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FRASS HAPPENS: CATERPILLAR FRASS INDUCED DEFENSE RESPONSES IN PLANTS

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by

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Abstract:

Caterpillar behaviors such as feeding, crawling, and oviposition are known to induce defenses in maize and other plant species. I examined plant defense responses to another important caterpillar behavior, their defecation. Fall armyworms (FAW, *Spodoptera frugiperda*), a major threat to maize (*Zea mays*), are voracious eaters and deposit copious amounts of frass in the enclosed whorl tissue surrounding their feeding site where it remains for long periods of time. FAW frass is composed of molecules derived from the host plant, the insect itself and associated microbes and hence provides abundant cues that may alter plant defense responses. I observed that proteins from FAW frass initially induced wound-responsive defense genes in maize; however, a pathogenesis-related (*pr*) defense gene was induced as the time after application increased. Elicitation of pathogen defenses by frass proteins was correlated with increased herbivore and reduced fungal pathogen performance over time. These responses differ from the typical plant response to oral secretions of the FAW. Fractionation of the proteins from caterpillar frass led to the identification of plant chitinase Pr4 and insect gut protein Insect Intestinal Mucin (IIM) that triggered pathogen and wound-induced defenses in maize, respectively. Application of purified Pr4 on maize plants induced pathogen defenses, suppressed herbivore defenses and increased performance of the herbivores on plants treated with Pr4. Immuno-depletion of the IIM resulted in an attenuation of herbivore-triggered wound response in maize and an elevation of pathogen defenses in maize leaves. Therefore in maize, Pr4 functions as an effector of herbivore defenses, while IIM acts as an elicitor of herbivore defenses. I also measured frass-induced defenses in host-herbivore systems where frass accumulates in enclosed feeding sites of the herbivore and compared them to those where it does not. Tomato fruitworm (*Helicoverpa zea* or TFW) frass from larvae fed on tomato (*Solanum lycopersicum*) leaves induced a sustained herbivore defense response in tomato leaves where frass does not accumulate close to feeding sites. However, TFW frass from larvae fed on tomato fruits induced pathogen defenses when injected in tomato fruits where frass does accumulate in close proximity to the herbivore’s feeding site. Therefore, frass-mediated elicitation of plant defenses are specific to host-herbivore
systems and depends on the insect and plant species involved, the diet of the herbivore, and the tissue on which it is deposited.
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Preface:

This dissertation contains experiments that were conducted by Dr. Saumik Basu at Dr. Joe Louis’ lab in University of Nebraska, Lincoln. In Chapter 5, the frass induced defense responses in maize to European corn borer frass were investigated by Dr. Saumik Basu. A letter of permission signed by Dr. Saumik Basu and Dr. Joe Louis granting the inclusion of their work in this dissertation is attached.
Dear Dr. Joe Louis and Dr. Saumik Basu,

I am completing a doctoral dissertation at The Pennsylvania State University entitled “Frass Happens: Caterpillar Frass Induced Defense Responses in Plants”. I would like your permission to use results obtained by you in your laboratory at University of Nebraska, Lincoln of induction of plant defenses in maize by European corn borer frass. With your approval I would like to include the results from this experiment in Chapter 5 of my thesis under the section entitled: “European corn borer frass sustains herbivore-induced defenses while suppressing pathogen-induced defenses in maize”

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If these arrangements meet with you approval, please sign this letter where indicated below and return it to me at you convenience. Thank you very much.

Sincerely,

Swayanjit Ray

PERMISSION GRANTED FOR THE USE REQUESTED ABOVE:

Dr. Saumik Basu

Dr. Joe Louis

Date: 11/18/2015

Date: 11/18/2015
Acknowledgements:

This dissertation is definitely one of my proudest accomplishments till now. However, none of this work would have been possible without the love and support of some the greatest people that I have worked with and my amazing ‘pack’ of friends. First, I would like to thank my dissertation adviser Professor Dawn S Luthe for being the best mentor that anyone can ever ask for. It’s with her guidance, constant support and encouragement I can call myself a researcher. I wish I can be as great a mentor and a scientist that she is some day in my career. I am indebted to Professor Gary W. Felton for his guidance, his funky titles for my presentations and papers and his subtle sense of humor which now shows in most of my presentations. This journey started with me being inducted into the Plant Biology family back in 2008 for which I am ever grateful to Professor Teh-Hui Kao and for his mentorship throughout my study. The Plant Biology program at Penn State has given me my best friends, colleagues, mentors and peers which I will treasure for life. I have to also thank my committee members Professor Surinder Chopra and Professor Ben McGraw for their support to help me finish this dissertation.

The more deserving of this dissertation is possibly my family than me. Being a researcher is a humbling experience each day, and I am blessed to have parents and grandparents who teaches me humility and patience everyday through their life example. Being one of a twin, also adds to this process of humility, no matter what I do, I am always aware that there is someone just like me who can do this as well but only better. So thanks to my brother for keeping me grounded and always making sure that I have a friend and I am never alone, since before birth.

I am one of the very lucky few on this planet who can boast to have two homes. To all my friends, colleagues and peers in State College, I have a home away from home because of you. I am ever so grateful for your friendship and all your support through this stupendous journey towards earning my doctorate. I treasure all your friendship dearly and thank you with all my heart.
Chapter 1. Introduction

The plant kingdom and the Class Insecta are the two most abundant taxa of living organisms on our planet. While green plants are the most abundant group of organisms in terms of biomass, insects outnumber them in the total number of species. More than half of the known insect species are phytophagous (Schoonhoven et al., 2005). Plants are known to put up an arsenal of defenses in response to insect herbivory (Felton, 2005a; Felton and Tumlinson, 2008; Howe and Jander, 2008). Plant defenses may also be induced by insect herbivory (Chen, 2008a). Insect-induced defenses in plants may be direct when the attacked plant tissues synthesize chemicals and enzymes that have toxic, antinutritional, or repellent effects on herbivores (Felton, 2005a; Chen, 2008a; Felton and Tumlinson, 2008). One example of such induced defenses is the synthesis of toxic proteins such as Mir1-CP found in some maize genotypes that degrades the lining of the caterpillar gut and retards its growth on the host (Pechan et al., 2002). Insects may also induce indirect defenses in plants when they emit volatiles in response to insect herbivory that attracts predators or parasites of the herbivore (Pare and Tumlinson, 1999; Walling, 2000).

To mount a successful defense response, plants are known to recognize the cues that are left by the insects during the act of herbivory (Acevedo et al., 2015; Schmelz, 2015). Although, mechanical damage is the most obvious cue of herbivory in plants, mechanical damage alone cannot explain the full spectrum of plant defenses that are induced by the insects (Felton and Tumlinson, 2008). Insect-related secretions such as oral secretions and the saliva that are deposited on the plants by herbivores contain chemical ‘cues’ for herbivory which can trigger defense responses in the plants (Bonaventure, 2014; Acevedo et al., 2015; Schmelz, 2015). While the oral secretion (or the regurgitant) originates from the foregut of most chewing insects, saliva is derived from the salivary glands and is deposited on the plants by both chewing and piercing/sucking insects (Felton et al., 2014). Insect-derived molecules that trigger herbivore defenses in plants have been isolated from the oral secretions of chewing insects such as the fatty acid conjugates volicitin and caeliferin, and proteins such as β-glucosidase and
inceptin (Mattiacci et al., 1995a; Alborn, 1997; Schmelz et al., 2006a; Alborn et al., 2007).
Enzymes from the caterpillar saliva such as glucose oxidase can induce herbivore defenses in plants (Tian et al., 2012). Feeding is only one of the behaviors of the insect on its host plant. The insect not only feeds on the host plant, it also lives on it (Schoonhoven et al., 2005). The host plants can also detect some of the other behaviors of the insect associated with it. A touch response by insect crawling on the host tissue trigger plant defenses (Peiffer et al., 2009a; Tooker et al., 2010). The deposition of secretion known as ‘egg secretions’ during oviposition may also put plants on an alert (Hilker and Meiners, 2006). Another fatty acid conjugate, bruchin derived from the egg secretion of insect has also been shown to induce plant defenses (Doss et al., 2000). Although, there are evidence of plant’s recognition of cues associated with most insect behaviors, relatively little is known about how plants perceive the most abundant and prolific insect behavior on plant, their defecation.

An insect’s life on the plant is a perilous one where it has to defend itself from not only the plant’s direct defenses, but also from the predators that are attracted by the plant’s volatile emissions. The zig-zag model that describes the ploy-counterploy between plants and pathogens (Jones and Dangl, 2006) has been adopted to explain the plant-herbivore interactions as well (Felton and Tumlinson, 2008; Felton et al., 2014; Acevedo et al., 2015). While the above mentioned cues that trigger herbivore defenses in plants have been referred to as ‘elicitors’ or HAMPs (Herbivore Associated Molecular Patterns), insect-derived molecules that can suppress the herbivore defenses in plants have been referred to as ‘effectors’ (Jones and Dangl, 2006; Felton and Tumlinson, 2008). Contrary to the story of herbivore defenses triggered by various elicitors from herbivore secretions, we have only scratched the surface on understanding the effector-mediated suppression of herbivore defenses in plants (Wu et al., 2012).

The regulation of plant defense pathways can be classified into two main pathways: the jasmonic acid (JA) primarily works against the onslaught of herbivore attack (Walling, 2000; Howe and Jander, 2008), while the salicylic acid (SA) works primarily against (Kunkel and Brooks, 2002; Glazebrook, 2005). These two pathways are known to be antagonistic to each other in most plant species possibly to the cost associated with activating both pathways at the
same time (Thaler et al., 2012; Van der Does et al., 2013). Interestingly, this antagonistic cross-talk between the two pathways has been harnessed by several pathogens and herbivores to work in their favor. The plant pathogen *Pseudomonas syringae pv. tomato* is known to synthesize a JA analog coronatine that can mediate a suppression of pathogen-induced defenses in the host thereby increasing the performance of the pathogen on the host (Block et al., 2005; Geng et al., 2012; Zheng et al., 2012). Similarly, whiteflies trigger pathogen defense responses in tomato and suppress herbivore defenses that increases the performance of the insects on the plants (Zarate et al., 2007). The oral secretion of Colorado potato beetle harbors endophytic bacteria that can be deposited on the host plants during feeding, suppresses the herbivore defenses and trick the host into believing that it is under attack from a pathogen (Chung et al., 2013). The effectors that mediate such suppression of herbivore defenses are not well characterized. Glucose oxidase, an enzyme isolated from the saliva of tomato fruitworm, was shown to suppress herbivore defenses in tobacco (Musser et al., 2002). Salivary ATPases and apyrases from the tomato fruitworm saliva has also been shown to suppress pathogen defenses in tomato (Wu et al., 2012). In a recent work, salicylic acid isolated from aphid honeydew was shown to suppress herbivore defenses in plants (Schwartzberg and Tumlinson, 2014).

The focus of this dissertation was to understand the plant defenses in response to the most abundant cue that the insect deposits on the plants, its frass. Frass-mediated plant defenses have been only shown in piercing/sucking mouth part insects (Schwartzberg and Tumlinson, 2014), and has been speculated to have a role in chewing insects as well (Schmelz, 2015). Frass from chewing insects was shown to harbor the herbivore defense hormone JA and another study shows that the insect avoids deposition of frass in order to detract predators (Weiss, 2003; Tooker and de Moraes, 2006). However, the effect of insect frass on plant defenses has not been studied. Frass-mediated defenses can play a significant role in plant-herbivore system where the insect feeds in enclosed host tissues and the frass accumulates over extended periods of time in close proximity of the feeding site. Cues from the frass may be perceived by the wounded host tissue leading to manipulation of plant defenses. Fall armyworm (*Spodoptera frugiperda* or FAW), a major pest of maize in the Americas, feeds in the enclosed whorl tissues
of maize where the frass accumulates for extended periods of time close to the feeding sites. My hypothesis was that frass from FAW larvae can manipulate plant defenses in maize. We measured defense related gene transcripts and accumulation of the plant defense hormones JA and SA in response to caterpillar frass. We observed that frass from FAW caterpillars suppressed the herbivore defenses temporally leading to the increased performance of the herbivore while activating the pathogen defenses and suppressing the growth of the fungal pathogen (Ray et al., 2015).

The second objective of this study was to identify the elicitor(s) and or effector(s) that are present in the frass that mediates the defense responses in plants. The elicitors and effectors that have been identified till date from herbivore secretions can of insect or plant origin. Frass-related elicitors or effectors can be not only from the insect or plants, but may also originate from the microbes growing in the frass or insect endosymbionts. While insect-derived molecules mediating plant defenses are amply known, there is an increasing evidence that plant derived molecules from insect secretions may be deposited on the host and recognized as ‘self’ leading to an elicitation of plant defenses (Heil, 2009). We fractionated proteins from FAW frass and identified proteins in FAW frass originating from both the insect and the plant that were involved in triggering the frass-mediated plant defenses in maize.

Another unsolved mystery of induction of plant defenses is the variable response of plants to the same insect cues. For example, oral secretions from tomato fruit worm (Helicoverpa zea) induces herbivore defenses in tomato but suppresses such defenses in tobacco (Musser et al., 2002; Tian et al., 2012). Induction of plant defenses may be different even with application of the same elicitor, such as glucose oxidase that suppresses direct defenses in tobacco, induces them in tomato and has no effect in maize (Musser et al., 2002; Tian et al., 2012; Louis et al., 2013b). Insect secretions are a blend of various molecules that can elicit variable and composite blends of plant defense responses. For example, glucose oxidase from tomato fruitworm saliva induces direct defenses in tomato while salivary ATPases suppress them (Tian et al., 2012; Wu et al., 2012). Therefore, the third objective of this study was to measure the frass-induced plant defenses in different host-herbivore systems, especially to compare frass-triggered defenses in host-herbivore systems where frass accumulates in enclosed feeding sites such as tomato.
fruitworm feeding on the tomato fruit and ones in which frass does not accumulate in enclosed feeding sites such as FAW feeding on rice leaves. My hypothesis is that frass-induced plant defenses are specific to host-herbivore systems and varies based on the composition of the frass, the insect depositing the frass, the tissue on which the frass is deposited and the diet of the insect. We measured frass-induced plant defenses by European corn borer (*Ostrinia nubialis*) frass in maize, tomato fruitworm frass (*Helicoverpa zea*) in tomato fruit and cabbage looper (*Trichoplusia ni*) frass in cabbage as examples of host-herbivore systems where frass accumulates on the host tissue over time. Contrastingly, we also measured frass-induced defenses in response to frass from tomato fruitworm frass on tomato leaves and FAW frass on rice leaves where frass does not accumulate on host tissues over time. My results suggest that frass-induced defenses in plants are specific to host-herbivore systems and depends on the composition of the frass, the tissue on which the frass is deposited and the insect and plant species that is involved.
Chapter 2. Maize Plants Recognize Herbivore-Associated Cues from Caterpillar Frass

Introduction:
Land plants are under constant threat of attack from herbivorous insects. Nearly 400,000 phytophagous insect species are known to live on 300,000 vascular plant species (Schoonhoven et al., 2005). When these herbivores feed on plants they not only cause mechanical damage, but often deposit substances that can manipulate the plant’s response to herbivory. These substances have been referred to as Herbivore-Associated Molecular Patterns (HAMPs), analogous to the Microbial (or Pathogen) Associated Molecular Patterns (MAMPs or PAMPs) that affect plant responses to pathogenic fungi or bacteria (Felton and Tumlinson, 2008; Mithöfer and Boland, 2008). Oral secretions such as regurgitant and those of salivary glands contain HAMPs that can elevate the wound response in plants (Alborn 1997; Felton and Eichenseer 1999; Felton and Tumlinson 2008; Halitschke et al. 2001; Louis et al. 2013; Musser et al. 2002; Schäfer et al. 2011; Tian et al. 2012). In addition to mechanical damage and HAMP deposition, it has been shown that herbivore behavior alters plant defenses. For example, feeding by sucking insects that pierce tissues induces both direct and indirect defenses (Bolwell and Wojtaszek, 1997; Korth and Dixon, 1997; Baldwin and Preston, 1999; Dempsey et al., 1999). Furthermore, it has been demonstrated that caterpillar crawling and even oviposition induces plant defenses (Alborn 1997; Bass et al. 2004; Doss et al. 2000; Hilfiker et al. 2014; Hilker and Meiners 2006; Kim et al. 2012; Little et al. 2007; Tooker et al. 2010). On the other hand, insect-mediated suppression of herbivore defenses has also been shown in several host-herbivore interactions implicating the presence of effector molecules in herbivore secretions (Chung et al. 2013; Musser et al. 2002; Wu et al. 2012; Zarate et al. 2007). In this study I focused on understanding plant defense responses to another herbivore behavior, their defecation.
When caterpillars such as fall armyworm (FAW, *Spodoptera frugiperda*) or tomato fruitworm (*Helicoverpa zea*) voraciously feed in enclosed spaces such as maize whorls or tomato fruits, a significant amount of frass accumulates in these tissues leaving abundant cues of herbivory. Since frass contains many insect, microbial and plant-derived molecules (Chen et al. 2005; Chen et al. 2007), it is probable that it contains cues that could be recognized by plants to elicit defensive responses. Recently, pea aphid honeydew has been shown to suppress accumulation of jasmonic acid (JA) and elevate salicylic acid (SA) in broad bean plants (Schwartzberg and Tumlinson 2014) and whitefly honeydew was shown to increase the accumulation of SA that subsequently suppressed JA-induced defenses in tomato (VanDoorn et al., 2015); however, plant defenses in response to chewing insect frass have not been studied.

While feeding on maize, FAW caterpillars not only wound the plant, but also deposit copious amounts of frass that typically accumulates inside the whorls (Fig 2.1), which could play a significant role in altering defense responses against the herbivore. In this study, I show that the performance of caterpillars fed on frass-treated plants increases with time. I also observed activation of pathogen defense pathway and reduced performance of a fungal pathogen in response to frass treatment of maize leaves.

**Materials and methods:**

*Plant and insect materials:* Maize (*Zea mays*, var. B73) plants were grown in glasshouse conditions with 16 hr light and 8 hr dark cycle in Hagerstown loam till they reached mid-whorl (V7-V8) development stage (Ritchie et al., 1998). FAW (*Spodoptera frugiperda*) eggs were obtained from USDA-ARS at Mississippi State University and reared on maize plants to collect frass.

*Frass collection and protein extraction:* Caterpillars were fed whorl leaves of B73 plants at V8 stage in 30 ml diet cups and frass was collected every 24 hr. The collected frass was homogenized in 1x-phosphate buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate) for 30 min at a ratio of 1 g (wet weight) of frass in 5 ml of PBS. After homogenization the slurry was filtered through Miracloth (EMD Millipore, USA) to remove the
debris and the extract was filter sterilized in 0.2-µm filters (EMD Millipore, USA). The sterile frass extract was then concentrated using centrifugal columns with a 3-kD molecular weight cut-off (Pall Life Sciences, USA) to remove small molecules present in the extract. Total protein in the frass extract was measured using the Bradford assay; 100 mg of whole frass yields approximately 20 µg of frass protein. Two of the outermost whorl leaves of maize at V8 stage were wounded using a wounding tool (as shown in Figure S1) and frass extract containing 20 µg of protein or 100 mg of whole frass was applied to each wound site. Leaf tissue around the wound site, approximately (1.5-2) cm² area, was collected for RNA extraction and phytohormone analysis.

