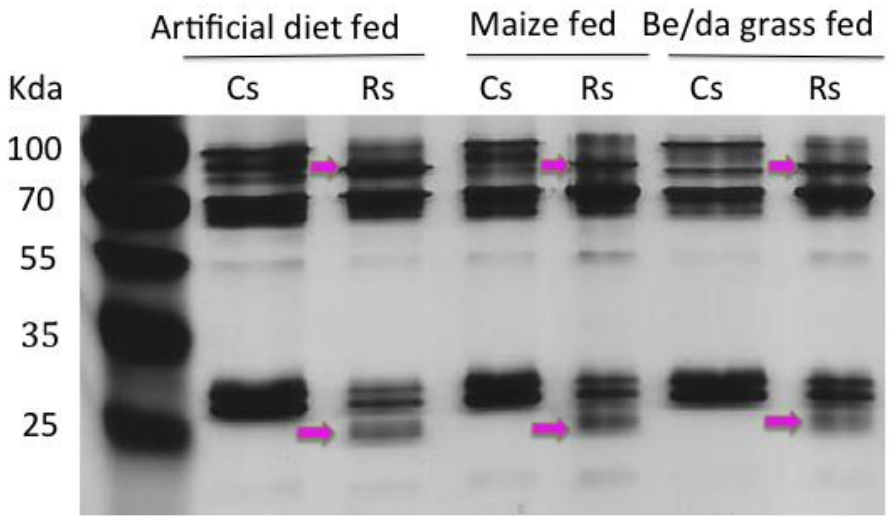

Figure 3-1. SDS PAGE of denatured proteins from the FAW strains fed on different diets. Cs = corn strain; Rs = rice strain. Arrows show different protein bands present in the rice strain but absent in the corn strain

Proteins identified in the FAW saliva

In the first iTRAQ plate, there were 315,667 MS/MS spectra generated, from which 4936 (1.5%) were identified at 95 % confidence, from these, 1840 were distinct peptides at > 95 % confidence. From these peptides, 80 different proteins were identified at > 99% confidence. In the second iTRAQ plate a total of 326,156 MS/MS spectra were taken, with 4133 (1.3 %) identified at > 95 % confidence. From these, 1250 distinct peptides were confidently identified (> 98.9 %) and used to identify 40 different proteins (> 99.8% confidence). After taking out the proteins associated with contaminants, there were 66 proteins left from plate 1 and 26 from plate 2. There were 12 new proteins

identified in plate 2 that were not identified in plate 1. Therefore, between the two plates there were 77 different proteins identified at high confidence level. From the additional label-free analyses, there were eight new proteins identified. Together, these analyses allowed the identification of 85 total proteins in the FAW saliva.

The proteins with higher peptide abundance were peroxinectin J (POX-J), glucose oxidase (GOX), and heat shock proteins (Fig. 3-2, Table 3-1). From the total number of proteins identified (85), about 16.5 % were oxidoreductases while the large majority (17.6 %) were uncharacterized proteins (Fig 3-3. Table 3-1).

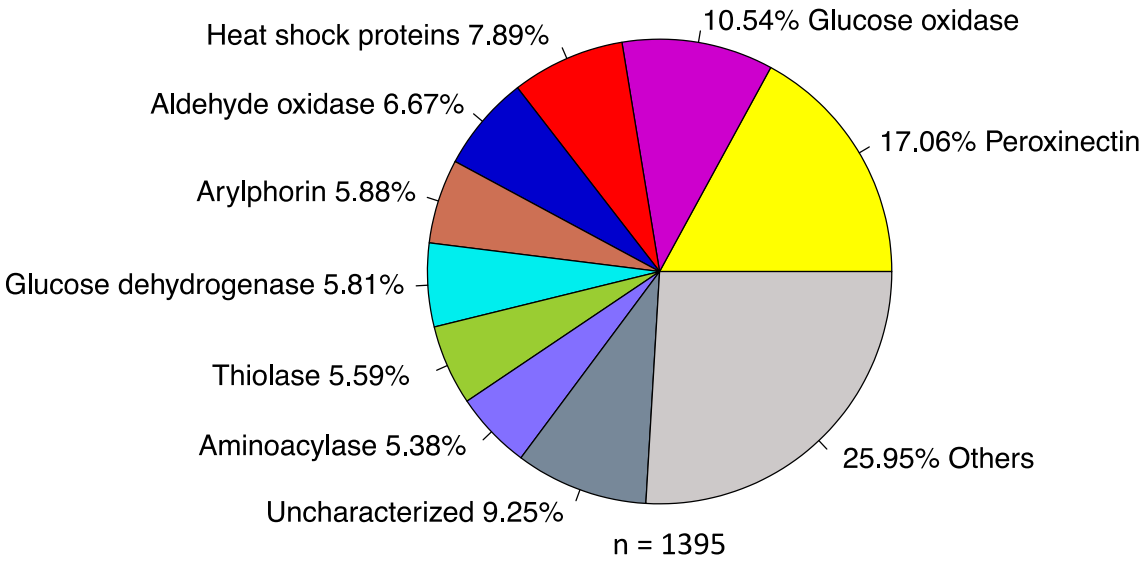

Figure 3-2. Total protein abundance of the FAW saliva. Percentages were calculated from the number of peptide counts for each protein identified

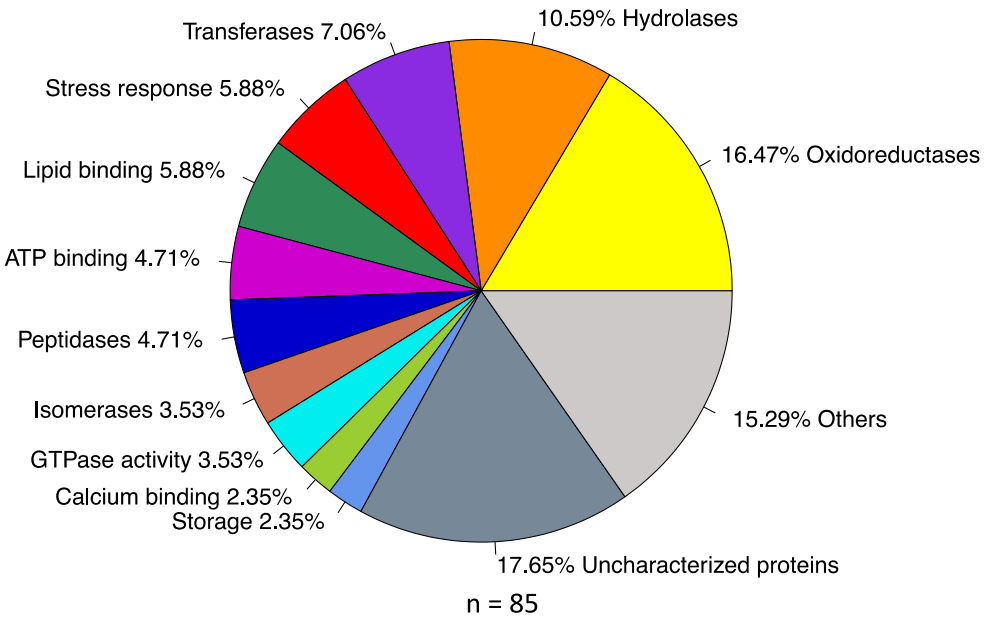

Figure 3-3. Biological function of the 85 salivary proteins identified in fall armyworm caterpillars. Function was assigned with QuickGo, the UniProt browser for gene ontology terms and annotations.

Differences in the salivary proteome of the FAW strains

With the label-free proteomic analyses there were six proteins differentially identified in the corn strain and five differentially identified in the rice strain (Table 3-2). Surprisingly, many of these proteins seem to be associated with pheromone production and only a few appear to have a meaningful function in relation to their host plants.

The labeled iTRAQ experiments did not allow identification of unique proteins for each strain, but there were several of them differentially expressed. When the corn and rice strain were compared, there were 11 proteins differentially expressed independent of the plant type they were reared on. The proteins upregulated in the corn strain were: POX-J, GOX, arginine kinase, FK506-binding protein, alpha-tubulin, protein disulfide-isomerase, translation elongation factor, lysozyme, annexin, and the heat shock cognate 70 protein; while arylphorin and a putative ecdysone oxidase were downregulated. These proteins (except for POX-J), were also differentially expressed when the two strains were compared after feeding on Bermuda grass; in addition to these, the putative chondroitin synthase protein was downregulated in the corn strain.

When the two strains fed on maize were compared, there were only two proteins differentially expressed, alpha-tubulin was upregulated in the corn strain while a putative ecdysone oxidase was upregulated in the rice strain. This putative ecdysone oxidase was highly upregulated in the rice strain feeding on maize compared with the rice strain feeding on Bermuda grass. For a full detail of these comparisons see appendix C**.**

Quantitative differences in the salivary proteome of the corn strain associated with different diets.

The protein composition of the FAW saliva plastically changed when feeding on different host plants. The salivary protein expression was more similar when caterpillar fed on maize and Bermuda grass than when they fed on tomato plants. Four proteins were upregulated in the saliva of Bermuda grass-fed caterpillars compared to the maize-fed ones, these proteins were: heat shock cognate 70 (two different proteins), protein disulfide isomerase and lysozyme. When comparing the salivary proteins of maize-fed with tomato-fed caterpillars, ten proteins were differentially expressed: POX-J, GOX, FK506-binding protein, yellow-d, and an uncharacterized protein were downregulated in tomato, while arylphorin, apolipophorin-3, prophenoloxidase, methionine-rich storage protein, and arginine kinase were downregulated in maize. Lastly, 12 different proteins changed when comparing the saliva of tomato-fed with Bermuda grass-fed caterpillars: POX-J, GOX, heat shock cognate 70, protein disulfide isomerase, lysozyme, and an uncharacterized protein were upregulated in Bermuda grass, while arylphorin, apolipoporin-3, prophenoloxidase, arginine kinase, hexamerin, and a methionine-rich storage protein were upregulated in tomato. See appendix D for full detail of these comparisons.

Effect of plant type and strain on the amount of protein in the FAW saliva

The plant type in which FAW caterpillars feed on has a strong effect on the amount of protein present in their saliva. The two FAW strains secreted saliva with higher protein content when feeding on Bermuda grass compared with maize. There were also differences between the FAW strains, with the rice strain secreting saliva with higher protein content than the corn strain (Fig. 3-4).

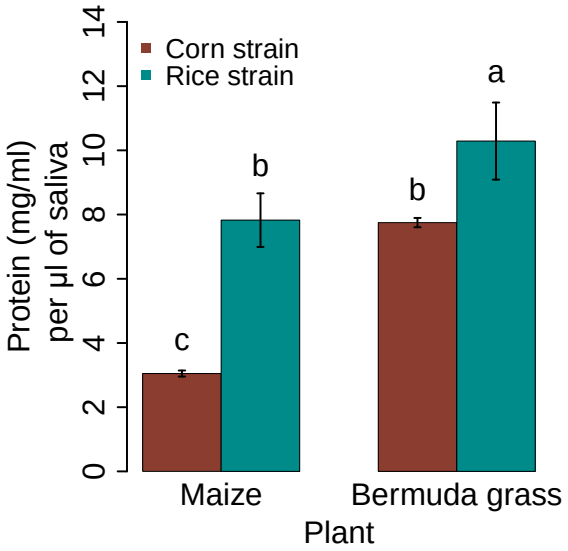

Figure 3-4. Amount of protein (mg/ml) in the secreted saliva of the FAW strains fed on maize and Bermuda grass. Values are untransformed means ± SEM. Different letters indicate significant differences obtained with ANOVA ($F_{3,8} = 16.84$, $P = 0.001$) following the Fisher test ($\alpha = 0.05$).

Discussion

In this study we identified several proteins present in the FAW saliva, many of which were differentially expressed between the two FAW strains. We also identified several changes in protein expression associated with diet type. The salivary proteome of the FAW shares numerous proteins previously identified in the saliva and salivary glands of other insect species (Celorio-Mancera *et al.* 2011; Tian *et al.* 2012; Harpel *et al.* 2015). Indeed 23% of the proteins identified in FAW were also present in the sialome of *Helicoverpa armigera* (Celorio-Mancera *et al.* 2011). To better understand the complexity of the FAW salivary proteome we categorized most of the identified proteins into six different functional groups: proteins involved in plant defense regulation, herbivore offense, insect immunity, detoxification, hydrolysis and other functions.

Proteins with potential role in plant defense induction

The saliva of the FAW is known to regulate defense responses in their host plants (Chuang *et al.* 2014) but the specific elicitors contained in these secretions are unknown. Some of the proteins identified in the FAW saliva with potential plant defense activity were GOX, heath shock proteins and thiolases. **GOX** is a well-known plant defense elicitor present in the saliva of several caterpillar species (Eichenseer *et al.* 2010). GOX uses glucose as a substrate to produce gluconic acid and hydrogen peroxide (Eichenseer *et al.* 1999). H_2O_2 act as a second messenger to activate defense-related pathways in plants including Jasmonic acid (JA), salicylic acid, abscisic acid and ethylene (Kerchev *et al.* 2012). The presence of this protein in the saliva of FAW caterpillars has been previously demonstrated (Eichenseer *et al.* 2010; Chuang *et al.* 2014). **Heat shock proteins (HSP)** are present in many organisms and are regularly induced by environmental stresses; they are also involved in protein metabolism, folding, and translocation (Wang *et al.* 2015). HSP are likely to be regulators of a variety of cellular processes as some of their substrates are hormone receptors, E3 ligases, kinases and transcription factors (Zhang *et al.* 2015). HSP 70 belongs to a protein family with an average molecular weight of 70 Kd and seem to be involved in insect resistance mechanisms to environmental stresses including high temperatures (Wang *et al.* 2015). HSP 70 also interacts with other proteins to form chaperone complexes that can have a variety of functions. It has been recently found that the jasmonate COI1 receptor in *Arabidopsis* is a substrate for the chaperon complexes SGT1b - HSP 70 - HSP 90 and therefore influence JA hormone signaling (Zhang *et al.* 2015). **Thiolase 2,** also called acetyl-Coenzyme A acetyltransferase catalyzes the formation of acetoacetyl-CoA from acetyl-CoA in the mevalonate pathway (Soto *et al.* 2011). This enzyme is involved in the synthesis of fatty acids, steroids, insect pheromones and juvenile hormones (Fujii *et al.* 2010; Noriega 2014; Brabcová *et al.* 2015). Thiolase 2 is the precursor for terpenoid biosynthesis in plants, which are a diverse group of primary and secondary metabolites including the plant hormones abscisic acid and brassinosteroids (Iriti & Faoro 2009). Terpenoids are also involved in plant defense responses to biotic and environmental stressors (Singh & Sharma 2015). Therefore, Thiolase 2 in the FAW saliva could be

indirectly involved in plant defense induction by synthesizing fatty acids with plant defense activity or directly by activating terpenoid biosynthesis in plants through the isoprenoid pathway.

Proteins involved in herbivore offense

Proteins identified with potential role in plant defense evasion were ecdysone oxidase, aminoacylase, POX, peroxiredoxin, GOX, canavanine hydrolase and beta-1-3 glucanases. **Ecdysone oxidase** is an oxidoreductase that uses ecdysone and oxygen as substrates to produce 3-dehydroecdysone and hydrogen peroxide. It is a common enzyme found in insects that breaks down the excess of ecdysteroids after repeated molts (Sun *et al.* 2012). This enzyme has also been found in the saliva of two other caterpillar species of the genus *Helicoverpa* but its role is unknown (Celorio-Mancera *et al.* 2011; Tian *et al.* 2012). Because plants produce ecdysteroid analogs to insect molting hormones that are able to affect insect development (Laekeman & Vlietinck 2013), the presence of ecdysone oxidase in insect saliva may help in the degradation of phytoecdysteroids. **Aminoacylases** are carboxypeptidases previously found in the gut and frass of heliothine lepidopterans. This enzyme hydrolases the fatty acid-amino acid conjugate (FAC) N-linolenoyl-L-glutamine (volicitin) into linolenic acid and free glutamine (Kuhns *et al.* 2012). Volicitin was first isolated from the oral secretions of *Spodoptera exigua* larvae and induces the emission of volatile compounds in maize; these volatiles recruit natural enemies of caterpillars (Alborn *et al.* 1997). In addition to volicitin, several other FACs have been identified in the oral secretions of caterpillars including FAW (Schmelz 2015). It is possible that the amynoacylase present in the FAW saliva degrades *in situ* the FACs that are secreted during caterpillar feeding as a mechanism to avoid plant defense induction. **POX** is an enzyme with peroxidase activity that can aid in the detoxification of reactive oxygen species (ROS) produced by plants in response to insect feeding or produced by the reaction of other insect derived enzymes with plant substrates. For example, POX can degrade the H_2O_2 produced by GOX and therefore reduce the downstream production of defensive compounds in plants. Likewise, **Peroxiredoxins** are

antioxidant enzymes that reduce hydrogen peroxide to water and alcohol and therefore protect cells from oxidative damage. These enzymes also act as chaperones and bind to other proteins and membranes suggesting possible roles in mediating signal transduction (Schulte 2011). **GOX** can also down regulate herbivore-induced defenses in some plants, GOX reduces the levels of the toxic alkaloid nicotine in tobacco plants induced by caterpillar feeding (Musser *et al.* 2002). **Canavanine hydrolase** breaks down L-canavanine to L-homoserine and hydroxyguanidine (Melangeli *et al.* 1997). L-Canavanine is a toxic non-protein amino acid present in legumes; its structural similarity with L-arginine facilitates its incorporation into proteins resulting in abnormal polypeptides (Igloi & Schiefermayr 2009). The enzyme canavanine hydrolase has been identified in the gut of *Heliothis virescens* conferring resistance to this insect to the toxic effects of L-canavanine in their diet (Melangeli *et al.* 1997). The canavanine hydrolase present in the saliva of FAW caterpillars is likely to aid in the hydrolysis of L-canavanine when this insect feeds on legumes. Lastly, **Beta-1-3 glucanases** are proteins induced in plants as a defense mechanism against pathogen infection (Balasubramanian *et al.* 2012). The exogenous application of this enzyme to plant wounded sites may trigger downstream responses to pathogens that could antagonize herbivore defensive pathways.

Immune related proteins

The FAW saliva has a large number of proteins involved in immune responses, these include POX, apolipophorin, prophenoloxidase, caspase, REPAT, scolexin, lysozyme, and glucose dehydrogenase. **Peroxinectines (POX)** belong to the peroxidase-cyclooxygenase superfamily and have peroxidase activity as well as cell adhesion properties (Johansson *et al.* 1995). The first POX was identified in blood cells of crayfish where it appears to mediate the immune responses of encapsulation and phagocytosis (Johansson 1999). POX contains the tripeptide Arg-Gly-Asp (*RGD*) adhesive motif which is recognized by integrins (proteins in the plasma membrane that are the main receptors for extracellular cell-adhesive ligands). In crayfish POX are stored in granules and secreted in an inactive form to the hemolymph where their peroxidase and cell

adhesion activity is activated by the presence of beta 1-3 glucans (Johansson *et al.* 1995; Johansson 1999). Furthermore, as sisters of cyclooxigenases, POX are likely to be involved in the synthesis of prostaglandins (immune regulating fatty acids) from eicosanoid fatty acids. Indeed, ten POX were recently identified in different tissues of *Spodoptera exigua* larvae; from these, POX-F and -H had close similarity to the peroxinectin *Pxt,* involved in the synthesis of prostaglandins in *Drosophila melanogaster* (Park *et al.* 2014). However, POX-F and -H from *S. exigua* lack the integrin binding sites present in *D. melanogaster* and Crustaceans *Pxt* genes (Park *et al.* 2014), suggesting that they may have a different mode of action or may not have cell adhesive properties. **Apolipophorins** are lipid-transporting proteins that play critical roles in insect immune responses (Whitten *et al.* 2004). These enzymes degrade microbial cell wall components, activate the expression of antimicrobial peptides, and are involved in the regulation of the prophenoloxidase pathway (Zdybicka-Barabas & Cytryńska 2013). **proPhenoloxidase** (proPO) is the inactive zymogen of phenoloxidase, an enzyme that catalyzes the oxidation of phenols to quinones, which upon polymerization become melanin. Melanization at wounded or infected sites is an immune response common in arthropods (Binggeli *et al.* 2014)**.** The activation of proPO to phenoloxidase is mediated by compounds of microbial origin including lipopolysaccharides, peptidoglycans and beta-1-3-glucans (Cerenius & Söderhäll 2004). The enzyme beta-1-3 glucanase (present in the FAW saliva) may regulate the activation of proPO by breaking down beta-1-3-glucans from invading fungi, bacteria or yeast (Christophides *et al.* 2002). **Caspase** proteins are peptidases that trigger programed cell death or apoptosis involved in both insect development and immune responses against foreign invaders (Courtiade *et al.* 2011). **REPAT** are proteins that get highly expressed in response to pathogens, the first REPAT protein was identified from the gut of *Spodoptera exigua* after infection with *Bacillus thuringiensis* and baculoviruses (Herrero *et al.* 2007). **Scolexins** are proteins found in insect hemolymph with coagulation activity in response to bacteria or viral infection (Finnerty *et al.* 1999). **Lysozymes** are well known antibacterial enzymes that hydrolyze glycosidic bonds in the peptidoglycan of bacteria cell walls, they are ubiquitously present in insects (Adamo 2004; Yang & Cox-Foster 2005). Lastly, **Glucose dehydrogenase**

plays an important role in insect immunity by mediating the encapsulation of fungal pathogens (Cox-Foster & Stehr 1994).

Detoxification proteins

The main proteins in this category are cytochrome P450, carboxyl esterases and aldehyde oxidases. **Cytochrome P450** mixed function oxidases are enzymes involved in the metabolism of endogenous compounds and biotransformation of xenobiotics (Cederbaum 2015). **Glutathione transferases** are a large family of multifunctional enzymes involved in the detoxification of xenobiotics (Salinas & Wong 1999). They are also involved in intracellular transport, biosynthesis of hormones, sterols and prostaglandins as well as regulation of cell death and protection against oxidative stress (Enayati *et al.* 2005; Laborde 2010). **Carboxyl ester hydrolases** are carboxyl/cholinesterases that catalyze the hydrolysis of carboxyl esters, they are widely distributed in insects and serve a variety of functions including resistance to insecticides and degradation of neurotransmitters, hormones and pheromones (Yan *et al.* 2009). Finally, **Aldehyde oxidases** are molybdenum containing enzymes with wide substrate specificity that oxidize aldehydes and heterocyclic rings (Garattini & Terao 2011). These enzymes have been found in insects where they appear to degrade aldehyde odorant compounds like pheromones and plant derived volatiles (Merlin *et al.* 2005). Moreover, due to their broad substrate specificity, these enzymes appear to be involved in insecticide resistance in the mosquito *Culex quinquefasciatus* (Coleman *et al.* 2002) and could also aid in the detoxification of plant toxins (Moriwaki *et al.* 1997).

Proteolytic enzymes

The enzymes in this category include glyceraldehyde-3-phosphate dehydrogenases, cathepsin, beta-1-3-glucanases, glucose dehydrogenase and lysozyme. **Glyceraldehyde-3-phophate dehydrogenase** is involved in carbohydrate metabolism but it is also involved in other processes including programed cell death, nuclear RNA transport and cytotoxicity of heavy metals (Chong & Ho 2013). **Cathepsins** are

proteolytic enzymes with a key role in the degradation of products from multiple processes in Lepidoptera insects including development, growth, metamorphosis and immunity (Saikhedkar *et al.* 2015). **Beta-1-3 glucanases** aid in digestion by breaking down plant tissues rich in glucanases (Levy *et al.* 2007). Lastly, glucose dehydrogenase and lysozyme are carbohydrate metabolizing enzymes (Kunieda *et al.* 2006).

Proteins with other functions

Under this category are proteins with diverse functions including protein folding, cell signaling, extracellular communication, sensory reception, calcium binding and proteins with unknown functions. **Arginine kinase** is an enzyme that catalyzes the transfer of a phosphoryl group from ATP to L-arginine to form phosphoarginine (Bragg *et al.* 2012). It is widely found in insects where it plays an important role in energy metabolism and transport of intracellular energy from mitochondria to sites of ATP consumption (Werr *et al.* 2009). **Hexamerins, arylphorin and methionine-rich** are known as storage proteins found in insect larvae that provide amino acids for adult protein synthesis (Leung *et al.* 1989; Wu 1993; Martins *et al.* 2010). Hexamerins also modulate the availability of juvenile hormones in social insects (Zhou *et al.* 2007; Martins *et al.* 2010). **Moesin** is a protein that binds to phosphatidylinositol 4,5-bisphosphate in the plasma membrane and regulates a variety of physiological and developmental functions in insects (Ben-Aissa *et al.* 2012). **Protein disulfide isomerases (PDI)** are proteins with multiple physiological roles; they are thiol-disulfide oxidoreductase, disulfide isomerases and redox-dependent chaperones (Ali Khan & Mutus 2014). They catalyze folding, assembly and posttranslational modification of proteins and also appear to be involved in cell-cell interactions (Goo *et al.* 2002; Galligan & Petersen 2012). **FK506-binding** proteins are enzymes also involved in protein folding (Kuzuhara & Horikoshi 2004). **Annexins** are calcium-regulated phospholipid binding proteins involved in membrane trafficking, calcium signaling and extracellular communication (Rescher & Gerke 2004; Gerke *et al.* 2005). **Arrestins** are proteins that regulate signal transduction at G protein-coupled receptor pathways (Merrill *et al.* 2002).

In insects they have been found to be involved in olfaction and visual sensory reception (Merrill *et al.* 2003, 2005). Arrestin genes have been identified in the salivary glands of *Anopheles gambiae* but their function is unknown (Das *et al.* 2010)**. CALNUC** are calcium nucleobinding proteins present in the golgi apparatus and cytoplasm of most cells; they are highly conserved proteins in different species and appear to be commonly secreted extracellular (Aradhyam *et al.* 2010). These proteins can also interact with G proteins suggesting a possible role in signal transduction. **Cyclic adenosine monophosphate (cAMP)-dependent protein kinases**, protein kinases are enzymes that phosphorylate other proteins modifying their activity and therefore regulating multiple cellular processes including signal transduction pathways (Hunter 1995). cAMP is an ubiquitous intracellular second messenger activated by G protein-coupled receptors; it is involved in many biological processes including intracellular signal transduction and cell communication (Glorian & Limon 2013). **Yellow** proteins have been found in insects, bacteria and fungi with mostly unknown function, although some yellow-like proteins appear to be involved in melanization and development (Xia *et al.* 2006; Ferguson *et al.* 2011). **Laminins** are cell adhesion ligands located in the extracellular matrix whose receptors are integrins (Johansson 1999). Although integrins have not been identified in plants, a more recent study shows that the protein Non-race-specific Disease Resistance1 (NDR1) located in the plasma membrane of Arabidopsis mediates plasma membrane cell wall adhesion similar to the animal analogs integrins and play a role in resistance to *Pseudomonas* in *Arabidopsis* (Knepper *et al.* 2011). Therefore, the laminin-like proteins present in the FAW saliva may have extracellular signal properties. **Chondroitin sulfate synthases** are enzymes that have glucuronyl transferase and galactosaminyl transferase activity and is involved in the synthesis of chondroitin sulfate, a glycosaminoglycan involved in diverse biological processes (Olson *et al.* 2006). In humans, chondroitin sulfate is expressed in cell surfaces and extracellular matrices, they link to a wide range of proteins and are important regulators of cell proliferation, extracellular matrix recognition and morphogenesis among other processes (Izumikawa *et al.* 2007). Chondroitin sulfate has been found in *Drosophila melanogaster*, the silk glands of *Bombix mori* and salivary glands and midgut in *Anopheles stephensi* where it serves a

variety of functions (Toyoda *et al.* 2000; Sinnis *et al.* 2007; Sugiura *et al.* 2013). Lastly, the **alpha and beta tubulin** proteins assemble together to form microtubules in eukaryotic cytoskeletons. The architecture of these microtubules and the sequences of their proteins are highly conserved in eukaryotes and their function include cellular transport of organelles and cell division (Janke 2014)

The FAW saliva is different for the two strains

Clear differences in the salivary proteome of the FAW strains were observed in protein gels (Fig. 1) and unique proteins for each strain were identified in label-free samples (Table 2). Within the most abundant "unique" proteins in the corn strain were the pheromone biosynthesis activating neuropeptide (PBAN) family. Insect neuropeptides regulate several physiological and behavioral processes during development, and reproduction, they are also involved in osmoregulation and water balance. The PBAN-like family has been found to stimulate sex pheromone biosynthesis in moths, control melanization and myotropic activity, and even egg diapause in *Bombix mori* (Altstein 2001). Neuropeptides play vital roles in orchestrating neural communication and appear to be involved in the regulation of task transitions in honeybee workers (Han *et al.* 2015). Another "unique" protein in the corn strain was the neurotransmitters serotonin 5-hydroxytryptamine (5-HT), which regulates physiological and behavioral processes through G protein-coupled receptors in vertebrates and invertebrates (Qi *et al.* 2014). Several types of 5-HT receptors have been identified in different insect tissues (Thamm *et al.* 2010; Blenau & Thamm 2011; Qi *et al.* 2014) including salivary glands (Troppmann *et al.* 2010; Watanabe *et al.* 2011; Röser *et al.* 2012). In the blowfly *Calliphora vicina*, 5-HT stimulates secretion in the salivary glands by activation of the inositol 1,4,5-triphosphate and cAMP pathways in the secretory cells (Röser *et al.* 2012). Paralytic peptides were also present in the saliva of the corn strain; these are short peptides (23 amino acids) found as inactive precursors in several insect tissues (Ishii *et al.* 2015). These are then converted to active forms (via proteolysis) that induce a variety of physiological reactions including the production of antimicrobial

peptides and phagocytosis related proteins, therefore mediating resistance to infection (Ishii *et al.* 2010; Song *et al.* 2015). Lastly, the saliva of corn strain caterpillars also contains canavanine hydrolase (discussed above), which may confer to these insects a selective advantage to feed on legumes compared with the rice strain. There were also a few proteins differentially identified in the saliva of rice strain caterpillars. Juvenile hormone esterase is an enzyme that catalyzes the hydrolysis of juvenile hormones (JH) and plays a critical role in the stabilization of JH hormone titers (Kamita & Hammock 2010). The presence of this hormone in the saliva is puzzling. The sensory neuron membrane protein (SNMP) is a transmembrane receptor that mediates lipid binding and transport and play important roles in insect chemoreception (Gu *et al.* 2014). In insects, SNMPs have been found in the receptor membranes of sex pheromone olfactory sensory receptors and appear to have a central role in odor detection (Rogers *et al.* 2001). Another protein identified in the rice strain was the cardioactive peptide (CAP) 23. Several CAPs have been identified in insects where they trigger an increase in hearth rate, modulate oviduct contractions and alter hindgut activity (Loi *et al.* 2001). It is possible (although speculative) that CAPs stimulate saliva secretion in salivary glands. Lastly, scolexin (described above) was another protein found in the rice strain, which appears to be involved in insect immunity.

In addition to the proteins that were differentially identified for each strain, there were several others that were differentially expressed independent of the diet in which caterpillars were reared. These proteins include plant defense elicitors like GOX and others with diverse functions. There were also proteins differentially expressed when these strains fed on different host plants and greater differences were found when the caterpillars were grown on Bermuda grass compared with maize. Due to the diversity of functions that these proteins appear to have, it is difficult to draw conclusions about possible adaptive changes in the saliva of these strains.

Our results also show that the total amount of protein in the saliva of the FAW strains is different and changes with the type of plant they feed on (Fig. 4). The saliva of

the rice strain appears to contain more protein than the saliva of the corn strain independent of the host plant they were fed on. This difference was greater in maize and may be correlated with the induction of higher levels of defense responses elicited by the rice strain caterpillars when feeding on this plant (Chapter 2). The two strains secreted saliva with higher protein content when feeding on Bermuda grass compared with maize. The factors triggering these differences in protein amount are unknown; we speculate that differences in water or protein content between the two plants may have an influence.

The salivary composition of the FAW changes with diet

Our results clearly show that the saliva composition of FAW caterpillars changes plastically with the type of diet. There were a few proteins differentially expressed in the saliva of the corn strain feeding on maize compared with Bermuda grass. However, there were many more proteins differentially expressed in the saliva from caterpillars fed on tomato compared with either maize or Bermuda grass. This is probably because the chemistry of maize and Bermuda grass is, to some degree, more similar than the chemical composition of tomato plants. In addition to their chemical composition, these plants may also have different protein and carbohydrate content, which could affect the composition of the FAW saliva. It has been shown that the activity of GOX in the salivary glands of *Spodoptera exigua* is affected by the amount of protein in the caterpillar diet (Babic *et al.* 2008). The FAW caterpillars appear to be well adapted to feed on maize and Bermuda grass (Chapter 2), but tomato is an unsuitable host for them. About 40% of the differentially expressed proteins have a role in immunity, indicating that diet type may directly affect the immune response of these caterpillars to pathogen infection.

Conclusions

The saliva of the FAW is a complex mixture of proteins involved in immunity, detoxification, proteolysis, host plant defense induction and evasion, signal transduction, membrane trafficking, and extracellular communication among other functions. The composition of the FAW saliva plastically changes with different diets but the factors

triggering these changes are unknown. Furthermore, the FAW strains have differences in their salivary proteome that may influence their host range. The results of this study indicate that the role of insect saliva in the regulation of plant defenses depends on the herbivore genetic makeup, the effect of diet on their saliva composition and the specific perception machinery of the host plant. Future research is needed to elucidate the function of these salivary proteins, by for example, silencing their encoding genes using genetic engineering techniques like RNAi or CRISPR.

Table 3-1. Proteins identified in the FAW saliva.