**RNA extraction, cDNA preparation and real-time PCR:** Leaf tissue was homogenized in liquid nitrogen using the GenoGrinder 2000 (OPS Diagnostics, USA) and RNA was extracted with TRIzol reagent (Life Technologies, USA) using the manufacturer’s protocol (100 mg tissue in 1 ml TRIzol). Genomic DNA was removed from the RNA by treatment with 2 units of DNaseI enzyme (New England Biolabs, USA) as suggested by manufacturer. The genomic DNA-free RNA was quantified using Nanodrop (Thermo-Fisher Scientific, USA). cDNA was made from 1 µg of total RNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) using Oligo-dT. Quantitative real-time PCR (qRT-PCR) was performed with Fast Start Universal SYBR Green Master Mix (Roche Applied Science, USA) with actin as reference gene. Gene-specific primers were designed with Primer Express 3.0 (Life Technologies, USA; details in Table S1). RNA extraction, cDNA synthesis and qRT-PCR were performed for each biological replicate separately, with each treatment having four biological replicates per time point. Gene expression levels in frass-treated, PBS buffer-treated and undamaged control plants were measured 4, 8, 24 and 48 hr after application.

**Data analyses:** Relative quantification for gene expression was analyzed with a two-factor ANOVA using time and treatment (frass, buffer or undamaged controls) as independent variables with SAS 9.1 (SAS Institute Inc., USA) software at p<0.05 level of confidence. However, there were interactions between time and treatment for all genes tested, hence a multiple comparison Tukey test was performed for each gene at p<0.05 level of confidence. The relative
quantification of gene expression after pronase digestion of frass proteins (Fig 2.5a,b) and frass or buffer treatment following FAW herbivory (Fig 2.6a-c) were analyzed with a multiple comparison Tukey test and tested for significance among treatments at p<0.05 using SAS 9.1. Absolute phytohormone quantities, caterpillar and fungal growth rate data were analyzed using one-way ANOVA and comparison of means using SAS 9.1 (SAS Institute Inc., USA) software at p<0.05 level of confidence with different treatments as the dependent variable each time point.

*Phytohormone extraction and measurement:* Plant tissue (100 mg) surrounding the wound site (1.5-2 cm²) was used to extract phytohormones as described in an earlier protocol (Schmelz et al., 2003; Schmelz et al., 2004). The phytohormones were analyzed by coupled GC-MS with isobutane chemical ionization with selected-ion monitoring (Helms et al., 2013). Amounts of JA and SA were quantified by comparison to internal standards of deuterated isotopes of both hormones at 10 ng/µl. The retention time, [M+H]+ m/z ions for the hormones were JA (cis-JA 8.7 min, 225; ²H₁-JA standard 8.48 min, 227) and SA (actual SA 4.97 min, 153; ³H₁-SA standard 4.73 min; 157). Hormones were extracted from four plants per treatment per time point.

*Protease digestion of proteins in frass extract:* To determine if the molecule(s) in frass mediating plant defenses are proteins, the extract was digested with 1:1 (w/w) protease cocktail Pronase (Sigma Aldrich, USA) overnight. Thereafter, the proteases were inactivated by boiling for 0.5 hr and the extract was concentrated using a 10-kD molecular weight cut off column (Pall Life Sciences, USA). I applied the retentate (larger than 10 kD), the eluent (smaller than 10 kD) and a combined fraction of both eluent and retentate from the column to wound sites on maize leaves. I also fractionated the undigested frass extract in a similar manner using the 10-kD columns and applied the retentate, eluent and combined fractions on maize leaves. Similarly, 1xPBS buffer was treated with pronase, heat inactivated and fractionated and applied to plants as controls. Relative abundance of transcript levels of *mpi* and *pr5* were measured for plants treated with the undigested frass fractions, pronase-digested frass fractions, buffer with inactivated pronase and undamaged control plants after 8 and 24 hr respectively with actin as reference gene. Relative quantification values were analyzed by one-way ANOVA at their respective time points (p<0.05).
Fall armyworm bioassay: Whorl leaves of six plants were treated with frass proteins or buffer for 8 and 24 hr, the tissue around the application site (3-4 cm on each side of wound) was collected and used for caterpillar feeding bioassays as described in Chuang et al. (Chuang et al., 2014b). Briefly, the plant tissues treated for either 8 or 24 hr were fed to 30 naïve first instar FAW for 4 days. Fresh tissue (after fresh application of frass and buffer) was supplied every day and older tissue was removed. Caterpillar mass was measured at beginning and end of the bioassay and used to calculate relative growth rates (RGR) as described earlier (Hoffmann and Poorter, 2002; Mohan et al., 2008). One-way ANOVA was performed to determine significance (p<0.05).

Fungal Bioassay: Cochliobus heterostrophus was cultured on potato dextrose agar (PDA) media for 2 weeks under lights (Yoder 1988). Fresh leaves from B73 maize at the V8 stage were treated with the frass extract, harvested 8 and 24 hr after treatment and placed on 150 mm petri dish with a 1% agar base with 0.01% kinetin. C. heterostrophus spores (100 µl at a concentration of $10^4$ spores per ml) was applied to one side of the leaf between two wound sites and the other side was treated with water (Fig 2.2 c, d). Inoculated leaves were incubated at 26˚C for 60 hr at 16 hr light and 8 hr dark cycles. The area infected by fungi was measured using Sigma Scan Pro 5.0 (SPSS, USA) at 60 hr after inoculation. Two leaves per biological replicate were analyzed for the infected area with three biological replicates per treatment at every time point. Significance of infection was measured by one-way ANOVA for the percentage of area infected by the pathogen (p<0.05).

Feeding with FAW and subsequent frass application and immunoblot analysis: Maize leaves were fed with fifth instar FAW caterpillars in inverted clip cages for 3 hr. While in these cages, larvae fed on the ventral side of the leaves, but deposited frass only in cages and not on the leaves (Fig. S4 a-c). Each plant was fed with three larvae for 3 hr and feeding sites were treated with buffer or 50 µg of frass protein for 4, 8, 24 or 48 hours. Four plants were used as biological replicates per treatment. Leaf tissue surrounding the cages (100 mg) was frozen in liquid nitrogen and homogenized using the GenoGrinder 2000 (OPS Diagnostics, USA). These samples were then used to extract RNA and qRT-PCR was performed as described before. Similarly,
these samples were also used to extract proteins with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Laemmli, 1970). Protein concentrations were determined by Non-interferring Protein assay kit (G-Biosciences, USA). Fifty μg of protein were loaded in each gel lane and separated by SDS-PAGE. Gels were blotted onto nitrocellulose membranes using the Panther Semi-Dry Electroblotter (Thermo Scientific, Owl, MA, USA). The blots were reversibly stained with Ponceau S (Salinovich and Montelaro, 1986) before immunoblot analysis to visualize protein transfer.

Polyclonal antibody to RIP protein was obtained from Dr. Rebecca Boston (North Carolina State University, Raleigh, NC, USA). Western blots were carried out using 1:10,000 diluted anti-RIP antibody and 1:10,000 diluted anti-rabbit secondary antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific, Rockford, IL, USA). Immunoreacting proteins were detected by chemiluminescence (West Femto Maximum Sensitivity Substrate, Thermo Scientific).

Results:

**Fall armyworm larvae perform better on plants treated with frass compared to buffer:**

When FAW larvae feed in the enclosed whorls of maize they mechanically damage the leaves and deposit frass that accumulates and remains in close contact with the wounded feeding sites over extended periods of time (Fig 2.1). Hence, I wanted to compare the performance of FAW caterpillars fed on leaves treated with frass to those with no frass treatment. I treated mechanically wounded maize leaves with frass extract or buffer only and harvested tissues at 8 and 24 hr time points to determine the effect of frass treatment on FAW growth. The harvested tissue was fed to naïve first instar FAW caterpillars and their relative growth rate was measured after 4 days. Naïve caterpillars showed no significant difference in growth when fed with leaves from plants treated for 8 hr (Fig 2.2a). However, they grew significantly faster when fed plant tissue that was treated with frass for 24 hr (Fig 2.2a). This strongly suggested that the longer–term exposure of plants to frass suppresses herbivore defenses leading to enhanced insect performance.
Frass protein-treated maize leaves inhibit the growth of Cochliobolus heterostrophus:

I also measured the performance of a fungal pathogen in response to frass treatment of maize leaves, in an effort to understand if the pathogen defense pathway was triggered at the time when herbivore defenses appeared to be suppressed. I inoculated maize leaves with Cochliobolus heterostrophus spores after 8 and 24 hr of frass treatment. C. heterostrophus is a necrotrophic fungus that causes southern leaf blight disease in maize and is characterized by eye-spot lesions on leaves (Yoder, 1988; Shanmugam et al., 2010). I inoculated undamaged maize leaves, those treated with the frass protein extract or buffer with equal amounts of fungal spores and measured necrotic lesion areas after 60 hr incubation. There was no difference in fungal growth on leaves treated with frass extract for 8 hr (Fig 2.2b, c); however when leaves were treated for 24 hr, fungal growth was significantly less than on the buffer-treated or undamaged control leaves (Fig 2.2b, d). It appears that maize plants respond to cues in FAW frass at 24 hr when resistance to herbivores was suppressed and pathogen resistance increased.

Fall armyworm frass attenuates the accumulation of maize herbivore defense gene transcripts and jasmonic acid levels while it elevates the abundance of pathogen defense gene transcript and salicylic acid over time:

To determine if there were temporal changes in maize defenses in response to frass application, I examined the expression levels of a suite of genes involved the herbivore defense pathway such as 12-oxophytodienoic acid reductase7 (opr7) involved in the JA biosynthesis pathway and wound-inducible maize protease inhibitor (mpi). Both opr7 and mpi are known markers for herbivore defense pathway (Chuang et al., 2014; Louis et al., 2013; Yan et al., 2012). Because frass is likely to remain in the maize whorl for relatively long periods of time, expression of the marker genes was measured from as early as 4 and 8 hr to as late as 24 and 48 hr after treatment.

opr7, encodes an enzyme in the JA biosynthesis pathway, the disruption of which leads to significant decreases in JA biosynthesis as well as an increased herbivore performance (Yan et al., 2012). The levels of opr7 transcripts were not significantly different between the frass and
buffer-treated plants at any of the times tested (Fig 2.3a) except at 24 hr. At 24 hr the frass treatment significantly suppressed the abundance of \textit{opr7} transcripts compared to buffer-treated plants (Fig 2.3a). When I determined the transcript levels of \textit{mpi}, an indicator of the wound response in maize, frass-treated plants had significantly higher abundance at 4 and 8 hr after application compared to buffer treatment, however, its abundance declined 8 hr after treatment (Fig 2.3b). I also measured the accumulation of \textit{cis}-JA in response to frass and buffer treatment at 4, 8, 24, 48 hr after application but, I did not detect \textit{cis}-JA in frass- or buffer-treated plants (Fig 2.3c). However, \textit{cis}-JA levels were lower in frass-treated plants compared to buffer treatment at 1 hr after application (Fig 2.3c).

Because I observed that the performance of a fungal pathogen on maize leaves decreased in response to frass at 24 hr, I measured the expression of a pathogenesis-related (\textit{pr}) protein transcript, \textit{pr5} at the 4, 8, 24 and 48 hr. Notably, \textit{pr5} expression was significantly higher than buffer-treated plants at 8, 24 and 48 hr after treatment (Fig 2.3d). Transcript levels of \textit{pr5} peaked 24 hr after frass application, when the abundance of \textit{opr7} transcript was suppressed (p<0.05).

\textit{Pr5} expression is regulated by SA (van Loon et al., 2006; van der Linde et al., 2012), thus SA accumulation in frass-treated leaves also was measured. Consistent with the \textit{pr5} expression data, SA levels peaked at 24 hr (Fig 2.3d, e) and were significantly higher in frass-treated plants compared to buffer-treated or undamaged plants at 4, 8 and 24 hr (Fig 2.3e). Taken together, these results indicate that expression of genes typically induced by herbivory increased up to 8 hr after frass treatment and then tapered off, whereas the SA-regulated gene \textit{pr5}, a marker for pathogen defense pathway, peaked at 24 hr.

\textbf{Wound-induced and pathogen defense responses were similar in maize treated with the frass extract or whole frass:}

The frass extract used in the previous experiments was concentrated with molecular cut-off columns and contained molecules larger than 3 kD. To determine if smaller molecules could be involved in regulating defense gene expression, I compared \textit{mpi} and \textit{pr5} transcript levels in response to the application of whole frass. There were no differences in \textit{mpi} transcript levels in
response to whole frass or the frass extract at 8 hr after application and both were higher than buffer-treated plants (Fig 2.4a). At 24 hr, pr5 transcript levels were lower in plants treated with whole frass than the frass extract, but both treatments had higher pr5 transcripts abundance compared to buffer-treated plants (Fig 2.4b). This suggests that the molecule(s) in FAW frass involved in inducing mpi and pr5 transcripts in maize is larger than 3 kD.

Proteins in caterpillar frass mediate defense responses in plants:

Analysis of the frass extract by SDS-PAGE indicated that a number of proteins were present in this fraction (Fig. S2). To determine if they were involved in mediating plant defenses, the frass extract was digested with a protease cocktail (Pronase) overnight, heat inactivated and concentrated using a 10-kD molecular cut off column as described in Materials and Methods. I applied the retentate, eluent and a combination of both fractions to wound sites on leaves. In addition, the frass extract not treated with Pronase was fractionated and applied to leaves in an identical manner. Transcript levels of mpi were measured in plants treated with these fractions at 8 hr, when mpi mRNA levels are typically the highest (Fig 2.3b). Changes in pr5 transcript levels in response to the same treatments were measured at 24 hr, when its expression levels peaked (Fig 2.3c).

Neither the eluent nor retentate from the Pronase-treated frass extract increased mpi transcript levels when compared with the buffer-treated control (Fig 2.5a). The combined retentate and eluent increased mpi mRNA abundance relative to the buffer-treated control, but it was not significantly different from the individual fractions. When plants were treated with fractions of the frass extract that was not treated with Pronase, the eluent had no effect on mpi transcript levels relative to the buffer-treated control, whereas the retentate and combined fractions significantly increased mpi transcript abundance (Fig 2.5a).

In the case of pr5 expression, the eluent from the Pronase-treated frass extract suppressed transcript levels relative to the buffer-treated control (Fig 2.5b). The retentate and the combined fractions had no effect on pr5 transcript abundance. When plants were treated with fractions of the frass extract that was not treated with Pronase, the eluent did not affect pr5
transcript abundance, but both the retentate and the combination of the two fractions significantly increased the abundance of pr5 transcripts (Fig 2.5b).

When SA accumulation in response to the eluent and retentate from the Pronase-treated frass extract was measured, I found that its levels increased relative to the buffer-treated control (Fig 2.5c). However, SA levels were higher when plants were treated with frass extract that was not treated with Pronase.

In summary, it appears that the molecules retained on the molecular cut-off column (>10 kD) from the frass extract that was not treated with Pronase were responsible for increasing the levels of both mpi and pr5 transcripts (Fig 2.5 a, b). If the frass protein extract was digested with Pronase prior to fractionation, neither the eluent nor retentate increased the abundance of these mRNAs. These results indicated that an intact frass protein(s) is (are) required to modulate the abundance of mpi and pr5 transcripts and SA levels in the frass-treated plants.

**FAW frass proteins suppresses accumulation of herbivore induced gene transcripts and defense protein RIP2 in maize leaves fed with FAW larvae:**

Since application of FAW frass to mechanically wounded maize leaves induced the accumulation of the transcripts of pathogen defense gene pr5 and SA over time, I wanted to investigate the effect of frass proteins in combination with FAW feeding and the associated deposition of oral secretions. Since it has been shown that FAW larvae deposit saliva on maize leaves during feeding (Chuang et al., 2014b), this experiment was designed to determine if frass proteins altered maize defenses in the presence of oral secretions. I applied FAW frass proteins or PBS buffer to feeding sites on leaves infested with FAW larvae for 3 hr in inverted clip cages, which prevented frass accumulation on the leaves (Fig. S4), and measured the transcript abundance of mpi, pr5 and rip2 over a period from 4 to 96 hr. RIP2 is herbivore-induced defense protein that has been shown to retard herbivore growth and both RIP2 and its transcripts accumulate in maize leaves within 30 min of caterpillar feeding (Chuang et al., 2014a).

At 4 and 8 hr rip2 transcripts were induced relative to the unwounded control, but there were no differences between FAW-fed plants treated with frass proteins or buffer (Fig 2.6c). At 8 hr
both *mpi* and *pr5* transcript abundance was significantly higher in FAW fed leaves treated with frass proteins compared to the buffer-treated controls (Fig 2.6a, c). At 24 hr, I observed a shift in gene expression patterns when the application of frass proteins to FAW feeding sites lowered *rip2* and *mpi* transcript abundance compared to that of buffer (Fig 2.6a, c). However, the abundance of pathogen-induced *pr5* transcripts was significantly higher in frass protein-treated leaves compared to the buffer-treated controls at this time (Fig 2.6b).

In addition, the abundance of RIP2 protein in maize leaves fed with FAW larvae for 3 hr and subsequently treated with FAW frass protein or buffer was determined by immunoblot analysis from 4 to 96 hr. Frass treatment diminished RIP2 accumulation at 4, 8 and 24 hr after application compared to buffer-treated leaves (Fig 2.6d). However at 48 hr RIP2 accumulation in frass or buffer-treated leaves was not different. These results suggest that the temporal shift from herbivore to pathogen defenses in response to FAW frass proteins occurs during herbivore feeding and concomitant deposition of oral secretions on the maize leaves.

**Discussion:**

Herbivore-associated mechanical damage due to feeding, oviposition, and crawling are known to induce plant defenses (Bricchi et al. 2010; Mithöfer et al. 2005; Hilker and Meiners 2006; Peiffer et al. 2009). However, mechanical wounding alone cannot account for the full spectrum of plant defense responses to herbivores. Biochemical cues from herbivores such as oral secretions arising from saliva and/or regurgitant are also recognized by plants adding further complexity to these interactions (Bonaventure et al. 2011; Felton and Tumlinson 2008; Mithöfer and Boland 2008). Several studies have shown heightened wound response in plants elicited by insect oral secretions (Alborn 1997; Erb et al. 2009; Louis et al. 2013; Schäfer et al. 2011; Tian et al. 2012). This has been attributed to insect-derived compounds that trigger plant immunity to herbivores called Herbivore Associated Molecular Patterns (HAMPs) that are analogous to the Pathogen Associated Molecular Patterns (PAMPs) (Erb et al., 2012). Until now several HAMPs, including volicitin, caeliferin, inceptin and β-glucosidase, have been isolated from the oral secretions of herbivores that induce anti-herbivore defenses in plants (Alborn et al. 2007; Felton and Tumlinson 2008; Mattiacci et al. 1995; Schmelz et al. 2006). There is also
evidence for herbivore-associated effector molecules that suppress such HAMP-triggered immunity (Chung et al. 2013; Musser et al. 2002; Zarate et al. 2007; Wu et al. 2012). Until now plant responses to herbivore cues primarily focused on oral secretions, however plant responses to frass, one of the most abundant herbivore cues, has not been extensively studied.