#	Uniprot Accession #	Protein name	Organism	# Peptides	Biological Function (GO)
1	tr|A0A088MGW5_SPOEX	POX-J	*Spodoptera exigua*	236	Peroxidase activity
2	tr|D9ZFI1_SPOEX	Glucose oxidase	*Spodoptera exigua*	147	Glucose oxidase activity
3	tr|Q9U5K4_SPOLT	Arylphorin subunit	*Spodoptera litura*	82	Storage
4	tr|A0A0N1IPT1_PAPMA	Glucose dehydrogenase	*Papilio machaon*	81	Flavin adenine dinucleotide binding
5	tr|A0A076FRM9_9NEOP	Aldehyde oxidase AOX3	*Sesamia inferens*	76	Catalyzes the oxidation of aldehydes into carboxylic acids
6	tr|G8EJ31_SPOFR	Aminoacylase-1	*Spodoptera frugiperda*	75	Aminoacylase activity / peptidase
7	tr|Q0MUU6_TRINI	Heat shock cognate 70 protein	*Trichoplusia ni*	71	ATP-Binding / Stress response
8	tr|D9ZFI5_SPOEX	Putative uncharacterized protein (Fragment)	*Spodoptera exigua*	48	
9	tr|M4M7W4_HELVI	Thiolase 2	*Heliothis virescens*	39	Transfer acyl groups other than amino-acyl groups
10	tr|M4M651_HELSB	Thiolase 2 (Fragment)	*Heliothis subflexa*	39	Transfer acyl groups other than amino-acyl groups
11	tr|S5M6C1_BOMMO	Actin 4	*Bombyx mori*	26	Nucleotide binding/ATP binding
12	tr|H9JKT2_BOMMO	Uncharacterized protein	*Bombyx mori*	25	

13	tr|A6YQV6_SPOFR	Beta-1_3-glucanase	*Spodoptera frugiperda*	20	Hydrolases O-glycosyl compounds
14	tr|Q95PD6_HELVI	Actin	*Heliothis virescens*	20	Nucleotide binding/ATP binding
15	tr|D3GDM6_SPOLI	Carboxylic ester hydrolase	*Spodoptera littoralis*	20	Carboxypeptidase activity
16	tr|H9J7W6_BOMMO	Uncharacterized protein	*Bombyx mori*	19	
17	tr|H9JGA4_BOMMO	Uncharacterized protein	*Bombyx mori*	16	
18	tr|M4PZR4_SPOFR	Arginine kinase	*Spodoptera frugiperda*	16	Arginine kinase activity
19	tr|S5FXH1_XESCN	Heat shock cognate 70	*Spodoptera littoralis*	15	ATP-Binding / Stress response
20	tr|A0S6A0_SPOFR	Prophenoloxidase subunit 1	*Spodoptera frugiperda*	14	Monooxygenase,oxidoreductase activity
21	tr|Q9U5K5_SPOLT	Methionine-rich storage protein	*Spodoptera litura*	14	Storage
22	tr|A0A0A7HBV4_9NEOP	Heat shock cognate 70 protein	*Sesamia inferens*	13	ATP-Binding / Stress response
23	tr|A0A0L7KZE8_9NEOP	Putative ecdysone oxidase	*Operophtera brumata*	11	Ecdysone oxidase activity
24	tr|L0GGU3_PLUXY	FK506-binding protein	*Plutella xylostella*	11	FK506-binding/prolyl isomerase activity
25	tr|C0H6N9_BOMMO	Putative cuticle protein	*Bombyx mori*	11	-
26	tr|Q8MUR5_CHOPR	Nucleoside diphosphate kinase	*Choristoneura parallela*	10	ATP binding/ synthesis of nucleoside triphosphates other than ATP
27	tr|G6DA51_DANPL	Yellow-d	*Danaus plexippus*	10	Unknown
28	tr|G6DDQ0_DANPL	Moesin	*Danaus plexippus*	10	Phosphatidylinositol-4,5-bisphosphate binding
29	tr|A0A0L7LP95_9NEOP	Putative venom acid phosphatase	*Operophtera brumata*	10	Integral component of membrane
30	tr|A0A0L7LJ52_9NEOP	Aldehyde oxidase AOX3 (Fragment)	*Operophtera brumata*	9	Catalyzes the oxidation of aldehydes into carboxylic acids
31	tr|A0A0F7QIE4_OSTFU	Aldehyde oxidase (Fragment)	*Ostrinia furnacalis*	8	Catalyzes the oxidation of aldehydes into carboxylic acid
32	tr|A0A0L7L6H5_9NEOP	Putative ecdysone oxidase	*Operophtera brumata*	8	Ecdysone oxidase activity
33	tr|M4Q0P2_SPOFR	Glyceraldehyde-3-phosphate dehydrogenase	*Spodoptera frugiperda*	7	Oxidoreductase/glucose hydrolysis
34	sp|O77248|APL3_SPOLT	Apolipophorin-3	*Spodoptera litura*	7	Lipid binding / transport of diacylglycerol (DAG)
35	tr|Q8T8B3_BOMMO	Beta-tubulin	*Bombyx mori*	7	GTPAse activity

36	tr|A0A0N1PF32_PAPMA	Elongation factor 1	*Papilio machaon*	7	Translational elongation
37	tr|X5F7Q0_9NEOP_	Alpha-tubulin (Fragment)	*Phyllonorycter ringoniella*	6	GTPAse activity
38	tr|A6YRR6_SPOEX	Hexamerine	*Spodoptera exigua*	6	Storage
39	tr|Q0MUU7_TRINI	Heat shock cognate 70 protein	*Trichoplusia ni*	6	ATP-Binding / Stress response
40	tr|A0A0U1VTU3_SPOLT	Protein disulfide-isomerase	*Spodoptera litura*	6	Protein disulfide isomerase activity
41	tr|Q86M26_SPOEX	Translation elongation factor 2	*Spodoptera exigua*	6	Translational elongation
42	tr|Q5F319_MANSE	Annexin (Fragment)	*Manduca sexta*	6	Calcium-dependent phospholipid binding
43	tr|CASP1_SPOFR	Caspase	Spodoptera frugiperda	6	Cysteine-type endopeptidase activity/Role in programmed cell death
44	tr|ARRH_HELVI	Arrestin homolog	*Heliothis virescens*	6	G-protein coupled receptor
45	tr|PBAN_AGRIP	PBAN-type neuropeptides	*Agrotis ipsilon*	6	Myostimulatory hormone activity
46	tr|I4DL30_PAPXU	Yellow-d	*Papilio xuthus*	5	Unknown
47	tr|A0A0N1IDJ4_PAPMA	Peroxiredoxin-4	*Papilio machaon*	5	Peroxidase/peroxiredoctin activity
48	tr|A0A0K8TUL7_EPIPO	Carboxylic ester hydrolase	*Epiphyas postvittana*	5	Carboxypeptidase activity
49	tr|S4PX64_9NEOP	Heat Shock Protein 21.4	*Helicoverpa armigera*	5	ATP-Binding / Stress response
50	tr|A0A0L7L701_9NEOP	Yellow-13	*Operophtera brumata*	5	Unknown
51	tr|Q9NJB0_SPOFR	CALNUC	*Spodoptera frugiperda*	4	Calcium ion binding
52	tr|D7NI45_HELAM	Glutathione S-transferase	*Helicoverpa armigera*	4	Detoxification of Reactive Oxygen Species
53	tr|G6DRS2_DANPL	cAMP-dependent protein kinase R2	*Danaus plexippus*	4	ATP binding/cAMP-dependent protein kinase activity
54	tr|Q587N4_BOMMO	Protein disulfide-isomerase	*Bombyx mori*	4	Protein disulfide isomerase activity
55	tr|H9JAF2_BOMMO	Uncharacterized protein	*Bombyx mori*	4	
56	tr|PBAN_AGRIP	V-type proton ATPase subunit B	*Heliothis virescens*	4	ATPAse activity
57	tr|CANHY_HE	Canavanine hydrolase	*Heliothis virescens*	3	Hydrolysis of L-canavanine

	LVI	(Fragments)			
58	tr|CP6B2_HELAM	Cytochrome P450 6B2	Helicoverpa armigera	3	Monooxygenase, Oxidoreductase
59	tr|CP6B7_HELAM	Cytochrome P450 6B7	Helicoverpa armigera	3	Monooxygenase, Oxidoreductase
60	tr|Q86FK1_SPOEX	Lysozyme	*Spodoptera exigua*	3	Glycoside hydrolase
61	tr|H9JYE6_BOMMO	Uncharacterized protein	*Bombyx mori*	3	
62	tr|D5G3F3_HELAM	Carboxyl/choline esterase CCE016d	*Helicoverpa armigera*	3	Carboxypeptidase activity
63	tr|I0B5W9_SPOLI	REPAT30	*Spodoptera littoralis*	3	Transferase
64	tr|S4NT73_9NEOP; tr|G6CSZ4_DANPL	Imaginal disc growth factor 1	*Pararge aegeria*	3	Imaginal disc development
65	tr|H9IZK9_BOMMO	Uncharacterized protein	*Bombyx mori*	3	
66	tr|Q9NL61_BOMMO	Annexin	*Bombyx mori*	2	Calcium-dependent phospholipid binding
67	tr|H9JXC7_BOMMO	Uncharacterized protein	*Bombyx mori*	2	
68	tr|A0A088MGF5_SPOEX	POX-C	*Spodoptera exigua*	2	Peroxidase activity
69	tr|A0A0L7LF88_9NEOP	Putative ca2+-binding actin-bundling protein	*Operophtera brumata*	2	Calcium binding
70	tr|H9JTZ0_BOMMO	Uncharacterized protein	*Bombyx mori*	2	
71	tr|G6DAA5_DANPL	Putative chondroitin synthase	*Danaus plexippus*	2	Glycosyltransferase
72	tr|H9J6E1_BOMMO	Uncharacterized protein	*Bombyx mori*	2	
73	tr|H9JLG2_BOMMO	Uncharacterized protein	*Bombyx mori*	1	
74	tr|H9JFZ8_BOMMO	Uncharacterized protein	*Bombyx mori*	1	
75	tr|A0A0L7LAX8_9NEOP	G patch domain containing 1	*Operophtera brumata*	1	Nucleic acid/metal ion binding
76	tr|R4X5G1_SPOFR	Beta-hexosaminidase	*Spodoptera frugiperda*	1	Beta-N-acetylglucosaminidase activity
77	tr|A0A0N0PAU4_PAPMA	Laminin-like protein epi-1	*Papilio machaon*	1	Cell adhesion / Protein binding

78	tr|S4PTE3_9NEOP	Cathepsin l	*Pararge aegeria*	1	Cysteine-type peptidase activity
79	tr|S4NTU9_9NEOP	Uncharacterized protein	*Pararge aegeria*	1	
80	tr|Q9NB88_AGRIP	Trypsin AiJ3 (Fragment)	*Agrotis ipsilon*	1	Serin protease
81	tr|S4PTF0_9NEOP	Carboxypeptidase E	*Pararge aegeria*	1	Peptidase activity
82	tr|Q2F5T8_BOMMO	Annexin	*Bombyx mori*	1	Calcium-dependent phospholipid binding
83	tr|H9JNX3_BOMMO	Uncharacterized protein	*Bombyx mori*	1	
84	tr|H9IRY8_BOMMO	Uncharacterized protein	*Bombyx mori*	1	
85	tr|SCLXB_HELVI	Scolexin B	*Heliothis virescens*	1	Serine-type endopeptidase activity

Table 3-2. Proteins differentially identified in the saliva of the FAW strains fed on maize. These identifications were obtained with label-free nano LC MS/MS. Cs = corn strain, Rs = rice strain.

Strain	**#**	**Uniprot Accession #**	**Protein name**	**Organism**	**# Peptides**
Cs	1	PBAN_AGRIP	PBAN-type neuropeptides	*Agrotis ipsilon*	6
Cs	2	PBAN_MAMBR	PBAN-type neuropeptides (Fragment)	*Mamestra brassicae*	4
Cs	3	PBAN_HELZE	PBAN-type neuropeptides	*Helicoverpa zea*	4
Cs	4	CANHY_HELVI	Canavanine hydrolase (Fragments)	*Heliothis virescens*	3
Cs	5	5HTR_HELVI	5-hydroxytryptamine receptor	*Heliothis virescens*	3
Cs	6	PAP2_SPOEX	Paralytic peptide 2	*Spodoptera exigua*	1
Rs	7	ESTJ_HELVI	Juvenile hormone esterase	*Heliothis virescens*	2
Rs	8	SNMP1_MAMBR	Sensory neuron membrane protein 1	*Mamestra brassicae*	3
Rs	9	CP23_SPOER	Cardioactive peptide CAP23	*Spodoptera eridania*	2
Rs	10	SCLXB_HELVI	Scolexin B OS=Heliothis virescens	*Heliothis virescens*	1
Rs	11	PBAN_HELAU	PBAN-type neuropeptides	*Helicoverpa assulta*	1

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Chapter 4

Phytohormones and small molecules in the fall armyworm saliva

Abstract

Plant defense responses induced by insect feeding are modulated by components present in insect saliva, oral secretions and frass. Several salivary proteins that regulate herbivore-induced defenses in plants have been identified; however, insect saliva is a complex mixture from which only its protein composition has been studied in a few insect species. The goal of this study was to identify other non-protein plant defense elicitors present in insect saliva. I used the fall armyworm (FAW), *Spodoptera frugiperda* and its host plants tomato, maize, Bermuda grass and rice as a model system. I tested the effect of protein-digested saliva or non-protein components on herbivore-induced defense responses in maize, rice and tomato. I identified the presence of phytohormones and lipids in the FAW saliva using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS). The results of this study show that non-protein components in the FAW saliva induced defense responses in different plant species. The saliva of this insect contains small amounts (< 5 ng per µl of saliva) of the phytohormones jasmonic acid, salicylic acid, benzoic acid, abscisic acid and linoleic acid. Nevertheless, treatment with similar phytohormone quantities detected in the FAW saliva downregulated late herbivore-induced defenses in tomato plants. In addition to phytohormones, lipid extractions from salivary glands showed the presence of more than 250 compounds from which putative identifications were assigned by an online search against the LipidMaps database. The presumptive identified compounds corresponded to glycerophospholipids, fatty acids and prostaglandins. The potential role of these compounds in plant immunity is discussed. Based on these results, I conclude that the

FAW saliva is a complex fluid that contains phytohormones and other small molecules besides the already known enzymatic plant defense elicitors.

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 SA is 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 directly activate the SA pathway by the secretion of plant hormones (Schwartzberg & Tumlinson 2014). Phytohormones have been identified in insect secretions including honeydew, regurgitant and frass (Tooker & De Moraes 2006; Dafoe *et al.* 2013; Schwartzberg & Tumlinson 2014). Some plant hormones have also been found in insect salivary glands (Tooker & De Moraes 2006; Suzuki *et al.* 2014), but it is unknown if they are secreted in their saliva or their potential role in plant defense induction.

Insects use phytohormones to regulate plant defenses. Feeding by the pea aphid, *Acyrthosiphon pisum,* downregulates JA-defense responses in *vicia faba*. This plant defense suppression appears to be mediated by SA present in their honewdew via induction of the SA pathway (Schwartzberg & 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 the

H. virescens larvae including their gut, regurgitant, salivary glands, frass and remaining body (Tooker & De Moraes 2006). Likewise, indole-3-acetic-acid (IAA) and its precursors were found in both the regurgitant and salivary glands of silkworms (Suzuki *et al.* 2014). Because some of these insect secretions like regurgitant, saliva and frass get in contact with plants during insect feeding, it is likely that their phytohormone content can elicit plant defenses.

In addition to phytohormones, insects also produce 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 & 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 the 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 (Pohnert *et al.* 1999; Alborn *et al.* 2007; Halitschke *et al.* 2001; Mori *et al.* 2003; 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 their protein composition. 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 have not always been triggered by the action of phytohormones, FACs or enzymes (Acevedo *et al.* 2015). For example, oral secretions from *Spodoptera littoralis* and *Pieris brassicae* downregulated the expression of several wound-inducible genes in *Arabidopsis thaliana*; evidence suggest 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) induces defense responses in maize (Chuang *et al.* 2014). Previous studies have shown that the two strains of this insect induce dissimilar plant defense responses due to differences in their saliva composition (Chapter 2 this thesis). The saliva of the FAW strains contains GOX, ATPases, and PLC, that are known plant defense elicitors (Chapter 2 & 3, Peiffer *et al.* unpublished); but their saliva also contains heat-resistant molecules that induce plant defenses (Chapter 2 this thesis). Therefore, the main goal of this study was to identify non-protein plant defense elicitors in the saliva of the FAW strains feeding on different diets. I specifically quantified the presence of phytohormones, lipids and other small molecules using Liquid Chromatography Mass Spectrometry (LC-MS). This study found BA, SA, LA, JA and ABA in the saliva of this insect; the exogenous application of these hormones in similar amounts found in caterpillar saliva regulated defenses in some plants. I also found a large number of putative glycerophospholipids, eicosanoid fatty acids, prostaglandins and other small molecules. The potential role of these compounds as plant defense elicitors will be discussed.

Methods

Insects

The 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, while 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 pair-mated in order to select the F1 individuals containing the corresponding mitochondrial marker that identify each strain (Nagoshi & Meagher 2003).

Plants

Maize plants (*Zea mays*, inbred line B73) were grown in Hagerstown loam soil until they reached the V8-V9 physiological stage. Bermuda grass (*Cynodon dactylon*) were grown in Hagerstown loam soil and used four weeks after germination (V8-V9 physiological stage). Rice plants (*Oryza sativa,* cultivar Nipponbare) were obtained from the USDA-ARS Dale Bumpers National Rice Research Center in Arkansas and grown as described in chapter 2. Tomato plants (*Lycopersicon esculentum,* cultivar Better Boy) were grown in Promix potting soil (Premier Horticulture Quakertown, PA, USA) in four-inch 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 hours light: 10 hours dark) at the Pennsylvania State University, University Park.

Plant defense responses

Plant defense responses to different treatments were evaluated by measuring the expression of JA defense-related genes and the activity of defense-related proteins using quantitative real-time PCR (qPCR) and biochemical assays, respectively. We measured the relative expression of the *Maize proteinase inhibitor (mpi)* gene in maize, the activity of trypsin protease inhibitors (TryPI) in rice, and the activity of polyphenol oxidase (PPO) and peroxidase (POX) in tomato plants. The activity of plant defensive enzymes was standardized by the total amount of protein in each sample. RNA extraction, cDNA synthesis, real time PCR and TryPI activity were performed as described in chapter 2. The activity of PPO was measured following Chung & Felton (2011).

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 the FAW saliva in induced plant defenses

Previous studies have shown that boiled saliva from the FAW strains induces defense responses in maize (Chapter 2), therefore I hypothesized that the FAW saliva contains other non-protein molecules that elicit plant defenses. To assess this I tested the effect of boiled salivary gland homogenates in the defense responses of maize, rice and tomato plants. I further tested the effect of protease-treated salivary glands, as well as protein-precipitated saliva in maize defense responses.

Plant treatment with boiled salivary glands

Salivary glands were dissected from two day-old last instar caterpillars 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 8,000 rpm for three minutes; the supernatant was recovered and further diluted in 1X PBS for plant treatment. Each plant was mechanically wounded and treated with 15-20 µg of either boiled (95 ^{o}C for 1- 4 hours) or non-boiled salivary gland homogenates. The effect of these treatments on plant defense responses was compared with wounding plus PBS and untreated controls.

Maize treatment with protease-treated salivary glands

Proteins were digested by incubating salivary gland homogenates with Pronase from *Streptomyces griseus* (Calbiochem cat # 53702) at 37 ^{o}C for 30 minutes, followed by a denaturation step at 95 ^{o}C for 45 min to inactivate the proteases. The concentration of salivary protein to protease was 10:1 µg. Plants were mechanically wounded and treated with either untreated or protease-treated salivary gland homogenates. The effect of these treatments on plant defense responses was compared with wounding plus PBS and untreated controls.

Maize treatment with protein-precipitated saliva

Saliva was collected from 2 day-old last instar FAW caterpillars fed in corn from egg hatch; caterpillars were chilled on ice for 45 minutes and immobilized into a metallic

haircliip, as they warmed up, their saliva was collected using a micropipette tip (VWR cat No. 53509-015) under a dissecting microscope (Olympus SZ30). 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 minutes 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 precipitated saliva or water.

SDS polyacrylamide gels (PAGE)

The saliva and salivary gland proteins 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 hours in a vertical electrophoresis camera (Biorad Mini-Protean #165800FC). The protein bands were then visualized by staining with silver nitrate.

Quantification of phytohormones in the saliva of the FAW strains

The quantification of phytohormones [JA, SA, BA, ABA, LA and cinnamic acid (CA)] in caterpillar saliva was carried out using HPLC/MS with isotope-labeled standards. Saliva was collected from caterpillars fed on plants and artificial diet (wheat germ) as described above and stored at – 80 ˚C until use. There were 3 biological replications for each FAW strain and diet combination (except for tomato in which only the corn strain was tested); each sample was composed of the pooled saliva quantities collected from 40 - 50 caterpillars. The saliva samples were diluted in MQ water to a final volume of 5 µl and their proteins precipitated overnight at – 20 ˚C by the addition of seven volumes (35 µl) of 100% methanol to which internal isotope-labeled standards had been added [1.4 µM of α13C SA (Campro Scientific # 2515.7-K-ME), d5 JA (CDN isotopes #D6936), d6 ABA (ICON #1001), d5 CA (CDN isotopes #D5284), BA ring $^{13}C_6$ (ICON #1C3089), and ^{13}C LA (TRC #L467500)]. The samples were then centrifuged at

20,000 rcf for 15 minutes 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 (100mm x 2.1mm 1.7 um particle size) maintained at 55 °C and a 20-minute aqueous acetonitrile gradient, at a flow rate of 250 ul/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 5600 (QTOF) TripleTOF 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 80V. The mass spectrometer 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 50V with a 20V spread. Data were acquired and analyzed using the Analyst software (Applied Biosystems). Phytohormones quantities were determined by the analysis of the Gaussian smoothed peak areas of each compound with respect to their corresponding isotopic standards. The *m/z* values of corresponding [M-H] ions, and the retention times for each labeled compound are in Table 4-1.

Table 4-1. Retention time and *m/z* values of isotope-labeled phytohormone standards.

Compound	**Formula**	**[M-H] *m/z***	**Retention time (min)**
Jasmonic acid	$C_{12}H_{13}D_5O_3$	214.1511	10.9
Salicylic acid	$^{13}CC_6H_6O_3$	138.0288	8.92
Abscisic acid	$C_{15}H_{14}D_6O_4$	269.1679	8.56
Benzoic acid	$^{13}C_6CH_6O_2$	127.0455	7.36
Linoleic acid	$C_{17}{}^{13}CH_{32}O_2$	280.2211	9.16
Cinnamic acid	$C_9H_3D_5O_2$	152.0725	9.01

Effect of phytohormones on plant defense induction

It has been shown that exogenous applications of phtyohormones modulate defense responses in plants (Bari & Jones 2009), therefore, I hypothesized that the phytohormones present in the FAW saliva could induce plant defense responses. To test this, I 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. Commercial phytohormones were diluted in 100% methanol to a concentration of 10 µM and then further diluted with MQ water to treat the plants. The 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 hours for tomato plants; 12, 24 and 48 hours for rice; and 24 hours for maize. In all cases each time point had a separate group of plants.

Lipidomics of the FAW salivary glands

Lipid analyses were performed with the aim to identify small molecules present in the FAW saliva with potential plant defense activity. Two samples (one for each FAW strain) of 50 labial salivary gland pairs were dissected from maize-fed caterpillars and homogenized in 300 µl of MQ water, centrifuged at 10,000 rpm for 5 minutes and the supernatant used for lipid extractions. Lipids were extracted from 250 µg of protein from each sample with the Folch lipid extraction protocol (Folch *et al.* 1957). The extracted fraction was resuspended in 100 µl of dichloromethane 10 µl injected for positive ion mode and 20 µl for negative ion mode.

For putative metabolite identification, the raw *.wiff* files were converted into *.mzXML* files using MSConverter software and analyzed using the Mzmine framework version 2.19 (Katajamaa & Orešič 2005; Pluskal *et al.* 2010). The mass detection was performed using the centroid algorithm; the chromatograms were built using a minimum

time span of 0.030 min with a minimum peak height of 2.7E3 and *m/z* tolerance of 0.04 ppm. Detected peaks were further deconvoluted using the wavelets algorithm (Tautenhahn *et al.* 2008) and deisotoped using an *m/z* tolerance of 0.02 and a retention time tolerance of 0.01 min. Peaks were then filtered to include those within a retention time range of 2-12 min and duration range of 0 - 2 min. Putative metabolite identification was performed by searching the filtered *m/z* values against the online LipidMaps database at the corresponding ionization mode (negative or positive) with a *m/z* tolerance of 0.04.

Experimental design and statistical analysis

Plant defense responses (gene expression) to the treatments (wounding plus boiled saliva, protease-treated saliva, precipitated saliva, PBS and untreated controls) were analyzed with one-way ANOVA following the post hoc tests of Tukey and Fisher at $\alpha = 0.05$. Differences in the phytohormone quantities found in the FAW caterpillar saliva were analyzed using a two-factor factorial design; the factors were *strain* (corn or rice) and *diet type* (artificial diet, maize, Bermuda grass and rice). The effects of *time* (specific for each plant type) and *treatment* (wounding plus phytohormone solution, blank or untreated controls) on plant defense responses (PPO, POX and trypsin PI activity) in time course experiments were analyzed using a two factor factorial design. Differences between peak areas of putative identified lipids for the corn and rice strains were descriptively analyzed by their corresponding ratios. All the statistical analyses were performed using the software Minitab 16 (Minitab Inc., State College, PA, USA) and all graphs were generated in R version 3.2.2 (Foundation for Statistical Computing, Vienna, Austria).

Results

Non-protein components in the FAW saliva induce defense responses in plants

To verify that the FAW saliva contains non-protein plant defense elicitors, I measured plant defense responses after wounding and treatment with saliva whose proteins where 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. 4-1 a-b). Conversely, in tomato plants, the application of salivary gland homogenates suppressed PPO activity to similar levels found in undamaged controls (Fig. 4-1 c). In all cases, plant defense responses elicited by non-boiled and boiled salivary gland homogenates were not statistically significant from each other ($P > 0.05$) indicating that some of the elicitors contained in the saliva of this insect are not heat sensitive. Likewise, when pronase-treated saliva was applied to wounded maize plants, the *mpi* gene expression was higher than the PBS treatment but not significantly different from the expression levels triggered by untreated saliva (Fig. 4-2). Similar results were obtained by the precipitation of salivary proteins with ethanol (Fig. 4-3). Together these results suggest that the FAW saliva contains non-protein plant defense elicitors that have not yet been identified.

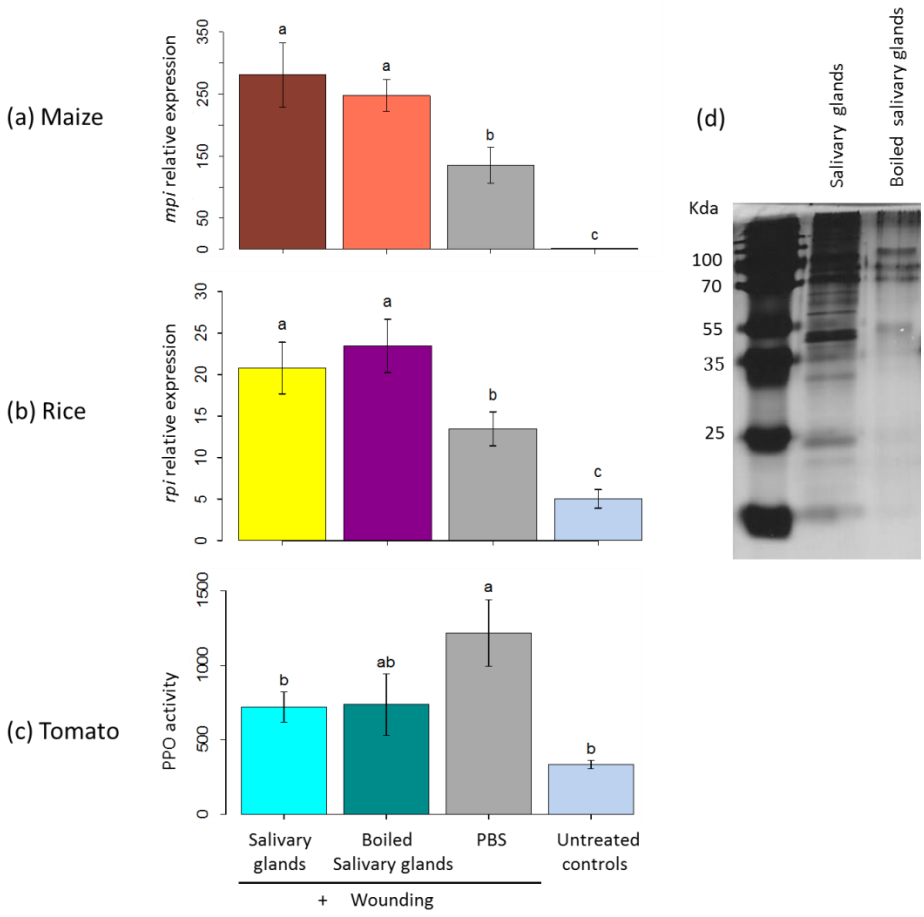

Figure 4-1. Plant defense response to wounding plus non-boiled or boiled salivary gland homogenates from FAW caterpillars. (a) *Maize proteinase inhibitor (mpi)* gene expression 24 hours after treatment ($F_{3,17} = 214.97$, P = 0.000; Tukey test; n = 4 - 5; log transformed data). (b) *Bowman-Birk proteinase inhibitor (rpi)* gene expression 24 hours after treatment ($F_{3,23} = 12.28$, P = 0.000; Fisher test; n = 5 - 6; log transformed data). (c) Polyphenol oxidase (PPO) activity 48 hours after treatment ($F_{3,20} = 4.07$, P = 0.021; Fisher test; n = 6; untransformed data). Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at $\alpha = 0.05$. Controls are undamaged plants. (d) SDS PAGE gel of boiled and non-boiled salivary gland homogenates showing protein degradation after heat treatment.

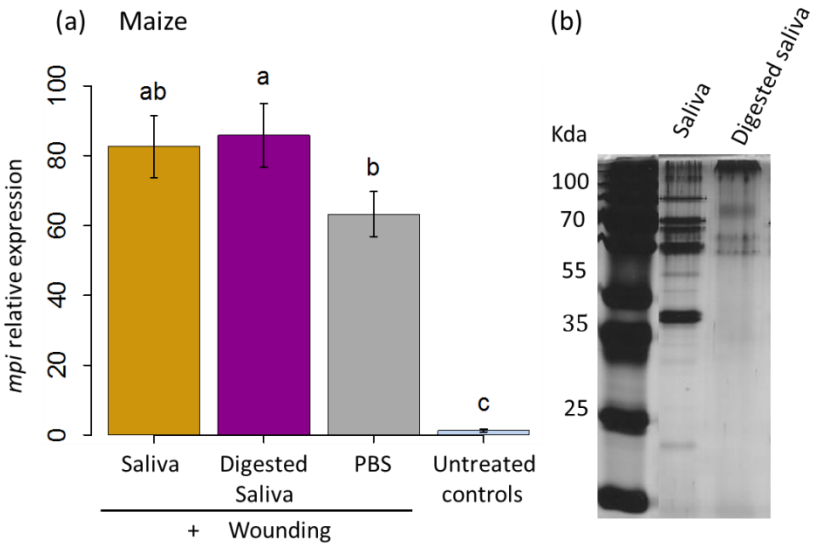

Figure 4-2. Maize defense response to wounding plus the application of saliva and protein-digested saliva (pronase-treated) from FAW caterpillars. (a) *Maize proteinase inhibitor (mpi)* gene expression 24 hours after treatment ($F_{3,19}$ = 181.93, P = 0.000; Fisher test; n = 5 - 6; log transformed data). (b) SDS PAGE gel showing protein degradation after pronase treatment. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α = 0.05. Controls are undamaged plants.

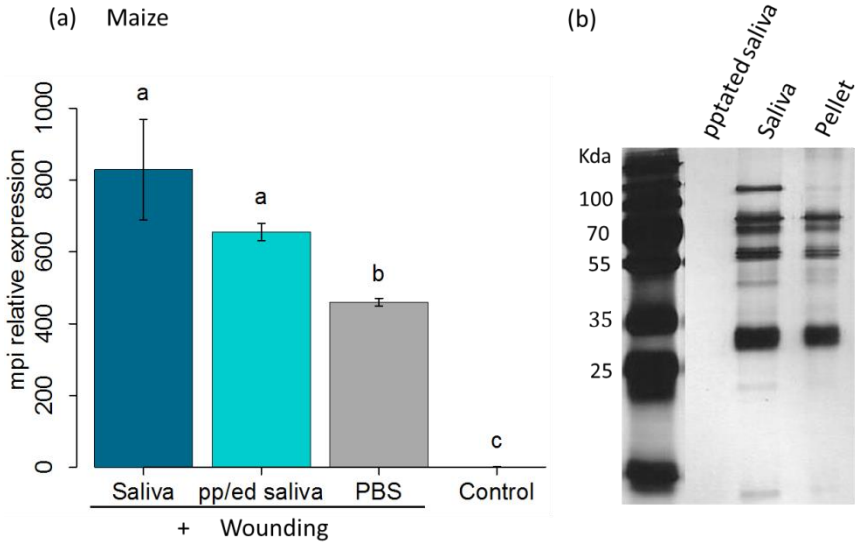

Figure 4-3. Maize defense response to wounding plus the application of saliva and protein-precipitated saliva from FAW caterpillars. (a) *Maize proteinase inhibitor (mpi)* gene expression 24 hours after treatment ($F_{3,15}$ = 420.32, P = 0.000; Fisher test; n = 4 - 5; log transformed data). (b) SDS PAGE gel showing the absence of protein bands after ethanol precipitation. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α = 0.05. Controls are undamaged plants.

The FAW saliva contains Phytohormones

As an attempt to identify non-protein salivary elicitors in the FAW saliva, this study identified and quantified the plant hormones JA, SA, BA, ABA, and LA using LC-MS. The most abundant plant hormones in the FAW saliva were BA and SA followed by LA; JA and ABA were also present at very low amounts (Fig. 4-4). 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 (Table 4-2). Rice-fed caterpillars contained greater amounts of SA and JA compared with caterpillars fed on maize, Bermuda grass, tomato and artificial diet. LA was present at greater amounts in both rice and Bermuda grass fed caterpillars while no differences among diets were observed for either BA or ABA (Fig. 4-4). LA was not detected in saliva from caterpillars grown in artificial diet. These results confirm the presence of non-protein elicitors in the FAW saliva.

Table 4-2. Effect of strain and diet factors on the phytohormone quantities detected in the FAW saliva.

Phytohormone	**Factor**	**F (treatment, error df)**	**ANOVA *p* value**
JA	strain	$F_{1,18} = 0.75$	0.399
	diet	$F_{4,18} = 7.05$	0.001*
SA	strain	$F_{1,18} = 0.01$	0.926
	diet	$F_{4,18} = 17.80$	0.000*
BA	strain	$F_{1,18} = 1.37$	0.257
	diet	$F_{4,18} = 2.85$	0.054
ABA	strain	$F_{1,18} = 1.68$	0.211
	diet	$F_{4,18} = 2.89$	0.052
LA	strain	$F_{1,18} = 2.39$	0.146
	diet	$F_{4,18} = 2.51$	0.023*

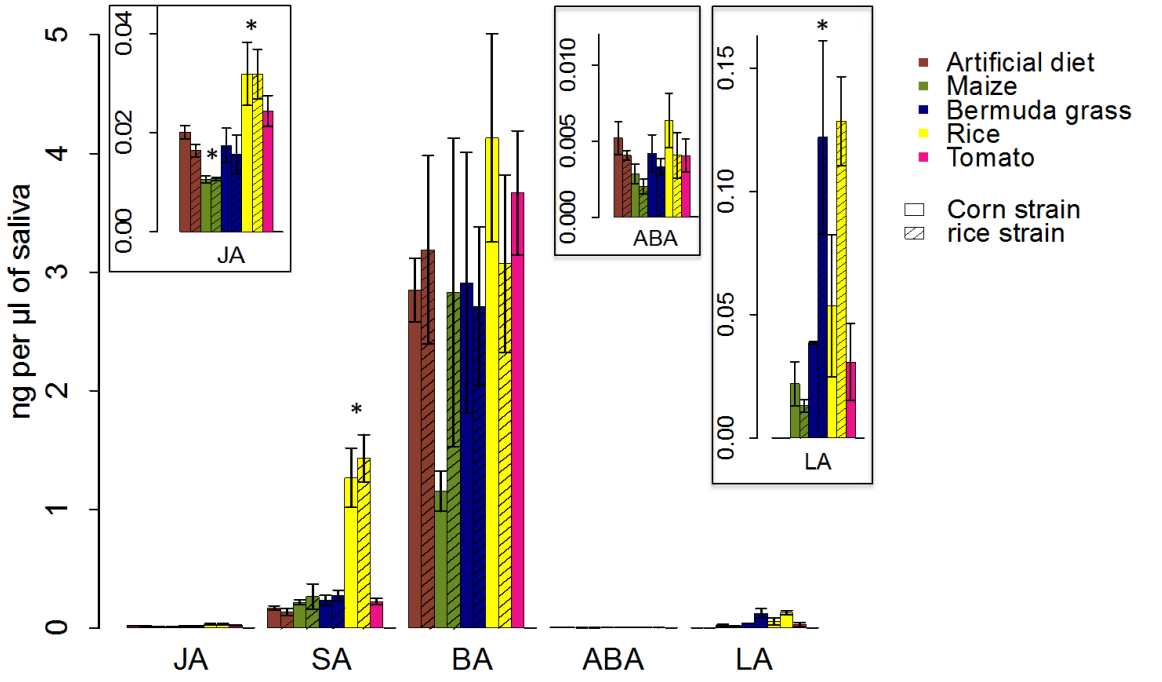

Figure 4-4. Phytohormone quantities in the saliva of FAW caterpillars grown in different diets. Values are untransformed means ± SEM. Asterisks indicate significant differences (α = 0.05) among diet types obtained by one way ANOVA and Tukey tests for each hormone: JA ($F_{4, 19}$ = 9.13 P = 0.000), SA ($F_{4, 19}$ = 20.71, P = 0.000), BA ($F_{4, 19}$ = 3.06 P = 0.042), ABA ($F_{4, 19}$ = 3.06 P = 0.042), and LA ($F_{4, 19}$ = 4.03 P = 0.016).

Phytohormone amounts present in the FAW saliva modulate defense responses in some plants

This study tested the effect of the phytohomones found in the FAW saliva on defense responses of tomato, rice and maize plants. Each wounded tomato plant was treated with either a mixture of hormones (0.0244 ng of JA, 0.2234 ng of SA, 3.67 ng of BA, 0.004 ng of ABA, and 0.0308 ng of LA; all diluted in an aqueous solution of 18% methanol) or a solution of 18% methanol. The activity of PPO and POX was significantly affected by both the treatments and the time points at which the samples were harvested (PPO time effect: $F_{3,97}$ =11.51, P = 0.000, PPO treatment effect: $F_{2,97}$ = 29.41, P = 0000; POX time effect: $F_{3,97}$ =39.93, P = 0.000, POX treatment effect: $F_{2,97}$ =179.64, P = 0.000). Therefore differences among treatments were analyzed for each time point using one-way ANOVA. The application of phytohormones induced higher (but not significantly

different) PPO activity than the water plus methanol solution at 48 and 72 hours after treatment. But at 96 hours, the activity of PPO was significantly suppressed by the application of the phytohomone mixture (Fig. 4-5). The same trend was found for POX, but there were no significant differences between phytohormones and water + methanol treatments at any time point tested (Fig. 4-6).

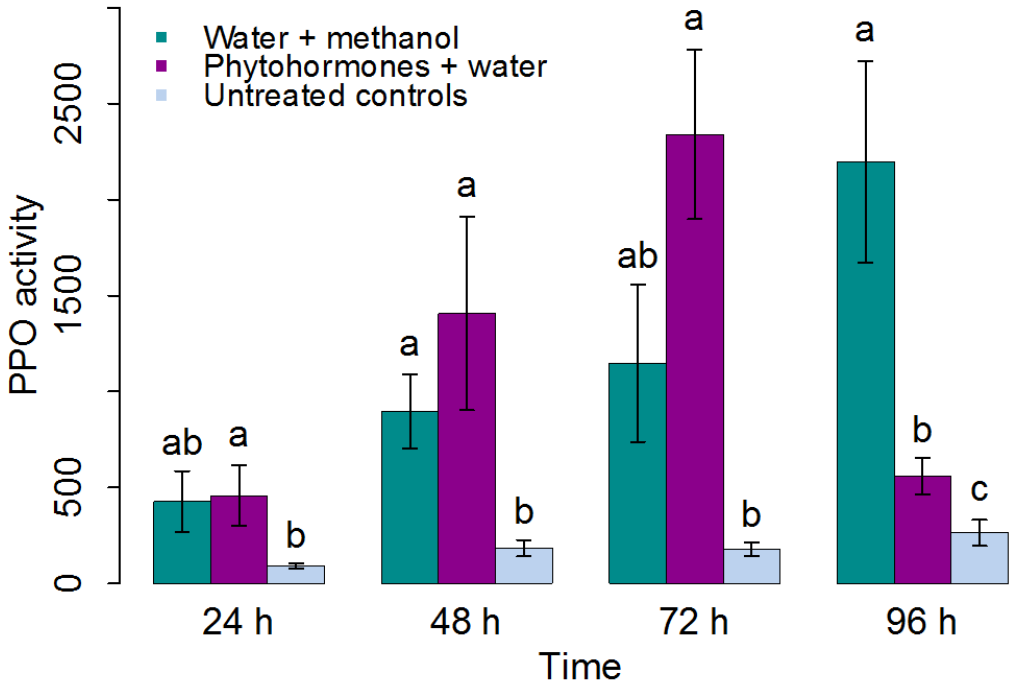

Figure 4-5. Polyphenol oxidase (PPO) activity in tomato plants treated with either a mixture of phytohormones or water plus methanol at different time points. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$ [24 hours ($F_{2,23} = 3.57$, P = 0.046), 48 hours ($F_{2,22} = 76$, P = 0.010), 72 hours ($F_{2,22} = 5.7$, P = 0.010), 96 hours ($F_{2,26} = 30.26$, P = 0.000)]. Controls are undamaged plants.

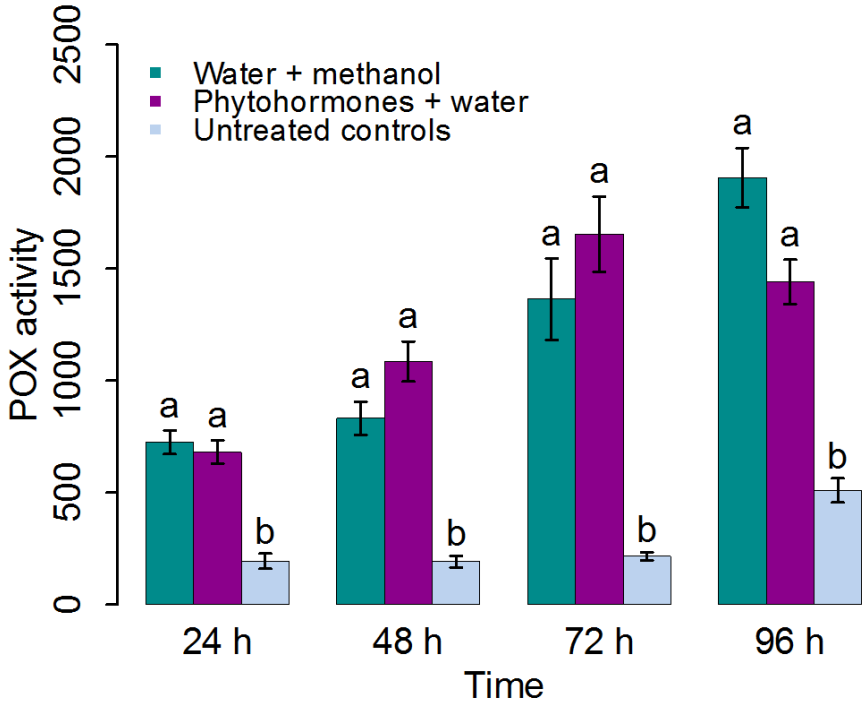

Figure 4-6. Peroxidase (POX) activity in tomato plants treated with either a mixture of phytohormones or water plus methanol at different time points. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$ [24 hours ($F_{2,21} = 41.8$, P = 0.000), 48 hours ($F_{2,22} = 73.46$, P = 0.000), 72 hours ($F_{2,22} = 50.79$, P = 0.000), 96 hours ($F_{2,26} = 68$, P = 0.000)]. Controls are undamaged plants.

Wounded rice plants were treated with a phytohormone mixture containing 0.032 ng of JA, 1.31 ng of SA, 3.86 ng of BA, 0.006 ng of ABA and 0.0724 ng of LA diluted in an aqueous solution of 8% methanol. There was a significant effect of both, treatment ($F_{2,70} = 25.5$ P = 0.000) and time ($F_{2,70} = 28.7$ P = 0.000) on the activity of trypsin PI. Individual ANOVAs for each time point followed by the Tukey multiple comparison test, did not show significant differences between the phytohormones and water + methanol treatment at any time point tested (Fig. 4-7).

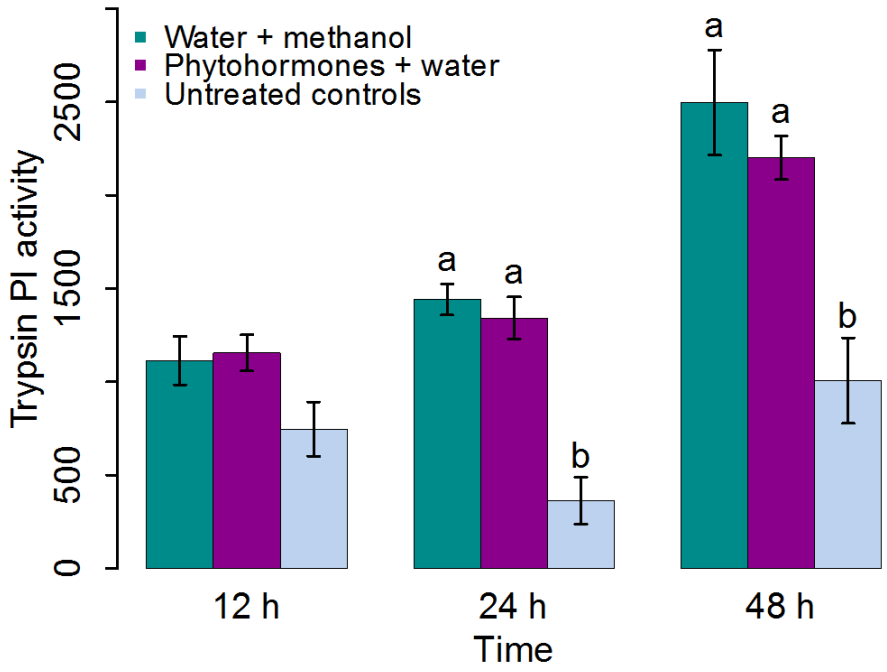

Figure 4-7. Trypsin proteinase inhibitor (Trypsin PI) activity in rice plants treated with either a mixture of phytohormones or water plus methanol at different time points. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following Tukey tests at $\alpha = 0.05$ [12 hours ($F_{2,24} = 2.99$, $P = 0.069$), 24 hours ($F_{2,21} = 16.18$, $P = 0.000$), 48 hours ($F_{2,21} = 11.10$, $P = 0.001$]. Controls are undamaged plants.

Wounded maize plants were treated with a mixture of phytohormones containing 0.0106 ng of JA, 0.2402 ng of SA, 1.99 ng of BA, 0.0024 ng of ABA, and 0.0175 ng of LA diluted in an aqueous solution of 13.3 % methanol. No significant differences were found between the phytohormones and the water plus methanol treatment at the only time point tested (24 hours) (Fig. 4-8).

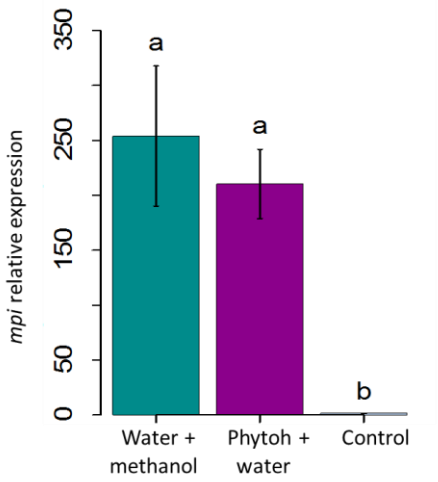

Figure 4-8. Maize proteinase inhibitor (*mpi*) gene expression 24 hours after phytohormone treatment. Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following a Tukey test at $\alpha = 0.05$ ($F_{2,10} = 7.94$, $P = 0.009$). Controls are undamaged plants.

The FAW salivary glands contain a large number of small molecules

Lipid extractions of salivary gland homogenates were analyzed with the purpose of identifying potential plant defense elicitors in the FAW saliva. Samples run in both negative and positive ionization modes showed a large number of well-defined peaks whose putative identity was assigned by an online data search against the LipidMaps database using the Mzmine framework (Fig. 4-9, 4-10). There were ~250 peaks with assigned putative ids from which 132 were selected for having known biological activity. The majority of these compounds (78.7%) corresponded to glycerophospholipids, while smaller fractions were classified as fatty acids and prostaglandins (Fig. 4-11). The relative quantities of some of these compounds were different for the FAW strains (Table 4-3) but the most remarkable differences appear to be associated with larger amounts of several glycerophosphocholines in the rice strain compared with the corn strain (Fig. 4-10). Although further efforts are required for a confident identification of these compounds, these results show the presence of several small molecules in the FAW saliva that could potentially regulate plant defense responses.

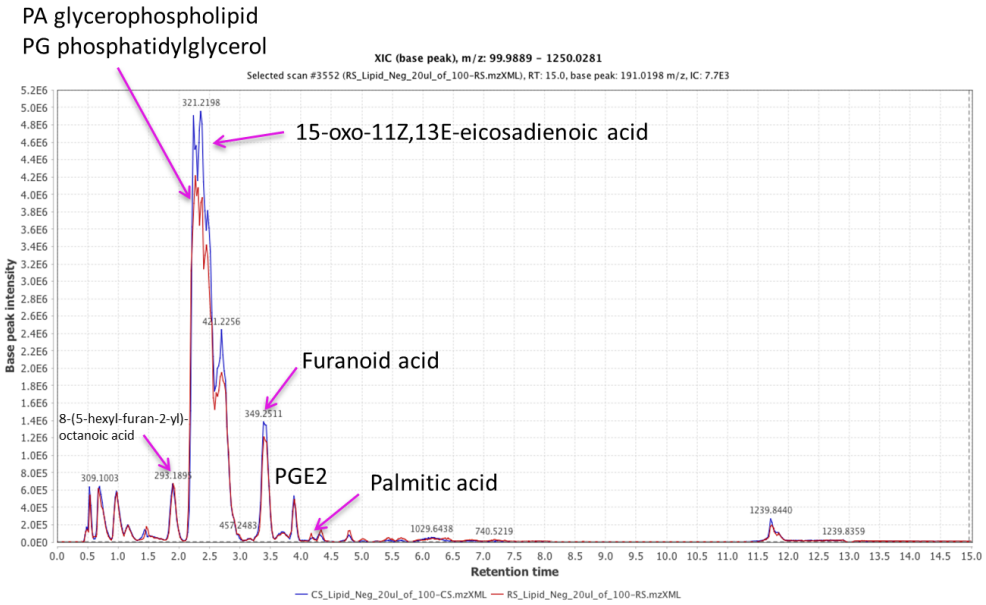

Figure 4-9. Total ion chromatogram in negative ionization mode from lipid extractions of corn (blue) and rice (red) strain salivary glands fed on maize. Putative peak identifications were obtained from a global search against the LipidMaps database using the Mzmine framework (Pluskal *et al.* 2010).

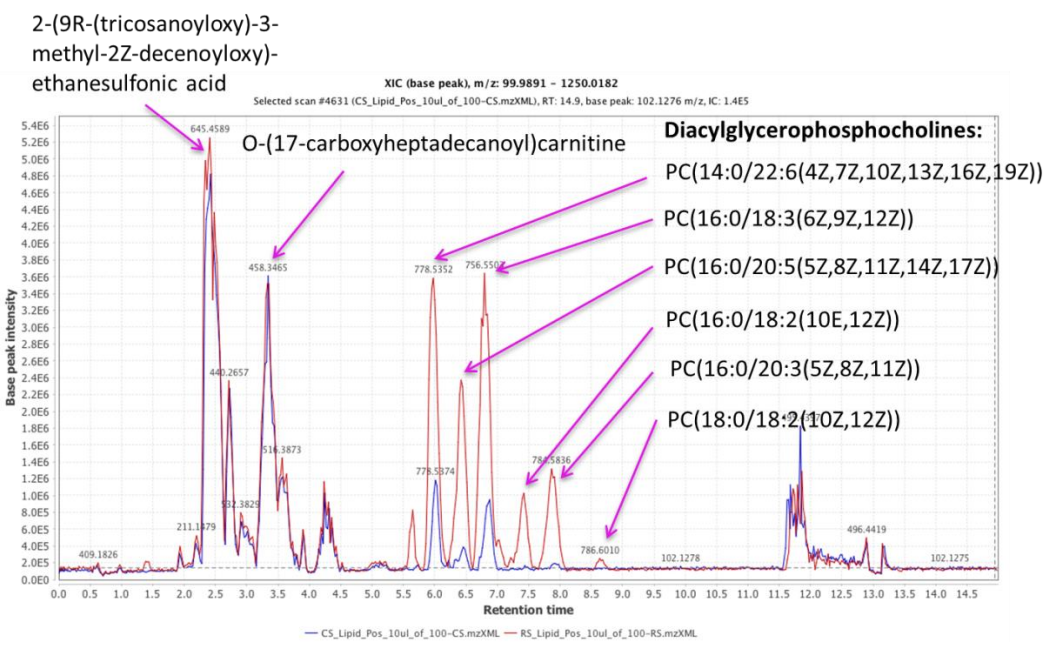

Figure 4-10. Total ion chromatogram in positive ionization mode from lipid extractions of corn (blue) and rice (red) strain salivary glands fed on maize. Putative peak identifications were obtained from a global search against the LipidMaps database using the Mzmine framework (Pluskal *et al.* 2010).

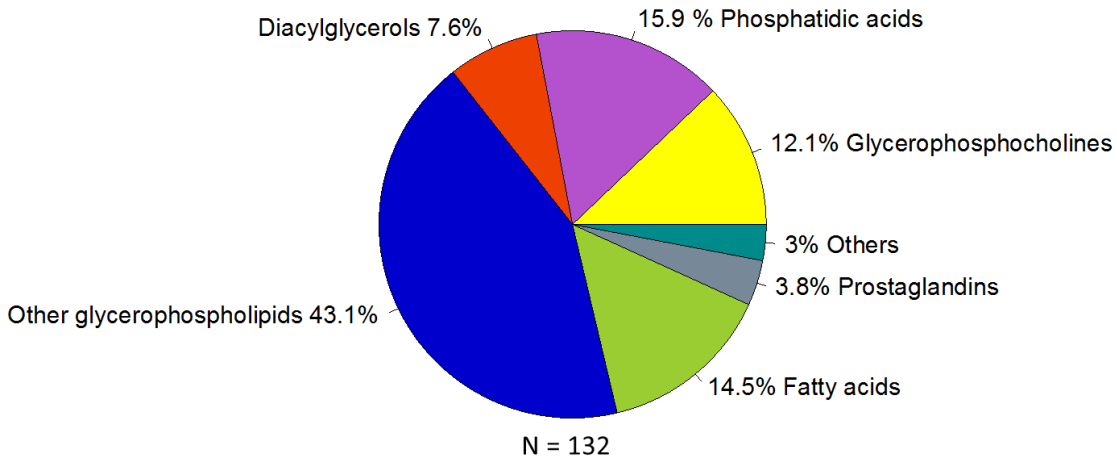

Figure 4-21. Classification of 132 putative compounds identified in lipid extractions of the FAW salivary glands.

Discussion

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

The results of this study show that caterpillar saliva of the lepidopteran fall armyworm contains non-protein molecules that regulate defense responses in plants. Plant treatment with saliva with heat-inactivated enzymes, digested and precipitated proteins, induced defense responses in maize, rice and tomato plants at similar levels found with untreated saliva, but higher than buffer-treated controls (Fig. 4-1 to 4-3). This was unexpected, because the FAW saliva also contains the enzymatic elicitors GOX and PLC (Chapter 2 this thesis), and therefore, untreated saliva should have induced different

plant defense response levels than boiled saliva where these elicitors were inactivated. A possible explanation for this is that the activity of GOX and PLC is affected by the freshness of the samples (Peiffer *et al.* unpublished) and the type of diet (Chapter 2 this thesis). Except for the experiments in tomato, care was not taken on using fresh saliva or salivary gland samples, which may have affected the activity of PLC. Moreover, the GOX activity levels in tomato-fed FAW caterpillars are very low compared with other diets (Acevedo *et al*. unpublished), which may explain why untreated salivary gland homogenates did not induce greater PPO activity than boiled samples or buffer-treated controls. Furthermore, enzymatic elicitors are also susceptible to degradation by other proteases that may be present in the FAW saliva, which may have also influenced these results. Together, these results along with previous studies indicate that the FAW saliva contains both protein and non-protein plant defense elicitors that interact with plants in a host-dependent manner.

Attempts to identify some of the non-protein elicitors in the FAW saliva, led us to screen for the presence of phytohormones. The saliva of this insect contains JA, SA, BA, ABA and LA; from these, BA and SA were found in greater amounts (Fig. 4-4). The plant benzoic acids, BA and SA, are precursors to several primary and secondary metabolites as well as plant defense responses against biotrophic pathogens (Zarate *et al.* 2007; Widhalm & Dudareva 2015). Previous studies have shown that benzoic acid or its conjugates influence SA accumulation in *Arabidopsis*, cucumber and tobacco plants (Doherty *et al.* 1988; Meuwly *et al.* 1995; Mauch-Mani & Slusarenko 1996; Dorey *et al.* 1997; Chong *et al.* 2001). Therefore, I hypothesized that exogenous application of these hormones through caterpillar saliva could induce defense responses in plants. The results of this study show that wounded tomato plants treated with a mixture of these phytohormones, at similar quantities detected in the FAW saliva, had lower activity of the anti-nutritional protein PPO four days after treatment compared with their respective controls (Fig. 4-5). In rice plants, the application of these hormones did not affect the activity of trypsin PI during the first two days after treatment, this could have been because rice contains high endogenous levels of SA (Silverman *et al.* 1995), and

therefore exogenous application of SA and BA at these small quantities (< 5ng) were not enough to trigger defense responses. Similarly, hormone treatment did not affect the expression levels of the maize proteinase inhibitor gene *mpi*, at the only time point tested (24 hours), it is possible that the expression of herbivore-induced genes in maize may be affected at later time points but, this remains to be tested. Alternatively, these hormones may influence plant defenses in concert with other constituents of the FAW saliva.

The quantities of some phytohormones detected in the FAW saliva were strongly influenced by the type of diet in which the caterpillars were grown. SA amounts were higher in the saliva of rice-fed caterpillars, probably because of the higher quantity of this hormone in rice tissues (Silverman *et al.* 1995). However, larger amounts of JA were also found in rice-fed caterpillars compared with other diet types. Similarly, the amounts of LA in saliva were higher when caterpillars grew in Bermuda grass and completely absent when grown in artificial diet, but diet had no effect on the quantities of BA and ABA. Previous studies have shown that the amounts of JA, BA and SA in diets are not associated with the quantities of these compounds detected in insect eggs (Tooker & De Moraes 2007). The amounts of JA were higher in insects than those found in their diets (Tooker & De Moraes 2005), suggesting that insects may be able to selectively sequester these compounds 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 growth (Werner *et al.* 2001; Dorchin *et al.* 2009; Straka *et al.* 2010; Zhao 2010; Tooker & Moraes 2011; Yamaguchi *et al.* 2012; Tanaka *et al.* 2013). These hormones are either synthesized by insects or by their symbions; 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*, seem to rely on endosymbiotic bacteria for the production of cytokinins (Kaiser *et al.* 2010). The associated bacteria of *P. blancardella*, induce the 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 the moth symbiotic bacteria are producing them (Giron *et al.* 2013).

In addition to phytohormones and proteins, the FAW saliva also appears to contain small molecules with potential effect on plant defense induction. A large number of compounds with putative identification were classified into three major groups: glycerophospholipids, fatty acids and prostaglandins (Fig. 4-11). Compounds within the first group are major constituents of cell membranes but some could potentially interact with plant defense signaling. For instance, diacylglycerols (DAG), phosphatidic acids (PA, PS) and phophatidylcholines (PC) are main constituents of the plant phosphoinositide/phospholipase C (PI/PLC) pathway that is activated in response to abiotic and biotic stresses (Canonne *et al.* 2011; Dong *et al.* 2012). These compounds are produced by the action of phospholipases on glycerophospholipids and act as secondary messengers for the downstream production of oxylipins and jasmonates (Canonne *et al.* 2011) involve in defense against some pathogens and herbivores. It has been shown that PA concentration increase in several plant species after pathogen attack (Dong *et al.* 2012), PA modulates the activity of several enzymes involved in membrane-trafficking, calcium signaling, oxidative burst and possible SA accumulation (Zhang & Xiao 2015). There is evidence that the FAW saliva contains PLC (Chapter 2 this thesis) whose hydrolytic activity on phospholipids could lead to the production of both DAG and PA. Exogenous application of caterpillar-derived PLC, induces defense responses in several plants (Chapter 2 this thesis), but it is unknown if exogenous treatment with DAG or PA could also modulate plant defense responses.

Fatty acids are other major presumptive lipid constituents of the FAW saliva. Several FACs have been identified in insect regurgitant and are potent plant defense elicitors (Tumlinson & Engelberth 2008). The majority of putative FACs identified in this study corresponded to eicosanoids. These FACs play a central role in insect immunity (Stanley *et al.* 2012), but also modulate plant defense responses (Dedyukhina *et al.* 2014). For instance, exogenous application of the eicosanoid arachidonic acid (AA) induced resistance to fungal infection in tomato (Savchenko *et al.* 2010) and other plants (Dedyukhina *et al.* 2014). AA is synthesized from phospholipids by the action of

phospholipases (Stanley & Kim 2014). The oxygenation of free AA by the action of oxygenases leads to the production of prostaglandins (PGs); which are biologically active compounds with a central role in insect immunity, reproduction and other physiological processes (Stanley & Kim 2014). The prostaglandin E2 (PGE2) has been identified in several blood-feeding species and appears to aid in food ingestion (Stanley & Kim 2014). High levels of PGE2 were identified in the salivary glands of ticks *Amblyomma americanum* and blowflies *Calliphora erythrocephala* where it appears to regulate the rates of saliva secretion (Stanley 2006; Stanley & Kim 2014). In addition to eicosanoids, other FACs identified in the FAW included sphingolipids and furan FACs, both with potential roles in plant immune responses by influencing cell death and oxidative stress (Berkey *et al.* 2012; Mawlong *et al.* 2014). Compounds regulating insect immunity may also affect plant signaling responses due to the striking similarities between the plant (oxylipins) and animal (eicosanoid) immune pathways (Savchenko *et al.* 2010). From these results I speculate that immune-related compounds in the FAW saliva may modulate defense responses in its host plants; however further research is needed to fully test this hypothesis.

In conclusion, this study has shown that, in addition to enzymatic elicitors, the FAW saliva contains non-protein compounds that modulate defense responses in different plants. We successfully identified and quantified five different phytohormones and have tested their role in plant defense induction. Experiments in tomato indicate that phytohormones in the FAW saliva suppress herbivore-induced defenses, which agree with the effect elicited by saliva treatment on this plant. It is unknown if these hormones are synthesized by these insects or are sequestered from their diet, future experiments aiming to elucidate these mechanisms are needed. Lastly, this study indicates that caterpillar saliva is a far more complex fluid than previously recognized due to the presence of an array of lipids.

Table 4-3. Putative metabolic compounds present in the FAW salivary glands.

Group	Subgroup	Molecular formula	Putative ID	m/z	Retention time	Rice strain Peak area	Corn strain Peak area	Ratio RS/CS
Glycerophospholipids		C28H55O12P	PI(19:0/0:0)	613.3457	3.89	76616.60	81418.24	0.94
		C49H81O13P	PI(18:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	907.5109	3.57	170941.14	185860.97	0.92
		C26H51O12P	PI(17:0/0:0)	585.3399	3.54	80934.43	91132.81	0.89
		C45H81O13P	PI(14:1(9Z)/22:2(13Z,16Z))	859.5325	5.44	448412.38	178097.60	2.52
		C43H77O13P	PI(14:0/20:3(8Z,11Z,14Z))	831.5038	5.02	255845.23	78857.49	3.24
		C39H69O13P	PI(12:0/18:3(6Z,9Z,12Z))	775.4305	3.62	432128.44	457612.41	0.94
		C18H35O10P	PG(6:0/6:0)	441.1782	2.35	159805.88	193141.55	0.83
		C28H45O9P	PG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)	555.3127	3.75	86843.74	81580.68	1.06
		C28H53O9P	PG(22:2(13Z,16Z)/0:0)	563.3434	3.33	66084.11	65668.05	1.01
		C26H49O9P	PG(20:2(11Z,14Z)/0:0)	535.2670	3.32	65229.70	66433.56	0.98
		C42H67O10P	PG(18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))	761.4246	3.38	69667.11	71130.96	0.98
		C38H75O10P	PG(18:0/14:0)	721.4686	3.42	133171.43	142218.94	0.94
		C40H69O10P	PG(14:1(9Z)/20:4(5Z,8Z,11Z,14Z))	739.4411	3.27	47659.66	47514.00	1.00
		C34H67O10P	PG(14:0/14:0)	665.4292	2.35	1135818.73	1365892.89	0.83
		C38H71O10P	PG(12:0/20:2(11Z,14Z))	717.4328	3.80	229.67	526.03	0.44
		C38H73O10P	PG(12:0/20:1(11Z))	719.4598	3.27	80323.56	116276.74	0.69
		C36H65O10P	PG(12:0/18:3(6Z,9Z,12Z))	687.4342	4.02	1089.91	229.38	4.75
		C35H65O10P	PG(12:0/17:2(9Z,12Z))	675.4335	3.27	98957.02	143748.85	0.69
		C33H65O10P	PG(12:0/15:0)	651.3861	3.32	68135.45	79016.70	0.86
		C41H70NO8P	PE(16:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))	734.4773	5.65	446437.28	166950.49	2.67
		C41H68NO8P	PE(14:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	732.4205	4.78	269.10	1145.29	0.23
		C27H53O12P	6-O-(1-O-stearoyl-sn-glycero-3-phosphono)-1D-myo-inositol	599.3254	3.75	501061.89	551568.83	0.91
	Glycerophosphocholines	C28H52NO7P	PC(20:3(8Z,11Z,14Z)/0:0)	544.3054	4.42	70046.17	65894.18	1.06
		C26H46NO7P	PC(18:4(9E,11E,13E,15E)/0:0)	514.3169	6.17	78328.41	200669.84	0.39
		C46H76NO8P	PC(18:3(6Z,9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z))	800.5447	6.80	291825.56	125365.58	2.33
		C47H78NO8P	PC(17:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	814.5601	6.81	451270.24	182604.23	2.47
		C41H72NO8P	PC(13:0/20:5(5Z,8Z,11Z,14Z,17Z))	736.4920	6.01	279129.61	64422.81	4.33

		C40H70NO8P	PC(12:0/20:5(5Z,8Z,11Z,14Z,17Z))	722.4958	3.42	47943.53	59271.13	0.81
		C46H76NO7P	1-(6-[5]-ladderane-hexanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphocholine	784.5139	5.55	121651.79	50379.26	2.41
	Phosphatidic acids	C23H43O7P	PA(20:2(11Z,14Z)/0:0)	461.2436	3.39	75655.99	90121.87	0.84
		C44H75O8P	PA(19:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	761.4882	5.24	91879.61	90118.43	1.02
		C39H61O8P	PA(18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))	687.3777	3.68	624290.09	677769.27	0.92
		C21H37O7P	PA(18:3(6Z,9Z,12Z)/0:0)	431.2157	2.03	144583.05	235444.30	0.61
		C33H65O8P	PA(18:0/12:0)	619.4156	11.74	1087038.61	1359615.56	0.80
		C20H37O7P	PA(17:2(9Z,12Z)/0:0)	419.1962	2.25	2788957.39	3052361.91	0.91
		C34H65O8P	PA(17:0/14:1(9Z))	631.4062	3.27	101682.54	129453.00	0.79
		C41H71O8P	PA(16:1(9Z)/22:4(7Z,10Z,13Z,16Z))	721.4404	3.73	580.42	670.28	0.87
		C19H39O7P	PA(16:0/0:0)	409.2178	4.19	67674.73	178944.73	0.38
		C31H57O8P	PA(14:1(9Z)/14:1(9Z))	587.3791	3.29	88158.19	99951.19	0.88
	Glycerophosphoserins	C45H78NO10P	PS(17:1(9Z)/22:4(7Z,10Z,13Z,16Z))	822.5282	5.95	218435.36	66300.54	3.29
		C41H70NO10P	PS(15:0/20:5(5Z,8Z,11Z,14Z,17Z))	766.4411	2.55	76261.95	84564.61	0.90
	Diacylglycerols	C47H70O5	DG(22:5(7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)[iso2]	713.4964	6.34	279951.59	122116.51	2.29
		C45H68O5	DG(20:5(5Z,8Z,11Z,14Z,17Z)/22:5(7Z,10Z,13Z,16Z,19Z)/0:0)[iso2]	687.5092	5.04	186.04	71.45	2.60
Fatty acids	Fatty acid amide	C23H38N2O4	N-linolenoyl-glutamine	405.2688	3.95	62601.94	71738.82	0.87
	Furan fatty acid	C22H38O3	Furanoid acid - F6	349.2510	3.39	9982186.01	11376867.15	0.88
	Eicosanoids	C26H38N2O3S	N-(4-benzenesulfonamide) arachidonoyl amine	457.2484	2.97	560724.16	570437.07	0.98
		C22H38O2	7,7-dimethyl-5Z,8Z,11Z-eicosatrienoic acid	333.2462	3.64	38746.28	43294.54	0.89
		C20H32O3	12-hydroxy-5Z,8Z,10Z,14Z-Eicosatetraenoic acid	319.2289	3.87	147334.51	147510.36	1.00
		C20H32O5	5,14,15-trihydroxy-6,8,10,12-Eicosatetraenoic acid	351.2465	3.74	580.42	932.74	0.62
	Other fatty acids	C18H35ClO3	9-chloro-10-hydroxy-octadecanoic acid	333.2138	3.91	989.15	1282.07	0.77
		C18H19BrO2	18-bromo-9E,17E-octadecadien-5,7,15-triynoic acid	345.0723	3.84	94300.87	120423.01	0.78
		C22H24O2	4,7,10,13,16-Docosapentaynoic acid	319.2059	2.14	64478.64	80167.46	0.80
		C17H28O5	(7Z)-14-hydroxy-10,13-dioxoheptadec-7-enoic acid	311.1770	2.21	39387.01	43202.49	0.91
		C18H36O2	Stearic acid	283.2634	4.80	686943.05	520354.98	1.32

		C16H32O2	Palmitic acid	255.2332	4.31	790374.77	827780.89	0.95
	Sphingolipids	C34H59NO15	Fumonisin B1	720.4145	4.19	35370.13	63287.40	0.56
	Prostaglandines	C22H34O4	16,16-dimethyl-PGA2	361.2601	3.29	44746.75	72218.69	0.62
		C21H34O5	15-methyl-15S-PGD2	365.2403	2.19	83631.39	110802.70	0.75
		C23H38O4	9-deoxy-9-methylene-16,16-dimethyl -PGE2	377.2823	3.89	2408222.94	2403848.52	1.00
	Flavonoids	C30H36O5	Sophoraisoflavanone C	475.2588	3.89	136963.78	154681.39	0.89
	sulfonic acid	C17H37NO4S	Capnine	350.2543	3.42	1852108.27	2037767.34	0.91

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Chapter 5

Fall armyworm armyworm-associated gut bacteria modulate plant defense responses

Abstract

Mechanical damage caused by insect feeding, along with components present in their saliva and oral secretions are known to induce defense responses in plants. This study investigated the role of caterpillar gut-associated microbes in plant defense induction. I specifically tested the influence of bacteria from oral secretions of the fall armyworm (FAW) *Spodoptera frugiperda*, on herbivore-induced defenses in tomato and maize plants. Bacteria from oral secretions of field-collected caterpillars were cultured in 2xYT media and individual isolates were identified using the Bruker Biotyper MALDI-TOF mass spectrometry technology and the 16S rRNA gene sequences. I identified seven different bacteria isolates all belonging to the family Enterobacteriacea. I tested the effect of these bacteria isolates on the induction of polyphenol oxidase (PPO), peroxidase (POX), trypsin inhibitor activity (TryPI) and the expression of a maize proteinase inhibitor (*mpi*) gene in tomato and maize, respectively. The effect of plant defense induction by these bacteria on caterpillar growth was tested by measuring the weight gain of young caterpillars feeding on plants pretreated with individual bacteria isolates. Two bacteria isolates, *Pantoea ananatis* and Enterobacteriaceae-1, suppressed PPO and TryPI on tomato, but induced *mpi* gene expression in maize. The plant defense responses to these bacteria isolates enhanced caterpillar growth in tomato, but diminished their growth on maize plants. I conclude that bacteria from the oral secretions of FAW influenced caterpillar performance on specific hosts by modulating defense responses in plants. These results highlight the importance of associated microbes mediating insect-plant interactions.