In this study, I applied frass to wounded maize leaves to tease out the effect of frass alone on wounded host tissue and to determine how it affected herbivore performance. My results indicated that frass-treated leaves increased herbivore performance (Fig.2a). Conversely, the performance of the fungal pathogen \( \text{C.heterostrophus} \) on frass-treated leaves was retarded compared to buffer-treated plants (Fig 2.2b-d). Because FAW larvae cannot avoid depositing frass in the enclosed whorl where they feed and damage leaves for extended periods of time, the frass-mediated induction of the pathogen defense pathway that appears to suppress insect defense pathways and improve herbivore performance makes sense from the ecological and evolutionary standpoint. Such herbivore-mediated pathogen defense triggering in the host plants by cues from Coleopteran and Hemipteran insects are known, but has not been previously reported for Lepidopterans (Chung et al. 2013; Zarate et al. 2007).

To better understand the mechanism that allows caterpillars grow faster on frass treated plants, I measured transcript levels of some gene markers of both the herbivore and pathogen defense pathway. Maize protease inhibitor (\( \text{mpi} \)) is a marker of herbivore-induced defenses that is downstream in the JA-signaling pathway (Shivaji et al., 2010). Herbivore secretions such as saliva of FAW and European corn borer (\( \text{Ostinia nubialis} \)) induce high levels of sustained \( \text{mpi} \) expression for up to 24 hr after application and is indicative of the elevated wound response due to herbivory (Chuang et al. 2014b; Louis et al. 2013). During the first 8 hr after application, caterpillar frass induced high levels of the wound response marker \( \text{mpi} \), however its transcript abundance waned after 8 hr (Fig 2.2b). The expression of \( \text{opr7} \), a marker for the JA biosynthesis pathway also was suppressed at 24 hr in response to frass treatment and was not significantly higher than the controls at any time points. Taken together, the reduced expression of \( \text{opr7} \) and \( \text{mpi} \) at 24 hr suggest that the typical caterpillar defense pathways of maize are suppressed and result in improved caterpillar performance at this time.
Notably, the abundance of pathogen-defense gene marker pr5, peaked at 24 hr after frass application (Fig 2.2 c, d) when the JA-related defense markers were suppressed. The JA-signaling pathway that is known to induce herbivore defenses may be antagonistic to the SA pathway, which is primarily involved in pathogen defenses (Howe and Jander, 2008; Robert-Seilaniantz et al., 2011; Thaler et al., 2012). SA levels steadily increased between 4 hr and 24 hr after frass treatment and peaked at 24 hr (Fig 2.3e) when transcript levels of the JA biosynthesis genes were suppressed (Fig 2.3a). I measured JA levels in response to FAW frass and found that they were suppressed in frass-treated plants compared to those treated with buffer (Fig 2.3c). Earlier studies in maize have demonstrated that FAW feeding induces JA accumulation and increased herbivore defenses at 24 hr after infestation (Shivaji et al., 2010). Furthermore, I have shown that JA-application induced the accumulation of mir1 transcripts encoding the insecticidal protein Mir1-CP at 24 hr, while SA-application suppressed mir1 transcript accumulation at the same time (Ankala et al., 2009). Recent studies have shown that SA accumulation mediates the suppression of JA signaling in Arabidopsis through degradation of JA-induced transcription factor ORA59 (Van der Does et al., 2013). This suggests that although plants initially detect frass as a cue of herbivory, its anti-herbivore defenses are not temporally sustained when the pathogen defense pathway is triggered. Whitefly feeding, beetle oral secretions and aphid honeydew were shown to have similar effects on the crosstalk between the two pathways (Chung et al. 2013; Schwartzberg and Tumlinson 2014; Zarate et al. 2007;).

The presence of frass on plant tissue is always preceded by caterpillar feeding that deposits oral secretions on the leaf, and this deposition of oral secretions and its subsequent activation of herbivore defense in plants have been well studied (Alborn 1997; Chuang et al. 2014b; Halitschke et al. 2001; Louis et al. 2013; McCloud and Baldwin 1997; Richard O. Musser 2005). FAW larvae are known to deposit saliva on the maize leaves that induces downstream herbivore defenses (Chuang et al., 2014b). To study the effect of frass-induced defenses in maize in the presence of such oral secretions, I allowed FAW larvae to feed on maize leaves for 3 hr in inverted cages that allowed larvae to feed and deposit oral secretions while preventing frass from contacting the leaves (Fig. S3). Subsequent application of frass or buffer to these caterpillar feeding sites showed an initial induction of herbivore defenses with mpi RNA levels
being induced higher in frass-treated plants compared to buffer-treated control (Fig 2.6a). However over time, these herbivore defenses were suppressed in frass-treated plants as seen by the decreases in *mpi*, *rip2* transcript abundance and RIP2 protein accumulation at 24 hr compared to buffer-treated controls (Fig 2.6a,c,d). On the other hand, at 24 hr the abundance of the pathogen defense marker, *PR5* peaked in response to frass treatment compared to the buffer-treated control after (Fig 2.6b). This suggests that the temporal shift of plant defenses from herbivore to pathogen defense in response to frass is independent of previous herbivory.

Of the herbivore-derived elicitors identified to date, three are derived from fatty acids and its conjugates, while inceptin and β-glucosidase are of proteinaceous nature (Felton and Tumlinson, 2008). The concentrated frass extract applied to plants was devoid of small molecules, so I tested the effect of frass prior to PBS extraction (whole frass). Whole frass induced the same or higher levels of wound and pathogen induced defenses as signified by *mpi* and *pr5* transcript levels, respectively compared to the frass extract in buffer (Fig 2.4a, b). This suggests that phytohormones in the frass (Tooker and de Moraes, 2006) did not appear to affect defense induction. Since the frass extract contained numerous proteins (Fig. S2), I tested their role in defense response induction. When frass proteins were digested and the resulting small peptides were removed, application of this extract to the plant did not increase *mpi* and *pr5* transcript levels at 8 and 24 hr, respectively, compared to buffer-treated plants (Fig 2.5a, b). Also, combinations of the protease-digested frass retentate and the eluent did not increase the levels of *mpi* or *pr5* transcripts as high as the treatment with undigested frass retentate (Fig 2.5a, b). Protease-digested frass proteins did not alter *pr5* transcript levels even when the retentate and eluent were combined (Fig 2.5b). This suggests that the molecules derived from herbivore frass that may act as elicitor(s) for wound and pathogen defenses in maize frass are proteinaceous in nature. In addition, the accumulation of SA that typically occurred at 24 hr was reduced when the digested extract was applied to the plants (Fig 2.5c). These peptides triggered higher SA accumulation than buffer-treated plants although the levels were lower than undigested frass extract (Fig 2.5c). These results suggest that the elicitor for pathogen defenses in FAW frass is a protein, that when degraded may induce SA accumulation, but an active protein is still required for the downstream activation of defense gene expression.
The presence of effectors which suppress plant defenses has been extensively studied in bacteria, fungi and nematodes (Bent and Mackey, 2007; Panstruga and Dodds, 2009), but the story of effector-mediated herbivore-defense suppression is only beginning to be unraveled (Chung et al., 2013). This study shows that FAW frass provides biochemical cues in the form of proteins that suppress herbivore defenses and increases herbivore performance on the plant. Since the frass protein extract used in my experiments was filtered through 0.2-µm filters, it is devoid of microorganisms, which were found to play a role in influencing plant defenses in a previous study (Chung et al., 2013). However, the effector-like molecule mediating anti-herbivore defense suppression in caterpillar frass may be a protein of plant, caterpillar or microbial origin that is present in the caterpillar gut. Herbivores such as silver spotted skipper larvae (Epargyreus clarus) launch frass pellets as far as 38 times its body length away from the feeding site to avoid leaving biochemical cues for predatory wasps (Weiss, 2003). In my study, I report the first host-herbivore system where protein(s) from frass suppresses the anti-herbivore defense in the plant, thereby increasing the performance of the herbivore on the host. Further studies unraveling the source and identity of the frass protein(s) that induces the pathogen defense are crucial for understanding this plant-insect dialogue that is mediated through fecal contamination of food by the herbivore.
Fig 2. 1. Accumulation of fall armyworm (FAW) frass in the maize whorl.

(a) Maize whorl showing accumulation of FAW frass (black arrow) after larval feeding for 24 hr. Frass accumulates in the enclosed space of maize whorl in close proximity to sites damaged by caterpillar feeding (b).
**Fig 2.2.** Effect of FAW frass extract-treated maize leaves on the growth rate of caterpillars and a fungal pathogen.

Fig 2.2 (a) Maize leaves were wounded and treated with FAW frass extract, PBS buffer (buffer-treated control) or left undamaged for 8 and 24 hr. Treated leaf tissue was fed to naïve FAW caterpillars and their relative growth rate (RGR) was measured after 4 days. RGR with different letters are significantly different from each other at each time point according to ANOVA analysis (p<0.05). Error bars indicate standard error of each group.
Fig 2.2 (b-d). The effect of FAW frass extract-treated maize leaves on growth of *Cochliobolus heterostrophus*.

Maize leaves were wounded and treated with FAW frass extract, PBS buffer (buffer-treated control) or left undamaged for 8 hr (b) and 24 hr (c) and treated with equal amounts of *C. heterostrophus* spore inoculum (upper half of midrib) or water (lower half of midrib). Fungal growth was measured as percentage of leaf area infected after 60 hr by determining the area of infected lesions (d). Percent leaf area with different letters at each time point are significantly different from each other according to ANOVA analysis (p<0.05). Error bars indicate standard error from the mean.
**Fig 2.3(c)**

*pr5*

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**Fig 2.3(d)**

Salicylic acid accumulation in leaf

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Fig 2. 3. Time course of maize defense gene expression and the accumulation of JA and SA in response to FAW frass extract.

Maize plants were wounded and treated with frass extract, PBS buffer (buffer-treated control) or left undamaged for 4, 8, 24 and 48 hr. Relative expression (RQ) for frass, buffer-treated and undamaged controls for opr7 (a), mpi (b), pr5 (c) was measured by qRT-PCR. Gene expression levels were normalized to actin and expressed as RQ (Relative Quantification). Data were analyzed by a two-way ANOVA and multiple comparison Tukey test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants (p<0.05) at the respective time points. Error bars indicate standard error from the mean. The accumulation of cis-jasmonic acid (JA) (e) and salicylic acid (SA) (d) in maize leaves in response to FAW frass extract was determined by wounding and treating maize plants with frass extract or PBS buffer. JA and SA levels were measured at the time points indicated. Total phytohormone content was measured relative to a deuterium labeled internal standard for each sample. Hormone levels were analyzed for significance at each time point by ANOVA and hormone levels of frass-treated samples that were significantly different from buffer or control samples (p<0.05) are signified by an asterisk at the respective time points. Error bars indicate standard error from the mean.
Fig 2.4. Expression of the maize defense genes mpi and pr5 in response to FAW frass extract and whole frass.

Maize plants were wounded and treated with whole frass, frass extract in PBS buffer, PBS buffer (buffer-treated control) or left undamaged and the relative expression (RQ) was measured using qRT-PCR for mpi at 8 hr (a) and pr5 at 24 hr (b). Expression levels were normalized to actin and data were analyzed for significance at each time point by ANOVA. RQ
values with different letters are significantly different (p<0.05). Error bars indicate standard error from the mean.

Fig 2.5. Pronase digestion of the frass extract reduces the expression of defense response genes and salicylic acid.
**Fig 2.5 (a,b)** The effect of FAW frass extract digested with a protease cocktail (Pronase) on mpi (a) and pr5 (b) transcript levels 8 and 24 hr after application respectively. Maize plants were treated with pronase-treated frass extract that was size-fractionated and contained molecules less than 10 kD (Eluate), greater than 10 kD (Retentate) or a combination of both fractions (Combined). In addition plants were treated with frass extract that was not treated with Pronase and size-fractionated and contained molecules less than 10kD (Eluate), greater than 10kD (Retentate) or a combination of both fractions (Combined). The controls were plants treated with PBS buffer containing inactivated pronase (Buffer) after wounding or undamaged plants. Relative expression (RQ) was determined by qRT-PCR and comparing expression levels to that of the reference gene, actin. The RQ values at each time point were separately analyzed by ANOVA and those with different letters are significantly different (p<0.05). Error bars indicate standard error from the mean at each group.

![Salicylic acid accumulation in leaf](image)

**Fig 2.5 (c).** Pronase digest of the frass extract alters SA accumulation. Effect of FAW frass extract digested with Pronase on salicylic acid (SA) accumulation in maize. Maize plants were treated with the undigested frass extract in PBS buffer, pronase-digested and size-fractionated frass extract containing molecules less than 10 kD (Eluate) and greater than 10 kD (Retentate). Controls were plants treated with PBS buffer containing heat treated Pronase after wounding.
or undamaged plants. Total SA content was measured relative to a deuterium labeled internal standard of SA for each sample. SA levels were analyzed for significance at each time point by ANOVA (p<0.05) and different letters are significantly different from each other. Error bars indicate standard error at each group.
**Fig 2.6.** Effect of FAW frass proteins on maize leaves after FAW feeding.

**Fig 2.6 (a-c).** FAW frass or PBS buffer (buffer-treated control) was applied to sites damaged by FAW larval feeding and the transcript abundance of *rip2 (a), mpi (b), pr5 (c)* was measured after 4, 8, 24 and 48 hr of frass application. Relative abundances of these genes were measured in comparison to undamaged controls and normalized against the endogenous actin mRNA transcript level. Transcript abundance of genes significantly different between buffer- and frass-treated controls are denoted by an asterisk (p<0.05, t test). Error bars indicate standard error from the mean.
**Fig 2.6 (d).** Immunoblot analysis of the defense protein RIP2 on maize leaves fed with FAW and treated with frass proteins or buffer (buffer-treated control) subsequently for 4, 8, 24 and 48 hr. Each lane contains a total of 50 µg protein pooled from four biological replicates; Ponceau S staining of Rubisco large subunit shows equal protein loading per lane.

**Supplemental Data:**

![Image](a) ![Image](b) ![Image](c)

**Fig S2.1. Wounding of maize whorl leaf with wounding tool and frass application.**

Maize whorl leaves at V8 stage were wounded using a wounding tool (a). Two outermost whorl leaves were wounded (b) and frass extract applied to each wound site (c).
**Fig S2.2.** Electrophoretic analysis (SDS-PAGE) of FAW frass proteins stained with colloidal silver.

Left lane contains the molecular mass markers and the right lane contains 10-µg of frass proteins.
**Fig S2. 3. Fall armyworm caterpillar feeding on maize leaves in inverted cages**

(a) Side view of inverted clip cages used to feed fall armyworm caterpillars; (b) Inverted clip cage with fall armyworm caterpillar eating maize leaf without depositing frass on the leaves; (c) Top view of inverted clip cages used to feed fall armyworm caterpillars on maize leaves.
### Table S2.1. List of primers for quantitative real time PCR for maize.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>NCBI Access Number</th>
</tr>
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<tr>
<td>Actin</td>
<td>GGAGCTCGAGAATGCAAGAGCAG</td>
<td>GACCTCAGGGCTCTGAACC</td>
<td>U60511.1</td>
</tr>
<tr>
<td>Maize Protease Inhibitor (MPI)</td>
<td>GCGGATTATCGCCCTAACC</td>
<td>CGTCTGGCGGACGATGTC</td>
<td>X78988</td>
</tr>
<tr>
<td>12-oxophytodienoate Reductase7 (OPR7)</td>
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<td>CGTCAGTCGCGTGGTGTGAT</td>
<td>NM_00111244.0.1</td>
</tr>
<tr>
<td>Pathogenesis-related gene 5 (PR5)(Morris et al., 1998)</td>
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<td>CGCACACAAATCCAGCTACG</td>
<td>U82201</td>
</tr>
<tr>
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<td>CTGCGCTGCCTGGATT</td>
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</tr>
</tbody>
</table>
Chapter 3. Turnabout is fair play: Maize chitinase Pr4 in fall armyworm frass induces pathogen defenses in maize

Introduction:

Insects are the most speciose group of organisms on the planet and more than half of insect species are phytophagous (Schoonhoven et al., 2005). Plants respond to insect herbivory with an arsenal of defenses that may be direct defenses when plants synthesize molecules that are detrimental for the insect pests, or may be indirect when plants emit volatiles after insect attack that recruit natural enemies of the insect pests (Chen, 2008b). The recognition of insect herbivory by plants has been attributed to the different insect behaviors such as feeding, crawling, oviposition and even defecation (Pechan et al., 2002; Felton and Tumlinson, 2008; Peiffer et al., 2009a; Hilfiker et al., 2014; Ray et al., 2015). Insect herbivory not only mechanically damages the host tissue, but also deposits insect secretions on plants such as insect saliva, regurgitant or frass (Felton and Tumlinson, 2008; Ray et al., 2015). The molecules that are deposited from these insect secretions and trigger herbivore defenses have been referred to as elicitors or HAMPs (Herbivore-associated Molecular Patterns) that are analogous to PAMPs (Pathogen-associated Molecular Patterns) (Bent and Mackey, 2007; Felton and Tumlinson, 2008; Acevedo et al., 2015; Schmelz, 2015). Plants may also recognize fragments of ‘self’ molecules that have been referred to as DAMPs (Damage-associated Molecular Patterns) (Karban and Shiojiri, 2009; Acevedo et al., 2015). There have been several studies identifying some of these HAMPs and DAMPs from the insect regurgitant and saliva (Acevedo et al., 2015; Schmelz, 2015). Similar to the model of pathogen-induced defense responses in plants, HAMP-mediated plant defenses may be suppressed by the other molecules or effectors in the herbivore secretions effectors (Felton and Tumlinson, 2008).

There exists a strong antagonism between the pathogen and herbivore defense pathways in most plant species (Thaler et al., 2012). The induction of herbivore defense pathway is often associated with a simultaneous suppression of the pathogen defense pathway and vice versa. While herbivore-induced defenses are primarily controlled by the jasmonic acid (JA) signaling pathway, the pathogen defenses pathway is modulated by salicylic acid (SA) (Thaler et al.,
Although, herbivory and insect associated elicitors of plant defenses typically trigger the JA pathway (Alborn et al., 1997; Musser et al., 2002; Alborn et al., 2007; Schmelz et al., 2007; Tian et al., 2012; Chuang et al., 2014b), insect secretions may suppress the herbivore defense pathway while simultaneously triggering the pathogen defense pathway (Zarate et al., 2007; Chung et al., 2013). For example, silverleaf whitefly herbivory induces pathogen defenses in tomato (Zarate et al., 2007), and Colorado potato beetle oral secretions harbor microbes from the beetle gut that suppress JA-induced defenses in tomato (Chung et al., 2013). These studies strongly imply that insect herbivores can harbor effector molecules that not only suppress herbivore-induced defenses in plants, but also trigger pathogen-induced defenses.