Introduction

Mechanical damage caused by feeding of chewing insects induces defense responses in plants. The magnitude of these responses is often modified by insect-derived cues present in their saliva, oral secretions (OS) or frass (Acevedo *et al.* 2015; Ray *et al.* 2015). The saliva of Lepidopteran larvae is mainly produced by the labial salivary glands and released extra orally through the spinneret (Felton *et al.* 2014). Some proteins present in the caterpillar saliva can directly interact with plants to enhance or suppress herbivore-induced defenses. Previous studies have shown that glucose oxidase (GOX), adenosine triphosphatases (ATPases) and a recently identified phospholipase C (PLC) modulate defense responses in several host plants species (Acevedo *et al.* 2015; Schmelz 2015). Different from saliva, oral secretions or regurgitant arise from the insects' foregut (Grant 2006) and contain fatty acid amino acid conjugates (FACs), insect and plant-derived enzymes, and microbes that modulate defense responses in plants (Schmelz 2015). Insect frass contains plant proteins, insect-derived proteins and microbes that regulate plant defenses (Ray *et al.* 2016). A large volume of work has been dedicated to identification of elicitors and effectors present in insect saliva and oral secretions but there is very little understanding on the effect of herbivore-associated symbionts in plant defense regulation.

Insects are hosts of diverse microbial communities that influence their interactions with other trophic levels and their environment. Some known services provided by insect-associated microorganisms include nutrient provisioning, synthesis of pheromone components, regulation of insect-immune responses and protection against parasites (Engel & Moran 2013; Douglas 2015). Microbes are essential for plant-feeding insects whose diet is generally low in nutrients and have high content of chemical defenses. Symbionts associated with phytophagous insects provide essential amino acids (Douglas 2015), aid in digestion (Visôtto *et al.* 2009) and the detoxification of toxic plant secondary metabolites including terpenes and phenolics (Hammer & Bowers 2015). For instance the phytophagous gypsy moth, *Lymantria dispar*, harbors symbiotic bacteria of

the genus *Acinetobacter* that appears to metabolize toxic phenolic glycosides from its host plant *Populus tremuloides* (Mason *et al.* 2016). In other cases the association with specific microbes restricts the insects' ability to utilize specific host plants; for example the fecundity of the pea aphid, *Acyrthosiphon pisum,* on white clover is highly dependent on the presence of the endosymbiont "pea aphid U-type" (Tsuchida *et al.* 2004). Similarly, the stinkbug *Megacopta punctatissima,* owe their ability to use crop legumes to their associated symbiont *Ishikawaella capsulate* (Hosokawa *et al.* 2007). Therefore, the capability of some phytophagous insects to exploit particular host plants seems to be, in part, mediated by their association with specific microorganisms.

Besides aiding nutrition and detoxification, microorganisms associated with plant-feeding insects can also regulate induced plant defenses. This regulation can be indirect by affecting herbivore physiology or behavior, which can, in turn, modify their perception by plants (Zhu *et al.* 2014). Alternatively, microbes present in insect oral secretions can directly interact with wounded plant tissues during insect feeding. Upon recognition by plants, these microorganisms can activate plant defensive pathways that could further affect the fitness of their herbivore host (Zhu *et al.* 2014). For example, the Colorado potato beetles *Leptinotarsa decemlineata,* harbor gut endosymbiotic bacteria that when released on tomato plants through oral secretions downregulate herbivore-induced defenses, which improves insect performance (Chung *et al.* 2013). Beetles do not possess salivary glands; therefore their oral secretions are mainly arising from their gut as regurgitant. In contrast, lepidopteran larvae release secretions from their salivary glands (saliva) and regurgitant from their foregut. Much work has characterized molecules present in lepidopteran oral secretions that regulate the induction of plant defenses, but thus far the role that microbes could play in these interactions has been neglected.

Lepidopterans constitute one of the largest phytophagous insect groups comprising about 180,000 described species, some of which are important agricultural pests (The Lepidoptera Taxome Project http://www.ucl.ac.uk/taxome). Surprisingly, studies of their gut microbiome are scarce, focusing on less than ten species: *Pieris rapae*

(Robinson *et al.* 2010), *Spodoptera littoralis* (Tang *et al.* 2012), *Helicoverpa armigera* (Priya *et al.* 2012) *Spodoptera exigua* (Ping *et al.* 2007), *Anticarsia gemmatalis* (Visôtto *et al.* 2009), *Plutella xylostella* (Indiragandhi *et al.* 2008) and *Lymantria dispar* (Broderick *et al.* 2004). Some of the Lepidoptera gut-associated bacteria promote plant growth (Indiragandhi *et al.* 2008), produce digestive proteases (Visôtto *et al.* 2009), metabolize toxic plant compounds (Mason *et al.* 2016) and appear to help in the hydrolysis or synthesis of the plant defense elicitors N-acyl amino acid conjugates (Ping *et al.* 2007). These studies hint at a potential role of lepidopteran gut symbionts in plant utilization and possibly plant defense regulation, but this role remains poorly explored.

In this study, I investigated the effect of bacteria isolated from the oral secretions of the polyphagous herbivore fall armyworm (FAW) *Spodoptera frugiperda* (Lepidoptera: Noctuidae) on herbivore-induced defenses of two host-plant species, tomato and maize. I used culture-dependent methods to identify microbes associated with field collected FAW caterpillars and tested the direct effect of these bacteria isolates on plant defense responses. I also tested the indirect effect of some of these isolates on activity and expression of insect salivary proteins. The results of this study show that bacteria from the FAW oral secretions modulated defense responses in plants. I identified seven different bacteria isolates from FAW oral secretions; of these, *Pantoea ananatis* and Enterobacteriaceae-1(*Serratia/Rahnella*) suppressed herbivore-induced defenses in tomato but induced defenses in maize. I found no effect of these isolates on the FAW salivary protein activity or protein expression. FAW caterpillars hosted and actively secreted microbial symbionts onto their host plants, but the extent to which the insect may benefit from hosting these specific microbes are largely dependent on their host plant species.

Methods

Insects

Fall armyworm caterpillars were collected in summer 2014 from susceptible non-Bt maize fields (*Zea mays* c.v Providence) at the Russell E. Larson Agricultural Research Center located at Rock springs, PA. This colony was then maintained in laboratory conditions (University Park, PA) feeding exclusively on corn leaves for 14 generations.

Plants

Tomato plants (*Solanum lycopersicum* c.v Betterboy) were grown in Promix potting soil (Premier Horticulture) and used for experiments when their 5^{th} leaf was fully expanded. Maize plants (*Zea mays* c.v B73 inbred line) were grown in Hagerstown loam soil and used at their V8-V9 physiological stage. Plants were grown under glasshouse conditions (14:10 hours of light: dark) at the Pennsylvania State University, University Park, PA.

Identification of bacteria

Oral secretions (or regurgitant) were obtained from field-collected caterpillars on the same day they were gathered from the field. The regurgitant was collected directly from the caterpillar's oral cavity using a 200 µl pipette. The regurgitant from each of the 14 caterpillars was further diluted 1:300 in sterile Milli-Q water, 100 µl of the mix were plated on sterile 2xYT [(0.016 g/ml of Bacto Tryptone (Becton Dickinson & Co. Sparks, MD, USA), 0.01 g/ml of Bacto Yeast extract (Becton Dickinson & Co. Sparks, MD. USA), 0.005 g of sodium chloride (BDH), and 0.014 g/ml of Agar (Bioserv. Newark, DE. USA)] agar plates and incubated overnight at 27 ^{o}C. Individual bacteria colonies were taken and sub cultured in 2xYT agar plates; after 24 hours a single individual colony was taken from each plate and grown overnight in 2xYT liquid media (without agar) at 27 $^{\circ}$C in a rotary shaker at 200 rpm. One volume of the liquid-grown bacteria was mixed with one volume of sterile 50% glycerol (EMD) and stored at -80 $^{\circ}$C for further use. Bacteria identification was carried out using two methods, the Bruker Biotyper matrix-assisted

laser desorption ionization-time of flight mass spectrometry, MALDI-TOF MS (Bruker Daltonics, Billerica, MA) system and the 16S ribosomal RNA gene sequencing.

For the Biotyper, bacteria isolates were prepared for analysis using a direct transfer method following a standard Bruker protocol (Schmitt *et al.* 2013). Briefly, individual colonies from overnight cultures (grown in 2xYT agar plates) were transferred onto a MALDI target plate using a wooden toothpick and allowed to dry; the cells were lysed by applying 1 µl of matrix solution [10 mg/ml of α-cyano-4-hydroxycinnamic acid (HCCA)] in 50% aqueous acetonitrile containing 2.5% of trifluoroacetic acid. The matrix-analyte mixture was allowed to dry and the resulting samples were used for the MALDI Biotyper data acquisition. A bacterial test standard (BTS; Bruker Daltonics) was used for instrument calibration and as a positive control. Matrix blank spots were included in each analysis to ensure that the target plate was thoroughly cleaned and there was no carryover signal. MALDI mass spectra were acquired on a Bruker Ultraflextreme MALDI TOF/TOF mass spectrometer in the linear, positive-ion mode. Spectra were processed using a factory default processing method for the Biotyper application and searched against a Bruker Taxonomy library containing 5,627 cellular organisms' entries using MALDI Biotyper Software version 3.1. The identification was carried out using manufacturer-recommended cutoff scores; scores ≥ 2.0 indicating identification to the species level, scores between 1.7 and 1.999 indicating identification to the genus level, and scores of < 1.7 indicating no identification.

The 16S rRNA sequences were analyzed in November 2014 using blast against the nucleotide database of the National Center for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov) and the Ribosomal Database Project Naive Bayesian rRNA (rdp) Classifier Version 2.1, using 95% confidence threshold (http://rdp.cme.msu.edu) (Wang *et al.* 2007). The PCR reaction contained 0.4 µM of each universal 16S rRNA primer (530 F 5'-GTG CCA GCM GCC GCG G-3' and 1392R 5'-ACG GGC GGT GTG TRC-3'), 12.5 µl of the GoTaq Green Master Mix (Promega), 2 µl of the overnight liquid-grown bacteria previously diluted 1:5 in sterile water, and 8.5 µl of MQ water for a total

volume of 25 µl. The PCR conditions had an initial denaturation step of 5 min. at 95 °C, followed by 30 cycles of 95 °C for 1 min, 57 °C for 30 sec, and 72 °C for 1 min. and a final extension step of 7 min. at 72 °C. The samples were cleaned up from remaining primers and nucleotides by incubating 5 µl of the PCR product with 2 µl of EXOSAP-IT (USB Corporation) at 37 °C for 15 min, followed by a denaturation step at 80 °C for 15 min to inactivate the EXOSAP-IT reagent. The PCR products were sequenced at the Penn State Genome Core Facility.

Effect of caterpillar gut bacteria on plant defense responses

I tested the effect of the FAW caterpillar gut bacteria on tomato and maize plants following the procedures reported in (Chung *et al.* 2013) with some modifications. First, plants were treated with field-collected caterpillars that were either pretreated with antibiotics (see below) or untreated controls. To ensure that all plants received the same amount of damage, the caterpillars were placed on clip cages (polypropylene with metallic micromesh screen, 23 mm diameter and 18 mm height) and removed after they ate the enclosed leaf area. Second, plants were mechanically wounded and treated with regurgitant obtained from field-collected caterpillars that were either pretreated with antibiotics or untreated controls. Third, mechanically wounded plants were treated with individual bacteria cultures (OD 600 = 0.1) grown overnight on 2xYT liquid media. The effect of each bacteria isolate on plant defense responses was compared against the effect of liquid media alone. And fourth, individual bacteria isolates were reintroduced into caterpillars (described below) that were further placed onto the plants. The leaf tissue (50-70 mg) around the feeding/damaged sites was harvested 24 and 48 hours later (for maize and tomato, respectively) in liquid nitrogen and stored at -80 °C for further analysis. Plant defense responses were measured by assessing the activity of defense-related proteins and by quantifying the expression of jasmonic acid defense-related genes. In tomato plants, we measured the activity of polyphenol oxidase (PPO), trypsin protease inhibitor (tryPI), and peroxidase (POX) using biochemical assays (described below). In maize plants we quantified the relative expression of the *maize proteinase inhibitor* (*mpi*) *gene* using quantitative real-time PCR (qPCR) (described below).

Antibiotic treatment

Caterpillars were treated with a cocktail of antibiotics containing 12.82 mg/ml of neomycin sulfate (MP Biomedicals. Santa Ana, Ca. USA), 64.1 mg/ml of aureomycin (Bioserv. Newark, DE, USA) and 3.85 mg/ml of streptomycin sulfate (Amresco. Solon, OH. USA) diluted in MQ water. 20 µl of the antibiotic cocktail were deposited as small drops onto a small maize leaf piece (~ a square inch) placed into an agar plate and air dried for 3-4 hours. Each caterpillar was allowed to eat only one treated maize leaf piece. Untreated caterpillars were fed with corn leaves treated with water.

Reintroduction of bacteria isolates into caterpillars

To closely study the effect of single bacteria isolates on plant defense induction, bacteria were reintroduced into caterpillars that were later allowed to feed on plants. Last instar caterpillars were first treated with antibiotics (as indicated above) to clean up their gut. The following day, caterpillars were fed twice with a small piece of artificial diet (0.1 g) containing 10 µl of bacteria (OD 600 = 1.0) resuspended in 10 mM of Magnesium Chloride ($MgCl_2$). The bacteria isolates were grown overnight in 2xYT liquid media, the bacteria suspension was centrifuged at 5,000 g for 10 minutes and the pellet resuspended in $MgCl_2$ (Chung *et al.* 2013). Control caterpillars were fed with diet and $MgCl_2$ only. After the diet was eaten (overnight), all caterpillars were transferred into a new cup and fed with an untreated squared inch of plant leaf before placing them into the plants (2 - 3 hours).

Effect of induced plant defenses on caterpillar's growth

I evaluated the effect of plant defense responses elicited by bacteria isolates from the FAW gut on caterpillar growth. Plants were mechanically wounded and treated with either liquid media or individual bacteria cultures (OD 600 = 0.1) grown over night on 2xYT liquid media. After 24 and 48 hours (for maize and tomato, respectively) the treated leaves were detached and used to feed 3rd instar FAW caterpillars for 6 days. The treated leaf tissue of each plant was used to feed three caterpillars and their average

weight gain used as an independent biological replicate for the statistical analysis. The caterpillar weight gain was calculated as the difference between initial and final weight.

Quantification of regurgitant in plant leaves

I quantified the amount of regurgitant secreted by the FAW caterpillars feeding on tomato and maize plants following the procedure described by (Peiffer & Felton 2009) caterpillars were grown from egg hatch on corn leaves and used at their last instar. The fluorescent dye was diluted in water and deposited onto the surface of the leaves as small drops using a micropipette, and allowed to dry for ~3 hours at room temperature in the dark; the leaf pieces were kept inside a petri dish containing 1% agar to avoid excessive dehydration. After the caterpillars ate the whole dye or water-treated leaf pieces, they were transferred into a new plastic cup and allowed to feed on new untreated leaves for about two minutes, these leaf pieces were used for detection of fluorescence. The amount of secreted regurgitant was measured using a standard curve.

Detection of specific bacteria on plant leaves

To verify that gut bacteria get in contact with the plant wounds during caterpillar feeding, FAW caterpillars with reintroduced *Pantoea ananatis*, a bacterium found in the FAW gut, were allowed to feed on the plants for 30 – 60 min. The tissue around the feeding sites was harvested with clean scissors and forceps, placed into a sterile 2 ml tube with 2xYT liquid media and incubated overnight at 27 $^{\circ}$C in a rotary shaker at 200 rpm. Negative controls were caterpillars treated with antibiotics and fed with $MgCl_2$. The presence of *P. ananatis* in bacteria cultures was assessed through PCR using the specific primers developed by Figueiredo & Paccola-Meirelles (2012). The PCR reaction contained 0.4 µM of each primer (ANAF: 5'-CGT GAA ACT ACC CGT GTC TGT TGC -3' and EC5: 5'-TGC CAG GGC ATC CAC CGT GTA CGC T3'), 12.5 µl of the GoTaq Green Master Mix (Promega), 2 µl of the overnight liquid-grown bacteria previously diluted 1:5 in sterile water, and 8.5 µl of MQ water for a total volume of 25 µl. The PCR conditions had an initial denaturation step of 5 min. at 95 $^{\circ}$C, followed by 35 cycles of 95 ^{o}C for 1 min, 60 $^{\circ}$C for 30 sec, and 72 $^{\circ}$C for 1 min. and a final extension step of 5 min. at

72 ˚C. The DNA fragments were visualized in a 2% agarose gel stained with SyBr green under UV light. The electrophoresis was run in 1% TAE at 75 V for 45 min.

Protein activity assays

The activity of PPO and tryPI were measured as previously described (Chung & Felton 2011). The tryPI activity was calculated as PI (%) = (1-(slope of sample/slope of Non inhibitor))*100 and the resulting activity values normalized by the amount of protein (mg) contained in the sample. The POX activity was assayed as described in (Bi & Felton 1995) with minor modifications, 50 mg of leaf tissue were grounded in liquid nitrogen and homogenized in 1.25 ml of 0.1 M potassium phosphate buffer (pH. 7.0) containing 5% of cross-linked polyvinylpyrrolidone (PVP) (Alfa Aesar, Ward Hill, MA) and centrifuged at 11,000 g for 10 min at 4 ˚C. 5 µl of the supernatant were mixed with 10 µl of 3% H_2O_2 and 190 µl of 3 mM guaiacol (MP Biomedicals). The change in absorbance was measured at 450 nm for 5 min. The GOX activity in the caterpillar salivary glands was measured following the protocol developed by (Eichenseer *et al.* 1999) and adjusted for a microplate reader. PLC enzymatic assays followed a protocol previously described (Kurioka & Matsuda 1976; Le Chevalier *et al.* 2015) and adapted for a microplate reader.

Effect of bacteria on the FAW salivary protein expression

Previous studies have shown that proteins in the FAW caterpillar saliva modulate defense responses in plants (Chuang *et al.* 2014); therefore, we tested if the presence of *P. ananatis* would affect the qualitative and quantitative protein expression in the saliva of this insect. *P. ananatis* was reintroduced into last instar caterpillars (following the procedure described above) and their saliva collected for proteomics analysis; a control saliva sample was collected from caterpillars treated with antibiotics and $MgCl_2$. FAW caterpillars were chilled on ice for 45 minutes and immobilized into a metallic hairclip, as they warmed up, their saliva was collected using a micropipette tip (VWR cat No. 53509-015) under a light microscope (Olympus SZ30). Two saliva samples each containing 10 µg of saliva and 0.3978 µg of protease inhibitor (Sigma P2714) diluted in 10 µl of MQ

water were used for proteomic analysis using isobaric tags for relative and absolute quantification (iTRAQ). The protein samples were prepared following the Pennsylvania State University College of Medicine Mass Spectrometry and Proteomics Core Facility standard protocol [**See Appendix B]** as described in chapter 3.

Presence of *P. ananatis* in caterpillar frass

Along with saliva and oral secretions, caterpillars deposit frass onto the plants during feeding. I tested if bacteria present in the caterpillars' regurgitant would also be present in the caterpillars' frass. Caterpillars with re-introduced *P. ananatis* were fed with corn leaves for 2 days, the caterpillars were transferred to clean cups twice and fresh leaves provided every 12 hours. At the end of the second day, fresh frass pellets were collected and placed on 2 ml tubes with 1.5 ml of 2xYT liquid media and incubated overnight at 27 ˚C in a rotary shaker at 200 rpm. The Following day, 1 µl of the liquid grown bacteria was transferred into a new tube with 1.5 µl of sterile liquid media and incubated overnight at the same conditions described above. 2 µl of the grown bacteria (diluted 1:5) were used for the detection of *P. ananatis* through PCR.

Bacteria from FAW in other caterpillar species

I collected other caterpillar species feeding on insect-susceptible corn fields (c.v Providencia) at the Russell E. Larson Agricultural Research Center, Rock Springs, PA. I collected the following lepidopteran species: *Ostrinia nubilalis*, *Agrotis ipsilon* and *Helicoverpa zea*. Bacteria from their regurgitant were cultured in the same way described above for FAW. The 16S region was amplified from individual bacteria cultures and sequenced. The sequences of bacteria genera corresponding to those found in the FAW were aligned using Clustal 1.6 with a gap opening penalty of 15 and a gap extension penalty of 6.66; the resulting sequence alignment was used to construct phylogenetic trees using the UPGMA hierarchical clustering method. The test of phylogeny was done with the bootstrap method with 1,000 replications using the software MEGA 5.0 (Tamura *et al.* 2011).

Pathogenicity of *P. ananatis* on plants

Plant leaves were infiltrated with liquid cultures of *P. ananatis* (OD600 = 0.1) grown overnight on 2xYT media or liquid media alone. The plants were kept in greenhouse conditions for five days, time at which the leaves were detached from the plants and photographed.

Effect of bacteria on caterpillar growth

I tested the effect of six bacteria isolates from the FAW regurgitant on the relative growth rate of FAW caterpillars. Caterpillar neonates were placed on artificial diet with antibiotics [streptomycin (5 mg/100ml) and aureomycin (100 mg/100 ml)] for five days, after that they were transferred to new cups containing 0.8 g of artificial diet (without antibiotics) inoculated with 50 µl of individual bacteria isolates diluted in 10 mM of $MgCl_2$ (OD 600 = 0.1). Controls received diet with $MgCl_2$ alone. Fresh diet with bacteria was provided every two days in clean cups. The caterpillars were weighed two and five days later and their relative growth rate calculated as {(*W2 –W1)/ [((W1+W2)/2)*d*]}; where *W1* is the initial weight, *W2* is the final weight and *d* is the number of days between measurements (Mohan *et al.* 2008).

RNA extraction, cDNA synthesis and Real time PCR

Leaf tissue (50 - 70 mg) frozen in liquid nitrogen was homogenized in a GenoGrinder 2000 (OPS Diagnostics, USA) and the total RNA extracted using a modified Trizol protocol [**See Appensix A].** Complementary DNA (cDNA) was synthesized from 1 µg of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) using Oligo-dT. Quantitative real time PCR (qPCR) was conducted using the 7500 Fast Real-Time PCR System (applied Biosystems) with SYBR green (Roche Applied Science, USA) as described in chapter 2. The specific primers used for the target and reference genes (*mpi* and *actin*, respectively) were the same ones reported in Ray *et al.* (2015).

Experimental design and statistical analysis

The plant defense responses (PPO, tryPI, POX activities and *mpi* gene expression) to different treatments (caterpillar feeding, application of caterpillar regurgitant and liquid bacteria isolates), the effect of bacteria reintroduction on the activity of GOX and PLC in the caterpillars' salivary glands, and the effect of plant defense responses on caterpillar weight gain were analyzed with one-way ANOVA following the post hoc tests of Tukey and Fisher at $\alpha = 0.05$. These statistical analyses were performed using Minitab 16 (Minitab Inc., State College, PA, USA) and all graphs were generated in R version 3.2.2 (Foundation for Statistical Computing, Vienna, Austria). The statistical analysis of quantitative iTRAQ was done as described in chapter 3.

Results

FAW associated gut bacteria suppressed herbivore-induced defenses in tomato plants but not in maize.

Feeding by FAW caterpillars induced significantly higher PPO activity and *mpi* gene expression compared with undamaged control plants in tomato and maize, respectively. In tomato, feeding by field-collected caterpillars treated with antibiotics induced significantly higher PPO activity than the untreated ones ($t = 2.71$, $P = 0.018$, $N = 8$) (Fig. 5-1 a). In maize, antibiotic-treated caterpillars induced similar transcript accumulation of the *mpi* gene than the untreated caterpillar controls ($t = 0.83$, $P = 0.428$, $N=6$) (Fig. 5-1 b). The same pattern of defense induction was found when plants were treated with regurgitant from these caterpillars (Fig. 5-1 c-d). These results indicate that bacteria present in the caterpillars' regurgitant were modulating the defense responses observed in some plants.

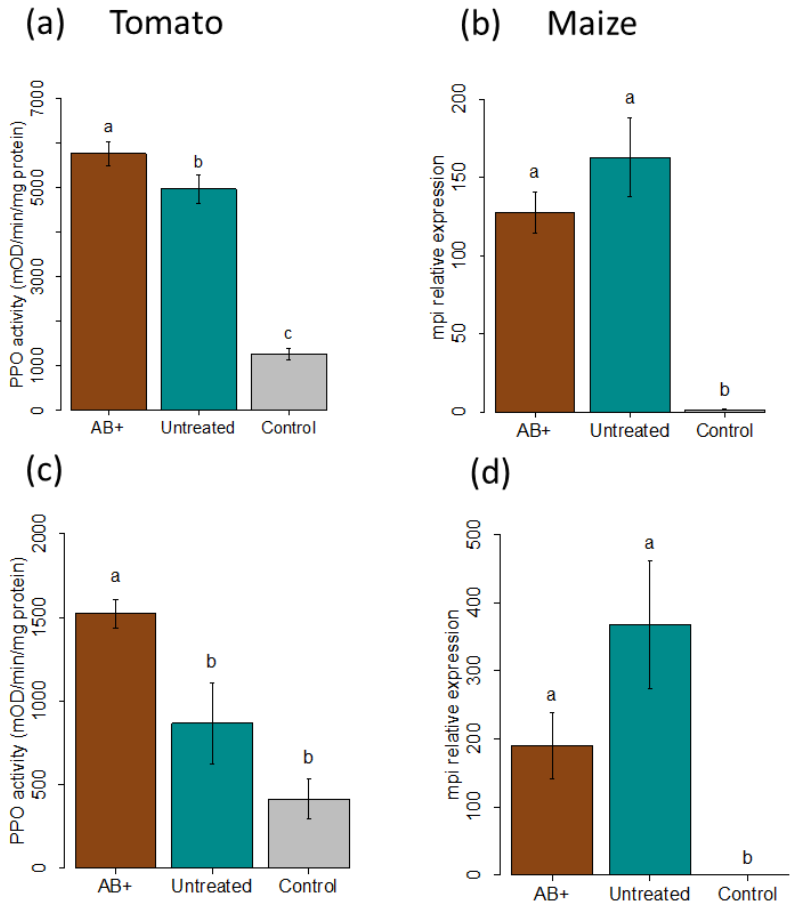

Figure 5-1. Plant defense response to feeding and regurgitant treatment from field-collected fall armyworm caterpillars treated (AB+) or untreated with antibiotics. (a) Polyphenol oxidase (PPO) activity in tomato plants fed by caterpillars ($F_{2,25}$ = 106.10, P = 0.000; n = 8 – 11; Fisher test). (b) *Maize Proteinase Inhibitor (mpi)* gene expression in maize plants treated with caterpillars ($F_{2,9}$ = 272.19, P = 0.000; n = 4; Fisher test; log transformed data). (c) PPO activity in tomato plants treated with caterpillar regurgitant ($F_{2,6}$ = 11.81, P = 0.0083; n = 3; Fisher test). (d) *mpi* gene expression in maize plants treated with caterpillar regurgitant ($F_{2,6}$ = 176.01, P = 0.000; n = 3; Tukey test). Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α=0.05. Controls are undamaged plants.

To identify bacteria present in the caterpillars' regurgitant, 15 bacteria samples (randomly picked) were sequenced. From those we identified five different bacteria genera within the family Enterobacteriaceae (Table 5-1). The percentage of identity was obtained with blast against the corresponding target sequences at NCBI. The *Pantoea* sp. isolate was further confirmed as *Pantoea ananatis* using specific primers (Figueiredo & Paccola-Meirelles 2012) and aligning the obtained sequence against the NCBI database using blast. The gene sequences obtained were deposited in GenBank and assigned accession numbers (Table 1). To further confirm that bacteria from the FAW gut were inducing defense responses, we applied individual bacteria isolates into wounded plants. In tomato plants, two isolates, *Pantoea ananatis* and Enterobacteriaceae-1 suppressed PPO and TryPI activity, but induced greater POX activity when compared with media-treated plants. *Raoultella* sp. and *Klebsiella* sp. had no effect on PPO but suppressed activity of POX and induced TryPI (Table 5-2). In maize plants all bacteria isolates (except for *Enterobacter* sp.2 and Enterobacteriaceae-2) induced higher *mpi* gene expression than the liquid media (Table 5-2).

Table 5-1. Bacteria isolates identified from the fall armyworm oral secretions.

Gen Bank Accession No.	**Name used in this paper**	**NCBI ID (% identity)**	**Rdp ID**	**Biotyper ID**	**Biotyper score**
KX161909	*Pantoea ananatis*	*Pantoea ananatis* (99%)	*Pantoea* sp.	*Pantoea ananatis*	2.443
KX161910	*Enterobacter* sp.1	*E. cloacae* *E. ludwigii* (99%)	*Enterobacter* sp.	*E. asburiae* *E. ludwigii*	2.304 2.264
KX161911	Enterobacteriaceae-1	*Rahnella aquatilis* *Serratia quinivorans* (99%)	*Serratia* sp.	*Rahnella aquatilis*	2.099
KX161912	*Raoultella* sp.	*R. ornithinolytica* (99%)	*Raoultella* sp.	*R. ornithinolytica* *R. planticola*	2.363 2.362
KX161913	*Klebsiella* sp.	*K. oxytoca* (97%)	Enterobacteriaceae	*K. oxytoca*	2.322
KX161914	*Enterobacter* sp.2	*Enterobacter* sp. (99%)	*Enterobacter* sp.	*E. cloacae* *E. asburiae*	2.031 2.029
KX161915	Enterobacteriaceae-2	*E. ludwigii* *Pantoea* sp. *Pantoea dispersa* (99%)	Enterobacteriaceae	**Pantoea agglomerans*	1.816

*Not reliable identification

Table 5-2. Plant defense response to the application of bacteria isolated from regurgitant of fall armyworm caterpillars. Asterisks (*) indicate significant differences from media-treated plants at $\alpha = 0.05$.

Plant treatment		Tomato						Maize	
	Bacteria isolate	PPO		POX		Trypsin PI		mpi	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Wounding +	*Pantoea ananatis*	870*	101	4103*	132	5481*	223	1538.4*	72.1
	Enterobacter sp.1	2720	385	3159	177	9826	899	1213*	212
	Enterobacteriaceae-1	991*	79.4	4515*	471	5548*	615	1963.3*	49.7
	Raoultella sp.	2951	390	2242*	123.2	16957*	1617	1138*	137
	Klebsiella sp.	3515	600	2513*	98.6	12951*	978	1241*	197
	Enterobacter sp.2	3102	286	3019	106	9347	615	1040	201
	Enterobacteriaceae-2	2862	174	3780	174	9092	935	813	119
	Media	2995	366	3435	200	8858	699	695	137
Untreated controls		776*	114	441.7*	89.2	1693*	358	2.1*	0.421

Plant defense responses triggered by FAW gut bacteria affected caterpillar growth

To test if the defense response elicited by bacteria from the FAW gut had any effect on caterpillar growth, plants were treated with individual bacteria isolates and the leaves used to feed young caterpillars. Caterpillars fed with detached leaves from tomato plants treated with *P. ananatis* and Enterobacteriaceae-1 gained more weight than those fed on tomato leaves treated with media (Fig. 5- 2 a). In maize, caterpillars gained less weight when fed on leaves previously treated with *P. ananatis* and Enterobacteriaceae-1 (Fig. 5-2 b). Caterpillars grown on leaves treated with *Enterobacter* sp.1 gained similar weight than those treated with media.

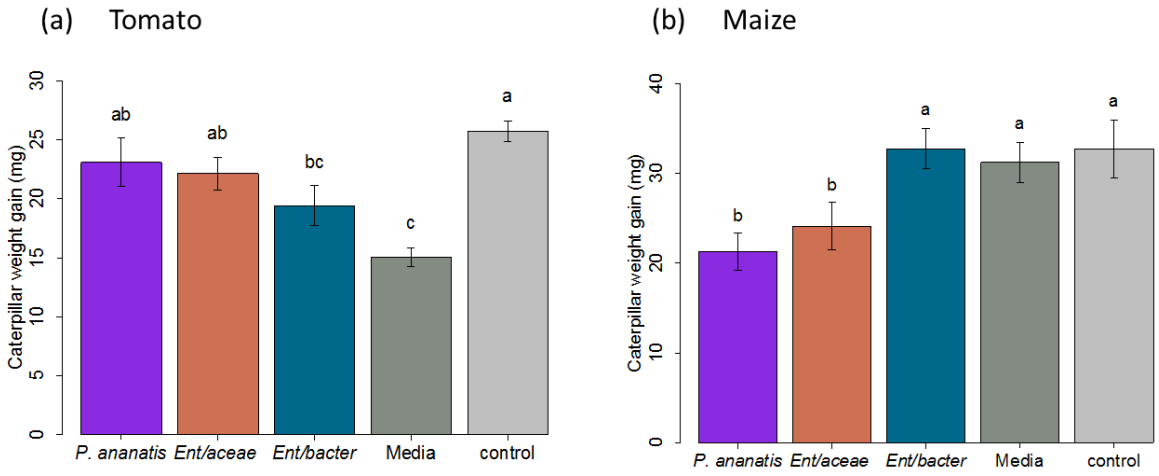

Figure 5-2. Caterpillar weight gain after feeding on plants treated with bacteria from the fall armyworm regurgitant. (a) Weight gain (mg) of caterpillars fed on treated tomato plants ($F_{4,45}$ = 8.98, P = 0.000; n = 10; Tukey test; log transformed data). (b) Weight gain (mg) of caterpillars fed on treated maize plants ($F_{4,25}$ = 6.24, P = 0.000; n = 4 - 8; Fisher test; log transformed data). Values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α = 0.05. *Ent/aceae* = *Enterobacteriaceae-1*, *Ent/bacter* = *Enterobacter sp.1*, controls are undamaged plants.

FAW caterpillars secreted regurgitant into plants during feeding

To verify that FAW caterpillars regurgitate while feeding on plants, we fed caterpillars with a florescent dye and quantified the amounts of regurgitant secreted on the feeding sites. All caterpillars tested (N = 10) regurgitated on the plants tested (Fig. 5-3). On average each caterpillar secreted 1.55 nl and 3.29 nl of regurgitant per feeding bout in maize and tomato respectively (Fig. 5-3)

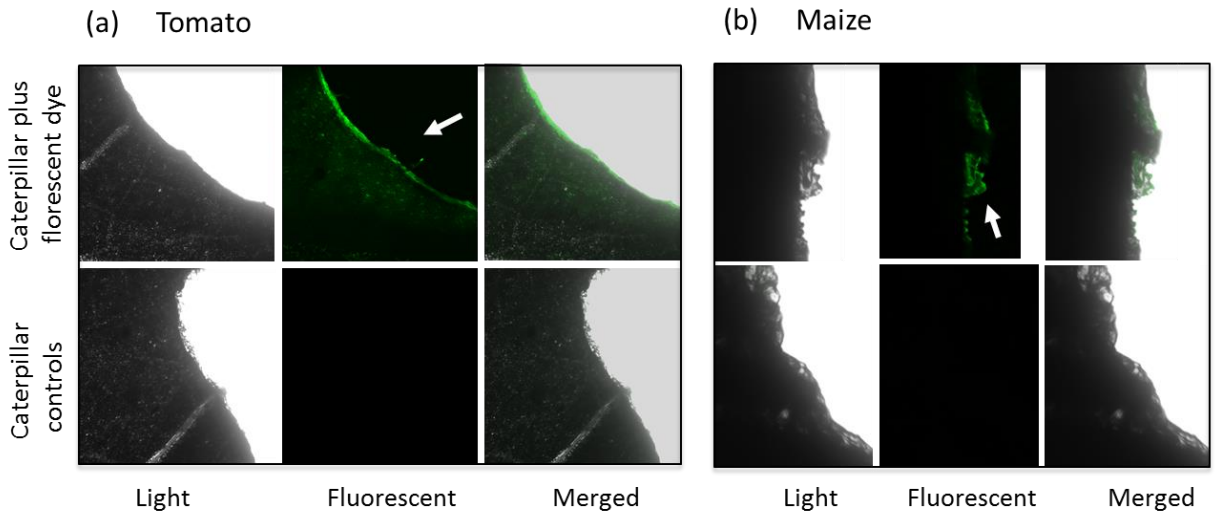

Figure 5-3. Detection of regurgitant secreted by fall armyworm caterpillars on (a) tomato and (b) maize leaves using fluorescent microscopy.