Although considerable work has been done on understanding plant responses to insect oral secretions, saliva and oviposition, relatively little is known about how insect feces induces plant defense responses. In a recent work it was shown that pea aphid honeydew contains salicylic acid (SA) that suppresses herbivore-induced defenses in plants (Schwartzberg and Tumlinson, 2014). In the previous chapter it was shown that frass from the chewing herbivore fall armyworm (FAW) can suppress herbivore defenses in maize over time, which leads to increased herbivory on the plant (Ray et al., 2015). This was an ecologically relevant finding since frass from FAW accumulates in the enclosed feeding site of the maize whorls where it remains near the insect feeding sites for extended periods of time. This also strongly suggested the presence of effector molecule(s) in the FAW frass that suppress herbivore defenses triggered by insect feeding. It was also observed that proteins in the FAW frass were responsible for inducing pathogen defenses in maize, thereby suppressing the herbivore defenses (Ray et al., 2015). Therefore, in this study proteins from FAW was fractionated in an effort to identify potential effector molecules in FAW frass that suppresses herbivore defenses. Since, the insect frass is composed of proteins that could be from the insect, plant or microbes present in the frass, MudPIT (Multidimensional protein identification technology) of the frass protein were performed on fractions that induced pathogen defenses and matched the peptides to protein databases from maize, lepidopoterans and microbes. My search identified the plant chitinases as possible effector molecules that induces pathogen defenses and suppresses herbivore defenses in maize. Plant chitinases are induced in response to insect herbivory and passes
through the insect gut to be deposited back into the host tissue (Han and Luthe, 2015). This may also be an example of ‘self-recognition’ of plants leading to the induction of plant defenses.

**Materials and Methods:**

**Plant and insect materials:** Maize (*Zea mays*, var. B73) plants were grown in glasshouse conditions with 16 hr light and 8 hr dark cycle in Hagerstown loam till they reached mid-whorl (V7-V8) development stage (Ritchie et al., 1998). *FAW* (*Spodoptera frugiperda*) eggs were obtained from USDA-ARS at Mississippi State University and reared on maize plants to collect frass. Frass was collected every 24 hr from caterpillars fed on V8 stage maize leaves. The collected frass was then homogenized in 1xPBS buffer (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate), filter sterilized and concentrated using centrifugal columns with a 3-kD molecular weight cut off (Pall Life Sciences, USA) as described earlier (Ray et al., 2015). Protein concentration in the frass extract was quantified by the Bradford assay.

**Fractionation of frass proteins using the Rotofor system:** Fractionation of frass proteins was performed in the standard Rotofor chamber after assembly of the apparatus as suggested by the manufacturer (Biorad Corporation, USA). Five milligrams of total frass protein in 1xPBS buffer (phosphate buffer saline) was loaded into the standard Rotofor chamber with 2% ampholytes (pH= 3 – 10; Biorad Corporation, USA) and 1.5 M urea. The electrolyte used for the anion exchange membrane was 0.1 N sodium hydroxide and 0.1 N phosphoric acid for the cation exchange membrane. Fractionation was performed at 4°C with a constant power of 12 watts until the voltage was stable for an hour. Twenty fractions were collected after the run and pooled to obtain ten fractions. The first two most acidic fractions were pooled to form fraction A, the next two formed B and so on. The fractions A-J were then concentrated using 3 kD centrifugal columns (Pall Life Sciences, USA) and the re-suspended in 1xPBS buffer. Protein concentration in each of these fractions were measured by the Bradford assay and 1 µg of total protein was applied to each wound site on maize leaves to test the effect of the fractionated frass proteins.
MudPIT analysis of frass protein fractions: Proteins from Rotofor fractions that induced plant defenses were identified by gel-free multidimensional protein identification technology (MudPIT). Proteins were alkylated with 10% (v/v) acetonitrile in 50 mM ammonium bicarbonate and digested with 1:20 (trypsin:protein) sequencing grade trypsin (Promega Corporation, USA) for 3 hr at 48°C. Digested peptides were dried in Speedvac (Thermo Fisher Scientific, USA) to completely remove acetonitrile and ammonium bicarbonate and the reaction was stopped with 0.1% trifluoroacetic acid. To identify the proteins bound to the chitin-binding beads from FAW frass, the beads with the bound proteins were boiled in 1X sample buffer for 10 min and loaded on a denaturing polyacrylamide gel (Laemmli, 1970). The gel was stained with Simply Blue Safe stain (Life Technologies, USA) overnight and destained in deionized water. The bands from the gel were then excised and treated with 200 µl of 200 mM ammonium bicarbonate in 50% (v/v) acetonitrile for 45 min at 37°C. The gel pieces were then reduced in 100 µl of 2 mM Tris-2-caboyxmethylphosphine (Sigma Aldrich, USA) for 15 min at 37°C and subsequently alkylated in 100 µl of 20 mM iodoacetamide in 25 mM ammonium bicarbonate (pH=8.0) for 25 min at 37°C in dark. The gel pieces were then washed thrice in 200 µl of 25 mM ammonium bicarbonate (pH=8.0) for 15 min. The gel pieces were then dried in a Speedvac and rehydrated in 70 µl of 0.02 µg/µl of sequencing grade trypsin (Promega Corporation, USA) in 10% acetonitrile (v/v), 40 mM ammonium bicarbonate (pH=8.0) and 0.1% noctylglucoside (1-O-n-Octyl-beta-D-glucopyranoside, Sigma Aldrich, USA) for at 48°C for 3 hr. After the incubation, the supernatant was removed and the reaction was stopped with 0.1% trifluoroacetic acid. Peptides obtained from both the gel-free and in gel digests were analyzed by electrospray in LC/MS/MS (ABSciex 5600 TripleTOF, SCIEX, USA). Data were analyzed using the Protein Pilot 5.0 (SCIEX, USA) and peptides were matched against maize (NCBI taxid id: 4577), Lepidoptera (NCBI taxid id: 7088), fungi (NCBI taxid id: 4751) and bacteria (NCBI taxid id: 2). Strength of matching the peptide fingerprint to particular database was measured by the Mascot score (Perkins et al., 1999; Koenig et al., 2008; Tang et al., 2008) and only matches with 95% or higher confidence for a peptide are reported in Tables 3.1-3.3.

Removal of chitin-binding proteins from frass: Frass proteins were incubated with chitin-binding magnetic beads (New England Biolabs, USA) in a ratio 5:1 (w:v) overnight at 4°C with constant
shaking. The tube containing this mixture was affixed on to a stand with a strong magnet attached to it and the supernatant that contained frass proteins devoid of chitin-binding proteins was collected with a pipette. Frass proteins containing chitinin-binding domains were attached to the remaining beads. The beads with bound proteins were washed five times in 1XPBS buffer. Twenty micrograms of total frass protein, an equivalent amount of frass proteins devoid of chitin-binding proteins and 1XPBS buffer were applied to maize leaves. Similarly, beads containing chitin-binding proteins from 20 µg of the total frass protein and an equivalent amount of beads without bound protein in 1XPBS buffer were applied to wound sites on the leaves. One hundred milligrams of leaf tissue around the wound site was collected for RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR).

**RNA extraction, cDNA preparation and real-time PCR:** One hundred milligram of leaf tissue was homogenized in liquid nitrogen using the GenoGrinder 2000 (OPS Diagnostics, USA) and RNA was extracted with 1 ml TRlzlol (Life Technologies, USA) as per manufacturer’s protocol. Genomic DNA was removed from the RNA by treatment with 2 units of DNasel enzyme (New England Biolabs, USA). The genomic DNA-free RNA was quantified using Nanodrop (Thermo-Fisher Scientific, USA) and 1 µg of total RNA was used to make cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) using Oligo-dT. Quantitative real-time PCR (qRT-PCR) was performed with Fast Start Universal SYBR Green Master Mix (Roche Applied Science, USA) with actin as reference gene. Gene-specific primers were designed with Primer Express 3.0 (Life Technologies, USA; details in Table S2.1). RNA extraction, cDNA synthesis and qRT-PCR were performed for each biological replicate separately, with each treatment having four biological replicates for Rotofor frass fraction or chitin bead treatments. Six plants per treatment were used when purified Pr4 was applied.

**Pr4 purification and chitinase enzyme assay:** Complementary DNA (cDNA) of maize chitinase Pr4 (NP_001150754.1) was cloned into the expression vector champion vector pET 102 (Life technologies, USA) using forward primer 5’-CACC CAGAACTGCGGGTGCGC-3’ and reverse primer 5’- GCAGGTGAGGTTGTTGCCCGGGT-3’. *Escherichia coli* cells (BL21 DE3) was transformed with the pET102 vector containing the cloned Pr4 sequence. Transformed *E. coli* cells were then induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) as suggested by the
manufacturer and cells were harvested and lysed with 1mg/ml lysozyme (Sigma Arldrich, USA). The expression vector added 6X-histidine tags to the expressed Pr4 and therefore, cell lysates were incubated with Ni-NTA agarose beads (Qiagen, USA) and Pr4 protein was eluted with 250 mM Immidazole, 300 mM sodium chloride and 50 mM sodium dihydrogen phosphate. The chitinase enzyme assay was performed with the eluted protein as described earlier (Peethambaran et al., 2009). In 96-well plates (fluorescence compatible), 100 µl of a mixture containing 1mg/ml of glycol chitosan, 12% acrylamide:bisacrylamide (37:1::v/v), 0.05% ammonium persulfate, 375mM Tris buffer (pH=8.8) was pipetted and the mixture was solidified with 10µl of TEMED. One hundred microliters of 0.05, 0.1 0.15, 0.2, 0.25 and 0.3 mg/ml of Pr4 protein was added to the wells containing glycol chitosan gels and incubated overnight. The wells were washed with water once the following day and incubated with 100µl of 3µg/ml calcofluor for 30 mins and washed again with water. Fluorescence was measured in spectrophotometer with an excitation wavelength of 356 nm and emission wavelength of 435 nm for 1 second. For each concentration of Pr4 tested, four technical replicates were measured.

**Fall armyworm bioassays:** The first three leaves of maize whorl in a V8 stage plant were wounded twice using a wounding tool (Ray et al., 2015). Each wound site was treated with 20 µg of purified Pr4 protein or equivalent volume of buffer or left undamaged. We used 18 biological replicates for each treatment, each of which were fed to 18 naïve FAW caterpillars for 2 days. Caterpillar mass was measured at beginning and end of the bioassay and used to calculate relative growth rates (RGR) as described earlier (Hoffmann and Poorter, 2002; Mohan et al., 2008). To observe the direct effect of Pr4 protein on FAW larval growth, 0.2 g of artificial diet was infused with 60 µg of purified PR4 protein or an equivalent volume of buffer and fed to thirty caterpillars. Diet pieces infused with Pr4 or buffer were replaced every two days and the larval weight were measured after 2, 4 and 6 days and the RGR for the larvae were measured. One-way ANOVA was performed to determine significance among treatments in both bioassays and means separation was done with Tukey’s test (p<0.05).

**Fungal Bioassay:** *Cochliobolus heterostropus* was cultured on potato dextrose agar (PDA) media for 2 weeks under lights (Yoder 1988). Fresh leaves from B73 maize at the V8 stage were
treated with 20 µg of purified Pr4 protein, an equivalent volume of buffer or left undamaged and harvested after 24 hr. Harvested tissue was placed on 150 mm petri dish with a 1% agar base with 0.01% kinetin. *C. heterostrophus* spores (100 µl at a concentration of 10⁴ spores per ml) were applied to one side of the leaf between two wound sites and the other side was treated with water (Fig. ....). Inoculated leaves were incubated at 26°C for 60 hr at 16 hr light and 8 hr dark cycles. The area infected by fungi was measured using Sigma Scan Pro 5.0 (SPSS, USA) at 60 hr after inoculation. Two leaves per biological replicate were analyzed for the infected area with six biological replicates per treatment. Amount of fungal colonization on the leaf was measured as a function of the percentage leaf area showing lesions. Significance of infection was measured by one-way ANOVA for the percentage of area infected by the pathogen and means separation was done using Tukey’s test (p<0.05).

**Results:**

**Proteins with basic and acidic isoelectric points in fall armyworm frass induced pathogen defenses in maize:**

In a previous study, it was shown that proteins from FAW frass triggered a temporal shift in plant defenses from herbivore to pathogen defenses in maize (Ray et al., 2015). In an attempt to identify the protein molecule(s) in FAW frass that trigger the pathogen defense pathway, the proteins in FAW frass were fractionated with the Rotofor® system (Biorad Corporation, USA). Ten frass fractions based on their isoelectric points ranging from a pH of 3 to 10 were obtained and named as fractions A – J, with frass proteins of isoelectric point of pH =3-3.5 in fraction ‘A’ and that successively increased to pH to pH=9.5-10 in fraction ‘J’. The rotofor fractions A – J were concentrated as described in Materials and Methods, applied to maize plants and the transcript abundance of the pathogen defense gene marker *pr5* was measured 24 hr after application. It was observed that fractions containing proteins with acidic isoelectric points (fraction B with pH of 3.5-4.0), mildly acidic isoelectric points (D with pH ranging from 5.5-6.0, fraction E with pH range of 6.5-7.0), with mildly basic isoelectric points (fraction F of pH 7.5) and a fraction with proteins having an basic isoelectric point (fraction I with pH of 8.5-9.0) induced higher *pr5* transcript abundance compared to the buffer treated plants (Fig. 3.1).
suggests that the proteins in FAW frass responsible for inducing pathogen defenses in maize were in the Rotofor fractions B, D, E, F and I.

**Identification of proteins from Rotofor fraction of frass inducing pathogen defense in maize shows the presence of maize chitinases:**
Frass protein fractions ‘B’ and ‘I’ induced the highest levels of pr5 RNA compared to buffer treated plants (Fig 3.1). To identify the proteins present in these fractions that could be responsible for inducing pathogen defense responses, MudPIT analyses of fractions ‘B’ and ‘I’ that increased pr5 transcript abundance higher than the buffer-treated controls were performed. The proteins identified in these fractions are summarized in Tables 3.1 and 3.3. Proteins in the fraction ‘A’ that failed to induce pathogen defense marker pr5 expression but was adjacent fraction B were also identified in a similar manner. It was speculated that there may be overlap of proteins from these two fractions because they were adjacent that would help us identify unique proteins responsible for inducing pathogen defenses. The fractions B contained several maize chitinases that included Pr4 (gi|226501166), Prm3 (gi|226501166) and Chitinase A (gi|226506778) as shown in Table 3.1. Beta-glucosidase (gi|162464369), maize lipoxygenases (gi|162463394), maize protease inhibitors (gi|226496089, gi|162459393) were also abundant in this fraction, but they were also quite abundant in the fraction A that did not induce pathogen defenses (Table 3.2). This suggested that chitinases in FAW frass could be triggering pathogen defenses in maize.

Interestingly, fraction ‘I’ which also induced pathogen defense gene marker pr5 abundance, showed presence of maize protease inhibitors and the insect gut protein IIM (insect intestinal mucin, (gi|379698934) as shown in Table 3.3. IIM is a known structural protein in the gut of most lepidopteran insects and binds to chitin along with other proteins known as peritrophins to form the peritrophic matrix (PM), which lines the food bolus and protects the caterpillar midgut. IIM protein belong to an extremely conserved group of proteins known as peritrophins and are highly glycosylated (Hegedus et al., 2009). The peptide sequence identified from the MudPIT analysis was from silk moth (*Bombyx mori*) but it also matched a FAW peritrophin protein (AAS89976.1). These proteins are known to bind chitin microfibrills and have conserves chitin binding domains (Hegedus et al., 2009). The presence of chitinases and IIM in the frass
protein fractions that induced pathogen defenses suggested proteins with chitin binding domains could be involved in inducing pathogen defenses.

**Chitin-binding proteins in FAW frass induced pathogen defenses in maize:**
Proteomic analysis of frass proteins identified maize chitinases and insect gut protein IIM as possible candidates for elicitors of pathogen defense present in caterpillar frass. While most plant chitinases have chitin binding domains, as was noted earlier, IIMs are known to have chitin binding property (Hegedus et al., 2009; Hamid et al., 2013). To better understand if FAW frass proteins with chitin binding domains induce pathogen defenses, the chitin-binding proteins from the frass proteins were removed and this fraction was applied to maize leaves and pathogen defenses were measured in comparison to buffer and FAW frass proteins containing-the chitin binding proteins. FAW frass depleted of chitin binding proteins failed to induce pr5 defense transcript in maize as compared to buffer or frass proteins containing chitin-binding proteins (Fig 3.2a). Notably, the frass protein fraction depleted of chitin-binding reduced pr5 expression (Fig 3.2a). Furthermore, when beads containing the chitin-binding proteins from the frass were applied to the plants, pr5 transcript abundance was significantly higher than in plants treated with the beads alone (Fig 3.2b). This strongly suggested that chitin-binding proteins present in the FAW frass were responsible for inducing pathogen defenses in maize.

**Proteomic analysis of chitin binding proteins in fall armyworm frass showed the presence of two maize chitinases, Pr4 and Chitinase A:**
The chitin-binding proteins from FAW frass that were bound to the beads in the previous experiment were boiled in sample buffer and analyzed by SDS-PAGE estimate of the number of proteins bound to the beads. We observed the presence of three major proteins at approximately 36 kD, 30 kD and 28 kD on the gel (Fig S3.1). Proteomic analysis of the proteins bound to these beads showed the presence of maize chitinases Pr4 (gi|226501166), Chitinase A (gi|259490577), chitinase precursor (gi|169234932) and Chitinase 2 (gi|226528230); however, IIM was not detected in the proteins that bound to these beads (Table 3.4). Chitinases are known to be induced in response to caterpillar herbivory and serves as a line of defense against the insect pests by digesting the chitin in the caterpillar gut (Hamid et al., 2013). Studies of
induction of chitinases in response to fungal attack in plants are also widely known (Schlumbaum et al., 1986). Maize contains a large chitinase gene family and some of these are induced by FAW herbivory (Han and Luthe, 2015). Since Pr4 was one of the most abundant proteins with a chitin-binding domain detected in FAW frass (Table 3.4), the transcript abundance of pr4 in response to caterpillar herbivory was measured. Transcripts of pr4 in plants fed with caterpillars for 24 hr were higher than control undamaged plants (Fig 3.2c). In maize, it appears that caterpillar herbivory results in the foliar accumulation of chitinases such as Pr4 that is consumed by the caterpillars and deposited back on to the plant in the frass. The frass Pr4 may be recognized by the plants leading to the induction of pathogen defenses.