Pantoea ananatis was secreted onto plants during FAW feeding

To confirm that *P. ananatis* was modulating plant defense responses triggered during caterpillar feeding, this bacterium was reintroduced into caterpillars and these allowed to feed on plants. In tomato, caterpillars with reintroduced *P. ananatis* induced less PPO activity than caterpillars treated with buffer ($t = -3.08$, $P = 0.0132$; $N = 5$-6) (Fig. 5-4 a). In maize, the gene expression levels of *mpi* induced by caterpillars with or without *P. ananatis* were not significantly different from each other ($t = -0.28$, $P = 0.779$; $N = 10$) (Fig. 5-4 b).

I verified that *P. ananatis* comes in contact with damaged plant tissue during caterpillar feeding by doing PCR on the bacteria grown close to the feeding sites. *P. ananatis* was detected on 100% ($N = 14$) of the tomato plants and 60% ($N = 10$) of the maize plants fed by caterpillars with the re-introduced bacteria. *P. ananatis* was not found on plants fed by caterpillars treated with antibiotics and $MgCl_2$ (Fig. 5-4 c-d).

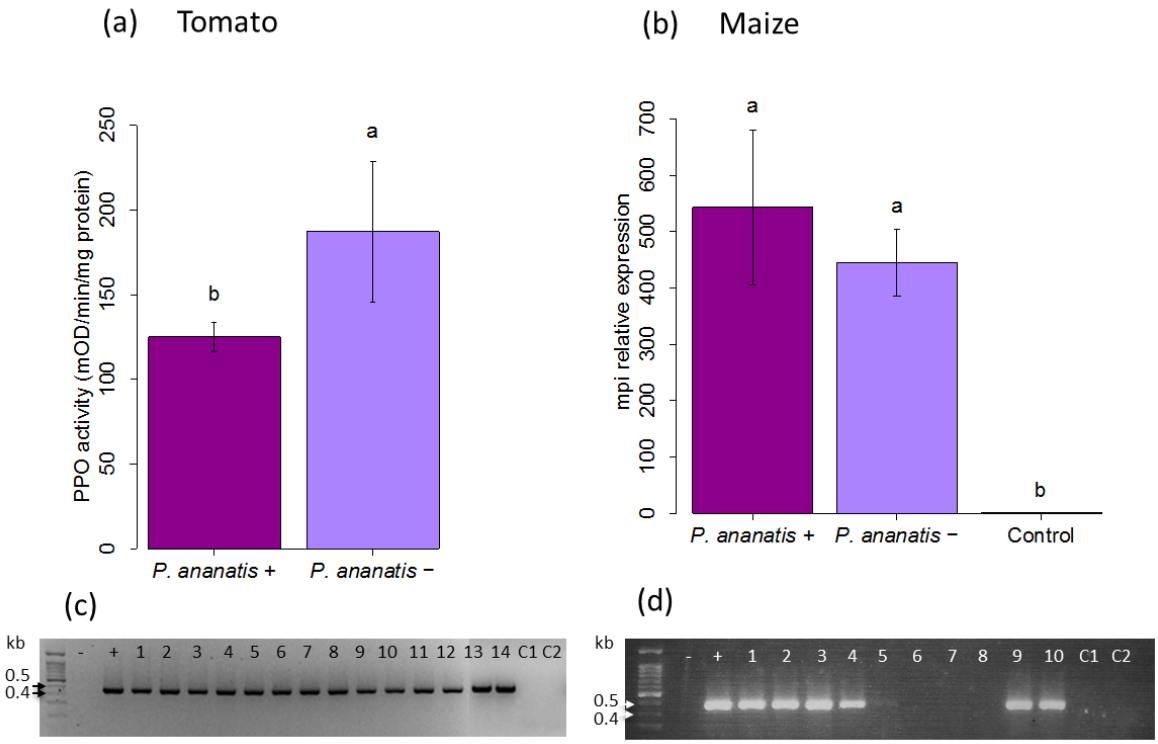

Figure 5-4. Plant defense response to caterpillar feeding with (*P. ananatis* +) or without (*P. ananatis* -) reintroduced *P. ananatis*. (a) Polyphenol oxidase (PPO) activity in tomato plants (t = -3.08, P = 0.0132; n = 5 – 6). (b) *Maize Proteinase Inhibitor (mpi)* gene expression in maize plants ($F_{2,27}$ = 528.98, P = 0.000; n = 10; Fisher test; log transformed data). (c) Presence of *P. ananatis* in tomato leaves fed by caterpillars with the reintroduced bacterium. Negative and positive PCR controls are depicted with (–) and (+) signs, lines 1-14 are regurgitant samples from caterpillars with reintroduced *P. ananatis*, while C1 and C2 are regurgitant samples from caterpillar controls. (d) Detection of *P. ananatis* in maize leaves fed by caterpillars with the reintroduced bacterium. (-) and (+) signs in the agarose gels are negative and positive PCR controls respectively. Lines 1-14 are regurgitant samples from caterpillars with reintroduced *P. ananatis*, while C1 and C2 are regurgitant samples from caterpillar controls. Bars are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α=0.05. Controls are undamaged plants.

Gut bacteria did not affect GOX and PLC activity in the FAW salivary glands

I tested the effect of *P. ananatis* and Enterobacteriaceae-1 on the activity of two salivary enzymes, GOX and PLC. The activity of these enzymes in salivary glands from caterpillars with reintroduced bacteria was not significantly different from the activity of caterpillars treated with $MgCl_2$ (Fig. 5-5).

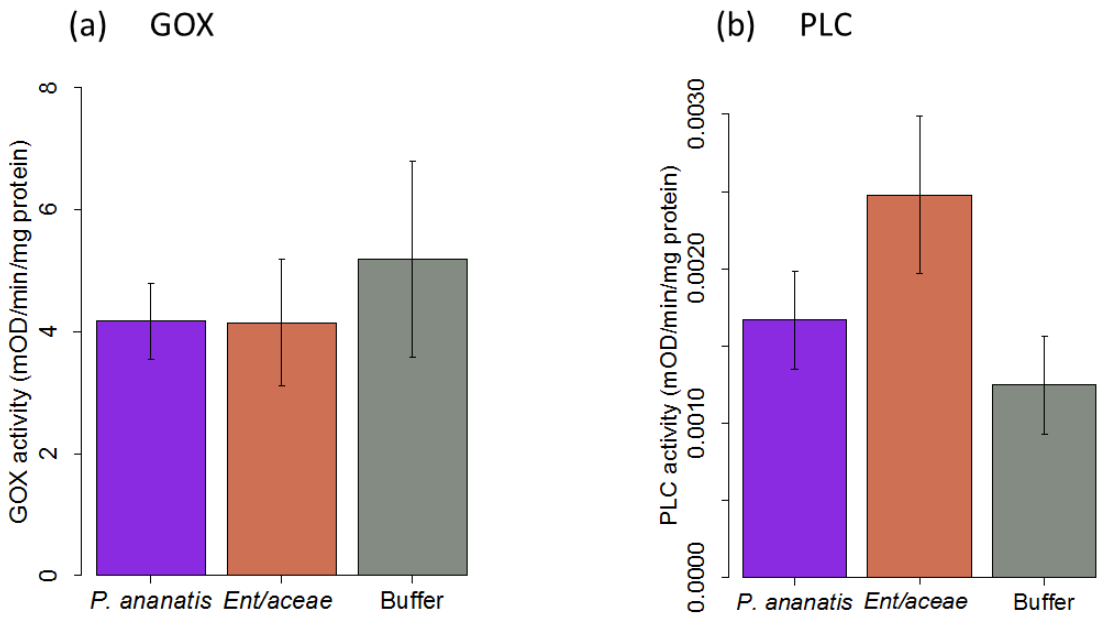

Figure 5-5. GOX and PLC activity in the fall armyworm salivary glands with reintroduced *P. ananatis*, *Enterobacteriacea-1 (Ent/aceae)* or buffer ($MgCl_2$). (a) GOX activity ($F_{2,16}$ = 0.27, P = 0.765; n = 5 – 7). (b) PLC activity ($F_{2,16}$ = 2.25, P = 0.138; n = 5 - 7). Bars are untransformed means ± SEM.

Gut bacteria did not affect quantitative protein expression in the FAW saliva

There were 4,936 spectra identified at 95% confidence out of 315,667 MS/MS spectra generated. From the spectra identified, 1840 were distinct peptides. The salivary protein abundances from caterpillars with reintroduced *P. ananatis* were not significantly different (qLFDR > 0.05) from caterpillars treated with antibiotics and $MgCl_2$ **(Appendix E)**.

Bacteria from FAW was present in other insect species feeding on maize

I tested if FAW bacteria were present on three different insect species found in corn fields: The corn earworm, *Helicoverpa zea*, the European corn borer, *Ostrinia nubilalis* and the black cutworm, *Agrotis ipsilon*. We found *P. ananatis* in caterpillars of the black cutworm, Enterobacteriacea-1 and *Enterobacter* sp.1 in caterpillars of the corn earworm (Fig. 5-6).

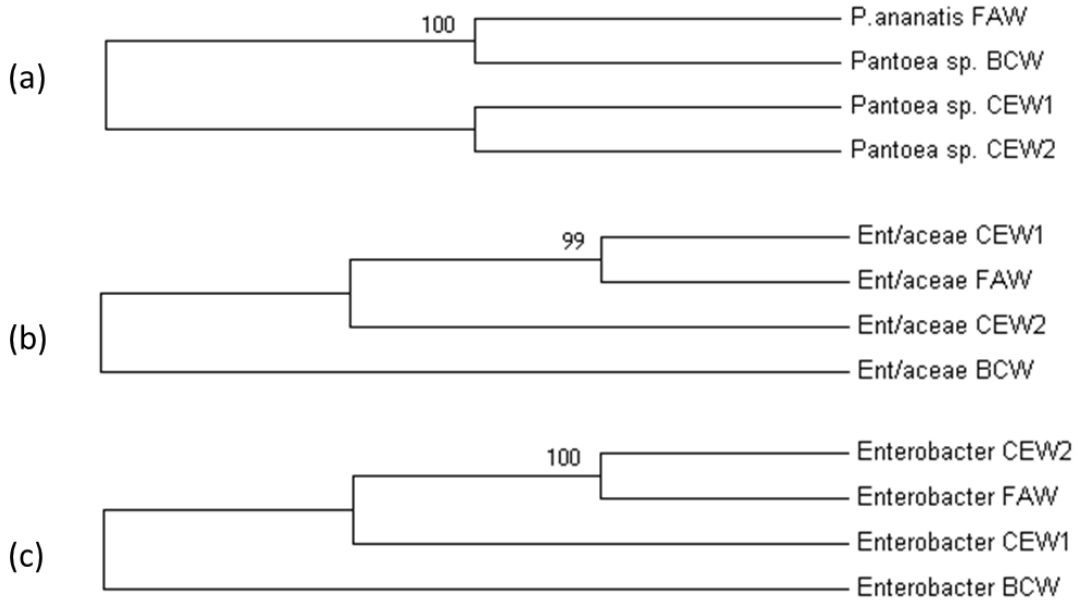

Figure 5-6. Phylogenetic trees of the 16S- rRNA gene sequences of different bacteria isolated from FAW caterpillars. (a) *Pantoea sp*., (Gen Bank accession numbers: KX161909, KX161916, KX16197, KX161918) (b) *Enterobacteriacea*-1 (KX161910, KX161920, KX161921, KX161919), and (c) *Enterobacter sp*.1 (KX161911, KX161922, KX161923, KX161924). The trees were built using the 16S rRNA sequences of bacteria isolates cultured from the regurgitant of three caterpillars species found in maize fields. FAW = fall armyworm, CEW = Corn earworm, BCW = black cutworm.

P. ananatis was found in the FAW caterpillar frass

During feeding, caterpillars deposit large amounts of frass that gets in contact with the plant wounded sites eliciting defense responses (Ray *et al.* 2015). We tested if the reintroduced *P. ananatis* was present in the caterpillar frass. This bacterium was found on 60% of the samples tested (N = 10) (Fig. 5-7).

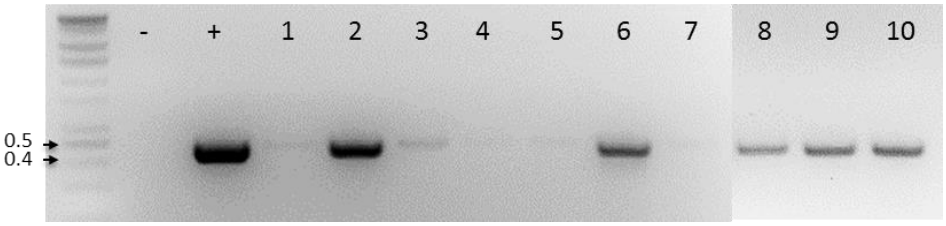

Figure 5-7. Presence of *P. ananatis* in frass from fall armyworm caterpillars with reintroduced bactera. (-) and (+) are negative and positive PCR controls respectively, lines 1-10 are frass samples from caterpillars with reintroduced *P. ananatis*.

P. ananatis induced hypersensitive-like response in tomato leaves

The *P. ananatis* strain found in the FAW gut may be phytopathogenic; I found a hypersensitive-like response on tomato leaves treated with bacteria, but not on leaves treated only with media. I observed no sign of pathogenicity in maize (Fig. 5-8).

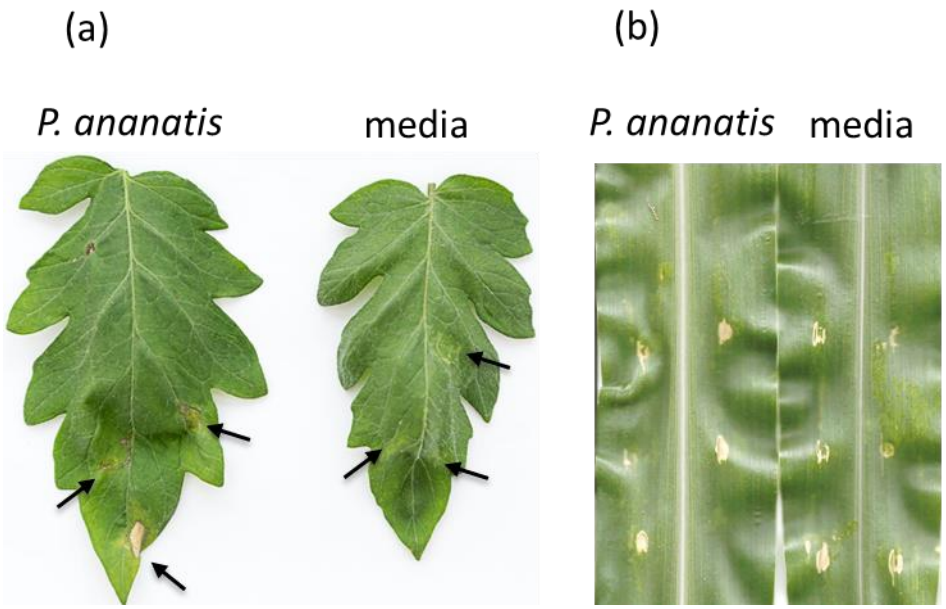

Figure 5-8. Hypersensitive-like response in (a) tomato and (b) maize plants five days after infiltration with *P. ananatis* and 2xYT media.

Bacteria from FAW oral secretions did not affect caterpillar growth

I tested the effect of bacteria isolates from FAW regurgitant on the relative growth rate of young caterpillars. Caterpillars grown on artificial diet containing bacteria gained the same weight than those fed on $MgCl_2$. There was one bacteria isolate, *Raoultella* sp., that enhanced caterpillar weight gain (Fig. 5-9).

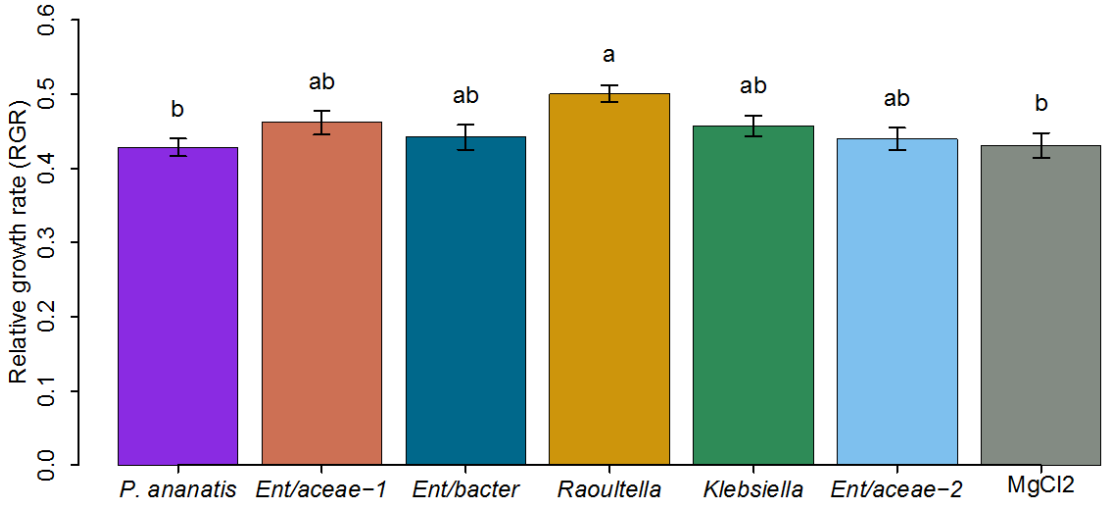

Figure 5-9. Relative growth rate (RGR) of fall armyworm (FAW) caterpillars grown on artificial diet supplemented with bacteria ($F_{6,203}$ = 2.9, P = 0.010; n = 30). Ent/aceae = Enterobacteriaceae, *Ent/bacter* = *Enterobacter sp.1*. Bars are untransformed means ± SEM.

Discussion

This study shows that symbiotic bacteria from the OS of FAW caterpillars modulate herbivore-induced plant defenses, which affect the performance of this insect species on specific hosts. Plant treatment with either field-collected caterpillars or their regurgitant induced less anti-herbivore defenses in tomato plants compared with caterpillars treated with antibiotics (Fig. 5-1 a-c). This suggests that in addition to fatty acids and hydrolytic enzymes (Schmelz 2015), the oral secretions of lepidopteran caterpillars also contain microbes able to modulate defense responses in plants. From the microbial community present in the oral secretions of wild FAW caterpillars, I identified five different bacteria genera belonging to the family Enterobacteriaceae, which is a common bacteria family present in phytophagous insects (Sugio *et al.* 2015). Five out of seven bacteria isolates elicited defense responses in tomato and maize upon their application to wounded leaves (Table 2). From these, *P. ananatis* and Enterobacteriaceae-1 (*Serratia/Rahnella*) suppressed herbivore defenses in tomato compared with controls

treated with growing media. Similar results were obtained when plants were fed by caterpillars to which *P. ananatis* had been re-introduced (Fig. 5-4 a). These results confirm that plant defenses were in fact being regulated by the presence of at least one of these bacteria isolates. However, it is possible that other bacteria taxa, not identified by our methods, could have contributed to the observed plant defense responses. The results of this study also show that FAW caterpillars actively secreted regurgitant on their feeding sites (Fig. 5-3). Furthermore, *P. ananatis* was deposited onto the plants during caterpillar feeding (Fig. 1c-d). This suggests that bacteria from the FAW oral secretions can easily get in contact with plant wounds where they can directly modulate defense responses. These results agree with a previous study in which bacteria associated with the phytophagous Colorado potato beetle, *Leptinotarsa decemlineata,* suppressed herbivore-induced defenses in tomato plants (Chung *et al.* 2013). But this study expands the relevance of gut associated microbes and plant defense regulation by lepidopteran caterpillars.

The effect of FAW-associated bacteria on plant defense responses is host-specific. In tomato plants, *P. ananatis* and Enterobacteriaceae-1 suppressed herbivore defenses; but in maize these bacteria isolates along with *Enterobacter* sp.1, *Raoultella* sp. and *Klebsiella* sp. induced transcript accumulation of the protease inhibitor gene *mpi* (Table 5-2). This could be due to differences in signal transduction pathways or receptor mediated recognition between these two host plants. Interestingly, when maize plants were treated with either wild caterpillars or caterpillars with reintroduced bacteria, the induction levels of the *mpi* gene were not significant different from controls (Fig. 5-1 b and 5-4 b). This could probably be explained by the low regurgitation rates of the FAW when feeding on maize (Fig. 5-3 b), or by adverse interactions of these bacteria with fragmented maize leaves inside the insects' gut chamber. It has been reported that diet type and their associated chemical defenses modify the composition of insect microbial communities in lepidopterans (Priya *et al.* 2012; Mason *et al.* 2015).

Members of the same bacteria genera identified in the oral secretions of FAW caterpillars have been reported in other Lepidoptera species, with the most common being *Pantoea* and *Enterobacter* (Broderick *et al.* 2004; Robinson *et al.* 2010; Priya *et al.* 2012; Tang *et al.* 2012). The few studies in Lepidopterans have shown a very low diversity in microbial composition of this group with some overlap of specific taxa (Sugio *et al.* 2015). It has been suggested that the highly alkaline conditions of Lepidoptera guts may negatively correlate with the levels of microbial diversity, and therefore may select for a few taxa able to tolerate these conditions (Engel & Moran 2013). Some of the bacteria isolates associated with FAW were also found in the regurgitant of *H. zea* and *A. ipsilon* feeding on maize in field conditions (Fig. 5-6). This suggests that these bacteria may have been acquired from their common host plant. Studies in other Lepidopterans have shown that a great proportion of their larvae gut bacteria is obtained from the host they feed on (Priya *et al.* 2012; Mason & Raffa 2014). However, the composition of gut bacteria communities are also influenced by the taxonomic group to which insects belong to (Colman *et al.* 2012), which may partially explain why none of the FAW associated bacteria was found in the European Corn Borer, *Ostrinia nubilalis*, which is from a different family, though the methods used in this study only partially surveyed the bacteria community of these insect species. Also, the effect of these bacteria on plant defense regulation may be different when associated with different caterpillar hosts species. It has been shown that not all caterpillars secrete oral secretions during their feeding activity (Peiffer & Felton 2009) which may be explained by differences in their gut morphology; caterpillars that usually regurgitate seem to have larger crops and smaller midguts than the ones that do not (Grant 2006).

The mechanisms by which the FAW-associated bacteria regulate plant defenses are unknown. The results of this study show that these bacteria 1) were present in the insect oral secretions, 2) regulated herbivore-induced defenses when applied to wounded plants, 3) were secreted onto the plants through the insect regurgitation, and 4) had no effect on either the activity or the protein expression of the FAW saliva. Therefore, these results suggest that either the bacteria themselves or bacteria-derived components are

eliciting specific plant defensive pathways. Some strains of *P. ananatis*, one of the defensive-suppressing bacteria in tomato plants, have been previously reported as pathogens of several plant species including maize and tomato (Coutinho & Venter 2009). The specific strain found in FAW induced a hypersensitive-like response in tomato and may be pathogenic (Fig. 5-8). *P. ananatis* has an extraordinary capacity of adapting to different living conditions; for example, it can have an endophytic, epiphytic, pathogenic or symbiotic association with their host plants (Coutinho & Venter 2009); it can also be found as a symbiont in insect guts (Wells *et al.* 2002; Murrell *et al.* 2003) and even as a human pathogen (De Baere *et al.* 2004). The ability of *P. ananatis* to live in insect guts facilitates its transmission to their host plants; it has been shown that this bacterium is vectored by the tobacco thrips, *Frankliniella fusca* (Wells *et al.* 2002). Insects are common vectors of plant pathogenic bacteria, for instance, *Pantoea stewarti*, the causing agent of the Stewart's wilt disease in maize, is vectored by the corn flea beetle *Chaetocnema pulicularia* possibly through their insect frass (Nadarasah & Stavrinides 2011). Although not tested in this study, *P. ananatis* could potentially be vectored by FAW caterpillars and transmitted through the insects' oral secretions and/or possibly frass. FAW can benefit from this association by the downregulation of plant defenses in some hosts without an apparent effect on its growth and development (Fig. 5-9). The pathogenicity of *P. ananatis* and *P. stewarti* seem to be regulated by the production of quorum-sensing (QS) signal molecules that activate the production of exopolysaccharides (EPS) and formation of biofilm leading to infection (Koutsoudis *et al.* 2006; Morohoshi *et al.* 2007). *P. ananatis* produces two QS molecules, N-acyl-L-homoserine lactone and N-(3-oxohexanoyl)-L-homoserine lactone (Morohoshi *et al.* 2007); from these, the former activates defense responses in tomato and *Arabidopsis thaliana* (Schuhegger *et al.* 2006; Schenk *et al.* 2014). FAW-associated bacteria is likely to be recognized by plants as a MAMP (Microbe Associated Molecular Patterns), inducing pathogen defenses that downregulate herbivore defenses by crosstalk of signal pathways.

I conclude that FAW gut-associated microbes regulate herbivore-induced defenses and enhance insect performance in tomato plants. *P. ananatis* and Enterobacteriaceae-1 (*Serratia/Rahnella*) were some of the defense-suppressing bacteria identified in the FAW oral secretions. These bacteria species did not alter salivary protein expression or the activity of insect salivary enzymes; therefore, they appear to directly regulate plant defenses upon their secretion through the insects' regurgitant and frass. This study contributes to the understanding of the mechanisms of plant defense regulation by lepidopteran caterpillars.

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Chapter 6

Physical plant defenses induced by fall armyworm herbivory and insect counter adaptation mechanisms

Abstract

Plants have a diverse array of structural defenses including leaf hairs, thorns, lacticifers and leaves strengthened with lignin, cellulose and minerals that constitute the first line of defense to insect herbivory. The goals of this study were to investigate the effect of herbivory on induction of trichomes and mineral deposition in plant leaves as well as counter insect adaptation mechanisms to overcome these defenses. I used the polyphagous insect fall armyworm (FAW) *Spodoptera frugiperda*, and tomato, maize, rice and Bermuda grass plants as a model system. Feeding by FAW caterpillars induced production of the glandular trichomes type VI in tomato, but induction was down regulated by unknown components in caterpillar saliva. Likewise, insect feeding induced accumulation of silica in maize and rice leaves but not in tomato. Deposition of silica in plant leaves induced caterpillar mandible wear in a dose-dependent manner. As an evolutionary adaptation to plant feeding, FAW caterpillars enrich the cutting edges of their mandible with zinc and chlorine to increase harness and reduce wear. FAW caterpillars were also able to plastically modify their morphology and development in response to leaf toughness. When feeding on hard rice leaves, these caterpillars increased the head size to house larger mandibular muscles and potentially increase their biting force. Furthermore, about 43% of rice-fed caterpillars underwent and additional molt before pupation. I conclude that FAW feeding modulates the induction of some plant physical defenses, which in turn induced plastic physiological and morphological changes in the insect. These results highlight the importance of physiological and morphological plastic adaptations of this insect to feed on different host plant species.

Introduction

Plants have evolved a variety of defense mechanisms to protect themselves against abiotic and biotic stressors including herbivores. Plant physical structures include trichomes, thorns, spines, lacticifers, lignification, and mineral deposition that are the first barriers against herbivorous insects (War *et al.* 2012). Plant chemical defenses include secondary metabolites, proteins and enzymes that have a negative effect on herbivores (Mithöfer & Boland 2012). Some of these defenses are constitutive, but some are induced by herbivory. The influence of insect feeding, their oral secretions and saliva on induction of plant chemical defenses has been extensively studied in a variety of plant-herbivore systems (Acevedo *et al.* 2015; Kaloshian & Walling 2015; Schmelz 2015; Stuart 2015), but their effect on induction of plant structural defenses is less well understood.

Plant leaf cuticles usually contain structural defenses in the form of hairs or trichomes that play important defensive roles against insect herbivores (Wagner *et al.* 2004; Glas *et al.* 2012). Trichomes are projections of the leaf epidermis that can be glandular or non-glandular; the former produce chemical compounds or store them in glands to be secreted, while the latter are non-secreting extensions of the leaf surface (Wagner *et al.* 2004). Glandular trichomes produce a variety of compounds including terpenes, phenolics, and flavonoid glycosides that can be highly toxic to herbivores (Wagner *et al.* 2004). Tomato plants contain seven types of trichomes from which at least four are glandular; their main constituents are acyl sugars, terpenes and methyl ketones (Glas *et al.* 2012; Bergau *et al.* 2015). The density of type VI trichomes is highly influenced by mechanical and insect damage (Tian *et al.* 2012). Maize plants contain three types of trichomes: macrohairs, prickle hairs and bicellular microhairs that serve as a physical defense mechanism against herbivory (Moose *et al.* 2004). Although the composition of these trichomes is unknown, their density appears to be induced after treatment with jasmonic acid, cytokinins and gibberellins (Maes & Goossens 2010). In

general, plant trichomes serve as physical barriers to insect feeding and oviposition by deterring, poisoning or killing herbivores (Valkama *et al.* 2003; Hanley *et al.* 2007).

Among other structural defenses, plants deposit minerals in their leaf surface (War *et al.* 2012). High concentration of certain minerals can directly affect the survival and development of herbivorous insects (Popham & Shelby 2006). One of the best examples is the accumulation of silicon dioxide or silica (Si); Si is up taken by the roots from the soil as silicilic acid and deposited in plant tissues as hydrated amorphous silica in special bodies known as phytoliths (Hunt *et al.* 2008). Phytoliths affect herbivores by increasing the abrasiveness of the leaves, wearing insect mandibles and deterring feeding. Besides this physical protection, upon damage, Si can also enhance production of chemical defense compounds and secondary metabolites effective against insects (Huitu *et al.* 2014). Si is accumulated at especially high amounts in grasses compared with dicots and its deposition is induced by mechanical damage and herbivory in several plant species (McNaughton & Tarrants 1983; Seastedt *et al.* 1989; Garbuzov *et al.* 2011; Reynolds *et al.* 2012).

The mechanical interaction with plants during feeding also elicits a variety of responses in insect attackers. Insect mandibular morphology and its musculature are often associated with the type of plant diet; grass feeders tend to have morphologically differentiated mandibles from those found in dicot-feeders (Clissold 2007). Insect mandibles are sclerotized structures mainly composed of chitin and proteins; adjacent chains of chitin are cross-linked by hydrogen bonds to form chitin microfibrils (Klowden 2008). The physical properties of insect mandibles are affected by cuticular thickness, chitin and protein content, arrangement of chitin fibers, level of hydration and metal deposition (Andersen *et al.* 1996; Klowden 2008). Some insects increase the hardness of their mandibles by incorporating minerals such as zinc, manganese, copper and calcium (Hillerton *et al.* 1984; Schofield *et al.* 2002; Cribb *et al.* 2005, 2008; Klowden 2008). These metals are mainly deposited in the cutting edges of the mandibles to enhance hardness and reduce abrasive wear during feeding (Schofield *et al.* 2002; Cribb *et al.*

2008). In addition to these adaptations, herbivores are also able to adjust their morphology, physiology and behavior to feed on a variety of host species. For instance, in response to laticifers, caterpillars of different species cut or trench latex containing veins before eating the distal part of the leaves; this behavior allows the insect to avoid the harmful sticky latex of their host plants (Dussourd & Denno 1994; Bernays *et al.* 2004; Darling 2007). The trenching behavior appears to be adaptive, as generalist caterpillars only trench plants containing latex and their weight gain is affected by their trenching capability (Dussourd & Denno 1994). Plastic morphological adaptations have also been observed in different insect species in response to plant feeding (Bernays *et al.* 1991). When feeding on hard grasses, caterpillars of *Pseudaletia unipuncta* had larger head mass, head width and greater area of mandibular adductor muscle attachments compared with caterpillars fed on soft wheat seedlings or artificial diet (Bernays 1986). Likewise, tough rye grass leaves induced development of larger heads in the grasshopper *Melanoplus femurrubrum* compared to those grown on red clover (Thompson 1992). Together, these studies illustrate the presence of host-specific insect counter adaptations to plant physical defenses.

The polyphagous herbivore fall armyworm (FAW) *Spodoptera frugiperda,* feeds on both dicots and monocots, and therefore faces the challenge of dealing with a diverse arrangement of both physical and chemical plant defenses. Previous work has shown that feeding by FAW caterpillars induce biochemical defense responses in several plants (Chapters 2 & 4 this thesis), but their feeding effect on the induction of physical plant defenses has not been addressed before. In this study I tested the effect of FAW herbivory on induction of trichomes and silicon accumulation in tomato, maize and rice plants. I also examined counter adaptation mechanisms used by this insect to overcome plant physical defenses.

The results of this study show that feeding by FAW caterpillars induces production of glandular trichomes in tomato, but this induction is downregulated by unknown components present in their saliva. FAW feeding also induced silicon

accumulation in rice and maize plants. In response to leaf toughness, caterpillars plastically increased their head capsule size to house larger mandibular muscles that could presumably increase their biting force. Furthermore, as a general adaptation to plant feeding FAW caterpillars accumulate minerals in their mandibles that aid in reducing wear during their feeding activity.

Methods

Insects

The FAW caterpillars were obtained from sweet corn fields at Hendy and Palm Beach County (South Florida) and the colony maintained in laboratory conditions at the USDA- ARS in Gainesville, Florida.

Plants

Maize plants (*Zea mays*, inbred line B73) were grown in Hagerstown loam soil until they reached the V8-V9 physiological stage. Rice plants (*Oryza sativa*, cultivar Nipponbare) were obtained from the USDA-ARS Dale Bumpers National Rice Research Center in Arkansas and grown as described in chapter 2. Bermuda grass (*Cynodon dactylon*) were grown in Hagerstown loam soil and used four weeks after germination (V8-V9 physiological stage). Tomato plants (*Lycopersicon esculentum,* cultivar Better Boy) were grown in Promix potting soil (Premier Horticulture Quakertown, PA, USA) in four-inch 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 hours light: 10 hours dark) at the Pennsylvania State University, University Park.

Energy Dispersive x-ray Spectroscopy (EDS) and elemental mapping

EDS and elemental mapping were carried out under low vacuum mode in a FEI Quanta 200 ESEM, equipped with a 10 mm silicon drift detector and Aztec software

version 2.3 (Oxford Instruments). The EDS measurements were done at a high voltage of 20 Kv, spot size of seven, and working distance of 12.5 mm under vacuum conditions of 70 Pa. EDS Id of representative areas of each sample were obtained with a process time of five and 500,000 counts using pulse pile up correction. These analyses were carried out at the Penn State Microscopy Facility.

Effect of FAW herbivory on the induction of leaf trichomes

I tested the effect of caterpillar feeding and their saliva on the density of trichomes in maize and tomato plants. Each plant, with five full-expanded leaves, was treated with two actively feeding last-instar caterpillars for 24 hours; after this time, the caterpillars were removed and the plants were allowed to grow for 10 more days. The effect of caterpillar saliva on the density of trichomes was tested by exposing plants to caterpillars with heat-cauterized spinnerets (ablated). Ten days after caterpillar damage, the 9^{th} leaf of each plant was harvested and used for trichome quantification. Two circular samples, each of 33.18 mm^2 for tomato, and 285.02 mm^2 for maize, were used for the quantification of trichomes under a dissecting microscope (Olympus SZ30). The density of trichomes in plants exposed to caterpillars was compared against untreated controls in a complete randomized design. Some of these leaf discs were prepared for Scanning Electron Microscopy (SEM) by immersing them in fixative solution (2.5 % glutaraldehyde, 1.5 % formaldehyde in 0.1 M sodium cacodylate buffer pH. 7.4), followed by dehydration through serial ethanol dilutions and critical point dried with liquid CO2. These leaf samples were then mounted in aluminum stubs with carbon tape and imaged in a FEI Quanta 200 ESEM.

Effect of FAW herbivory on silicon accumulation in plant leaves

The effect of FAW herbivory on the leaf accumulation of silicon was tested in maize, rice and tomato plants. Each plant, with five full-expanded leaves, was treated with two actively feeding last-instar caterpillars for 24 hours; after this time, the caterpillars were removed and the plants were allowed to grow for 10 more days. The new regrowth leaves were harvested, dried to constant weight at 55 $^{\circ}C$ and grounded to

powder for mineral analysis. The amount of silica was quantified using the molybdenum blue method reported in Diogo & Wydra (2007). The effect of FAW herbivory on plant silicon accumulation was compared with mechanical damage and untreated control plants in a complete randomized design. Leaf samples for silicon mapping were immersed in fixative solution, dehydrated and critical point dried (as explained above). Samples were mounted in aluminum stubs with carbon tape and imaged following the EDS and elemental mapping conditions explained above.

Effect of silicon on caterpillar mandible wear

I tested the effect of silicon on caterpillar mandible wear in two different ways; first, by feeding caterpillars with plant leaves containing different amounts of silica, and second, by incorporating silica into artificial diets at different concentrations. For the plant treatments, caterpillars were grown on detached maize leaves (in 1 oz plastic cups containing 1 ml of 3 % agar) for their first five instars, newly molted six-instar caterpillars were then transferred to new cups containing either artificial diet or detached leaves from maize, Bermuda grass and rice for three days. For the diet treatments, caterpillars were grown in artificial diet (wheat germ) for their first five instars. Newly molted six-instar caterpillars were transferred to cups containing artificial diet with three different concentrations (0 %, 2.5 % and 5 %) of silicon dioxide (Sigma S5631). After three days of feeding, the caterpillar mandibles were dissected and stored at – 20 $^{\circ}$C until use. Thawed samples were dehydrated through serial ethanol dilutions and critical point dried with liquid CO_2. The samples were then mounted in aluminum stubs with carbon tape and imaged in a FEI Quanta 200 ESEM.

Elemental analysis of the FAW mandibles

The elemental composition of the FAW mandibles was characterized using Energy Dispersive x-ray Spectroscopy or EDS. Mandibles extracted from last instar caterpillars grown on either maize leaves or artificial diet were dehydrated through serial ethanol dilutions and critical point dried with liquid CO_2. For elemental mapping the samples were then mounted in aluminum stubs with carbon tape. For EDS the mandibles

were immersed in Spurr's resin, polymerized for three days at 60 ˚C and cut transversally with a microtome 10 µm from their tips. EDS and elemental mapping were carried out following the conditions described above.

Time course mineral accumulation in the FAW mandibles

To identify the time at which mineral deposition occurs in the FAW mandibles I imaged samples at different time points in the caterpillar development. Caterpillars were grown in artificial diet for their first five instars and transferred to new empty cups at their pre-molting stage. The time of molting was registered and each set of 10 caterpillars were frozen at -20˚C at time zero (freshly molted) and then every 30 minutes for eight hours. Caterpillars were them thawed and their mandibles dissected, dehydrated with serial ethanol dilutions, dried with liquid CO_2 and imaged in an FEI Quanta 200 ESEM using a backscatter detector. To find out if mineral deposition occurs in caterpillars of early instars, mandibles were dissected from neonates, first and second instar caterpillars and processed in the same manner.

Morphological adaptations of FAW caterpillars to feed on tough leaves

The effect of diet on the caterpillars' head capsule size was studied by growing caterpillars (from egg hatch) on detached leaves of maize and rice. The head capsule size of 6^{th} instar caterpillars (2 days after molting) was measured with a caliper. In a separate experiment, caterpillars were grown on maize and rice leaves and the number of molts and head capsule size was recorded to test if they would undergo additional molts. Ten caterpillar heads from these experiments were cut out and immersed in fixative solution for at least 24 hours. After this time, the heads were dehydrated with serial dilutions of ethanol and critical point dried with liquid CO_2. Dry samples were fixed to aluminum stubs using carbon tape and the mandibular muscles dissected under a dissecting microscope (Olympus SZ30). After dissection some of these samples were imaged in a FEI Quanta 200 ESEM.

Statistical analysis

The effect of caterpillar feeding on the density of plant trichomes and the amount of silicon accumulated was analyzed with one-way ANOVA following the post hoc tests of Tukey and Fisher at $\alpha = 0.05$. Likewise, the effect of plant type on caterpillar head size was analyzed with one way ANOVA. These statistical analyses were performed using Minitab 16 (Minitab Inc., State College, PA, USA) and graphs were generated in R version 3.2.2 (Foundation for Statistical Computing, Vienna, Austria).

Results

The FAW saliva downregulated the production of glandular trichomes in tomato but not in maize

Tomato plants have seven types of glandular and non-glandular leaf trichomes (Glas *et al.* 2012). From those the glandular type VI trichomes are regularly induced by insect feeding (Tian *et al.* 2012). Therefore, in this study I measured the density of type VI trichomes (Fig 6-1 a) in tomato plants. In maize leaves, I observed two types of trichomes: prickle hairs and long macrohairs (Fig. 6-1 b); in this study I only measured the density of macrohairs because they were easier to visualize under a dissecting microscope. Feeding by FAW caterpillars induced production of leaf trichomes in tomato at greater levels than plants mechanically damaged with scissors. However, damage by ablated caterpillars (impaired to salivate) induced greater number of trichomes than damage by intact caterpillars (able to salivate) (Fig. 6-2 a). These results suggest that components in the FAW saliva down regulate the induction of glandular type VI trichomes in tomato plants. Conversely, caterpillar feeding and mechanical damage had no effect on density of long trichomes in maize plants, suggesting than these trichomes are not inducible.

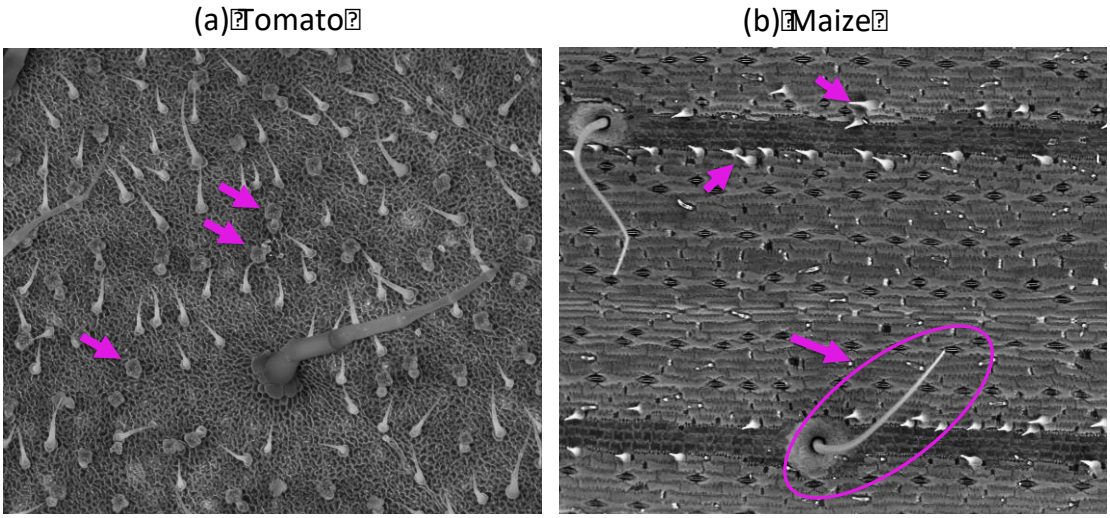

Figure 6-1. Scanning Electron Micrograph (SEM) of plant trichomes. (a) Leaf trichomes in tomato, arrows point to glandular type VI trichomes. (b) Leaf trichomes in maize, arrows point to prickle hairs, while the ellipse indicates the macrohairs counted in this study.

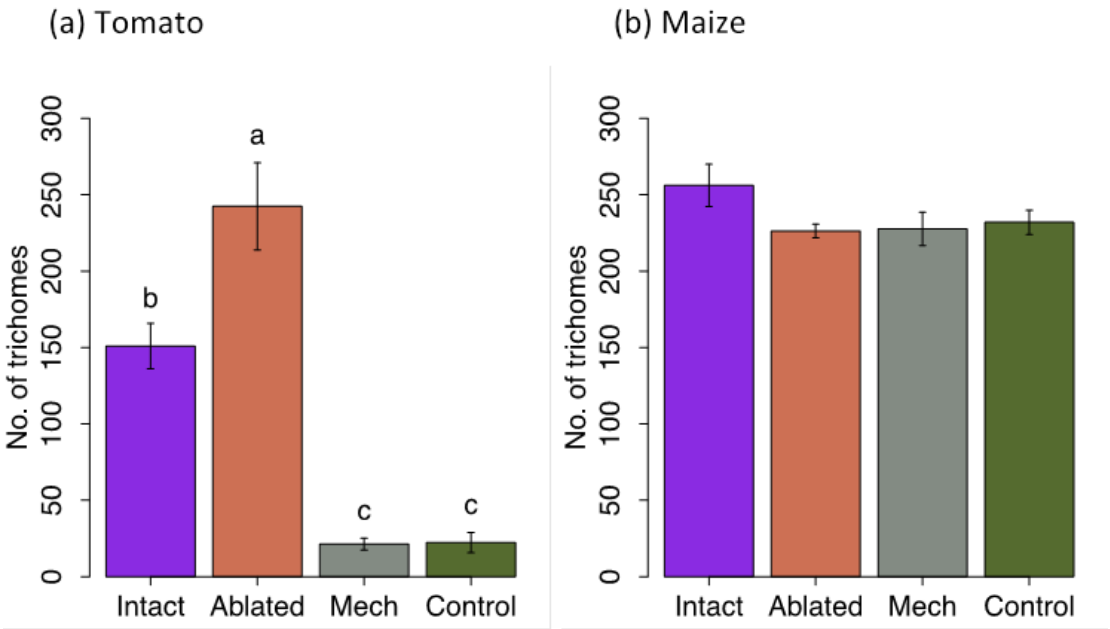

Figure 6-2. Effect of FAW herbivory on the induction of leaf trichomes in tomato and maize plants. (a) Glandular trichomes type VI in tomato ($F_{3,25}$ = 37.30, P = 0.0001; Tukey test; n = 5 - 9; untransformed data). (b) Long trichomes in maize plants ($F_{3,19}$ = 1.97, P = 0.153; n = 5 – 7; untransformed data). Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α = 0.05. Controls are undamaged plants.

FAW herbivory induces the accumulation of silicon in maize and rice but not in tomato

Rice and maize accumulate silicon dioxide in the epidermis of their leaves and tips of trichomes (Fig. 6-3 & 6-4). When damaged by FAW caterpillars both maize and rice increased amounts of silica in their leaf tissues compared with undamaged controls but at similar levels as plants mechanically damaged with scissors (Fig. 6-5 a-b). In tomato plants there was no effect of any of the treatments in the amount of silicon deposited in their leaves (Fig. 6-5 c).

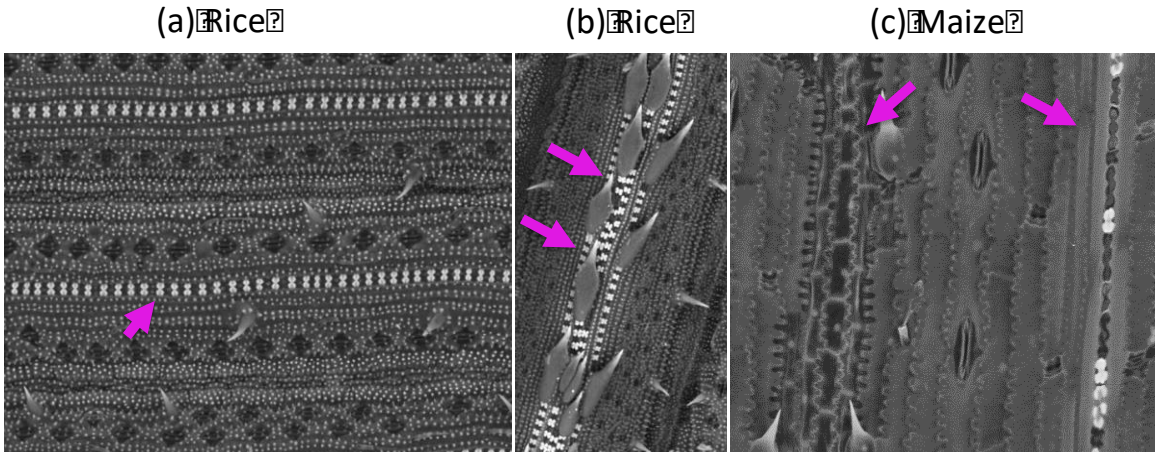

Figure 6-3. Silicon accumulation in plant leaves depicted as white structures with backscatter Scanning Electron microscopy (SEM). (a) Silicon bodies in the epidermis or rice leaves (arrow). (b) Silicon accumulation at the tips of trichomes in rice leaves. (c) Silicon accumulation in the epidermis and tips of trichomes in maize leaves.

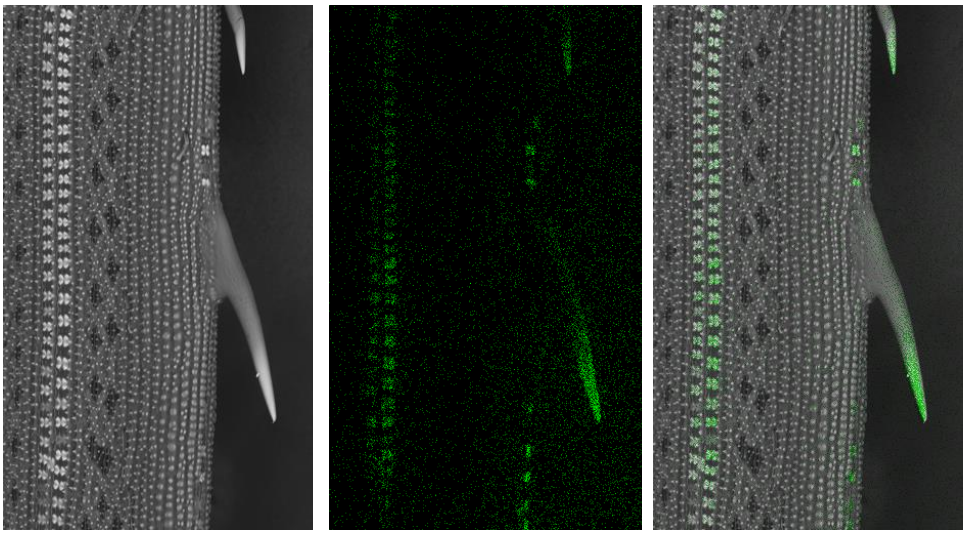

Figure 6-4. Map of silicon accumulation (green areas) in rice leaves obtained with Energy Dispersive x-ray Spectroscopy (EDS).

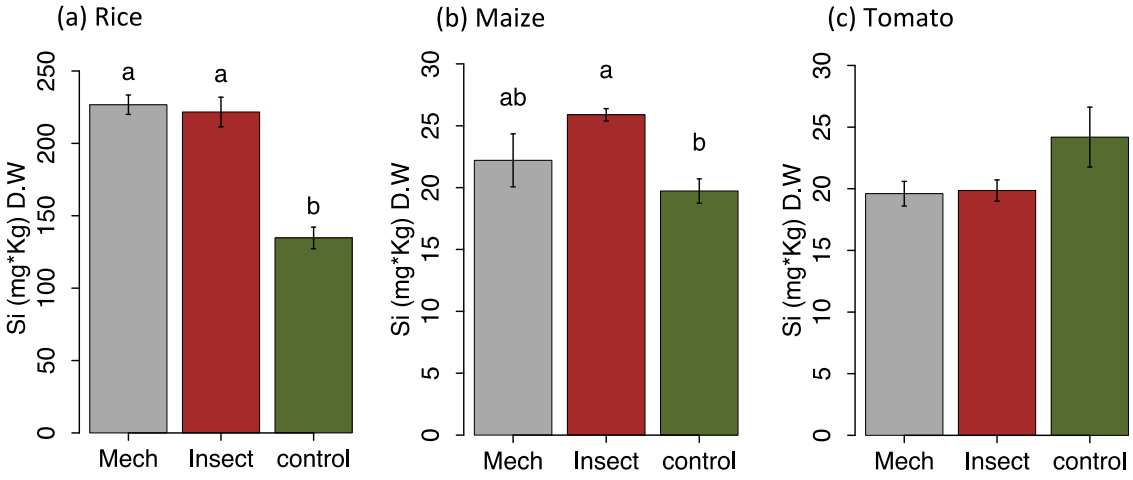

Figure 6-5. Effect of FAW herbivory on silicon accumulation in plant leaves. (a) Rice ($F_{2,6}$ = 39.53, P = 0.0001; Tukey test; n = 3). (b) Maize ($F_{2,12}$ = 4.92, P = 0.012; Fisher test; n = 5). (c) Tomato ($F_{2,17}$ = 2.49, P = 0.112; n = 6 - 7). Bar values are untransformed means ± SEM; different letters indicate significant differences obtained with ANOVA following post hoc tests at α = 0.05. Controls are undamaged plants.

Plant silicon accumulation wears caterpillar mandibles

Higher levels of mandible wear were observed in caterpillars fed on rice and Bermuda grass leaves compared with those fed on maize and artificial diet (Fig. 6-6). Rice and Bermuda grass plants have higher silica content in their leaves (Fig. 6-7) compared to maize. Therefore levels of mandible wear visually correlated with higher silica content in plant leaves. To further confirm the effect of silicon on mandible wear, artificial diet was supplemented with three doses of silicon oxide (0 %. 2.5 % and 5%). The mandibles from caterpillars fed on diets with 5 % SiO_2 were more worn compared with those fed on either 2.5 % SiO_2 or artificial diet without silicon (Fig. 6-8). Therefore, these results confirm the detrimental effect of silicon on caterpillar mandible wear.

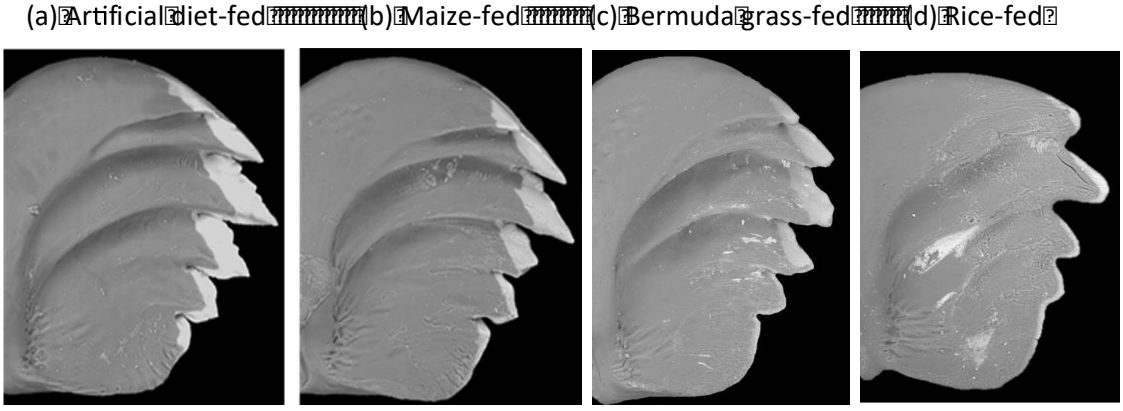

Figure 6-6. Backscatter scanning electron micrographs of FAW mandibles from caterpillars fed on different diets. The white outer areas indicate different elemental composition.

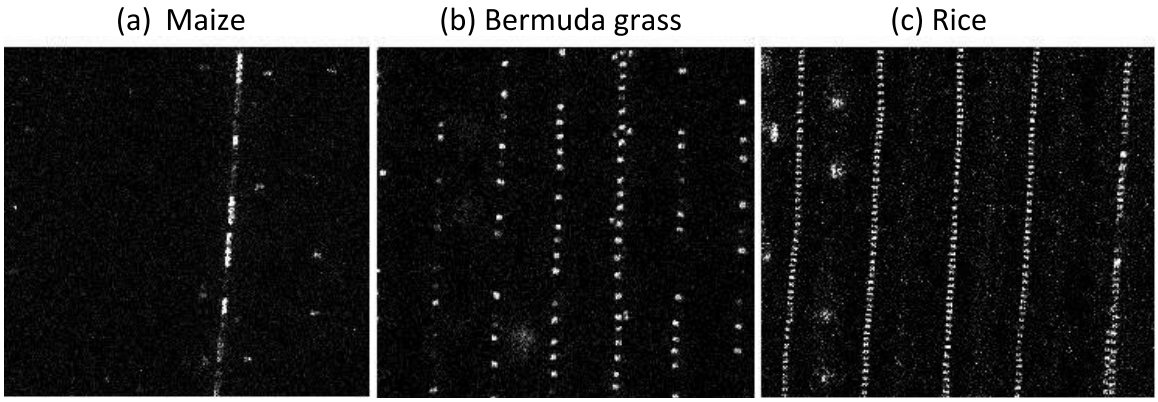

Figure 6-7. Map of silicon accumulation (white dots) in maize, Bermuda grass and rice leaves obtained with Energy Dispersive x-ray Spectroscopy (EDS).

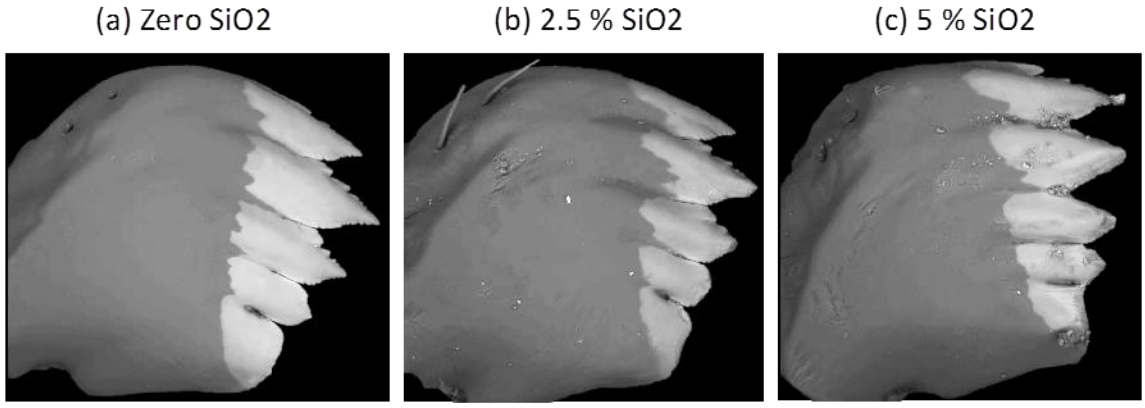

Figure 6-8. Backscatter scanning electron micrographs of FAW mandibles from caterpillars fed on diets containing different concentrations of silicon dioxide. The white outer areas indicate different elemental composition.

The FAW mandibles are enriched with minerals

Backscatter SEM images clearly showed differences in elemental composition (white outer areas) between the tips and the rest of the mandibles (Fig. 6-6 and 6-8). Therefore, further analyses were carried out to identify the elemental composition of these areas using EDS. The elemental spectrum obtained from cross-sectioned mandible tips showed the presence of zinc, chlorine and small amounts of calcium and sulfur (Fig. 6-9). Subsequent elemental mapping confirmed that zinc and chlorine are mainly accumulated at the tips of the mandibles (Fig. 6-10). The accumulation of these minerals was observed in caterpillar of different instars (Fig. 6-11), indicating that mineral

deposition occurs during or after each larval molt. Additional experiments were then carried out to identify the time at which these elements are deposited in the mandibles during the caterpillars' development. Time course experiments showed that the mandibles of newly molted 6th instar FAW caterpillars did not contain minerals; the elemental deposition took place between 1.5 and four hours after molting occurred (Fig. 6-12). Furthermore, zinc and chlorine were deposited together at the same time (not shown). Together, these results suggest that mineral deposition in caterpillar mandibles plays a major role in their feeding activity.

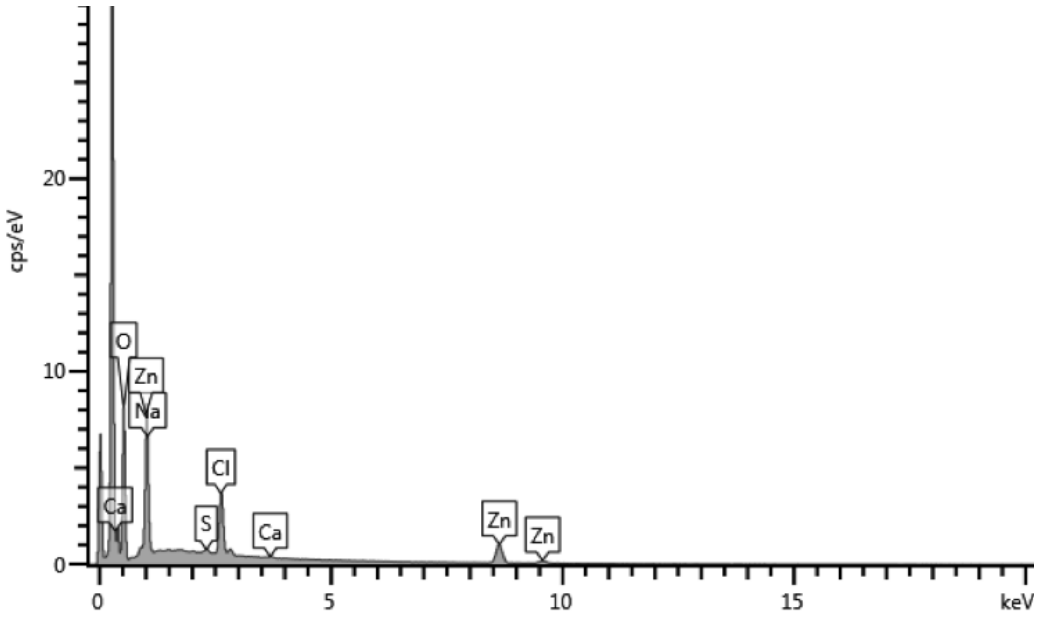

Figure 6-9. Elemental composition spectrum of cross sectioned mandible tips in the FAW obtained with Energy dispersive x-ray spectroscopy.

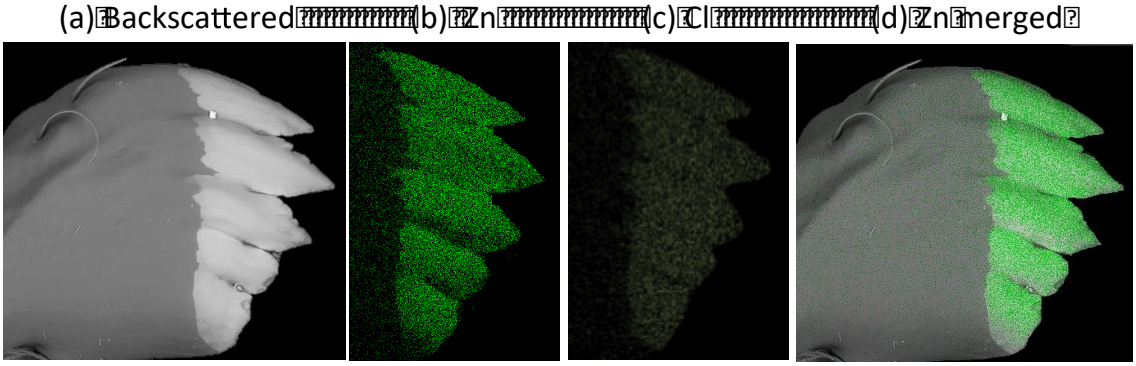

Figure 6-10. Scanning electron micrographs of FAW mandibles. (a) Backscattered image showing different elemental composition in the mandible tips (white areas). (b) Zinc map showing a higher concentration of this element in the mandible tips (green area). (c) Chlorine map displaying a larger concentration of this element in the mandible tips (yellowish area). (d) merged backscatter and Zn map images. Elemental mapping was obtained with Energy Dispersive x-ray Spectroscopy (EDS).

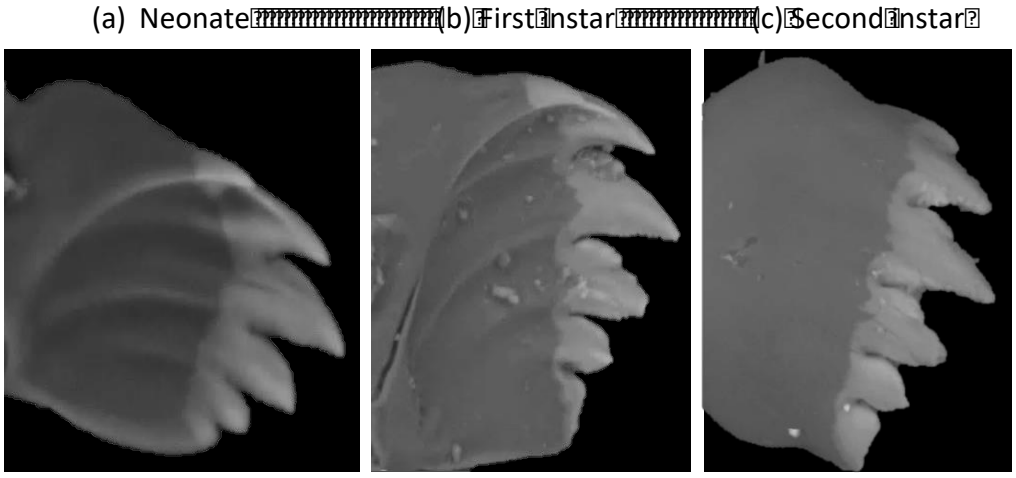

Figure 6-11. Zinc accumulation in the mandibles of FAW caterpillars at different developmental stages (different instars). Pictures are Backscatter electron micrographs; white outer areas correspond to zinc deposition as confirmed with elemental mapping (not shown).

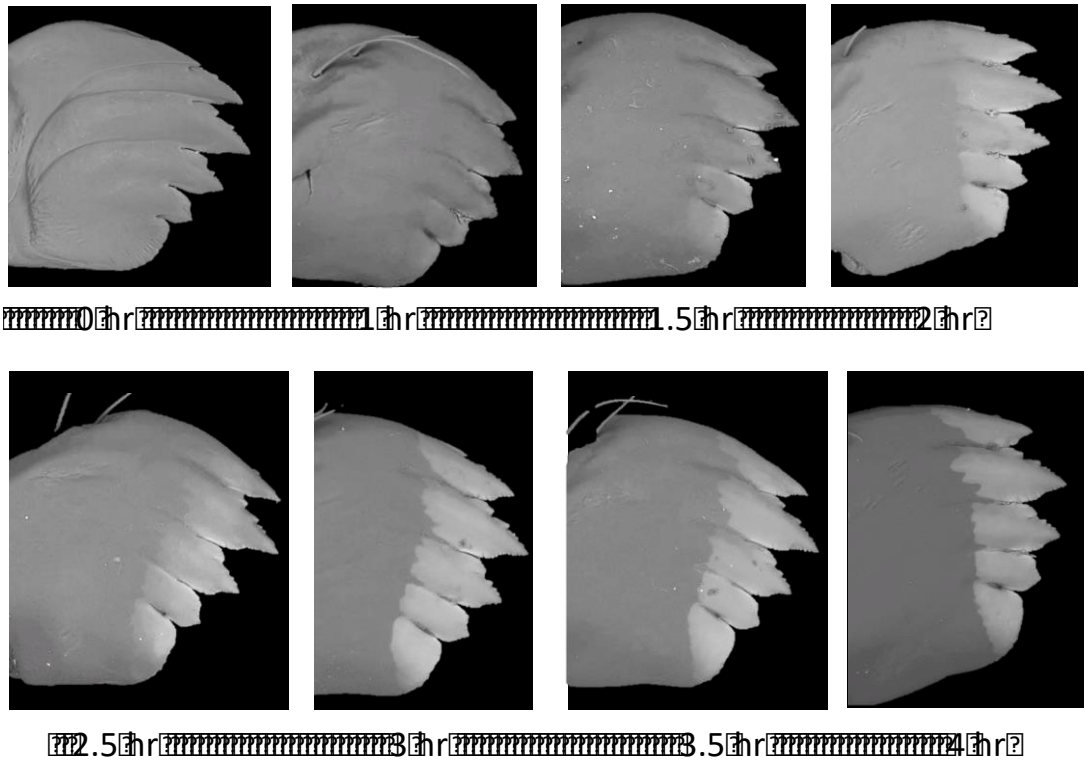

Figure 6-12. Time course of zinc accumulation in the FAW mandibles. Zero hours correspond to freshly molted 6th instar FAW caterpillars. Pictures are Backscatter electron micrographs; white outer areas correspond to zinc deposition as confirmed with elemental mapping (not shown).

The FAW plastically modifies its physiology and morphology to feed on tough leaves

FAW caterpillars are able to change the size of their heads when feeding on different plants. The head capsule size of 6th instar caterpillars was around 2.6 ± 0.0186 mm when feeding on maize; but, when feeding on rice their average head capsule sizes were 2.83 ± 0.0277 mm. These differences were significant ($F_{1,56} = 67.03$; $p < 0.0001$; n = 27 - 31) at $\alpha = 0.05$. Furthermore, when feeding on rice, 42.6 % of the larvae observed (n = 57) underwent an additional molt with head capsule sizes around 2.92 ± 0.1854 mm. To further identify the biological significance of having bigger larval heads, several caterpillars were dissected and the internal morphology of their heads examined. The bulk volume of caterpillar heads was occupied by mandibular and maxillary muscles (Fig. 6-13). Caterpillars with bigger heads have larger mandibular muscles that could

potentially increase their biting force. Moreover, caterpillars that underwent an additional molt grew both larger mandibles and larger attached muscles that allowed these larvae to ingest the necessary amount of food before pupation (Fig. 6-14).

Figure 6-13. Heads of 6th instar FAW caterpillars. (a) Light image and (b) Scanning electron micrograph showing the internal mandibular muscles housed in the caterpillar head.

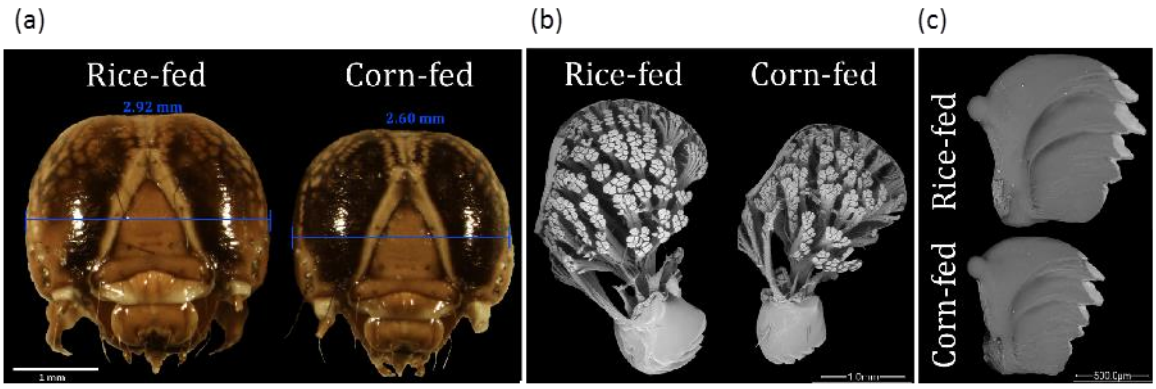

Figure 6-14. Differences in development of FAW caterpillars feeding on maize and rice. (a) Different head capsule sizes of the 7th (rice-fed) and 6th (corn-fed) instars of FAW caterpillars. (b) Scanning electron micrographs showing different size of mandibles and attached muscles from the 7th (rice-fed) and 6th (corn-fed) instars of FAW caterpillars. (c) Scanning electron micrographs showing differences in mandible size of the 7th (rice-fed) and 6th (corn-fed) instars of FAW caterpillars.

Discussion

The FAW is a highly polyphagous insect that feeds on more than 80 different plant species that vary in their chemistry and structural defenses. Our previous studies have shown that this insect is able to regulate induction of biochemical defenses in different host plant species through proteins and small molecules present in their caterpillar saliva and through associated symbiotic bacteria (Chapter 2 - 4). In this study we show that FAW feeding also regulates induction of plant physical defenses, some of which trigger development of plastic morphological and developmental responses in this insect.