**Pr4 has chitinase activity, induces pathogen defenses and suppresses herbivore defenses in maize:**
The Pr4 that was expressed in *E. coli* and purified exhibited chitinase activity as shown in Fig S2. Purified Pr4 was applied to maize leaves and the expression of the pathogen defense marker *pr5* and herbivore defense marker *mpi* were measured. Pr4 induced the expression of *pr5* higher than the buffer treated control while it suppressed the expression of *mpi* in maize leaves (Fig 3.3a, b). This is another classic example of the antagonism between these two defense pathways present in plants. *Mpi* is controlled by the herbivore-induced jasmonic acid (JA) pathway while *pr5* induction is mediated by the pathogen- induced salicylic acid (SA) pathway. These results indicate that Pr4 is the one of the proteins from FAW frass that is responsible for inducing the pathogen defense pathway and suppressing herbivore defenses in maize.

**Pr4 application suppresses the growth of fungal pathogen in maize leaves while it increases the performance of FAW caterpillars:**
Since induction of *pr5* in maize leaves upon Pr4 application was observed, the growth of fungal pathogen *Cochliobolus heterostroptus* on maize leaves treated with Pr4 was measured and compared buffer-treated controls and undamaged leaves. *C.heterostroptus* is a known maize pathogen that causes Southern leaf blight. Plants treated with Pr4 showed much lower fungal colonization (only 34% leaf area colonized) compared to buffer-treated plants (nearly 60% leaf area colonized) (Fig 3.4a, b). Fungal growth was more than twice as high in undamaged leaves when compared to those treated with Pr4. Therefore, it can concluded that Pr4 application on
maize leaves can not only induce pathogen defense gene expression, but also retards the growth of fungal pathogen.

Pr4 application on maize leaves, also suppressed the expression of herbivore/wound-induced *mpi* transcript abundance. Therefore, FAW caterpillars leaves treated with Pr4 were fed to naïve FAW caterpillars and their growth rate were compared to those fed with buffer-treated leaves or undamaged leaves. Caterpillars reared on leaves treated with Pr4 had a growth rate similar to those fed undamaged leaves (Fig 3.4c). However, the larvae that consumed the buffer-treated tissue showed significantly lower growth rate compared to those that fed on Pr4-treated or undamaged leaves. This strongly suggests that not only does Pr4 suppress herbivore/wound-induced defense gene expression, but also suppresses the defense phenotype leading to enhanced growth of the caterpillars.

**Fall armyworm larvae show a transient growth suppression in response to purified Pr4:**

Thus far, it has been shown that caterpillar feeding induces the accumulation of active chitinases in maize, one of which is Pr4. The Pr4 protein then passes through the caterpillar gut and is detected in its frass, which when deposited on the leaves induced pathogen defense responses. I also determined Pr4 induced by FAW feeding altered caterpillar growth. Therefore, the active Pr4 enzyme was mixed with artificial diet, fed to naïve caterpillars and FAW growth rate was measured over 6 days. Interestingly, FAW larvae showed a slower growth rate when fed Pr4-containing diet after 2 days (Fig. 3.5). However, as the bioassay time increased, larvae fed on Pr4 treated diet showed no change in growth rate compared to buffer-treated controls at 4 and 6 days (Fig. 3.5). This suggests that caterpillar growth is only transiently affected by Pr4. Therefore, the FAW larvae are not only unaffected by the accumulation of Pr4 in the leaves in response to herbivory, but concentrate it in their frass, deposit it on the host and trigger pathogen defenses, thereby increasing herbivore performance.

**Discussion:**

Insect secretions such as saliva or regurgitant are shown to harbor several elicitors that can trigger herbivore defenses in plants. Insect-derived elicitors of herbivore defenses such as volicitin and caeliferin that were isolated from insect oral secretions and bruchins that were
identified in the oviposition secretion of adult moths are all fatty acid conjugates that can induce herbivore defenses in plants (Alborn et al., 1997; Doss et al., 2000; Alborn et al., 2007). On the other hand, elicitors such as inceptin, a fragment of plant ATP synthase, and β-glucosidase are proteinaceous in nature and were isolated from insect oral secretions (Mattiacci et al., 1995b; Schmelz et al., 2006b; Schmelz et al., 2007). Glucose oxidase is another protein that is abundant in the saliva of tomato fruitworm (Helicoverpa zea) and may induce herbivore defenses in tomato or suppress them in other plants such as tobacco (Musser et al., 2002; Tian et al., 2012). I observed that proteins from FAW frass induced the pathogen defense pathway over time (Ray et al., 2015). Since, frass is one of the most abundant cues that herbivores leave on their host, I fractionated the FAW frass proteins based on their isoelectric points and applied them to maize leaves. I found two fractions that had the greatest effect in increasing expression of the pathogen defense marker pr5 in maize (Fig 3.1).

Proteomic analysis of these fractions led us to infer that plant chitinases and insect gut protein insect intestinal mucin (IIM) are FAW frass proteins that may be involved in inducing pathogen defenses in maize (Table 3.1, 3.3). IIM belongs to class of structural proteins called peritrophins that are found in the PM of the caterpillar gut (Hegedus et al., 2009). The PM is a highly conserved acellular matrix in most insect guts that separates the food bolus from the midgut. It is composed of chitin microfibrills, which are bound together several classes of proteins called peritrophins that have several conserved domains including a chitin-binding domain (Hegedus et al., 2009). Since both fractions of FAW frass proteins that induced pr5 contained proteins that bind chitin, I removed these proteins from the caterpillar frass. Application of chitin-binding protein depleted frass to maize leaves resulted in attenuation of pathogen defenses (Fig 3.2a) while the beads with the bound protein were able to induce the defense (Fig 3b).

Proteomic analysis of the chitin-binding beads after incubation with FAW frass indicated that Pr4 and chitinase A were abundant in this fraction. The chitin-binding fraction did not contain IIM, therefore, this study focused on the chitinases.

Chitinases are enzymes that degrade chitin either to its oligomers (endochitinases) or to dimers and monomers (exochitinases). All chitinases belong to family 18 and 19 glycosyl hydrolases and plants chitinases have been classified into six classes (Hamid et al., 2013). Plant chitinases
have been shown to accumulate in response to pathogen attack as well by herbivory (Kasprzewska, 2003; Han and Luthe, 2015). When I applied the maize chitinase Pr4 that was present in both the Rotofor fraction and attached to the chitin binding beads, it induced the expression of the pathogen defense gene marker pr5 (Fig 3.3a) and simultaneously suppressed the expression of wound-induced mpi transcript abundance (Fig 3.3b). Consistent with the transcript abundances, the fungal pathogen C.heterostrospus showed lower growth on the Pr4 treated plants compared to the buffer-treated ones (Fig 3.4 a,b). On the other hand, Pr4 treatment suppressed the wound response in plants and naïve FAW caterpillars grew faster on plants treated with Pr4 protein compared to the ones treated with buffer (Fig 3.4c) suggesting that Pr4 from the frass of FAW caterpillars can mediate a suppression of the wound-induced defense response. Therefore, in reference to our previous study (Ray et al., 2015), it can be said that Pr4 is one of the proteins in FAW frass that suppresses the wound-induced response and activates the pathogen defenses in maize. This also is direct evidence of the typical antagonism between plant defense pathways mediated by an herbivore that activates pathogen defenses and suppresses herbivore defenses. Such suppression of herbivore defenses and the concomitant activation of pathogen defenses by herbivory has been previously shown in tomato by whiteflies and Colorado potato beetles (Zarate et al., 2007; Chung et al., 2013). Pr4 also joins the list of other proteins such as apyrases and glucose oxidase that are deposited by the herbivore on the host plant and suppress the plant’s herbivore defenses (Musser et al., 2002; Wu et al., 2012).

Notably, chitin oligomers are a known elicitor and PAMP involved in triggering the pathogen defense pathway in plants (Kombrink et al., 2011). The receptors that bind chitin oligomers and induce pathogen defenses in rice and Arabidopsis thaliana have also been identified (Tanaka et al., 2012; Wan et al., 2012; Kouzai et al., 2014). However, there has been no previous report that chitinases may be involved in eliciting plant defenses. For the first time I show that a plant chitinase can act as an effector of herbivore-induced defenses when deposited on the plant in herbivore frass. The mechanism by which Pr4 induces pathogen defenses and suppresses the wound-induced defenses in plants is story beyond the scope of this dissertation. However, I speculate that plants may perceive Pr4 as a self-protein and induce defenses in response to it.
Self to non-self-recognition have been studied plant reproduction and development (Gruntman and Novoplansky, 2004; Williams et al., 2015). Self-recognition by damage associated by caterpillar herbivory has also been shown in plants (Heil, 2009; Karban and Shiojiri, 2009). Pr4 from caterpillar frass may be identified as self by the maize plant and trigger its pathogen defenses.

Maize and its herbivore pests such as FAW have co-evolved for thousands of years. The deposition of frass on damaged host tissue by the herbivore to suppress the wound-induced defenses in its host could be a strategy that the herbivore has developed during the course of evolution. I observed that pr4 transcripts were induced in response to caterpillar herbivory in an effort to suppress the herbivore growth. However, the herbivore had a transiently reduced growth rate 2 days after feeding in Pr4 their diet, after which Pr4 had no effect on growth rate (Fig 3.5). This further supports the notion that over the course of evolution, FAW caterpillars have developed a mechanism whereby it is not affected by the host chitinase Pr4 and can accumulate them in the frass. In turn, Pr4 in the frass in turn can suppress the damage-associated defenses in the host, induce pathogen defense and increase the long-term performance of the herbivore on the host.
Table 3. 1 MudPIT analysis of Rotofor fraction ‘B’

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### Table 3. 4 MudPIT of chitin-binding proteins in fall armyworm frass

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Figure 3.1. Maize defense gene expression in response to protein fractions from Rotofor separation of frass proteins.

Maize leaves were wounded and treated with 10 protein fractions (A-J) or PBS buffer for 24 hr and the pathogen defense gene marker pathogenesis-related protein5 (pr5) transcript abundance was measured by quantitative real-time PCR (qRT-PCR). Transcript abundance of pr5 was normalized to that of the reference gene actin and expressed as relative quantification (RQ). Data were analyzed by a one-factor ANOVA and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated (p<0.05). Error bars indicate standard error from the mean.
Figure 3.2. Pathogen defense gene expression in maize in response to frass proteins depleted of chitin-binding proteins and pr4 transcript abundance in response to caterpillar herbivory in maize.

**Fig 3.2 (a,b):** Maize leaves were wounded and treated with (a) frass protein, (a) frass protein devoid of chitin-binding proteins, (a) PBS buffer, (b) chitin binding beads (beads) in PBS buffer, (b) the beads with the chitin binding proteins from fall armyworm frass (bound protein) or left undamaged for 24 hr and pathogen defense gene marker pathogenesis-related protein5 (pr5) transcript abundance was measured by quantitative real-time PCR (qRT-PCR). Transcript abundance of pr5 was normalized to that of the reference gene actin and expressed as relative quantification (RQ). Data were analyzed by a one-factor ANOVA and mean separation was calculated by multiple comparison Tukey’s test. RQ values of
frass-treated gene expression marked with an asterisk are significantly different from buffer-treated (p<0.05). Error bars indicate standard error from the mean.

**Fig 3.2 (c):** Maize chitinase *pr4* transcript abundance in plants fed with fall armyworm.

Maize leaves (var. B73) were infested with fall armyworm caterpillars for 24 hr and maize chitinase gene *pr4* transcript abundance was measured by quantitative real-time PCR (qRT-PCR). Transcript abundance of *pr5* was normalized to that of the reference gene *actin* and expressed as relative quantification (RQ). Data were analyzed by a one-factor ANOVA and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated (p<0.05). Error bars indicate standard error from the mean.
Figure 3.3. Transcript abundance of maize defense genes in response to Pr4 application.

Maize leaves were wounded and treated with purified Pr4 protein or buffer for 24 hr. Maize pathogen defense related *pathogenesis related protein 5* (*pr5*) (a) and herbivore defense-related *maize protease inhibitor* (*mpi*) (b) transcript abundance was measured by quantitative real-time PCR (qRT-PCR). Transcript abundance of *pr5* and *mpi* was normalized to that of the reference gene *actin* and expressed as relative quantification (RQ). Data were analyzed by a one-factor ANOVA and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with
an asterisk are significantly different from buffer-treated (p<0.05). Error bars indicate standard error from the mean.

**Figure 3.** The effect of Pr4-treated maize leaves on the growth of Cochliobolus heterostrophus and fall armyworm larvae

**Fig 3.4 (a,b):** Maize leaves were wounded and treated with Pr4, buffer or left undamaged for 24 hr and treated with equal amounts of *C. heterostrophus* spore inoculum on upper half of midrib or water on the lower half of midrib (a). Fungal growth was measured as percentage of leaf area infected after 60 hr by determining the area of infected lesions (b).
Percent leaf area with different letters at each time point are significantly different from each other according to ANOVA analysis (p<0.05). Error bars indicate standard error from the mean.

**Fig 3.4 (c):** Effect of Pr4-treated maize leaves on the growth rate of naïve caterpillars.

Maize leaves were wounded and treated with Pr4, buffer or left undamaged for 24 hr. Treated leaf tissue was fed to naïve FAW caterpillars and their relative growth rate (RGR) was measured after 3 days. RGR with different letters are significantly different from each other at each time point according to ANOVA analysis (p<0.05). Error bars indicate standard error of each group.
Figure 3.5. Effect of purified Pr4 on the growth rate of fall armyworm caterpillars.

Naïve first instar fall armyworm caterpillars were fed artificial diet infused with Pr4 or buffer and relative growth rate (RGR) of the caterpillars were measured at 2, 4 and 6 days. Asterisks indicate difference in RGR between the treatments at each time point according to ANOVA analysis (p<0.05). Error bars indicate standard error of each group.

Fig S3.1: Denaturing polyacrylamide gel showing chitin binding proteins in FAW frass
Fig S3.2: Standard curve showing Pr4 chitinase activity.
Chapter 4. Inside Out: Insect Gut Protein in Caterpillar Frass Induces Wound Defense in Maize

Introduction:
Plants can thwart insect herbivory by inducing direct defenses such as the accumulation of toxins detrimental to insect growth or indirectly defend themselves by the emission of volatiles to attract natural enemies of its herbivore (Chen et al., 2007). However, the induction of such defenses is downstream of the plant’s recognition that it is under attack. Although, plants can induce defenses in response to the damage associated with insect herbivory, the full spectrum of plant defenses in response to insect herbivory cannot be achieved by damage alone (Felton and Tumlinson, 2008). Insect derived secretions such as oral secretions, saliva and even frass that is deposited during herbivory are known to trigger plant defenses (Alborn, 1997; Felton and Eichenseer, 1999; Acevedo et al., 2015; Ray et al., 2015). Several insect-derived molecules have been identified that induce plant defenses and are named elicitors or HAMPs (Herbivore Associated Molecular Patterns) (Bonaventure et al., 2011; Acevedo et al., 2015; Schmelz, 2015). Similarly, upon feeding damage, plants may also produce DAMPs (Damage Associated Molecular Patterns) that trigger plant defenses (Heil, 2009; Acevedo et al., 2015). Such DAMPs or HAMPs may induce the herbivore defense pathway or suppress it by activating the pathogen defense pathway (Bonaventure et al., 2011; Acevedo et al., 2015; Schmelz, 2015). In Chapter 2 it was shown that proteins from fall armyworm (*Spodoptera frugiperda*) frass can transiently induce a wound response before activating a sustained pathogen defense response on the host (Ray et al., 2015). This strongly implied the presence of proteinaceous HAMPs and DAMPs in fall armyworm (FAW) frass.

In this study, fractionation of proteins from FAW frass were continued in an attempt to identify the protein(s) that may be responsible for the transient frass-induced wound response in maize. The insect gut protein, Insect Intestinal Mucin (IIM) was identified from the frass fraction that elicited a wound response in maize leaves in Chapter 3. IIM is associated with the peritrophic matrix (PM), a structure that provides a physical barrier between the food bolus and the insect midgut (Hegedus et al., 2009). It was hypothesized that parts of the PM are shed with
the frass pellets and PM-associated proteins such as IIM could be involved in eliciting wound-induced defenses. My results show that indeed PM proteins are involved in triggering wound-induced defenses in plants. Since, IIM is one such PM protein, IIM from the insect frass was immune-depleted. This resulted in the attenuation of wound-induced defenses while enhancing the pathogen-induced defenses in plants. This suggests that not only is IIM a HAMP present in the insect frass, but may also be the only HAMP that can have a dual function of triggering the wound-induced response and suppressing the pathogen-induced defenses.

**Materials and Methods:**

**Plant and insect materials:** Maize (Zea mays, var. B73) plants were grown in glasshouse conditions with 16 hr light and 8 hr dark cycle in Hagerstown loam till they reached mid-whorl (V7-V8) development stage (Ritchie et al., 1998). FAW (Spodoptera frugiperda) eggs were obtained from USDA-ARS at Mississippi State University and reared on maize plants to collect frass. Frass was collected every 24 hr from caterpillars fed on V8 stage maize leaves. The collected frass was then homogenized in 1xPBS buffer (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate), filter sterilized and concentrated using centrifugal columns with a 3-kD molecular weight cut off (Pall Life Sciences, USA) as described earlier (Ray et al., 2015). Protein concentration in the frass extract was quantified by the Bradford assay. Fifth instar FAW larvae feeding on B73 leaves were dissected to collect the peritrophic matrix (PM). Thirty PMs were homogenized in 500 µl of 1xPBS buffer and 20 µl of the PM homogenate was applied to each wound site on B73 leaves, four wound sites per plant.

**Frass protein fractionation and identification:** Fractionation of frass proteins was performed in the standard Rotofor chamber as described in the previous chapter. Twenty fractions were collected after the run and pooled to obtain ten fractions as before. The first two most acidic fractions were pooled to form fraction A, the next two formed B and so on. All of the fractions were concentrated using 3 kD centrifugal columns (Pall Life Sciences, USA) and the resuspended in 1xPBS buffer. Fraction ‘i1’ and ‘i2’ are the two original fractions that were pooled to obtain fraction ‘I’. Protein concentration in each of these fractions were measured by the Bradford
assay and 1 µg of total protein was applied to each wound site on maize leaves to test the effect of the fractionated frass proteins. Proteomic analysis was performed at proteomics core facility at Hershey Medical Center, PA as described in the previous chapter. Multidimensional Protein Identification (MudPIT) was performed for fractions ‘i1’ and ‘i2’ separately by electrospray in LC/MS/MS (ABSciex 5600 TripleTOF, SCIEX, USA).