Feeding by FAW caterpillars induced production of glandular type VI trichomes in tomato plants compared to controls, but this induction was down regulated by components present in caterpillar saliva (Fig. 6-2 a). These results agree with previous experiments in which the application of salivary gland homogenates from FAW caterpillars to wounded tomato plants down regulated the activity of polyphenol oxidase (Acevedo *et al*, unpublished). Therefore, salivary components in FAW appear to suppress both biochemical and physical defenses in tomato plants. However, contrasting results were found with another noctuid species, *Helicoverpa zea*, in which caterpillar saliva induced production of higher number of trichomes in tomato plants compared with controls; this induction appears to be elicited by the salivary enzyme glucose oxidase (Tian *et al.* 2012). Even though FAW saliva also contains glucose oxidase, activity levels are very low when this insect feeds on tomato compared with other diets (Acevedo *et al*, unpublished). Interestingly, FAW feeding triggered production of greater number of trichomes than plants damaged with scissors, even though effort was made to prune the same amount of foliage as insect feeding (Fig. 6-2 a). This difference could probably be explained by plant recognition of herbivore-derived elicitors in the insect oral secretion or frass or by the plant recognition of repetitive insect damage. It has been shown that recurring mechanical damage to maize plants induce a different array of plant responses when compared to single wound events (Bricchi *et al.* 2010). In contrast to tomato, I

observed no induction of macrohair trichomes in maize that received mechanical or insect treatment (Fig. 6-2 b). This suggest that leaf macrohairs are not inducible in B73 maize plants because both wounded and untreated controls had the same number of trichomes. These results indicate that FAW feeding and its associated elicitors modulate physical defenses in plants in a host-specific manner.

Unlike tomato where most trichomes are glandular, maize and rice plants have trichomes enriched with silicon (Fig. 6-3 & 6-4). These plant species, and grasses in general accumulate large amounts of silicon dioxide in the epidermis of their leaves and other tissues (Ma & Yamaji 2006; Van Soest 2006). Silica deposition is essential for increasing strength and plant rigidity, but it is also a critical component of plant defenses against both abiotic and biotic stresses including herbivores (Currie & Perry 2007). Due to its importance in plant defense, Si accumulation is inducible by herbivory (McNaughton & Tarrants 1983; Seastedt *et al.* 1989; Garbuzov *et al.* 2011; Reynolds *et al.* 2012). Our results show that FAW feeding induced higher accumulation of Si in maize and rice compared with untreated controls (Fig. 6-5 a-b). However, the Si amounts induced by FAW herbivory were not different from the ones induced by mechanical damage, suggesting that herbivore-derived elicitors probably do not play a major role in plant Si deposition. In tomato plants, FAW feeding and mechanical damage did not induce different Si deposition compared with untreated controls (Fig. 6-5 c), probably because plants were grown on potting soil with presumably low amounts of Si. Even though, tomato plants are considered low Si accumulators, infestation with *Ralstonia solanacearum* and Si supplementation resulted in higher Si accumulation in stems and roots compared with *R. solanacearum* infection without the addition of Si (Diogo & Wydra 2007). Si accumulation increases abrasiveness of plant leaves decreasing leaf digestibility and causing mandible wear, which in turn reduces insect growth and development (Clissold 2007; Kvedaras *et al.* 2009). In FAW, visual levels of mandible wear were directly proportional to the amounts of Si present on their diet (Fig. 6-6 to 6-8). However, levels of mandible wear were much lower than those observed in plant-fed caterpillars, indicating that silicon alone does not account for the entire levels of

mandible wear observed. Although Si increases abrasiveness, leaf toughness is highly influenced by the content of lignin and cellulose (Westbrook *et al.* 2011).

To reduce mandible wear, caterpillars increase the hardness of their mandibles by incorporating metals. As an extension of the exoskeleton, insect mandibles are mainly made out of cross-linked chitin layers (Klowden 2008). The strength of their mandibles is highly dependent on the sclerotization levels and the deposition of minerals such as Zn, Mn, Cu, and Ca (Cribb *et al.* 2008; Klowden 2008). FAW mandibles are mainly enriched with Zn and chlorine (Fig. 6-10). Zn content is highly correlated with mandibular hardness in termites, ants and the marine worm *Nereis virens* (Schofield *et al.* 2002; Broomell *et al.* 2006; Cribb *et al.* 2008). In other species, halogens are usually co-located with metals in cuticular structures (Schofield 2001; Lichtenegger *et al.* 2003; Schofield *et al.* 2003; Birkedal *et al.* 2006). For instance, chlorine has been found with zinc in different ratios for the *Nereis* worm and termites (Lichtenegger *et al.* 2003; Cribb *et al.* 2008). Furthermore, the ratio of metal to halogen seems to influence mandibular hardness (Cribb *et al.* 2008). In FAW mandibles, deposition of Zn and Cl occurs within the first four hours after molting (Fig. 6-12), which appears to coincide with the time that caterpillars started eating (personal observation). Studies in the scorpions *Vaejovis spinigeris* and *Centruroides exilicauda* showed that Zn deposition happened 90 hours after ecdysis; in ants (*Tapinoma sessile*) Zn deposition occurred within the first 100 hours after molting (Schofield *et al.* 2003). Deposition of Zn in cuticular structures has been found in several insect orders including Lepidoptera, Orthoptera, Phasmidae, Hymenoptera, some Coleoptera species and at least one species of Diptera (Hillerton & Vincent 1982; Fontaine *et al.* 1991; Morgan *et al.* 2003). However, cuticular hardening in insects is not always dependent on Zn deposition; for example, adults of the jewel beetle *Pseudotaenia frenchi* contain Mn and Cl at the cutting edges of their mandibles, but their larvae, which has equivalent mandible hardness to their adult forms, are deprived of metals (Cribb *et al.* 2010).

In addition to mandible hardening by mineral deposition, FAW caterpillars are also able to plastically modify their morphology and development in response to leaf toughness. When feeding on rice leaves, which have more Si and are harder tissues, FAW caterpillars increased the size of their heads and some underwent an additional molt. Larger heads contained larger mandibular muscles to potentially increase biting force; greater musculature is probably the result of excessive caterpillar biting work to fracture hard leaves (Bernays *et al.* 1991). Speculatively, this increase in muscle mass could have also triggered the extra molt observed by compromising oxygen supply. Support for this hypothesis comes from recent studies showing that the fixed tracheal system in a given insect instar fails to supply the oxygen demand as body mass increases, and therefore induces molting by a size-sensing mechanism (Callier & Nijhout 2011). Together, these results illustrate the extraordinary physiological and morphological plasticity of this insect to feed on different host plants.

Based on the results of this study, I conclude that feeding by FAW caterpillars modulates the induction of plant structural defenses in various plant species. It induces the production of glandular trichomes in tomato and the accumulation of Si in maize and rice. In response to leaf hardness, FAW caterpillars modify their head morphology and development to increase their mandibular muscle mass and grow larger mandibles. The mandibles of this insect are enriched with Zn and Cl as evolutionary strategies to reduce mandible wear during plant feeding. This study highlights the importance of insect developmental and morphological plasticity to facilitate counter adaptations in response to plant structural defenses.

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Chapter 7

Discussion

This short discussion focuses on answering the questions presented in chapter 1, presents some general conclusions and future research directions.

Polyphagous insects trigger different defense responses in different host plants

The results of my studies with FAW show that the response to insect mechanical damage and associated elicitors is different for each plant species (Fig. 7-1). Mechanical damage caused by caterpillar feeding induced defense responses in all plants species tested, but the levels of defense induction were modulated by herbivore-associated microbes and molecules present in insect saliva and oral secretions. The only case in which FAW feeding did not induce plant defenses was in Bermuda grass treated with corn strain caterpillars; in these plant species, the effect of saliva was strong enough to down regulate induction of trypsin proteinase inhibitors to similar levels found in undamaged controls (Chapter 2). The effect of the salivary enzyme glucose oxidase (GOX) on plant defense regulation in tomato appears to be small compared with the effect of non-protein salivary components (small molecules). As illustrated in figure 7-1, untreated saliva down regulated the activity of anti-nutritional protein in this plant despite its GOX content. This can be explained by the low expression and activity levels of GOX in the FAW saliva when feeding on tomato (Chapter 3; Acevedo *et al.*, unpublished). In general the defense responses triggered by FAW, its secretions and associated microbes were similar in maize and rice but notably different in tomato and Bermuda grass (Fig. 7-1). This contrast in defense responses among different plant species may be explained by differences in mechanisms of receptor-mediated recognition (Schmelz *et al.* 2009), differences in the availability of enzyme substrates and/or differences in hormonal crosstalk between plant defense pathways.

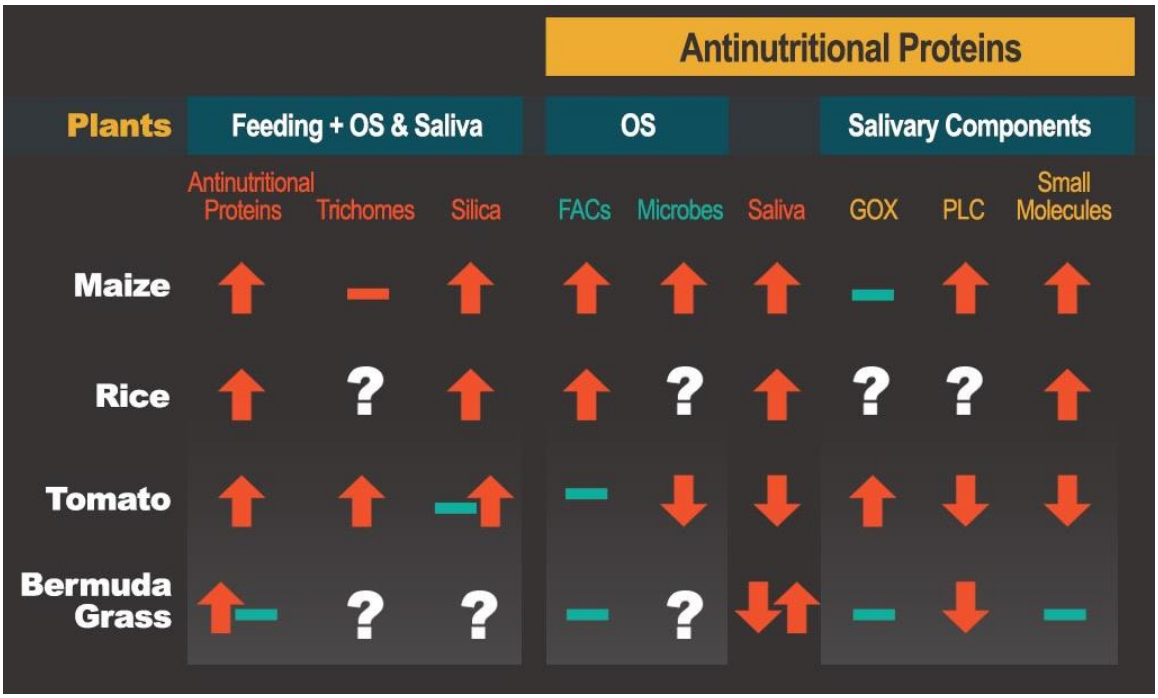

Figure 7-1. Effect of FAW feeding and its associated secretions (OS and saliva) on induction of structural and biochemical defenses of its host plants maize, rice, tomato and Bermuda grass. From left to right: feeding by caterpillars of the FAW induces (↑) production of anti-nutritional proteins in all host plants; in Bermuda grass, the effect is dependent on the insect strain. Caterpillar feeding also induces production of trichomes in tomato but not in maize (−). Likewise, FAW feeding induces deposition of silica in maize and rice; in tomato, silica accumulation depends on whether or not the plants are supplemented with this element (Acevedo *et al.*, unpublished). When these plants were mechanically wounded and treated with oral secretions (OS) from plant-fed caterpillars, which contain fatty acids (FACs) and other proteins, there was up regulation of anti-nutritional proteins in maize and rice, but no effect was observed in tomato and Bermuda grass. However, when these plants were treated with FAW OS containing microbes there was down regulation (↓) of anti-nutritional proteins in tomato but induction of proteinase inhibitors in maize. When plants were mechanically wounded and treated with saliva or salivary glands from FAW caterpillars, there was up regulation of defense responses in maize and rice but not in tomato; the effect of saliva on Bermuda grass was dependent on the insect strain. Mechanical damage and treatment with GOX, a component of FAW saliva, induced greater activity of anti-nutritional proteins in tomato but no effect was observed in maize or Bermuda grass. Mechanical damage and treatment with phospholipase C (PLC) down regulated the production of anti-nutritional proteins in tomato and Bermuda grass, but up regulated defenses in maize. Lastly, treatment with boiled saliva or saliva with precipitated proteins (depicted as Small Molecules in the graph) induced production of anti-nutritional proteins in maize and rice, down regulated their production in tomato and had no effect of Bermuda grass. Arrows up indicate induction, arrows down indicate suppression, dash indicates no effect and question marks indicate that it has not been tested.

The effect of induced plant defenses in insect host preference

The polyphagous FAW can feed and develop on different plant species but it is mostly associated with grasses including maize, Bermuda grass, sorghum and rice. In my studies, the caterpillars of this insect developed faster and gained greater weight when feeding on maize and Bermuda grass compared with rice and tomato (Chapter 2; Acevedo *et al.*, unpublished). Although preference was not tested in this dissertation, there is accumulating evidence that FAW is mostly associated with grasses in field conditions and occasionally associated with tomato (Barlow & Kuhar 2009). This host plant preference is positively correlated with insect performance on these plants (Chapter 2). However, feeding by this insect species induced defenses in three of the four plant species tested in this study (not Bermuda grass); therefore, the correlation of insect host preference with induced plant defenses appears to be weak. When comparing plant defense responses triggered by the FAW strains, I found greater induction of herbivore defenses by the rice strain in maize compared with the corn strain. These results negatively correlate with the host association of this strain in field conditions and therefore, suggest that induced plant defenses may play a role in insect preference. However, my results in Bermuda grass were opposite; the rice strain induced greater herbivore defense responses than the corn strain, even though the rice strain appears to be preferentially associated with Bermuda grass in field conditions. Furthermore, no differences in defense induction were found in rice after feeding by the two strains (Chapter 2) despite multiple reports of the rice strain associated with rice crops in the field. Consequently, my results do not show a clear relationship between induced defenses and host plant association of the FAW strains in field conditions. However, they do show a clear negative correlation between induced plant defenses and insect performance (growth; Chapter 2 & 5). A recent study in a closely related species, *Spodoptera littoralis* feeding on 28 different plant species, showed a significant positive association between caterpillar performance and its host preference, but this study ignored plant defenses (Kempel *et al.* 2015).

In field conditions, insects are exposed to a complex environment where induced plant defenses are but one of the factors that possibly influence their host association. Other factors influencing insect host preference are: host availability, host nutritional value, host chemical and physical composition, and the risk of predation when associated with particular hosts (Kempel *et al.* 2015). The host-plant association of the FAW strains may also be affected or driven by differential pressure of natural enemies. A recent study has shown that the generalist parasitic wasps *Euplectrus platyhypenae* (Hymenoptera: Eulophidae) had higher fitness on host FAW larvae fed on maize than fed on stargrass, *Cynodon plectostachyus*. Adult wasp recovery was especially low from rice strain caterpillars reared on stargrass (Hay-Roe *et al.* 2013). It would be interesting to test if differences in plant volatile release would have an effect on the recruitment of natural enemies for the strains.

Additionally, the differential host-plant association of the FAW strains in field conditions may also be influenced by their associated symbionts. In chapter 5, I showed that FAW-associated bacteria differentially modulate defense responses in different plant species but we did not include the two FAW strains. In field conditions, these strains may harbor different microbial communities that could influence their host plant association. The effect of symbionts in insect host shifts has been documented in other insect species. For example, in Japan the plant association of the pea aphid (*Acyrthosiphon pisum)* host races on either vetch (*Vicia sativa*) or white clover (*Trifolium repens*) is affected by presence of the bacterium pea aphid U-type symbiont (PAUS). Aphid fitness is significantly improved by PAUS infection when feeding on white clover (Tsuchida *et al.* 2004). In another study, two closely related stinkbug species, *Megacopta punctatissima* and *Megacopta cribraria* can feed and reproduce on crop legumes only if they harbor a specific genotype of the symbiont '*Candidatus* Ishikawaella capsulata*'* (Hosokawa *et al.* 2007). Although the specific mechanisms by which these bacteria symbionts affect host fitness were not investigated in these studies, it would not be surprising if among other effects, these bacteria also influence the induction of defense responses in their insect host plants. It has been recently demonstrated that endosymbiotic bacteria harbored by

phytophagous insects can manipulate plant defenses to benefit its insect host (Chung *et al.* 2013).

The composition of insect secretions changes with host plant type

The results of this dissertation show that diet has a significant influence on the salivary composition of FAW larvae. Although the proteomic profile of insect saliva is genetically regulated, diet modifies both the expression and activity of salivary proteins (Chapter 2 & 3). This is especially important in the study of insect-plant interactions because some salivary proteins like GOX, PLC and ATPases regulate plant defense induction (Musser *et al.* 2002; Wu *et al.* 2012, Peiffer *et al.* unpublished). In addition to changes in salivary proteins, diet also influenced the composition of phytohormones in FAW saliva and may also affect the quantities of other small molecules (Chapter 4). Furthermore, diet influenced the total protein concentration present in a given volume of saliva (Chapter 3) and may also influence secretion rates. All of these changes are potentially important for the insect because they modify how plants perceive them. However, it is unknown if these physiological changes are driven by plant chemistry or are insect plastic adaptations to regulate plant defenses and maximize their survival in a given host. My results indicate that these salivary changes are different between insect genotypes and therefore the plastic variation of insect saliva is likely to be regulated by interactions of the plant chemical composition and the insect genetic makeup.

Changes in insect saliva associated with different diets have been found in other insect species. The tomato fruitworm, *Helicoverpa zea* had higher protein concentration and GOX activity in their salivary glands when feeding on tobacco compared with tomato and cotton (Peiffer & Felton 2005). These salivary changes are likely to favor insect fitness because GOX decreases the levels of nicotine in tobacco (Musser *et al.* 2002) but induces the production of proteinase inhibitors in tomato (Tian *et al.* 2012). Likewise, the activity of GOX in salivary glands of the beet armyworm, *Spodoptera exigua*, was higher in caterpillars fed on artificial diet compared with those fed on *Medicago truncatula* (Merkx-Jacques & Bede 2005). The activity of GOX was positively

associated with the amount of glucose and protein present in *S. exigua* diet (Babic *et al.* 2008; Hu *et al.* 2008). These studies strongly suggest plastic variations in the biochemical composition and secretion of insect saliva associated with diet.

Polyphagous herbivores overcome plant defenses using a variety of mechanisms

The results of this work indicate the presence of several strategies used by FAW larvae to cope with plant defenses; these include effector salivary proteins, detoxification enzymes, sequestration of plant hormones, and modifications in head morphology and development. The salivary enzyme PLC down regulates herbivore defenses in Bermuda grass (Chapter 2) and tomato (Peiffer *et al.* unpublished). This defense suppression is likely to favor caterpillar growth on these plants. There were also several detoxification enzymes found in the FAW saliva that can potentially influence the caterpillar's ability to feed on particular host plants (Chapter 3). FAW caterpillars are also able to sequester and perhaps synthesize plant hormones that are released back into the plants to down regulate defenses. I show that benzoic acid and salicylic acid were present in the FAW saliva and treatment with these compounds down regulated herbivore defenses in tomato (Chapter 4). Lastly, FAW caterpillars increased their head capsule size when feeding on Bermuda grass and rice plants; larger heads housed larger mandibular muscles that could potentially increase the biting force needed to feed on tough leaves. In rice, FAW caterpillars had an extra molt allowing for the growth of new mandibles and larger heads potentially useful in food consumption. Yet another strategy is the association with microbes, I show that bacteria in the FAW oral secretions down regulate herbivore defenses in tomato upon secretion into plant wounds (Chapter 5). A recent study has shown that plant chitinases in the FAW frass suppress herbivore defenses in maize (Ray *et al.* 2016). These results illustrate several mechanisms by which a polyphagous caterpillar overcomes biochemical and physical defenses in different host plants.

Conclusions

Insect saliva is an important regulator of plant defenses that can potentially influence the insect's ability to feed on different host plants. FAW saliva contains both protein and non-protein plant defense elicitors whose composition changes plastically with the type of diet. Herbivorous insects use a variety of mechanisms to overcome plant defenses including the release of effector molecules in their secretions, plasticity in their morphology and development and association with microbes. In light of these results I suggest that one of the main strategies used by polyphagous insects to utilize plants with divergent chemistry and physical structures lays in their physiological, morphological and behavioral plasticity.

Research impact

This dissertation made important contributions to the field of insect plant interactions. To my knowledge, this is the first study to report differences in plant defense induction by populations of the same insect species. It also highlights the role of salivary secretions as modulators of inducible physical and chemical plant defenses. It quantified, for the first time, the presence of plant hormones in secreted saliva and their effect in plant defense regulation; it also suggest the presence of small molecules in caterpillar saliva as active plant defense elicitors. In addition, it presents the first quantitative proteomic analysis of secreted insect saliva associated with different host plant species. This is probably the most comprehensive study of secreted saliva in Lepidoptera insects that has been done so far. In addition, this dissertation documents the effect of microbes in caterpillar oral secretions on plant defense regulation, documents the mineral composition of the FAW mandibles and presents some plastic morphological adaptations of FAW caterpillars to feed on different hosts. The results of this dissertation significantly improve understanding of the factors influencing the ecological interactions between a polyphagous insect and some of its host plants.

Further directions

The results presented in this dissertation lay the groundwork for a variety of studies. For example, the salivary proteome of the FAW contains a large amount of proteins with intriguing functions; the use of biochemical methods and gene silencing techniques could be useful on determining their biological function. In addition to proteins, insect saliva also contain small molecules that could be identified using metabolomics. The results of this dissertation also present a potentially good model system (maize and tomato) to study differences in perception mechanisms of herbivore-derived elicitors between monocots and dicots.

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Appendix A

Modified trizol protocol for RNA extraction

The total RNA was extracted using a modified trizol protocol: 1ml of TRIzol reagent (Life technologies, USA) was added to the homogenized tissue, hand-shaked and incubated at room temperature (rm) for 5 minutes. The samples were then centrifuged at 12000 rpm for 10 min at 4 ºC, the supernatant was transferred into a new tube. 200 µl of chloroform (EMD Millipore CX 1054) were added, thoroughly mixed by vortex for 30 sec, and incubated for 2 min at rm. After 15 min of centrifugation at 12000 rpm (4 ºC), 350 µl of the aqueous phase were transferred into a new 1.5 ml tube; the nucleic acids were precipitated by the addition of 250 µl of isopropyl alcohol (EMD Millipore PX 1835), and 250 µl of a salt precipitation solution (0.8 M sodium citrate; 1.2 M NaCl) for 10 min at rm. The samples were then centrifuged at 12000 rpm for 10 min (4 ºC), the supernatant was discarded and the pellet washed by adding 1 ml of 75% ethanol (200 proof Koptec), followed by 5 min of centrifugation (4 ºC) at 7500 rpm; the supernatant was discarded and the pellet air dried for 10 min at rm. The samples were further treated with 130 µl of water + 50 µl of LiCl 8 M (L7026, Sigma) to eliminate DNA residues and incubated overnight at 4 ºC. After 15 min of centrifugation at 10000 rpm (4 ºC), the supernatant was discarded and the RNA further precipitated by adding 100 µl of water, 10 µl of sodium acetate (3 M, pH. 5.2), and 250 µl of 100% ethanol, followed by 2 hours of incubation at -20 ºC. The pellet was recovered by 15 min of centrifugation at 15000 rpm (4 ºC) and washed twice with 1 ml of 75 % ethanol. The resulting RNA pellet was air-dried and re-suspended in 50 µl of water. The RNA was quantified in a nanodrop 2000 C (Thermo-Fisher Scientific, USA).

Appendix B

iTRAQ sample preparation protocol

The protein samples were prepared following the Pennsylvania State University College of Medicine Mass Spectrometry and Proteomics Core Facility standard protocol adapted from the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). We used the applied Biosystems iTRAQ 8-plex reagents (cat #4390811). The two saliva samples for this experiment were run along with 6 more FAW saliva samples from another experiment. Each sample was labeled with a unique isobaric tag using the following protocol. To each sample containing 10 µg of protein diluted in 10 µl of MilliQ water, we added 20 µl of dissolution buffer [0.5 M triethylammoniumbicarbonate (TEAB, SIGMA 17902) pH 8.5, diluted in water]. After that we added 1 µl of the denaturant (2% SDS) and vortexed the samples. Each sample was reduced by adding 1 µl of 110 mM tris-(2-carboxyethyl) phosphine (TCEP) (Pierce #20490), vortexed, spun and incubated at 60 °C for 1 hr. 1 µl of freshly prepared 84 mM solution of iodoacetamide (Sigma # A3221-10vl) were added to each sample, vortexed, spun and incubated in the dark at room temperature for 30 minutes (tubes wrapped in foil). The samples were then digested with sequencing grade trypsin (Promega # V511 resuspended in 50 mM acetic acid) by adding 2.5 µg to each sample, vortexed, spun and incubated overnight at 48 ºC. To each of the 8 samples, one iTRAQ labeled tag [(113, 114, 115, 116, 117, 118, 119, 121) each resuspended in 50 µl of isopropanol] was added and well mixed. The samples were spun and incubated at room temperature for 2 hours. After that 100 µl of Milli-Q water were added to each tube to quench the iTRAQ reaction, samples were incubated at room temperature for 30 minutes. The contents of all 8 iTRAQ Reagent-labeled samples were combined into one tube, mixed and dried. 100 µl of water were added, mixed, spun and dried completely for three times. Lastly, the sample was resuspended in 500 µl of cation exchange buffer-load (12 mM ammonium formate in 25% acetonitrile at pH 2.5-3.0).

Appendix C

Quantitative proteomic analysis of the saliva from the FAW strains fed on different host plants

				Corn strain Vs Rice strain				Cs maize Vs Rs maize			
#	Uniprot Accession #	Protein name	# peptides	log2 ratio	std	p value	FDR < 0.05	log2 ratio	std	p value	FDR < 0.05
1	tr|A0A088MGW5_SPOEX	POX-J	236	0.436	0.157	0.006	**0.027**	0.580	0.704	0.410	0.875
2	tr|D9ZFI1_SPOEX	Glucose oxidase	147	0.671	0.177	0.000	**0.002**	0.254	0.704	0.719	0.979
3	tr|Q9U5K4_SPOLT	Arylphorin subunit	82	-0.445	0.147	0.002	**0.014**	-0.298	0.176	0.091	0.353
4	tr|A0A0N1IPT1_PAPMA	Glucose dehydrogenase	81	1.438	0.707	0.042	0.430	1.525	0.707	0.031	0.282
5	tr|A0A076FRM9_9NEOP	Aldehyde oxidase AOX3	76	0.137	0.143	0.335	0.594	0.213	0.705	0.762	0.992
6	tr|G8EJ31_SPOFR	Aminoacylase-1	75	-0.292	0.223	0.191	0.434	-0.741	0.706	0.294	0.820
7	tr|Q0MUU6_TRINI	Heat shock cognate 70 protein	71	2.161	0.169	0.000	**0.000**	1.554	0.179	0.000	0.000
8	tr|D9ZFI5_SPOEX	Putative uncharacterized protein (Fragment)	48	0.002	0.705	0.998	1.000	0.055	0.705	0.937	0.999
9	tr|M4M7W4_HELVI	Thiolase 2	39	-1.181	0.708	0.095	0.642	-1.739	0.711	0.014	0.197
10	tr|M4M651_HELSB	Thiolase 2 (Fragment)	39	-0.816	0.706	0.248	0.882	-1.053	0.707	0.137	0.708
11	tr|S5M6C1_BOMMO	Actin 4	26	0.446	0.238	0.061	0.188	0.387	0.269	0.150	0.459
12	tr|H9JKT2_BOMMO	Uncharacterized protein	25	-0.100	0.707	0.888	1.000	-0.162	0.708	0.819	0.992
13	tr|A6YQV6_SPOFR	Beta-1_3-glucanase	20	0.290	0.710	0.683	1.000	0.632	0.713	0.375	0.861
14	tr|Q95PD6_HELVI	Actin	20	0.607	0.708	0.392	0.965	0.734	0.711	0.302	0.820
15	tr|D3GDM6_SPOLI	Carboxylic ester hydrolase	20	-0.954	0.714	0.181	0.823	-0.565	0.715	0.429	0.875
16	tr|H9J7W6_BOMMO	Uncharacterized protein	19	0.266	0.747	0.721	1.000	0.304	0.761	0.690	0.978
17	tr|H9JGA4_BOMMO	Uncharacterized protein	16	1.105	0.728	0.129	0.729	0.960	0.738	0.193	0.782

18	tr|M4PZR4_SPOFR	Arginine kinase	16	1.152	0.282	0.000	**0.001**	0.917	0.711	0.197	0.782
19	tr|S5FXH1_XESCN	Heat shock cognate 70	15	0.361	0.711	0.612	1.000	0.508	0.714	0.477	0.875
20	tr|A0S6A0_SPOFR	Prophenoloxidase subunit 1	14	-0.059	0.278	0.831	0.906	0.093	0.401	0.816	1.000
21	tr|Q9U5K5_SPOLT	Methionine-rich storage protein	14	-0.429	0.203	0.034	0.125	-0.420	0.264	0.112	0.403
22	tr|A0A0A7HBV4_9NEOP	Heat shock cognate 70 protein	13	0.575	0.258	0.026	0.100	-0.180	0.312	0.563	0.867
23	tr|A0A0L7KZE8_9NEOP	Putative ecdysone oxidase	11	-0.245	0.297	0.409	0.654	-1.618	0.718	0.024	0.263
24	tr|L0GGU3_PLUXY	FK506-binding protein	11	0.820	0.286	0.004	**0.021**	0.905	0.713	0.204	0.787
25	tr|C0H6N9_BOMMO	Putative cuticle protein	11	0.570	0.710	0.422	0.968	0.801	0.714	0.262	0.820
26	tr|Q8MUR5_CHOPR	Nucleoside diphosphate kinase	10	0.992	0.712	0.164	0.773	1.210	0.717	0.091	0.562
27	tr|G6DA51_DANPL	Yellow-d	10	0.443	0.718	0.537	0.993	0.633	0.725	0.383	0.869
28	tr|G6DDQ0_DANPL	Moesin	10	0.322	0.723	0.656	1.000	0.138	0.724	0.849	0.992
29	tr|A0A0L7LP95_9NEOP	Putative venom acid phosphatase	10	0.284	0.734	0.699	1.000	0.440	0.748	0.556	0.930
30	tr|A0A0L7LJ52_9NEOP	Aldehyde oxidase AOX3 (Fragment)	9	0.052	0.716	0.942	1.000	-0.048	0.722	0.947	0.999
31	tr|A0A0F7QIE4_OSTFU	Aldehyde oxidase (Fragment)	8	-0.283	0.715	0.693	1.000	-0.741	0.722	0.305	0.820
32	tr|A0A0L7L6H5_9NEOP	Putative ecdysone oxidase	8	-1.196	0.256	0.000	**0.000**	-0.964	0.342	0.005	**0.046**
33	tr|M4Q0P2_SPOFR	Glyceraldehyde-3-phosphate dehydrogenase	7	0.644	0.722	0.372	0.965	1.260	0.731	0.085	0.540
34	sp|O77248|APL3_SPOLT	Apolipophorin-3	7	-1.049	0.715	0.143	0.729	-1.264	0.721	0.080	0.540
35	tr|Q8T8B3_BOMMO	Beta-tubulin	7	-0.305	0.719	0.671	1.000	0.169	0.723	0.815	0.992
36	tr|A0A0N1PF32_PAPMA	Elongation factor 1	7	0.123	0.715	0.863	1.000	0.443	0.719	0.538	0.915
37	tr|X5F7Q0_9NEOP	Alpha-tubulin (Fragment)	6	1.548	0.304	0.000	**0.000**	1.196	0.416	0.004	**0.041**
38	tr|A6YRR6_SPOEX	Hexamerine	6	-0.351	0.310	0.258	0.522	0.176	0.417	0.673	0.977
39	tr|Q0MUU7_TRIN	Heat shock cognate 70 protein	6	1.945	0.742	0.009	0.203	1.874	0.738	0.011	0.173
40	tr|A0A0U1VTU3_SPOLT	Protein disulfide-isomerase	6	0.915	0.271	0.001	**0.006**	1.396	0.721	0.053	0.409
41	tr|Q86M26_SPOEX	Translation elongation factor 2	6	0.872	0.247	0.000	**0.004**	0.162	0.735	0.826	0.992
42	tr|Q5F319_MANSE	Annexin (Fragment)	6	0.543	0.726	0.454	0.968	0.584	0.733	0.426	0.875
43	tr|I4DL30_PAPXU	Yellow-d	5	0.254	0.729	0.727	1.000	-0.632	0.737	0.391	0.875
44	tr|A0A0N1IDJ4_PAPMA	Peroxiredoxin-4	5	0.323	0.731	0.659	1.000	0.052	0.743	0.944	0.999
45	tr|A0A0K8TUL7_EPIPO	Carboxylic ester hydrolase	5	1.973	0.740	0.008	0.203	1.876	0.741	0.011	0.173
46	tr|S4PX64_9NEOP	Heat Shock Protein 21.4	5	0.774	0.761	0.310	0.930	0.808	0.777	0.299	0.820

47	tr|A0A0L7L701_9NEOP	Yellow-13	5	0.359	0.816	0.660	1.000	-0.321	0.846	0.704	0.979
48	tr|Q9NJB0_SPOFR	CALNUC	4	1.198	0.747	0.109	0.686	1.301	0.771	0.091	0.562
49	tr|D7NI45_HELAM	Glutathione S-transferase	4	0.830	0.882	0.347	0.954	0.330	0.903	0.715	0.979
50	tr|G6DRS2_DANPL	cAMP-dependent protein kinase R2	4	-1.042	0.740	0.159	0.770	-0.726	0.750	0.333	0.846
51	tr|Q587N4_BOMMO	Protein disulfide-isomerase	4	1.564	0.739	0.034	0.413	1.634	0.755	0.030	0.282
52	tr|H9JAF2_BOMMO	Uncharacterized protein	4	-0.878	0.728	0.228	0.864	-0.671	0.734	0.361	0.855
53	tr|Q86FK1_SPOEX	Lysozyme	3	1.219	0.472	0.010	**0.044**	0.229	0.663	0.730	0.993
54	tr|H9JYE6_BOMMO	Uncharacterized protein	3	0.203	0.731	0.781	1.000	-0.008	0.742	0.991	1.000
55	tr|D5G3F3_HELAM	Carboxyl/choline esterase CCE016d	3	0.139	0.728	0.848	1.000	-0.037	0.738	0.960	1.000
56	tr|I0B5W9_SPOLI	REPAT30	3	0.761	0.743	0.306	0.930	0.961	0.768	0.211	0.790
57	tr|S4NT73_9NEOP	Imaginal disc growth factor 1	3	0.391	0.741	0.598	1.000	0.117	0.756	0.877	0.992
58	tr|H9IZK9_BOMMO	Uncharacterized protein	3	-0.558	0.740	0.451	0.968	-1.091	0.759	0.151	0.740
59	tr|Q9NL61_BOMMO	Annexin	2	1.334	0.363	0.000	**0.002**	0.625	0.763	0.413	0.875
60	tr|H9JXC7_BOMMO	Uncharacterized protein	2	-0.972	0.754	0.197	0.848	-1.340	0.777	0.085	0.540
61	tr|A0A088MGF5_SPOEX	POX-C	2	0.118	0.764	0.877	1.000	0.276	0.792	0.728	0.982
62	tr|A0A0L7LF88_9NEOP	Putative ca2+-binding actin-bundling protein	2	-0.399	0.763	0.601	1.000	-0.148	0.794	0.852	0.992
63	tr|H9JTZ0_BOMMO	Uncharacterized protein	2	0.205	0.935	0.826	1.000	1.073	0.934	0.251	0.817
64	tr|G6DAA5_DANPL	Putative chondroitin synthase	2	-1.712	0.760	0.024	0.326	-0.903	0.788	0.252	0.817
65	tr|H9J6E1_BOMMO	Uncharacterized protein	2	-1.533	0.810	0.059	0.494	-1.896	0.827	0.022	0.248
66	tr|H9JLG2_BOMMO	Uncharacterized protein	1	-0.136	0.751	0.856	0.924	0.917	0.878	0.296	0.663
67	tr|H9JFZ8_BOMMO	Uncharacterized protein	1	-0.691	0.960	0.471	0.972	-0.829	0.977	0.396	0.875
68	tr|A0A0L7LAX8_9NEOP	G patch domain containing 1	1	0.702	0.763	0.358	0.959	0.242	0.792	0.760	0.992
69	tr|R4X5G1_SPOFR	Beta-hexosaminidase	1	-0.905	1.054	0.391	0.965	-1.488	1.231	0.227	0.799
70	tr|A0A0N0PAU4_PAPMA	Laminin-like protein epi-1	1	0.815	1.107	0.462	0.969	0.956	1.339	0.475	0.875
71	tr|S4PTE3_9NEOP	Cathepsin l	1	0.393	1.678	0.815	1.000	-0.247	2.258	0.913	0.999
72	tr|S4NTU9_9NEOP	Uncharacterized protein	1	0.171	1.682	0.919	1.000	0.454	1.667	0.785	0.992
73	tr|Q9NB88_AGRIP	Trypsin AiJ3 (Fragment)	1	0.623	0.801	0.437	0.968	0.316	0.854	0.711	0.979
74	tr|S4PTF0_9NEOP	Carboxypeptidase E	1	0.158	0.762	0.836	1.000	0.082	0.789	0.918	0.999
75	tr|Q2F5T8_BOMMO	Annexin	1	-0.503	1.030	0.625	1.000	-0.278	1.172	0.812	0.992