**Scanning electron micrographs of fall armyworm frass:** Frass was collected from fifth instar FAW caterpillars fed on B73 leaves and fixed in 1.5% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate overnight at 4°C. The frass pellets were washed three times in 0.1M sodium cacodylate for 2 mins each and incubated with 1% osmium tetroxide in the dark for 2 hr at room temperature. Frass pellets were again washed three times in 0.1M sodium cacodylate for 2 min each and dehydrated by incubating them serially in 25%, 50%, 70%, 85%, 95% and pure ethanol for 10 min each. Frass samples were stored in 100% ethanol overnight at 4°C. The following day, samples were dried in a critical point drier and sputter coated with gold particles. Coated samples were then visualized using the scanning electron microscope at the Microscopy Core facility in Penn State, University Park, PA.

**Immunoblot analysis and immunodepletion of IIM protein from FAW frass:** Fifty micrograms of frass proteins were loaded to a SDS-PAGE gel and run for 1 hr at 200 constant volts. Gels were blotted onto nitrocellulose membranes using the Panther Semi-Dry Electroblotter (Thermo Scientific, Owl, MA, USA). The blots were reversibly stained with Ponceau S (Salinovich and Montelaro, 1986) before immunoblot analysis to visualize protein transfer. Polyclonal antibody to IIM protein was obtained from Dr. Ping Wang (Cornell University, Ithaca, NY, USA). Western blots were carried out using 1:10,000 diluted anti-IIM antibody and 1:10,000 diluted anti-rabbit secondary antibody conjugated with horseradish peroxidase (Thermo Fisher Scientific, Rockford, IL, USA). Immunoreacting proteins were detected by chemiluminescence (West Femto Maximum Sensitivity Substrate, Thermo Scientific).

To immune-deplete IIM from the FAW frass, 500 µg of FAW frass was incubated with 1:10,000 anti-IIM antibody overnight at 4°C. One ml of anti-rabbit secondary antibody fused to agarose beads (Rockland Antibodies, PA, USA) was added to the mixture containing FAW frass proteins
and anti-IIM antibody and incubated overnight at 4°C. The agarose beads were centrifuged the following day at 10,000g for 15 min at 4°C and the supernatant was recovered. Fifty microgram proteins from the immune-depleted supernatant of FAW frass and 5 µl of agarose beads with bound IIM were then boiled in 1xsample buffer separated by SDS-PAGE gel and analyzed on a Western blot as described above.

**Treatment of plants with frass, PM and quantification of defense related genes in maize:** Leaves of V8 stage maize plants were treated with 20 µg of frass protein, or an equivalent volume from which IIM was precipitated, or 20 µl of the PM homogenate, or 1xPBS buffer on each leaf wound site to measure transcript abundance of defense-related genes in maize. Four plants were treated per treatment and transcript abundance of defense-related genes such as *maize protease inhibitor (mpi)*, *pathogen-related protein 5 (pr5)* and *ribosome inactivating protein 2 (rip2)* was measured. One hundred milligrams Leaf tissue around the wound site, approximately (1.5-2) cm² area, was collected and homogenized in liquid nitrogen using the GenoGrinder 2000 (OPS Diagnostics, USA) and RNA was extracted with TRlzol reagent (Life Technologies, USA) using the manufacturer’s protocol (100 mg tissue in 1 ml TRlzol). Genomic DNA was removed from the RNA by treatment with 2 units of DNasel enzyme (New England Biolabs, USA) as suggested by manufacturer. The genomic DNA-free RNA was quantified using Nanodrop (Thermo-Fisher Scientific, USA). cDNA was made from 1 µg of total RNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) using Oligo-dT. Quantitative real-time PCR (qRT-PCR) was performed with Fast Start Universal SYBR Green Master Mix (Roche Applied Science, USA) with actin as reference gene. Gene specific primers were the same as those in a previous study (Ray et al., 2015). Relative quantification of gene expression was analyzed with a one-factor ANOVA using time and treatment as independent variables and means separation was performed by Tukey’s test with SAS 9.1 (SAS Institute Inc., USA) software at p<0.05 level of confidence.
Results:

Proteins in fall armyworm frass with basic isoelectric points trigger wound-induced response in maize:

In Chapter 2 it was shown that proteins from FAW frass enhance the expression of wound-induced as well as pathogen-induced defenses in maize (Ray et al., 2015). While the wound-induced defenses are transient, the pathogen-induced defenses were sustained for longer periods of time. In Chapter 3, it was also demonstrated that Rotofor fractions of frass proteins with acidic isoelectric points triggered pathogen-induced defenses in maize (Fig 4.1). In this study, the induction of wound-induced responses in maize to the Rotofor fractions was examined in an attempt to identify FAW frass proteins that trigger the transient wound-induced response seen earlier. Relative transcript levels of the wound-induced defense gene marker mpi (maize protease inhibitor) that is a marker of herbivore-induced defenses in maize (Louis et al., 2013b; Chuang et al., 2014b) was measured. When the Rotofor fractions of frass proteins were applied to maize leaves, I observed that fraction ‘I’, which containing proteins with basic isoelectric points increased mpi transcript levels higher than buffer-treated plants (Fig 4.1a). Since fraction ‘I’ consisted of two fractions (i1 and i2) that were pooled, I applied these fractions separately to determine if there were differences in mpi induction. The transcript abundance of mpi was higher in ‘i1’ than in buffer-treated plants (Fig 4.1b), while fraction ‘i2’ did not significantly increase mpi mRNA levels (Fig 1b). This suggests that the elicitor for wound-induced defenses is present in fraction ‘i1’.

Proteomic analysis of Rotofor fraction that enhance the wound-induced response contains the caterpillar gut protein Insect Intestinal Mucin:

Data in the previous chapter showed MudPIT (Multidimensional Protein Identification Technology) analysis of the Rotofor fraction ‘I’ and indicated that it contained the caterpillar gut protein Insect Intestinal Mucin or IIM (Table 3.3). In this study, MudPIT analysis of both fraction ‘i1’ and ‘i2’ were performed to identify proteins that were different between the two fractions that could be responsible for triggering the observed wound-induced defenses (Fig 4.1b). I detected the presence of maize protease inhibitors (gi|350536017, gi|162460972) in both fractions ‘i1’ and ‘i2’ suggesting that these proteins are not involved in triggering the wound-
induced response (Table 4.1, 4.2). However, the most abundant protein in fraction ‘i1’, which was capable of triggering the wound-induced response, was *Bombyx mori* IIM (gi|379698934) (Table 4.1). Since, I was unable to identify an IIM protein candidate in fall armyworm using the protein query of *Bombyx mori* IIM, I used the result from *Bombyx mori* IIM and searched the nucleotide database of *Spodoptera*. My search found a partial cDNA sequence of fall armyworm peritrophin gene (AY581894.1). The translated product this cDNA sequence harbors a region of 15 amino acid sequence (TEQCDWPENVDCGDR) that was extremely similar to the *Bombyx mori* IIM peptide (TWQCDWPENVECGDR) identified in the MuDPIT analysis. IIM proteins that contain several conserved similar domains are present in almost all lepidopterans (Hegedus et al., 2009). My results suggest that one of these conserved regions present in FAW frass could be involved in wound-induced defense elicitation in maize.

**Fall armyworm frass contains peritrophic matrix pieces and a 55 kD IIM fragment:**

Insect intestinal mucin (IIM) is a component of the peritrophic matrix (PM) in the caterpillar gut (Hegedus et al., 2009), therefore, I wanted to know if the caterpillar defecates parts of the PM with the frass pellets. I collected frass from FAW larvae fed on maize leaves and examined them by scanning electron microscopy (SEM). I observed peritrophic matrix fragments at the edges of the frass pellets (Fig 4.2a, b), which suggests that the IIM protein is present in these PM fragments. In the caterpillar gut, IIM binds to the chitin microfibril network along with other proteins known as peritrophins to form the PM (Hegedus et al., 2009). The intact IIM is heavily glycosylated and measures ~130 kD in FAW (Fescemyer et al., 2013). Since, I observed PM in the caterpillar frass and speculated that it contained IIM, I tested for its presence in frass by immunoblot analysis. The results indicated the presence of a ~55 kD IIM fragment instead of the 130 kD protein present in the caterpillar gut (Fig 4.2c). Therefore, it appears that caterpillar frass contains digested fragments of their gut protein that could be recognized by the plant as a wound-inducible elicitor.

**Peritrophic matrix of caterpillars marginally triggers wound-induced response in maize:**

Since, I detected the presence of PM fragments in frass, I wanted to measure plant defense responses to the PM and its associated proteins. I dissected the PM from FAW caterpillars fed on maize, applied it to leaves and measured the abundance of *mpi* transcripts after 8 hr when
frass-induced wound responses are known to peak (Ray et al., 2015). The mRNA levels of mpi showed a statistically significant increase in plants treated with the PM compared to buffer-treated controls (Fig 4.3a). However, mpi transcript abundance was higher plants treated with frass proteins than the PM, which is consistent with previous results showing that total frass proteins increased mpi expression 8 hr after application (Ray et al., 2015). When pr5 expression in response to PM and frass proteins was tested at 24 hr after application, the PM did not enhance pr5 expression (Fig. 3b), which suggests that PM proteins cannot induce the pathogen defense pathway.

I also measured the trypsin protease inhibitor (TPI) activity in plants treated with PM and frass for 8 and 24 hr. Trypsin protease inhibitors (TPI) are known to be induced in response to caterpillar herbivory and retard caterpillar growth on their host (Felton, 2005b; Chung and Felton, 2011; Acevedo et al., 2015). I did not observe any change in the TPI activity in plants treated with PM, frass or buffer at 8 hr, which was contrary to the trend of protease inhibitor (mpi) transcript accumulation at this time (Fig 4.3c). However at 24 hr, frass-treated plants had suppressed TPI activity compared to buffer-treated plants (Fig 4.3d). The suppression of TPI activity supports my previous results that showed a suppression of herbivore defenses in response to frass proteins at 24 hr. In contrast to the frass protein application, there were no changes in TPI activity in plants treated with buffer or PM (Fig 4.3d). This suggests that FAW PM proteins and frass trigger increased expression of herbivore-induced mpi mRNA at early time points, but the proteins encoded by this and other protease inhibitor genes do not accumulate until later.

**Removal of IIM protein from fall armyworm frass attenuates wound-induced responses and elevates pathogen-induced responses:**

Proteins from the PM, of which IIM is a part, as well as the Rotofor fraction that contained IIM increased mpi transcript abundance in maize. Therefore, I immuno-depleted IIM from FAW frass proteins (Fig 4.4a) and applied the fraction devoid of IIM to maize plants to determine if its absence affected herbivore defense induction. At 8 hr, the abundance of mpi transcripts in plants treated with frass proteins devoid of IIM was than lower than that of plants treated with total frass proteins (Fig 4.4b). This suggests that IIM is needed to enhance the wound-induced
defense response in maize at 8 hr. I also measured the transcript abundance of herbivore-induced defense marker rip2 (ribosome inactivating protein 2) at 8 hr after the application of IIM-depleted frass proteins (Chuang et al., 2014a). The abundance of rip2 transcripts was similar in buffer-treated and frass protein-treated plants, but was higher than the unwounded control plants. The absence of IIM suppressed the rip2 RNA levels compared with these treatments and suggests that IIM is needed to maintain rip2 transcript levels that are induced by wounding (Fig 4.4e).

On the other hand, it appears that IIM suppresses the pathogen defense response that occurs 24 hr after frass protein application. At this time, the expression of the pathogen defense marker pr5 RNA was elevated in plants treated with IIM-depleted frass proteins. In addition, IIM-depleted-frass proteins also induced pathogen defenses at 8 hr when frass proteins typically have no effect on pathogen defenses (Fig 4.4d) (Ray et al., 2015). This suggests that IIM in frass may be a suppressor of frass-mediated pathogen defenses in maize, and its removal from elevates pr5 expression.

The effect of IIM depletion on the expression of TPI activity also was determined at 8 and 24 hr. At 8 hr both wounding plus buffer and wounding plus frass protein application increased TPI activity over the unwounded control (Fig. 4f). The absence of IIM prevented the wound-induced enhancement of TPI activity at 8 hr. At 24 hr there were no differences between the undamaged plants or the wounding plus buffer treatments (Fig. 4e), but wounding plus frass application suppressed TPI activity in a manner similar to that shown in Fig. 3d and supports the finding that frass proteins suppress herbivore defenses at 24 hr (Ray et al., 2015). The depletion of IIM from the frass proteins further enhances this suppression and suggests that it is needed for enhancing TPI activity. Hence, it is seems IIM enhances frass-induced herbivore defenses and suppresses pathogen-induced defenses in maize.

**Discussion:**

Several insect-derived molecules that are called elicitors or HAMPs (Herbivore Associated Molecular Patterns) have been shown to induce plant defenses (Mattiacci et al., 1995b; Alborn, 1997; Doss et al., 2000; Schmelz et al., 2006b; Alborn et al., 2007; Schäfer et al., 2011; Tian et
Although most of these compounds are derived from insect secretions such as the saliva and oral secretions, some of these elicitors originate from the plant (Alborn, 1997; Alborn et al., 2007; Heil, 2009; Tian et al., 2012). For example, the fatty acid portion of the fatty acid conjugated elicitor volicitin is derived from the plant when the insect disrupts the cell membrane lipids (Paré et al., 1998; Heil, 2009). Similarly, another elicitor inceptin is fragment of the plant ATP synthase digested in the insect gut and deposited on the host plant with the insect oral secretions (Schmelz et al., 2006b; Schmelz et al., 2007). Plant defenses are also known to be induced by the application of leaf extract or ‘leaf juice’, which suggests that the presence of plant-derived molecules in such extracts may lead to damaged-self recognition (Turlings et al., 1993; Mattiacci et al., 1995b). In this study I show the presence of an insect-derived protein (IIM) in caterpillar frass that enhances wound-induced defenses in maize (Fig 4.4b).

My previous study demonstrated that proteins from caterpillar frass induced a transient wound response in maize (Ray et al., 2015). Therefore, I fractionated proteins from FAW frass and identified IIM in one fraction that induced the wound responses (Fig 4.1a, b and Table 4.1). IIM is an insect gut protein which belongs to the peritrophin family of proteins that bind chitin microfbrils to form the peritrophic matrix (PM) present in most Lepidopteran insect larvae (Hegedus et al., 2009). The PM serves a physical barrier that separates the insect midgut from the food bolus and its disruption has been shown to be detrimental to the insects (Pechan et al., 2002). However, the insect is known to regenerate the PM throughout its larval life cycle and excrete the “used” PM in the frass (Hegedus et al., 2009). This and the presence of IIM in the FAW frass led us to speculate that IIM is embedded in PM in insect frass. SEM analysis confirmed that PM fragments were in the FAW frass (Fig 4.2a, b). Immunoblot analysis indicated that ~55kD IIM fragment was present in the FAW frass proteins (Fg 2c). Application of PM proteins on maize leaves showed an induction of wound responses (Fig 4.3a) further emphasizing that PM proteins such as IIM could be a involved in triggering this defense in maize. The PM is known to harbor chitin and since frass contains PM as well, one may speculate that chitin (or its oligomers) could be involved in triggering the observed plant defenses since chitin is a known plant defense elicitor (Kaku et al., 2006; Hegedus et al., 2009). However, I
have shown that proteins from caterpillars enhanced the frass-induced plant defenses in maize and removal of proteins abolished such responses (Ray et al., 2015). Hence, I immuno-depleted IIM from FAW frass (Fig 4.4a) and tested the effects of the resulting protein mixture on maize defenses.

When frass proteins devoid of the IIM fragment where applied to the plant there was an attenuated wound-induced response (Fig 4.4b, e, and f). In addition, immuno-depletion of IIM from frass elevated the frass-triggered pathogen defenses (Fig 4.4c, d and g), which strongly suggests that IIM also suppresses pathogen-induced defenses in maize. The antagonism between the jasmonic acid (JA) regulated herbivore defense pathway and the salicylic acid (SA) regulated pathogen defense pathway is well known in plants (Thaler et al., 2012). In a previous study, I have shown that such an antagonism occurs when FAW frass proteins are applied to maize plants (Ray et al., 2015). From this study it can be concluded that the transient peak of frass-induced wound response in maize is elicited by the insect gut protein IIM. IIM may also mediate crosstalk between the herbivore and the pathogen defense pathway by suppressing frass-induced pathogen defenses at later time points.
Table 4. 1 MudPIT of fraction 'i1'

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession number</th>
<th>Source species</th>
<th>Peptide count (95%)</th>
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<tbody>
<tr>
<td>Insect intestinal mucin precursor</td>
<td>gi</td>
<td>379698934</td>
<td>Bombyx mori</td>
</tr>
<tr>
<td>Subtilisin-chymotrypsin inhibitor Cl-1B</td>
<td>gi</td>
<td>350536017</td>
<td>Zea mays</td>
</tr>
<tr>
<td>Subtilisin-chymotrypsin inhibitor homolog1</td>
<td>gi</td>
<td>162460972</td>
<td>Zea mays</td>
</tr>
<tr>
<td>Sidekick-like protein</td>
<td>gi</td>
<td>512900304</td>
<td>Bombyx mori</td>
</tr>
<tr>
<td>Uncharacterized protein LOC100273479 precursor</td>
<td>gi</td>
<td>226496763</td>
<td>Zea mays</td>
</tr>
<tr>
<td>Maize LOC101739898 precursor</td>
<td>gi</td>
<td>512936725</td>
<td>Zea mays</td>
</tr>
<tr>
<td>Endoribonuclease Dcr-1-like</td>
<td>gi</td>
<td>512889762</td>
<td>Bombyx mori</td>
</tr>
<tr>
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<td>170097445</td>
<td>Laccaria bicolor</td>
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<tr>
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<tr>
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<td>gi</td>
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Table 4. 2 MudPIT of fraction ‘i2’

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<th>Peptide count (95%)</th>
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<tbody>
<tr>
<td>Subtilisin-chymotrypsin inhibitor Cl-1B</td>
<td>gi</td>
<td>350536017</td>
<td>Zea mays</td>
</tr>
<tr>
<td>Subtilisin-chymotrypsin inhibitor homolog1</td>
<td>gi</td>
<td>162460972</td>
<td>Zea mays</td>
</tr>
<tr>
<td>exoglucanase1 precursor</td>
<td>gi</td>
<td>212274863</td>
<td>Zea mays</td>
</tr>
<tr>
<td>2-keto-4-pentenoate hydratase</td>
<td>gi</td>
<td>497826988</td>
<td>Oceanicola sp.</td>
</tr>
<tr>
<td>Uncharacterized protein LOC100136885</td>
<td>gi</td>
<td>167860184</td>
<td>Zea mays</td>
</tr>
</tbody>
</table>
Figure 4.1a Maize defense gene expression in response to fractionated frass proteins.

(a) Maize leaves were wounded and treated with 10 protein fractions (A-J) or PBS buffer for 8 hr and the transcript abundance of the wound defense marker *Maize protease inhibitor* (*mpi*) was measured by quantitative real-time PCR (qRT-PCR). 

(b) The Rotofor fraction ‘I’ that induced highest *mpi* transcript abundance was pooled from two original fractions ‘i1’ and ‘i2’, which were separately applied to leaves *mpi* transcript abundance was measured with buffer-treated plants as control. Transcript abundance
of pr5 was normalized to that of the reference gene actin and expressed as relative quantification (RQ). Data were analyzed by a one-factor ANOVA and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk or having different letters are significantly different from buffer-treated (p<0.05). Error bars indicate standard error from the mean.

**Figure 4.2** Scanning electron micrograph of FAW frass and immune-detection of IIM in FAW frass.

(a,b) Frass from fifth instar FAW larvae fed on B73 plants was collected and observed under scanning electron microscope. Images show the presence of peritrophic matrix (red arrows).

(c) Proteins from FAW frass were run on a denaturing polyacrylamide gel and IIM protein was detected as a ~55kD fragment by immune-detection using IIM antibody. Ponceau S staining of nitrocellulose membrane shows effective transfer of proteins to the membrane.
mpi expression in maize

Relative Quantification (RQ)

Control  Buffer  PM  Frass

Fig 4.3a
**Fig 4.3b**

**pr5 expression in maize**

![Graph showing relative quantification (RQ) of pr5 expression in maize.](image)

**Fig 4.3c**

**Trypsin protease Inhibitor Activity 8 hr**

![Graph showing activity per mg of protein.](image)
Maize plants were treated with the peritrophic matrix (PM) homogenate from fifth instar FAW larvae, frass proteins or buffer on wound sites or left undamaged (control). Transcript abundance of the defense-related genes *maize protease inhibitor or mpi* (a) and *pathogenesis related protein 5 or pr5* (b) were measured after 8 and 24 hr respectively and normalized to the expression of an endogenous gene *actin* and expressed as relative quantification (RQ). Trypsin protease inhibitor (TPI) activity was also measured for the same treatments after 8 hr (c) and 24 hr (d). Data were analyzed by a one-factor ANOVA and mean separation was calculated by multiple comparison Tukey’s test. RQ values and TPI activity with different letters are significantly different each other (p<0.05). Error bars indicate standard error from the mean.
Figure 4.4 (a): Immunodetection of IIM protein showing immuno-depletion of IIM from FAW frass. (b-g) Transcript abundance and trypsin protease inhibitor activity of defense-related genes in response to IIM depletion from FAW frass

(a) FAW frass was depleted of IIM proteins by immune-precipitation. Proteins from immune-depleted FAW frass, total FAW frass and the IIM precipitate were run on a denaturing polyacrylamide gel and a western blot was performed for IIM protein. PonceauS staining shows equal loading of proteins on the gel and equal transfer to membrane.

 mpi expression in maize at 8hr
**Fig 4.4c**

*pr5 expression in maize at 24hr*

![Graph showing pr5 expression in maize at 24hr](image)

**Fig 4.4d**

*pr5 expression in maize at 8hr*

![Graph showing pr5 expression in maize at 8hr](image)
Fig 4.4e

*rip2* expression in maize at 8hr

![Bar chart showing relative quantification (RQ) for Control, Buffer, Frass, and Frass-IIM.](chart)

Fig 4.4f

Trypsin protease Inhibitor Activity in maize at 8hr

![Bar chart showing activity per mg of protein for Control, Buffer, Frass, and Frass-IIM.](chart)
Fig 4.4 (b-g): Transcript abundance and trypsin protease inhibitor activity of defense-related genes in response to IIM depletion from FAW frass.

Maize leaves were treated with proteins from FAW frass, frass protein devoid of IIM (Frass-IIM) or buffer on wound sites or left undamaged (control). Transcript abundance of maize defense genes \textit{mpi} (b) and \textit{ribosome inactivating protein2} or \textit{rip2} (e) were measured at 8 hr while that of \textit{pr5} was measured at 8 hr (c) and 24 hr (d) and normalized to that of an endogenous gene \textit{actin} and expressed as relative quantification (RQ). Trypsin protease inhibitor (TPI) activity was measured for the same treatments at 8 hr (f) and 24 hr (g). RQ and TPI activity were for treatments were analyzed by one-way ANOVA and means separation was performed by Tukey’s test. RQ and TPI activity values with different letters are significantly different from each other (p<0.05).
Chapter 5. Lessons from the far end: Caterpillar frass induced plant defenses in maize, rice, cabbage and tomato.

Introduction:

Plants deploy a sentinel of induced and constitutive defenses to thwart insect attack (Chen, 2008a). Plant defense responses to insect herbivory have been attributed to several insect behaviors such as feeding, crawling, oviposition and even defecation (Alborn et al., 1997; Mithöfer et al., 2005; Felton and Tumlinson, 2008; Peiffer et al., 2009b; Kim et al., 2012; Hilfiker et al., 2014). Insect feeding is associated with deposition of oral secretion and saliva on plant tissue, which leaves chemical cues of herbivory that induces defenses in host plants (Alborn, 1997; Musser et al., 2002; Schmelz et al., 2006b; Alborn et al., 2007; Schäfer et al., 2011). Induction of plant defenses in response to such chemical cues (or elicitors) has been known to be specific to host-herbivore systems. For example, oral secretions from tomato fruit worm (*Helicoverpa zea*) induces herbivore defenses in tomato but suppresses such defenses in tobacco (Musser et al., 2002; Tian et al., 2012). Induction of plant defenses may be different even with application of the same elicitor, such as glucose oxidase that suppresses direct defenses in tobacco, induces them in tomato and has no effect in maize (Musser et al., 2002; Tian et al., 2012; Louis et al., 2013b). Insect secretions are a blend of various molecules that can elicit variable and composite blends of plant defense responses. For example, glucose oxidase from tomato fruitworm saliva induces direct defenses in tomato while salivary ATPases suppress them at the same time (Tian et al., 2012; Wu et al., 2012). Therefore, it is fair to say that the complexity of induced plant defenses in response to insect herbivory is highly specific to the composition of the herbivore secretion deposited on the plant, the plant species on which it is deposited and the insect that deposits the secretion.

My previous studies have shown that in host-herbivore system such as maize and fall armyworm, frass from the herbivore induces pathogen defense over time in maize leaves where it accumulates close to feeding sites (Ray et al., 2015). However, there are several host-herbivore systems where frass does not accumulate in enclosed feeding structures over
extended periods of time and may only briefly come in contact with the wound site. I hypothesized that frass-induced defenses in plants are variable and specific to the host-herbivore system. To test my hypothesis, I measured both herbivore and pathogen-induced plant defenses in response to caterpillar frass. Since, caterpillar frass is more likely to accumulate in host-herbivore systems with enclosed feeding habits, I measured plant defenses in such host-herbivore systems such as European corn borer (ECB) frass in maize, cabbage looper (CL) frass in cabbage and tomato fruit worm (TFW) frass in tomato fruit. I also measured frass-induced plant defenses in host-herbivore systems where frass does not accumulate in close proximity to insect feeding sites such as fall armyworm (FAW) frass on rice leaf or TFW on tomato leaves. Although frass will not be naturally present in these host-herbivore systems for extended periods of time in nature, I measured plant defenses in response to frass in these systems over a four day period to compare these results with those from previous studies where frass accumulated in close proximity to feeding sites for extended periods of time (Ray et al., 2015).

**Materials and Methods:**

**Plant material:** Maize (Zea mays var.B73), tomato (Solanum lycopersicum var.Microtom), rice (Oryza sativa var. Nipponbare) and white cabbage (Brassica oleracea var. oleracea Platinum dynasty) were grown glasshouse conditions with 16:8 hr light:dark cycle. Maize plants were grown in field soil until they reached the V8 stage (Ritchie et al., 1998). Cabbage and tomato plants were grown to their desired stages in Promix-HP potting mix containing mycorrhizae (Premier Tech Home and Garden, Ontario, Canada). Rice seeds were germinated first on filter paper with 16:8 hr light:dark cycle and then transplanted in Metro-mix 360 (Sungro, MA, USA). All plants were fertilized with Osmocote Plus (Scotts, OH, USA) once after potting and rice plants were additionally fertilized with 5% ammonium sulfate solution weekly and once with slow release iron chelator 1% Sprint 330 (Hummert International, MO, USA) after potting. Maize seeds (var.B73) were obtained from USDA-ARS in Mississippi State University, USA; rice seeds were obtained from USDA-ARS Dale Bumpers National Rice Research Center in Arkansas; white cabbage seeds (var.Platinum dynasty) were purchased from Seminis (MO, USA).
Insects, collection and extraction of frass: Fall armyworm (*Spodoptera frugiperda*), cabbage looper (*Trichoplusia ni*) and European corn borer (*Ostrinia nubialis*) larvae were reared on rice, white cabbage and maize leaves, respectively in plastic cups. Frass from the larvae was collected each day, plastic cups were cleaned and fresh plant tissue was given to the larvae. Tomato fruitworm (*Helicoverpa zea*) were fed both on tomato leaves and green fruits in plastic cups. Frass was collected each day and fresh tissue supplied to the larvae for feeding. Frass obtained from the different larvae species was homogenized in 1x-phosphate buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate) for 30 min at a ratio of 1 g (wet weight) of frass in 5 ml of PBS. The homogenized frass slurry was then filtered through Miracloth (EMD Millipore, USA) to separate the insoluble debris. The soluble frass extract was then filter sterilized using a 0.2 µm filters (EMD Millipore, USA). The sterilized frass extract were concentrated using a 3-kD molecular weight cut-off column (Pall Life Science, USA). Protein concentration in the frass extract were measured using a Bradford protein quantification assay (Bradford, 1976). Twenty micrograms of total frass protein were applied to each wound site on leaves of the plants tested or injected into tomato fruit.

Treatment of plant material with frass extract: Whorl leaves of V8 stage maize plants were treated with frass or buffer on wound sites as described (Ray et. al, 2015). Rice plants were grown for 30 days after germination when plants typically had three tillers. The leaves of all tillers were wounded with a hole-punch and frass or buffer was applied. Cabbage plants were grown for 30 days to the four-leaf stage, then the leaves were wounded with the maize wounding tool (Ray et al., 2015) and treated with either frass proteins or buffer. Tomato plants were grown for 4 weeks, leaves were wounded with hole-punch and then treated with frass proteins or buffer. Fruit treatments were done on unripe tomato fruits on 6-week-old plants by injecting frass proteins or buffer into the fruit using a 10µl pipette tip.

RNA extraction and cDNA preparation: Leaf and fruit tissues were homogenized in liquid nitrogen using GenoGrinder 2000 (OPS Diagnostics, USA) and RNA was extracted with TRIzol reagent (Life Technologies, USA) using the manufacturer’s protocol (100 mg tissue in 1 ml TRIzol). Genomic DNA was removed from the extracted RNA by treating it with 2.5M lithium
chloride overnight at 4°C. The precipitated RNA was then washed with 75% ethanol twice and re-suspended in nuclease-free water. The genomic DNA-free RNA was quantified using a Nanodrop (Thermo-Fisher Scientific, USA). One microgram of total RNA was then used to prepare cDNA using Oligo-dT with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). RNA extraction and cDNA synthesis were performed for each biological replicate separately. Four biological replicates for each treatment were used for maize plants, five for each treatments in cabbage and tomato plants and seven replicated for each treatment in rice per time point.

**Quantitative real-time PCR:** Quantitative real-time PCR (qRT-PCR) was performed with Fast Start Universal SYBR Green Master Mix (Roche Applied Science, USA). Gene-specific primers were designed with Primer Express 3.0 (Life Technologies, USA; details in Table S5.1). Relative quantification (RQ) of the target gene expression was calculated by the delta-delta Ct method (Livak and Schmittgen, 2001) using *actin* as an endogenous gene for maize and rice, *ubiquitin* for tomato and *glyceraldehyde-3-phosphate dehydrogenase* for cabbage. Gene expression levels in frass-treated, PBS buffer-treated and undamaged control plants were measured 4, 24, 48 and 96 hr after application for the respective target genes tested.

**Trypsin protease inhibitor assay:** Trypsin protease inhibitor assay was performed on tomato leaves and fruits tissue treated with 20 µg of frass for 4, 24, 48 and 96 hr. One hundred milligram of plant tissue was ground in Genogrinder (OPS Diagnostics, USA) as described above and homogenized in 1.25 ml of buffer (0.046M Tris Buffer pH=8.1, 0.012M calcium chloride) containing 5% insoluble polyvinyl-pyrrolidone (Chung and Felton, 2011). Samples were centrifuged at 11,000g for 10 mins and 10 µl the supernatant was mixed with 80 µl of extraction buffer and 10 µl of 1 mM trypsin (Sigma Aldrich, USA). The mixture was incubated at room temperature for 10 mins and 100 µl of the substrate (2mM p-toluene-sulfonyl-L-arginine methyle ester, Sigma Aldrich) was added and the optical density (OD) was measured for 5 mins at 247 nm. Trypsin protease inhibitor (TPI) activity per milligram of protein was calculated by the formula, PI = [1-(A/B)]/P where A represents the trypsin activity of the sample, B represents the maximum trypsin activity in a sample where only extraction buffer was added (no inhibitor
and P is mg of protein added to measure TPI activity. The protein concentration of each sample was measured separately using Bradford assay (Bradford, 1976).

**Data analyses:** RQ values for gene expression and trypsin protease inhibitor (TPI) activity were analyzed with a two-factor ANOVA using time and treatment (frass, buffer or undamaged controls) as independent variables with SAS 9.2 (SAS Institute Inc., USA) software at p<0.05 level of confidence. However, there were interactions between time and treatment for all genes/TPI activity tested, hence a multiple comparison Tukey test was performed for each gene at p<0.05 level of confidence for each set of host and insect-frass systems tested.

**Results:**

**European corn borer frass sustains herbivore-induced defenses while suppressing pathogen-induced defenses in maize:**

European corn borer (ECB) herbivory is known to induce defenses in crops such as maize and tomato (Houseman et al., 1992; Louis et al., 2013b). Oral secretions and saliva of ECB have been implicated in harboring elicitors (and/or effectors) that can trigger direct and indirect herbivore defenses in plants (Louis et al., 2013a; Louis et al., 2013b). However, defenses triggered by the presence of elicitors (and/or effectors) from ECB frass have not been studied. I measured the transcript abundance of herbivore-induced *lipoxygenase3* (*lox3*) and *maize protease inhibitor* (*mpi*) in response to ECB frass proteins at 4, 24, 48 and 96 hr. I also measured pathogen-induced *pathogenesis related defense protein5* (*pr5*) transcript abundance in maize leaves treated with ECB frass proteins at the same time points (van Loon et al., 2006; van der Linde et al., 2012). *Lox3* encodes *lipoxygenase3* in the jasmonic acid (JA) biosynthesis pathway and is a hallmark for herbivore-induced early defense in maize. Frass-treated leaves showed higher abundance of *lox3* transcripts compared to controls at 4 hr, however frass treatment suppressed its transcript abundance at 48 hr (Fig 5.1a). Another herbivore-induced defense gene *mpi*, showed higher transcript accumulation in leaves treated with frass at 4, 24 and 96 hr compared to control (Fig 5.1b). Notably, the marker for pathogen defense *pr5* showed higher transcript levels only at 24 hr in frass-treated maize leaves compared to buffer-treated controls; however, frass treatment suppressed *pr5* abundance at 48 and 96 hr compared to buffer-
treated controls (Fig 5.1c). This suggests that frass-induced plant defenses in maize may be insect-specific since these results are in contrast to the results obtained with fall armyworm frass that increased the expression of pr5 in maize between 8 and 48 hr (Ray et al., 2015).

**Cabbage looper frass triggers an oscillating pattern of herbivore defenses while the pathogen induced-defenses steadily increases over time:**

I measured caterpillar frass-induced plant defenses in cabbage by cabbage looper (CL) frass proteins. This represents another naturally occurring plant-herbivore host-plant system where frass is likely to accumulate in an enclosed structure. Transcript abundance of herbivore defense-related genes such as lipoxygenase (lox) that is involved in the JA biosynthesis pathway in the Brassicaceae and induced by caterpillar herbivory by lepidopteran pests was measured (Zheng et al., 2007). I also determined transcript levels a trypsin protease inhibitor (tpi) that is downstream of the JA pathway and known to retard CL growth in cabbage (Broadway and Colvin, 1992). I measured transcript abundance of both lox and tpi in response to CL frass proteins and buffer at 4, 24, 48 and 96 hr. The JA biosynthesis precursor lox, showed higher transcript abundance in frass-treated plants only at the early time point of 4 hr compared to buffer-treated plants (Fig 5.2a). After this, lox transcripts steadily declined in response to frass treatment in comparison to buffer-treated cabbage at 24 and 48 hr and was not different in frass- and buffer-treated plants at 96 hr. However, lox transcript levels in both buffer- and frass-treated plants increased dramatically at 96 hr, which suggests that lox expression is stimulated by wounding. tpi transcript accumulation showed an oscillating pattern of induction and suppression in response to frass- treatment. Transcript levels of tpi in frass-treated plants were higher than buffer-treated plants at 4 hr, while they were suppressed at 24 hr. Interestingly, the frass-treated plants showed higher induction of tpi transcripts at 48 hr that was followed by suppression at 96 hr (Fig 5.2b). This oscillating pattern of induction followed by suppression of herbivore-induced plant defenses fits the Z-scheme model of effector triggered immunity that is widely accepted in plant-pathogen interaction and has been hypothesized in plant-herbivore interactions as well (Jones and Dangl, 2006; Felton and Tumlinson, 2008).

The *pathogenesis-related protein1* (*pr1*) gene has been shown to be induced in cabbage in response to pathogen attack (Park et al., 2005). Transcript abundance of *pr1* in response to CL
frass proteins in cabbage was measured (Fig. 2c). The pr1 transcript levels were slightly induced at 4 hr and suppressed at 24 hr. However, the expression of pr1 transcripts increased dramatically at 48 and 96 hr and was significantly higher than the buffer-treated controls. This suggests that there could be a shift to an enhanced pathogen defense pathway at these later time points.