76	tr|H9JNX3_BOMMO	Uncharacterized protein	1	-4.237	1.695	0.012	0.225	-4.906	2.275	0.031	0.282
77	tr|H9IRY8_BOMMO	Uncharacterized protein	1	-0.283	0.770	0.713	1.000	-0.277	0.806	0.731	0.982

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				Cs Bermuda vs Rs Bermuda				**Maize plant Vs Bermuda plant**			
#	Uniprot Accession #	Protein name	# peptides	log2 ratio	std	p_value	FDR < 0.05	log2 ratio	std	p_value	FDR < 0.05
1	tr|A0A088MGW5_SPOEX	POX-J	236	0.221	0.161	0.169	0.370	-0.032	0.703	0.963	1.000
2	tr|D9ZFI1_SPOEX	Glucose oxidase	147	0.836	0.184	0.000	**0.000**	0.598	0.704	0.396	1.000
3	tr|Q9U5K4_SPOLT	Arylphorin subunit	82	-0.449	0.160	0.005	**0.028**	0.131	0.150	0.385	0.917
4	tr|A0A0N1IPT1_PAPMA	Glucose dehydrogenase	81	0.925	0.714	0.195	0.745	0.655	0.707	0.354	1.000
5	tr|A0A076FRM9_9NEOP	Aldehyde oxidase AOX3	76	0.262	0.157	0.095	0.261	0.527	0.705	0.454	1.000
6	tr|G8EJ31_SPOFR	Aminoacylase-1	75	-0.010	0.249	0.968	0.996	0.296	0.706	0.675	1.000
7	tr|Q0MUU6_TRINI	Heat shock cognate 70 protein	71	2.455	0.182	0.000	0.000	-0.401	0.168	0.017	0.235
8	tr|D9ZFI5_SPOEX	Putative uncharacterized protein (Fragment)	48	-0.081	0.708	0.909	1.000	-0.004	0.705	0.996	1.000
9	tr|M4M7W4_HELVI	Thiolase 2	39	-0.010	0.716	0.989	1.000	0.487	0.709	0.492	1.000
10	tr|M4M651_HELSB	Thiolase 2 (Fragment)	39	-0.182	0.714	0.798	0.997	0.729	0.707	0.303	1.000
11	tr|S5M6C1_BOMMO	Actin 4	26	0.454	0.244	0.063	0.196	-0.341	0.242	0.159	0.775
12	tr|H9JKT2_BOMMO	Uncharacterized protein	25	0.063	0.713	0.930	1.000	-0.205	0.707	0.772	1.000
13	tr|A6YQV6_SPOFR	Beta-1_3-glucanase	20	-0.472	0.725	0.515	0.948	0.489	0.711	0.492	1.000
14	tr|Q95PD6_HELVI	Actin	20	0.332	0.720	0.644	0.970	0.452	0.709	0.524	1.000
15	tr|D3GDM6_SPOLI	Carboxylic ester hydrolase	20	-1.586	0.738	0.032	0.424	0.036	0.716	0.960	1.000
16	tr|H9J7W6_BOMMO	Uncharacterized protein	19	0.566	0.776	0.466	0.929	-0.348	0.745	0.640	1.000
17	tr|H9JGA4_BOMMO	Uncharacterized protein	16	1.073	0.770	0.164	0.716	0.035	0.731	0.962	1.000
18	tr|M4PZR4_SPOFR	Arginine kinase	16	1.284	0.348	0.000	**0.002**	0.755	0.710	0.288	1.000
19	tr|S5FXH1_XESCN	Heat shock cognate 70	15	-0.168	0.728	0.817	1.000	0.305	0.713	0.668	1.000
20	tr|A0S6A0_SPOFR	Prophenoloxidase subunit 1	14	-0.112	0.322	0.729	0.902	0.194	0.301	0.519	0.917

21	tr|Q9U5K5_SPOLT	Methionine-rich storage protein	14	-0.419	0.231	0.070	0.208	0.006	0.214	0.979	1.000
22	tr|A0A0A7HBV4_9NEOP	Heat shock cognate 70 protein	13	1.069	0.283	0.000	**0.002**	-0.515	0.268	0.054	0.470
23	tr|A0A0L7KZE8_9NEOP	Putative ecdysone oxidase	11	-0.216	0.306	0.479	0.746	0.381	0.299	0.203	0.797
24	tr|L0GGU3_PLUXY	FK506-binding protein	11	0.705	0.313	0.024	0.102	0.219	0.294	0.457	0.917
25	tr|C0H6N9_BOMMO	Putative cuticle protein	11	0.020	0.724	0.978	1.000	0.144	0.711	0.839	1.000
26	tr|Q8MUR5_CHOPR	Nucleoside diphosphate kinase	10	0.503	0.730	0.491	0.930	0.373	0.714	0.601	1.000
27	tr|G6DA51_DANPL	Yellow-d	10	-0.010	0.742	0.990	1.000	-0.390	0.719	0.587	1.000
28	tr|G6DDQ0_DANPL	Moesin	10	0.012	0.769	0.987	1.000	0.830	0.726	0.253	1.000
29	tr|A0A0L7LP95_9NEOP	Putative venom acid phosphatase	10	-0.194	0.772	0.802	0.997	-0.142	0.735	0.847	1.000
30	tr|A0A0L7LJ52_9NEOP	Aldehyde oxidase AOX3 (Fragment)	9	0.246	0.739	0.739	0.972	0.422	0.718	0.556	1.000
31	tr|A0A0F7QIE4_OSTFU	Aldehyde oxidase (Fragment)	8	0.420	0.728	0.564	0.962	-0.191	0.715	0.789	1.000
32	tr|A0A0L7L6H5_9NEOP	Putative ecdysone oxidase	8	-1.291	0.286	0.000	**0.000**	-0.201	0.277	0.469	0.917
33	tr|M4Q0P2_SPOFR	Glyceraldehyde-3-phosphate dehydrogenase	7	-0.623	0.756	0.410	0.919	0.180	0.724	0.803	1.000
34	sp|O77248|APL3_SPOLT	Apolipophorin-3	7	-0.536	0.739	0.468	0.929	0.495	0.717	0.490	1.000
35	tr|Q8T8B3_BOMMO	Beta-tubulin	7	-1.368	0.759	0.071	0.540	0.432	0.722	0.549	1.000
36	tr|A0A0N1PF32_PAPMA	Elongation factor 1	7	-0.335	0.737	0.650	0.970	0.541	0.716	0.450	1.000
37	tr|X5F7Q0_9NEOP	Alpha-tubulin (Fragment)	6	1.725	0.339	0.000	**0.000**	-0.442	0.332	0.182	0.780
38	tr|A6YRR6_SPOEX	Hexamerine	6	-0.719	0.369	0.051	0.170	0.212	0.327	0.516	0.917
39	tr|Q0MUU7_TRIN	Heat shock cognate 70 protein	6	1.497	1.091	0.170	0.720	-0.684	0.738	0.353	1.000
40	tr|A0A0U1VTU3_SPOLT	Protein disulfide-isomerase	6	1.363	0.279	0.000	**0.000**	-0.847	0.290	0.003	0.087
41	tr|Q86M26_SPOEX	Translation elongation factor 2	6	1.312	0.289	0.000	**0.000**	-0.163	0.270	0.548	0.926
42	tr|Q5F319_MANSE	Annexin (Fragment)	6	0.400	0.758	0.598	0.962	0.241	0.728	0.741	1.000
43	tr|I4DL30_PAPXU	Yellow-d	5	1.563	0.762	0.040	0.428	0.774	0.730	0.289	1.000
44	tr|A0A0N1IDJ4_PAPMA	Peroxiredoxin-4	5	0.909	0.787	0.248	0.780	0.075	0.735	0.919	1.000
45	tr|A0A0K8TUL7_EPIPO	Carboxylic ester hydrolase	5	1.864	0.843	0.027	0.424	0.846	0.753	0.261	1.000
46	tr|S4PX64_9NEOP	Heat Shock Protein 21.4	5	0.037	0.827	0.964	1.000	0.268	0.765	0.726	1.000
47	tr|A0A0L7L701_9NEOP	Yellow-13	5	1.350	0.963	0.161	0.713	-0.139	0.830	0.867	1.000
48	tr|Q9NJB0_SPOFR	CALNUC	4	0.767	0.805	0.340	0.841	0.215	0.749	0.774	1.000
49	tr|D7NI45_HELAM	Glutathione S-transferase	4	1.526	1.197	0.202	0.762	-0.078	0.913	0.932	1.000

50	tr|G6DRS2_DANPL	cAMP-dependent protein kinase R2	4	-1.602	0.822	0.051	0.488	0.473	0.748	0.527	1.000
51	tr|Q587N4_BOMMO	Protein disulfide-isomerase	4	1.184	0.797	0.138	0.703	0.163	0.742	0.826	1.000
52	tr|H9JAF2_BOMMO	Uncharacterized protein	4	-1.299	0.802	0.105	0.634	0.390	0.733	0.594	1.000
53	tr|Q86FK1_SPOEX	Lysozyme	3	1.513	0.488	0.002	**0.012**	-1.494	0.508	0.003	0.087
54	tr|H9JYE6_BOMMO	Uncharacterized protein	3	0.416	0.782	0.595	0.962	-0.338	0.735	0.645	1.000
55	tr|D5G3F3_HELAM	Carboxyl/choline esterase CCE016d	3	0.471	0.781	0.547	0.962	0.776	0.732	0.289	1.000
56	tr|I0B5W9_SPOLI	REPAT30	3	0.419	0.789	0.595	0.962	-0.336	0.743	0.651	1.000
57	tr|S4NT73_9NEOP	Imaginal disc growth factor 1	3	0.898	0.823	0.275	0.780	0.178	0.748	0.812	1.000
58	tr|H9IZK9_BOMMO	Uncharacterized protein	3	0.421	0.802	0.599	0.962	0.166	0.743	0.823	1.000
59	tr|Q9NL61_BOMMO	Annexin	2	1.290	0.413	0.002	**0.012**	-0.442	0.408	0.279	0.865
60	tr|H9JXC7_BOMMO	Uncharacterized protein	2	-0.167	0.846	0.843	1.000	0.085	0.760	0.911	1.000
61	tr|A0A088MGF5_SPOEX	POX-C	2	-0.203	0.875	0.816	1.000	0.134	0.772	0.862	1.000
62	tr|A0A0L7LF88_9NEOP	Putative ca2+-binding actin-bundling protein	2	-0.879	0.866	0.310	0.817	-0.348	0.769	0.651	1.000
63	tr|H9JTZ0_BOMMO	Uncharacterized protein	2	-0.488	1.073	0.649	0.970	-0.398	0.915	0.664	1.000
64	tr|G6DAA5_DANPL	Putative chondroitin synthase	2	-3.159	0.846	0.000	**0.017**	-0.716	0.765	0.349	1.000
65	tr|H9J6E1_BOMMO	Uncharacterized protein	2	1.440	1.387	0.299	0.808	0.854	0.852	0.316	1.000
66	tr|H9JLG2_BOMMO	Uncharacterized protein	1	-0.498	1.007	0.621	0.824	0.852	0.820	0.298	0.883
67	tr|H9JFZ8_BOMMO	Uncharacterized protein	1	-0.416	1.579	0.792	0.997	0.940	1.053	0.372	1.000
68	tr|A0A0L7LAX8_9NEOP	G patch domain containing 1	1	1.622	0.871	0.063	0.529	-0.326	0.770	0.672	1.000
69	tr|R4X5G1_SPOFR	Beta-hexosaminidase	1	0.000	0.703	1.000	1.000	-1.255	1.036	0.226	1.000
70	tr|A0A0N0PAU4_PAPMA	Laminin-like protein epi-1	1	0.531	1.368	0.698	0.972	-0.126	1.078	0.907	1.000
71	tr|S4PTE3_9NEOP	Cathepsin l	1	1.674	1.726	0.332	0.831	-0.139	1.505	0.926	1.000
72	tr|S4NTU9_9NEOP	Uncharacterized protein	1	0.000	0.703	1.000	1.000	0.698	1.997	0.727	1.000
73	tr|Q9NB88_AGRIP	Trypsin AiJ3 (Fragment)	1	1.235	0.940	0.189	0.745	-0.484	0.806	0.548	1.000
74	tr|S4PTF0_9NEOP	Carboxypeptidase E	1	0.310	0.874	0.723	0.972	0.644	0.770	0.403	1.000
75	tr|Q2F5T8_BOMMO	Annexin	1	-0.952	1.442	0.509	0.948	0.366	1.054	0.728	1.000
76	tr|H9JNX3_BOMMO	Uncharacterized protein	1	-2.900	1.782	0.104	0.634	0.393	1.528	0.797	1.000
77	tr|H9IRY8_BOMMO	Uncharacterized protein	1	-0.295	0.875	0.736	0.972	-0.530	0.775	0.494	1.000

Continued

#	Uniprot Accession #	Protein name	# peptides	Rs maize Vs Rs Bermuda				Cs maize Vs Cs Bermuda			
				log2 ratio	std	p_value	FDR < 0.05	log2 ratio	std	p_value	FDR < 0.05
1	tr|A0A088MGW5_SPOEX	POX-J	236	-0.304	0.162	0.060	0.404	0.197	0.161	0.221	0.672
2	tr|D9ZFI1_SPOEX	Glucose oxidase	147	0.444	0.184	0.016	0.152	0.155	0.187	0.409	0.903
3	tr|Q9U5K4_SPOLT	Arylphorin subunit	82	-0.040	0.159	0.801	0.976	0.135	0.176	0.443	0.917
4	tr|A0A0N1IPT1_PAPMA	Glucose dehydrogenase	81	0.291	0.715	0.683	1.000	0.948	0.707	0.180	1.000
5	tr|A0A076FRM9_9NEOP	Aldehyde oxidase AOX3	76	0.151	0.170	0.377	0.921	-0.081	0.164	0.622	0.935
6	tr|G8EJ31_SPOFR	Aminoacylase-1	75	0.459	0.257	0.074	0.441	-0.019	0.294	0.950	1.000
7	tr|Q0MUU6_TRINI	Heat shock cognate 70 protein	71	-0.054	0.177	0.758	0.970	-0.936	0.183	0.000	**0.000**
8	tr|D9ZFI5_SPOEX	Putative uncharacterized protein (Fragment)	48	-0.153	0.707	0.829	1.000	-0.349	0.284	0.220	0.672
9	tr|M4M7W4_HELVI	Thiolase 2	39	1.245	0.711	0.080	0.765	-0.346	0.717	0.629	1.000
10	tr|M4M651_HELSB	Thiolase 2 (Fragment)	39	1.137	0.709	0.109	0.809	0.256	0.711	0.719	1.000
11	tr|S5M6C1_BOMMO	Actin 4	26	-0.093	0.240	0.697	0.970	-0.125	0.271	0.643	0.945
12	tr|H9JKT2_BOMMO	Uncharacterized protein	25	-0.067	0.711	0.925	1.000	-0.299	0.711	0.674	1.000
13	tr|A6YQV6_SPOFR	Beta-1_3-glucanase	20	-0.106	0.719	0.883	1.000	0.949	0.719	0.187	1.000
14	tr|Q95PD6_HELVI	Actin	20	0.222	0.716	0.757	1.000	0.648	0.714	0.364	1.000
15	tr|D3GDM6_SPOLI	Carboxylic ester hydrolase	20	-0.677	0.722	0.348	1.000	0.345	0.726	0.635	1.000
16	tr|H9J7W6_BOMMO	Uncharacterized protein	19	-0.175	0.800	0.827	1.000	-0.544	0.737	0.460	1.000
17	tr|H9JGA4_BOMMO	Uncharacterized protein	16	-0.175	0.783	0.823	1.000	-0.157	0.727	0.829	1.000
18	tr|M4PZR4_SPOFR	Arginine kinase	16	0.170	0.717	0.812	1.000	1.262	0.717	0.078	1.000
19	tr|S5FXH1_XESCN	Heat shock cognate 70	15	-0.057	0.721	0.937	1.000	0.640	0.721	0.375	1.000
20	tr|A0S6A0_SPOFR	Prophenoloxidase subunit 1	14	0.149	0.315	0.636	0.970	0.237	0.401	0.554	0.935
21	tr|Q9U5K5_SPOLT	Methionine-rich storage protein	14	-0.043	0.229	0.852	0.986	-0.060	0.265	0.820	1.000
22	tr|A0A0A7HBV4_9NEOP	Heat shock cognate 70 protein	13	0.173	0.286	0.546	0.970	-1.089	0.308	0.000	**0.017**
23	tr|A0A0L7KZE8_9NEOP	Putative ecdysone oxidase	11	3.084	0.731	0.000	**0.001**	0.246	0.300	0.413	0.903
24	tr|L0GGU3_PLUXY	FK506-binding protein	11	-0.008	0.315	0.979	1.000	0.295	0.318	0.353	0.841

25	tr|C0H6N9_BOMMO	Putative cuticle protein	11	-0.259	0.720	0.719	1.000	0.540	0.717	0.452	1.000
26	tr|Q8MUR5_CHOPR	Nucleoside diphosphate kinase	10	0.038	0.727	0.958	1.000	0.642	0.721	0.374	1.000
27	tr|G6DA51_DANPL	Yellow-d	10	-0.705	0.734	0.337	1.000	-0.086	0.732	0.907	1.000
28	tr|G6DDQ0_DANPL	Moesin	10	0.583	0.747	0.435	1.000	0.823	0.735	0.263	1.000
29	tr|A0A0L7LP95_9NEOP	Putative venom acid phosphatase	10	-0.519	0.762	0.496	1.000	0.228	0.753	0.762	1.000
30	tr|A0A0L7LJ52_9NEOP	Aldehyde oxidase AOX3 (Fragment)	9	0.505	0.731	0.490	1.000	0.208	0.730	0.775	1.000
31	tr|A0A0F7QIE4_OSTFU	Aldehyde oxidase (Fragment)	8	0.375	0.725	0.605	1.000	-0.791	0.726	0.276	1.000
32	tr|A0A0L7L6H5_9NEOP	Putative ecdysone oxidase	8	-0.106	0.285	0.711	0.970	0.043	0.350	0.902	1.000
33	tr|M4Q0P2_SPOFR	Glyceraldehyde-3-phosphate dehydrogenase	7	-0.790	0.740	0.286	0.982	1.081	0.744	0.146	1.000
34	sp|O77248|APL3_SPOLT	Apolipophorin-3	7	0.857	0.727	0.238	0.894	0.101	0.733	0.890	1.000
35	tr|Q8T8B3_BOMMO	Beta-tubulin	7	-0.342	0.736	0.642	1.000	1.130	0.740	0.127	1.000
36	tr|A0A0N1PF32_PAPMA	Elongation factor 1	7	0.090	0.729	0.902	1.000	0.784	0.728	0.282	1.000
37	tr|X5F7Q0_9NEOP	Alpha-tubulin (Fragment)	6	-0.140	0.384	0.716	0.970	-0.782	0.378	0.038	0.341
38	tr|A6YRR6_SPOEX	Hexamerine	6	-0.389	0.356	0.275	0.900	0.525	0.426	0.218	0.672
39	tr|Q0MUU7_TRIN	Heat shock cognate 70 protein	6	0.675	1.111	0.544	1.000	0.254	0.729	0.727	1.000
40	tr|A0A0U1VTU3_SPOLT	Protein disulfide-isomerase	6	-0.273	0.313	0.383	0.923	-1.507	0.276	0.000	**0.000**
41	tr|Q86M26_SPOEX	Translation elongation factor 2	6	0.367	0.304	0.227	0.815	-0.823	0.321	0.010	0.162
42	tr|Q5F319_MANSE	Annexin (Fragment)	6	0.095	0.758	0.901	1.000	0.267	0.736	0.717	1.000
43	tr|I4DL30_PAPXU	Yellow-d	5	1.581	0.764	0.039	0.500	-0.298	0.740	0.687	1.000
44	tr|A0A0N1IDJ4_PAPMA	Peroxiredoxin-4	5	0.454	0.771	0.556	1.000	-0.341	0.757	0.653	1.000
45	tr|A0A0K8TUL7_EPIPO	Carboxylic ester hydrolase	5	0.750	0.838	0.371	1.000	0.813	0.749	0.278	1.000
46	tr|S4PX64_9NEOP	Heat Shock Protein 21.4	5	-0.316	0.836	0.706	1.000	0.696	0.778	0.371	1.000
47	tr|A0A0L7L701_9NEOP	Yellow-13	5	0.523	1.027	0.610	1.000	-0.769	0.839	0.359	1.000
48	tr|Q9NJB0_SPOFR	CALNUC	4	0.049	0.802	0.952	1.000	0.548	0.776	0.480	1.000
49	tr|D7NI45_HELAM	Glutathione S-transferase	4	0.442	1.204	0.713	1.000	-0.752	0.852	0.378	1.000
50	tr|G6DRS2_DANPL	cAMP-dependent protein kinase R2	4	0.174	0.752	0.817	1.000	0.946	0.820	0.249	1.000
51	tr|Q587N4_BOMMO	Protein disulfide-isomerase	4	-0.047	0.802	0.953	1.000	0.306	0.751	0.684	1.000
52	tr|H9JAF2_BOMMO	Uncharacterized protein	4	0.159	0.743	0.831	1.000	0.855	0.788	0.278	1.000
53	tr|Q86FK1_SPOEX	Lysozyme	3	-0.770	0.547	0.159	0.678	-2.233	0.586	0.000	**0.007**

54	tr|H9JYE6_BOMMO	Uncharacterized protein	3	-0.161	0.772	0.835	1.000	-0.513	0.754	0.496	1.000
55	tr|D5G3F3_HELAM	Carboxyl/choline esterase CCE016d	3	1.026	0.763	0.179	0.815	0.497	0.754	0.510	1.000
56	tr|I0B5W9_SPOLI	REPAT30	3	-0.561	0.791	0.478	1.000	-0.151	0.771	0.845	1.000
57	tr|S4NT73_9NEOP	Imaginal disc growth factor 1	3	0.539	0.807	0.505	1.000	-0.246	0.770	0.749	1.000
58	tr|H9IZK9_BOMMO	Uncharacterized protein	3	0.892	0.782	0.254	0.904	-0.601	0.780	0.441	1.000
59	tr|Q9NL61_BOMMO	Annexin	2	0.132	0.823	0.872	1.000	0.142	0.427	0.740	0.972
60	tr|H9JXC7_BOMMO	Uncharacterized protein	2	0.453	0.786	0.564	1.000	-0.569	0.827	0.491	1.000
61	tr|A0A088MGF5_SPOEX	POX-C	2	-0.193	0.816	0.813	1.000	0.331	0.840	0.693	1.000
62	tr|A0A0L7LF88_9NEOP	Putative ca2+-binding actin-bundling protein	2	-0.737	0.824	0.372	1.000	-0.019	0.837	0.982	1.000
63	tr|H9JTZ0_BOMMO	Uncharacterized protein	2	-1.062	1.257	0.398	1.000	0.469	0.920	0.610	1.000
64	tr|G6DAA5_DANPL	Putative chondroitin synthase	2	-1.642	0.772	0.034	0.462	0.421	0.857	0.623	1.000
65	tr|H9J6E1_BOMMO	Uncharacterized protein	2	2.394	1.327	0.071	0.759	-0.400	0.880	0.649	1.000
66	tr|H9JLG2_BOMMO	Uncharacterized protein	1	0.659	0.921	0.474	0.970	2.074	0.968	0.032	0.341
67	tr|H9JFZ8_BOMMO	Uncharacterized protein	1	1.147	1.198	0.339	1.000	0.733	1.419	0.605	1.000
68	tr|A0A0L7LAX8_9NEOP	G patch domain containing 1	1	0.364	0.836	0.663	1.000	-1.016	0.828	0.220	1.000
69	tr|R4X5G1_SPOFR	Beta-hexosaminidase	1	0.000	0.703	1.000	1.000	-1.751	1.215	0.149	1.000
70	tr|A0A0N0PAU4_PAPMA	Laminin-like protein epi-1	1	-0.339	1.556	0.828	1.000	0.086	1.115	0.938	1.000
71	tr|S4PTE3_9NEOP	Cathepsin l	1	0.822	2.603	0.752	1.000	-1.100	1.140	0.335	1.000
72	tr|S4NTU9_9NEOP	Uncharacterized protein	1	0.000	0.703	1.000	1.000	0.850	2.021	0.674	1.000
73	tr|Q9NB88_AGRIP	Trypsin AiJ3 (Fragment)	1	-0.025	0.934	0.979	1.000	-0.943	0.861	0.273	1.000
74	tr|S4PTF0_9NEOP	Carboxypeptidase E	1	0.758	0.834	0.363	1.000	0.530	0.831	0.523	1.000
75	tr|Q2F5T8_BOMMO	Annexin	1	0.029	1.227	0.981	1.000	0.703	1.396	0.614	1.000
76	tr|H9JNX3_BOMMO	Uncharacterized protein	1	1.396	0.846	0.099	0.809	-0.610	2.763	0.825	1.000
77	tr|H9IRY8_BOMMO	Uncharacterized protein	1	-0.539	0.840	0.521	1.000	-0.521	0.842	0.536	1.000

Appendix D

Quantitative proteomic analysis of the saliva from the corn strain fed on different diets

#	Protein name	# peptides	Cs Tomato vs Cs Bermuda				Cs Maize vs Cs Tomato				Cs Maize Vs Cs Bermuda			
			Log 2 ratio	std	p_value	FDR < 0.05	log2 ratio	std	p_value	FDR < 0.05	log2 ratio	std	p_value	FDR < 0.05
1	POX-J	151	-1.01	0.17	0.000	**0.000**	1.26	0.18	0.000	**0.000**	0.20	0.16	0.221	0.672
2	Arylphorin subunit	82	2.95	0.19	0.000	**0.000**	-2.97	0.20	0.000	**0.000**	0.13	0.18	0.443	0.917
3	Heat shock cognate 70 protein	71	-0.70	0.20	0.000	**0.004**	-0.27	0.20	0.181	0.598	-0.94	0.18	0.000	**0.000**
4	Glucose oxidase	58	-1.29	0.21	0.000	**0.000**	1.52	0.21	0.000	**0.000**	0.15	0.19	0.409	0.903
5	Aldehyde oxidase AOX3	50	-0.33	0.18	0.059	0.222	0.29	0.19	0.135	0.525	-0.08	0.16	0.622	0.935
6	Apolipophorin-3	33	3.84	0.32	0.000	**0.000**	-3.83	0.34	0.000	**0.000**	-0.08	0.35	0.819	1.000
7	Putative uncharacterized protein (Fragment)	32	-1.72	0.32	0.000	**0.000**	1.38	0.35	0.000	**0.002**	-0.35	0.28	0.220	0.672
8	Actin-4	26	0.63	0.26	0.015	0.070	-0.74	0.28	0.009	0.080	-0.13	0.27	0.643	0.945
9	Yellow-d	23	-0.81	0.32	0.012	0.063	1.36	0.35	0.000	**0.003**	0.40	0.29	0.168	0.672
10	Putative ecdysone oxidase	21	-0.04	0.32	0.910	1.000	0.25	0.32	0.446	0.884	0.25	0.30	0.413	0.903
11	Aminoacylase-1	17	0.08	0.34	0.823	1.000	-0.15	0.37	0.684	1.000	-0.02	0.29	0.950	1.000
12	Prophenoloxidase subunit 1	14	1.85	0.33	0.000	**0.000**	-1.44	0.42	0.001	**0.010**	0.24	0.40	0.554	0.935
13	Methionine-rich storage	14	2.01	0.25	0.000	**0.000**	-2.01	0.29	0.000	**0.000**	-0.06	0.27	0.820	1.000
14	Protein disulfide-isomerase	14	-1.74	0.28	0.000	**0.000**	0.29	0.30	0.337	0.805	-1.51	0.28	0.000	**0.000**
15	Heat shock cognate 70	13	-0.50	0.31	0.113	0.345	-0.67	0.35	0.052	0.286	-1.09	0.31	0.000	**0.017**
16	FK506-binding protein	9	-0.70	0.34	0.038	0.155	1.02	0.35	0.003	**0.039**	0.29	0.32	0.353	0.841
17	Putative ecdysone oxidase	8	-0.27	0.33	0.418	0.709	0.53	0.40	0.181	0.598	0.04	0.35	0.902	1.000
18	Nucleoside diphosphate kinase	8	-1.00	0.39	0.011	0.063	0.53	0.42	0.201	0.621	-0.02	0.36	0.964	1.000
19	Translation elongation factor 2	7	-0.56	0.32	0.080	0.277	-0.10	0.37	0.788	1.000	-0.82	0.32	0.010	0.162
20	Alpha-tubulin (Fragment)	6	-0.79	0.40	0.050	0.194	0.09	0.45	0.837	1.000	-0.78	0.38	0.038	0.341
21	Arginine kinase	6	1.17	0.35	0.001	**0.010**	-1.27	0.41	0.002	**0.027**	0.04	0.36	0.909	1.000
22	Hexamerine	6	1.53	0.41	0.000	**0.002**	-0.86	0.45	0.058	0.305	0.53	0.43	0.218	0.672
23	Annexin	5	-0.50	0.48	0.301	0.589	0.31	0.55	0.579	1.000	0.14	0.43	0.740	0.972
24	Lysozyme	3	-1.69	0.61	0.005	**0.038**	-0.33	0.72	0.642	1.000	-2.23	0.59	0.000	**0.007**
25	CALNUC	3	-0.85	0.47	0.069	0.253	0.93	0.50	0.065	0.321	-0.19	0.42	0.654	0.945
26	Uncharacterized protein	1	-1.21	1.39	0.383	0.681	3.29	1.34	0.014	0.108	2.07	0.97	0.032	0.341

Appendix E

Quantitative proteomic analysis of the FAW with and without reintroduced *Pantoea ananatis*

Protein name	# of peptides	(Pantoea +) vs (Pantoea -) log2 ratio	std	p-value	FDR
POX-J	421	0.159	0.427	0.710	1.000
glucose oxidase	257	-0.645	0.452	0.154	1.000
heat shock cognate 70 protein	168	0.135	0.674	0.841	1.000
aminoacylase 1	104	-0.299	0.431	0.488	1.000
V-type ATP synthase	101	-0.095	0.566	0.867	1.000
unknown_ partial	84	-0.044	0.697	0.950	1.000
heat shock protein 70	38	1.114	0.590	0.059	1.000
actin	30	-0.279	0.544	0.608	1.000
antennal esterase CXE15	30	-0.576	0.504	0.253	1.000
arginine kinase	26	0.311	0.472	0.510	1.000
translation elongation factor 2	25	0.408	0.499	0.414	1.000
beta-1_3-glucanase	22	-0.033	0.645	0.959	1.000
apolipophorin-III	16	-0.148	0.534	0.781	1.000
annexin IX	13	0.412	0.524	0.431	1.000
carboxyl/choline esterase	13	0.189	0.697	0.786	1.000
carboxyl/choline esterase	13	0.058	0.696	0.934	1.000
Kettin1 protein	13	0.765	0.687	0.266	1.000
hypothetical protein	12	0.307	0.946	0.745	1.000
alpha-tubulin	11	0.366	0.491	0.456	1.000
elongation factor 1-alpha	10	0.376	0.570	0.509	1.000
hemolin	10	1.323	0.559	0.018	0.531
thioredoxin peroxidase	10	-0.008	1.244	0.995	1.000
14-3-3 zeta protein	9	0.268	0.546	0.623	1.000
beta-tubulin	9	1.130	0.476	0.018	0.531
trehalase	9	-0.299	0.459	0.515	1.000
carboxylesterase	8	-1.264	1.159	0.276	1.000
glyceraldehyde-3-phosphate	8	-0.271	0.571	0.635	1.000

dehydrogenase					
POX-C	8	-0.130	0.693	0.851	1.000
C-MYC	7	2.274	1.781	0.202	1.000
GABA-B receptor type 1_ partial	7	0.272	0.713	0.703	1.000
abnormal wing disc-like protein	6	-0.441	0.597	0.460	1.000
arylphorin subunit	6	0.364	0.667	0.585	1.000
glucosidase II alpha-subunit	6	0.475	0.504	0.346	1.000
ribosomal protein L31_ partial	6	1.181	1.024	0.249	1.000
small heat shock protein	6	-0.941	0.633	0.137	1.000
60S ribosomal protein L15	5	-0.574	1.181	0.627	1.000
cytochrome P450 CYP6B43	5	-1.164	0.696	0.095	1.000
enolase	5	0.308	0.606	0.612	1.000
thiolase 2	5	-0.839	0.697	0.228	1.000
C-type lectin	4	0.283	0.807	0.726	1.000
CALNUC	4	0.087	0.696	0.901	1.000
chemosensory protein 20	4	-0.482	0.524	0.358	1.000
chitin synthase B	4	0.225	0.557	0.687	1.000
heat shock protein 90	4	-0.389	0.594	0.512	1.000

VITA

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Education

2010-2016 Ph.D., Entomology with a minor in Statistics. College of Agricultural sciences and Eberly College of Science. The Pennsylvania State University. University Park, PA.

2000-2005 B.S., Agronomy, Department of Agronomy, College of Agricultural Sciences. Universidad de Caldas, Caldas, Colombia.

Research Experience

08/2010 - 08/2016 **Graduate Research Assistant/Fellow.** Department of Entomology. The Pennsylvania State University. University Park, PA 16802. USA.
05-2011 – 07/2011 **Summer Intern.** Institute for Bioscience and Biotechnology Research. University of Maryland, 9600 Gudelsky Drive Rockville, MD.
02/2007 - 06/2010 **Research Associate**. Colombian Center for Coffee Research (Cenicafé). Chinchiná, Colombia.
08/2006 – 10/2006 **Visiting Student.** University of Florida, Tropical Research and Education Center (UF-TREC). Homestead, Florida USA.
06/2004 – 07/2006 **Research Student.** Colombian Center for Coffee Research (Cenicafé). Chinchiná, Colombia.

Selected Publications

Acevedo FE, Rivera-Vega LJ. Chung SH, Ray S, and Felton GW. (2015) Cues from Chewing Insects—the Intersection of DAMPs, HAMPs, MAMPs and Effectors. *Current Opinion of Plant Biology* (26):80-86.

Chuang W, Ray S, **Acevedo FE**, Peiffer M, Felton G, Luthe DS. (2014) Herbivore cues from the fall armyworm (*Spodoptera frugiperda*) larvae trigger direct defenses in maize. *Molecular Plant-Microbe Interactions* 27 (5): 461-470.

Teaching Assistant

BMMB 852 Applied Bioinformatics. Penn State University, summer, 2015
ENT 202 The Insect Connection. Penn State University, spring, 2012
ENT 313 Principles of Entomology. Penn State University, spring, 2011 and spring 2012

Selected Grants and Awards

2015 Northeast Sustainable Agriculture and Education (SARE) Graduate Student Grant.
2015 Ralph O. Mumma Outstanding Graduate Award. Department of Entomology. Penn State University.
2013 College of Agricultural Sciences Competitive Grant. Penn State University.
2010 Fulbright Fellowship. Colombian Fulbright association and US Department of State, Bogota, Colombia