**Fall armyworm frass steadily induces herbivore defenses in rice while suppressing pathogen defenses:**

Fall armyworm (FAW) is a generalist herbivore that feeds on several important crop species including rice. It has been shown earlier that frass proteins from FAW caterpillars fed on maize trigger a pathogen defense response in maize where the herbivore deposits its frass in the enclosed feeding sites of the whorls (Ray et. al, 2015). In this study plant defenses triggered by frass proteins from FAW larvae that fed on rice were measured. The rice-FAW interaction is a host-herbivore system where frass does not accumulate in close proximity to feeding sites. RNA levels of JA biosynthesis-related lipoxygenase gene (lox) have been shown to be induced by Spodoptera litura feeding in rice (Xu et al., 2003). I observed that lox had higher transcript abundance in frass protein-treated plants only at 24 hr after application compared to buffer treated plants (Fig 5.3a). However, at 48 hr after application, frass-treated plants showed a suppression of lox transcript abundance compared the controls (Fig 5.3a). A Bowman-Birk type rice protease inhibitor (rpi) downstream of the JA signaling pathway has been shown to be induced by beet armyworm herbivory in rice (Venu et al., 2010). Transcript abundance of this herbivore-induced rpi was weakly induced in frass-treated plants compared to buffer-treated plants at 24 hr, but it soared in frass-treated plants at 48 hr. Rice shows a strong SA-JA crosstalk and is known to regulate the SA-JA antagonism through NPR1 (non-expressor of pathogenesis-related protein1) protein that is an early marker for the salicylic acid (SA) pathway and plays a critical role in pathogen defense mechanisms in rice (Chern et al., 2005; Yuan et al., 2007; Thaler et al., 2012). NPR1 overexpression in rice is also known to increase its susceptibility to herbivores (Yuan et al., 2007). I measured the transcript abundance of npr1 in response to FAW frass in rice. Notably, frass-treated plants showed a higher abundance of npr1 transcript compared to buffer treated plants at 24 hr, however, at 48 hr when the herbivore-induced rpi
transcript peaked, \textit{npr1} transcript abundance was suppressed (Fig 5.3c). This strongly suggests that FAW frass proteins trigger sustained herbivore defenses while suppressing pathogen-induced defenses in host-herbivore system where frass does not accumulate in close proximity to feeding sites.

**Tomato fruitworm frass induces sustained herbivore defenses in tomato leaf tissue while having no effect on pathogen defenses:**

As another example of a host-herbivore system where frass does not accumulate in host plant tissue, I measured plant defenses in tomato leaves in response to frass proteins from tomato fruit worm (TFW) fed on tomato leaves. Lipooxygenase D (\textit{lox D}), involved in JA biosynthesis, and \textit{Protease inhibitor 2} (\textit{pin2}) downstream of the JA pathway are hallmarks of herbivore induced defense genes in tomato (Peiffer et al., 2009b; Tian et al., 2012). TFW frass proteins caused weak suppression and a subsequent induction of \textit{loxD} transcripts at 4 hr and 48 hr, respectively, compared to buffer-treated tomato leaves (Fig 5.4a). However, \textit{pin2} transcript abundance increased appreciably in frass-treated leaves compared to those treated with buffer at all time points (Fig 5.4b). I also measured transcript abundance of a SA-induced pathogen defense gene \textit{pathogenesis related protein 1-p4} [\textit{pr1-(p4)}] in response to frass protein on leaves (Chung et al., 2013). Transcript abundance of \textit{pr1-(p4)} was same in both frass- and buffer-treated tomato leaves at all time points tested (Fig 5.4c). This strongly suggests that proteins in TFW frass when applied to a tomato leaf tissue, where frass does not accumulate close to feeding sites in nature, induces only herbivore defenses in plants without triggering pathogen defenses as seen earlier with ECB, CL or FAW frass (Fig 5.1c, 5.2c, 5.3c).

**Tomato fed tomato fruitworm frass shows an initial suppression of herbivore defenses followed by a subsequent induction in tomato fruits:**

To better understand if TFW frass-induced defenses are altered when TFW feeds on the enclosed tomato fruit, I collected frass proteins from TFW fed on fruit, injected it into the fruit and measured the same defense gene transcripts as for tomato leaves. The herbivore-induced JA biosynthetic marker \textit{loxD} showed higher transcript levels in frass-treated plants compared to buffer-treated ones only at the later time points of 48 and 96 hr (Fig 5.5a). However, expression of the downstream JA-induced herbivore defense gene \textit{pin2} was suppressed in frass-injected
tomato fruits compared to buffer treated ones at 4 and 24 hr. But, pin2 RNA levels were higher in TFW frass-injected fruits compared to buffer control at 48 and 96 hr (Fig 5.5b). Compared to buffer-treated fruits, the pathogen-induced SA marker gene pr1-p4 was suppressed by frass treatment at 24 and 48 hr after frass injection. However, frass treatment induced higher pr1-p4 transcripts compared to buffer 96 hr (Fig 5.5c). Taken together, the results from TFW frass treatment on tomato leaves and fruits strongly suggests that the frass-induced defense response depends not only on the diet of the defecating herbivore but also on the tissue where they deposit their frass.

**Tomato fruitworm frass induces trypsin protease inhibitor activity in tomato leaves, but suppresses it in tomato fruit:**

In an attempt to understand if changes in defense gene transcript abundance affect biochemical content of the plant tissues in response to frass, I chose to measure trypsin protease inhibitor (TPI) activity in tomato fruits and leaves in response to TFW frass. Protease inhibitors that are induced in response to insect herbivory in a number of plants prevent the digestion of proteins in the insect gut thereby increasing the demand for essential amino acids and retarding insect growth (Felton, 2005b; Chung and Felton, 2011). These two host-herbivore system served as good representatives of systems where frass accumulates and remains in the enclosed fruit and where frass is only briefly in contact with feeding sites on the leaves. Trypsin protease inhibitor has been shown to correlated with pin2 transcript abundance (Chung and Felton, 2011) TPI activity was higher in tomato leaves treated with tomato leaf-fed frass at 4 and 24 hr compared to the buffer treated controls (Fig 5.6a). However, after 24 hr the TPI activity was the same in both frass and buffer-treated leaves. Similarly, in fruits injected with frass from TFW fed on fruits, the TPI activity was lower compared to fruits injected with buffer at 4, 24 and 96 hr (Fig 5.6b). TPI activity was induced in leaves, but suppressed in fruits at 4 and 24 hr and these results were followed the same overall trend pin2 transcript induction at these time points (Fig 5.4b, 5.5b). This suggests that not only the transcript abundance but also the biochemical composition of the plants was altered in response to frass application.
Discussion:

Herbivore-induced plant defenses can be specific to insect cues depending on the plant species and the insect depositing cues on the plant (Karban and Baldwin, 1997; Acevedo et al., 2015). The repertoire of plant defense compounds that are induced in response to two different insects, tobacco hornworm (*Manduca sexta*) and beet armyworm (*Spodoptera exigua*) herbivory, in tobacco are different from each other (Voelckel and Baldwin, 2004). Alternatively, herbivore cues such as saliva from the same insect tomato fruitworm (*Helicoverpa zea*) suppresses herbivore induced defenses in tobacco, but induces such defenses in tomato (Musser et al., 2002; Tian et al., 2012). Although, most of these studies are focused on caterpillar oral secretions or saliva, little is known about frass-induced plant defenses in various host-herbivore systems. This is of particular importance in understanding the complexity of host-herbivore interactions, since the composition of frass can change depending on the plant tissue the herbivore consumes. Furthermore, the host’s response to the frass may also vary depending on the tissue where it is deposited and the length of time it is in contact with the tissue.

My previous study demonstrated that in a host-herbivore system such as fall armyworm and maize where frass accumulates in the host tissue over time, herbivore defenses are suppressed and pathogen defenses are induced (Ray et al., 2015). In this study, I measured the transcript abundance of both pathogen- and herbivore-induced genes in three more additional systems where frass accumulates in the host’s enclosed tissues, viz., ECB frass in the maize whorl, CL frass on cabbage leaves and TFW frass inside the tomato fruit. I measured herbivore induced *lipoxygenase3* (*lox3*) and *maize protease inhibitor* (*mpi*) transcript abundance (Louis et al., 2013b; Chuang et al., 2014b), in response to ECB frass in maize leaves. Similar to FAW frass-induced defenses in maize, ECB frass also induced higher *lox3* and *mpi* transcripts compared to buffer-treated plant at 4 hr after application (Fig 5.1a, b). However, *mpi* transcript abundance was higher in plants treated with ECB frass at 24 and 96 hr as compared to buffer-treated ones (Fig 5.1b). On the other hand, the pathogen-induced *pr5* transcript levels were only higher at 24 hr and then were suppressed compared to buffer-treated controls at both 48 and 96 hr (Fig
5.1b, c). This is in contrast to my previous study where FAW frass caused a temporal shift in defenses from herbivore to pathogen defenses in maize and pr5 transcript abundance steadily increased after 24 hr of frass application (Ray et al., 2015).

Cabbage looper also feeds in relatively enclosed spaces on cabbage leaves and their frass can stick to the host leaves for long periods. My study revealed that the pathogen induced-pr1 transcript levels steadily increased at 48 and 96 hr after frass application (Fig 5.2c). Notably, the herbivore-induced JA precursor lox showed reduced transcript levels at both 24 and 48 hr in response to frass treatment (Fig 5.2a). Another herbivore- induced gene transcript tpi, showed an oscillating pattern of induction at 4 hr (and 48 hr) followed by suppression at 24 (and 96 hr) that is reminiscent of the Z-scheme model of effector-triggered immunity. The Z-scheme is a widely accepted model in plant-pathogen interactions and has been proposed to be important in plant-herbivore interactions as well (Jones and Dangl, 2006; Felton and Tumlinson, 2008). In general, CL frass treatment suppressed herbivore defenses over time while activating pathogen defenses. This was similar to the pattern found in the TFW-tomato fruit interaction where frass also accumulates in an enclosed host tissue. TFW frass injected into fruits induced a temporal shift towards pathogen defenses by highly inducing pr-1(p4) transcript accumulation in fruits at 96 hr (Fig 5.5c). This is in contrast to the expression of pin2, a maker for herbivore resistance. pin2 transcript abundance was suppressed at 4 and 24 hr in frass-injected fruits and this was mirrored by TPI protease inhibitor activity at these time points (Fig 5.5b, 5.6b). At 48 and 96 hr pin2 transcript levels were higher than those of buffer-injected tomato fruits, but TPI activity in fruits was lower at these time points (Fig 5.6b). In general pin2 expression in response to herbivory is generally several thousand-fold higher than the unwounded control, but in this case the induction was approximately 200-fold greater (RQ value of 238.47 at 48hr and 260.04 at 96hr). Consequently, pin2 transcript levels may not have reached the threshold needed to be translated in protein. Although CL frass on cabbage leaves and TFW frass in tomato fruits activated the pathogen defense pathway, I cannot conclude that all host-herbivore systems where frass accumulates in enclosed host feeding sites can induce pathogen defense pathway since I did not see this trend in maize when ECB frass was applied on maize leaves (Fig 5.1 a-c).
As examples of host-herbivore systems where frass is in contact with the host tissue for a limited amount of time, I measured frass-induced defenses of FAW frass on rice leaves and TFW frass on tomato leaves. In rice, the transcript abundance of herbivore-induced \textit{lipoxygenase} (\textit{lox}) increased in response FAW frass at 24 hr and then was suppressed at 48 hr (Fig 5.3a). Transcript abundance of \textit{rice protease inhibitor} (\textit{rpi}), a gene that is further downstream of \textit{lox} in the herbivore defense pathway, was slightly higher in frass treated plants at 24 hr, but dramatically increased at 48 hr compared to buffer treated plants (Fig3b). Interestingly, the pathogen-induced \textit{npr1} gene was suppressed in response to frass at 48 hr when \textit{rpi} was at its peak (Fig 5.3c). This is contrary to frass-induced defenses of the same herbivore (FAW) in maize where pathogen defenses were induced over time (Ray et al., 2015). In tomato leaves, TFW frass consistently induced much higher abundance of the herbivore-induced \textit{pin2} transcripts from 4 to 96 hr (Fig 5.4b). The \textit{pin2} transcript abundance also correlated with higher protease inhibitor activity at 4 and 24 hr in frass-treated (Fig 5.6a). Interestingly, the pathogen defense marker in tomato \textit{pr-1(p4)}, which was induced in response to TFW frass in fruits, was not induced on leaves at any of the time points tested (Fig 5.4c, 5c). These results are particularly interesting since TFW frass obtained from leaf fed larvae induces the herbivore defenses in leaves, while the frass from the same herbivore when fed on fruits induces the antagonistic pathogen defenses in tomato fruits. This strongly suggesting that frass induced defenses are specific to host-herbivore systems and are different even for the same herbivore and the same host when the feeding habits are altered.

Herbivore frass is composed of a complex blend of biomolecules arising from the insect, plant and microbes present in the herbivore gut or growing on the frass itself. I have only scratched the surface on understanding how endophytic symbionts in the insect gut that can alter plant defenses (Chung et al., 2013). Similarly, very little is known about herbivore frass-induced defenses in plants (Schwartzberg and Tumlinson, 2014; Ray et al., 2015). In this study, I present an overview of how frass-induced defenses can alter host defenses depending on the insect depositing the frass, the host plant and the tissue on which the frass is deposited. The composition of the frass is likely to change depending on the host tissue where the herbivore feeds. The change frass composition could alter the herbivore-associated cues deposited on the
host. Another level of added complexity for frass-induced defenses is the recognition of herbivore cues by the plant host. Induction of plant defenses can be tissue specific (Karban and Baldwin, 1997; Erb et al., 2012), which could explain why the TFW frass-induced defenses in tomato fruits and leaves are such stark opposites. Finally, frass-induced defenses appear to be a function of time and may change from an initial induction of herbivore defenses to pathogen defenses as the time of exposure to frass increases. This was the case for CL frass on cabbage. While in other cases there was either a sustained herbivore defense (TFW frass applied to tomato leaves) or sustained pathogen defense (TFW frass injected into tomato fruit). Taken together, I conclude that frass-induced defenses on host plants are extremely complex and specific to the host-herbivore system and considerable work needs to be done to understand the mechanism of frass induced defense elicitation in plants.

![Fig 5.1(a)](image-url)
Maize leaves (var. B73) were wounded and treated with either frass proteins or PBS buffer or left undamaged for 4, 24, 48 and 96 hr. Relative expression (RQ) of lipoxygenase3 or lox3 (a), maize protease inhibitor or mpi (b) and pathogenesis-related protein5 or pr5 (c) were measured
by quantitative real-time PCR (qRT-PCR) by normalizing transcript abundance to that of the reference gene actin. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants (p<0.05) at the respective time points. Error bars indicate standard error from the mean.

**Fig 5.2(a)**

*lox in cabbage*

- **Undamaged Control**
- **Wound+Buffer**
- **Wound+Frass**

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Cabbage (var. Platinum dynasty) plants were wounded and treated with either frass proteins or PBS buffer or left undamaged for 4, 24, 48 and 96 hr. Relative expression (RQ) of lipoxygenase or lox (a), trypsin protease inhibitor or tpi (b) and pathogenesis-related protein1 or pr1 (c) were
measured by quantitative real-time PCR (qRT-PCR) by normalizing transcript abundance of target genes to that of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants (p<0.05) at the respective time points. Error bars indicate standard error from the mean.

Fig 5.3(a)  

*ox in rice

- Undamaged Control
- Wound+Buffer
- Wound+Frass

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Figure 5.3 Defense gene expression in rice in response to fall armyworm frass.

Rice (cv. Nipponbare) plants were wounded and treated with either frass proteins or PBS buffer or left undamaged for 4, 24, 48 and 96 hr. Relative expression (RQ) of rice lipoxygenase or lox (a), rice protease inhibitor or rpi (b) and non-expressor of pathogenesis-related protein1 or npr1
were measured by quantitative real-time PCR (qRT-PCR) by normalizing transcript abundance to that of actin. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants (p<0.05) at the respective time points. Error bars indicate standard error from the mean.
Figure 5.4 Defense gene expression in tomato leaves in response to tomato fruitworm frass fed on tomato leaves.

Tomato (var. Microtoms) leaves were wounded and treated with either frass proteins or PBS buffer or left undamaged for 4, 24, 48 and 96 hr. Relative expression (RQ) of lipoxigenaseD or
loxD (a), protease inhibitor2 or pin2 (b) and pathogenesis-related protein1 (p4) or pr-1(p4) (c) were measured by quantitative real time PCR (qRT-PCR) by normalizing transcript abundance to that of the house-keeping gene ubiquitin. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants (p<0.05) at the respective time points. Error bars indicate standard error from the mean.

Fig 5.5(a)  
loxD in tomato fruit

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- Undamaged Control
- Wound+Buffer
- Wound+Frass
**Figure 5.5** Defense gene expression in tomato fruits in response to tomato fruitworm frass fed on tomato fruits.

Tomato (var. Microtoms) fruits were injected with either frass proteins or PBS buffer or left undamaged for 4, 24, 48 and 96 hr. Relative expression (RQ) of lipoygenaseD or loxD (a),
protease inhibitor2 or pin2 (b) and pathogenesis-related protein1 (p4) or pr-1(p4) (c) were measured by quantitative real-time PCR (qRT-PCR) by normalizing transcript abundance to that of the house-keeping gene ubiquitin. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants (p<0.05) at the respective time points. Error bars indicate standard error from the mean.

Fig 5.6(a)
Trypsin protease inhibitor (TPI) activity in tomato leaf

![Graph showing trypsin protease inhibitor (TPI) activity in tomato leaf. The graph compares undamaged control, wound+buffer, and wound+frass treatments at 4hr, 24hr, 48hr, and 96hr time points. Asterisks indicate significant differences (p<0.05) between treatments at the respective time points. Error bars represent standard error from the mean.]
Figure 5.6 Trypsin protease inhibitor (TPI) activity in tomato leaves and fruits in response to tomato fruitworm frass (TFW) fed on tomato leaves and fruits, respectively.

Tomato (var. Microtoms) fruits were injected with either frass proteins or buffer and leaves were treated with frass proteins or buffer or left undamaged for 4, 24, 48 and 96 hr. TPI activity was measured spectrophotometrically in both leaves (a) and fruits (b) and expressed as TPI activity per milligram of protein. Data were analyzed by a two factor ANOVA with time and treatment as independent variables and mean separation was calculated by multiple comparison Tukey’s test. RQ values of frass-treated gene expression marked with an asterisk are significantly different from buffer-treated or undamaged control plants (p<0.05) at the respective time points. Error bars indicate standard error from the mean.
**Table S5. 1** List of primers used for quantitative-real time PCR (qRT-PCR).

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</table>
Chapter 6. Conclusion and future directions

Plant defenses in response to various herbivore behaviors such as feeding, crawling, oviposition are known to leave chemical cues on the plant (Felton and Tumlinson, 2008; Howe and Jander, 2008; Peiffer et al., 2009a; Hilfiker et al., 2014; Acevedo et al., 2015). These chemical cues may either be elicitors that induce herbivore defenses or effectors that suppress these defenses (Felton and Tumlinson, 2008; Acevedo et al., 2015). Herbivore-associated elicitors and effectors have been identified from herbivore oral secretions and saliva that are deposited on the host plant during feeding (Acevedo et al., 2015; Schmelz, 2015). Oviposition-related ’egg secretion’ are also known to induce herbivore defenses in plants (Hilker and Meiners, 2006; Hilfiker et al., 2014). Movement of caterpillars on the plant breaks trichomes that can be sensed by the plants (Peiffer et al., 2009a). In this study I show that the most abundant cue of herbivory deposited on the plant by a chewing herbivore, the frass, can trigger plant defenses.

One of the key findings of this work shows that FAW frass-mediated plant defenses temporally shift from a transient induction of herbivore defenses to a sustained induction of pathogen defenses in maize. FAW caterpillars perform better on the maize plants treated with frass since JA and the downstream JA induced defenses were suppressed as shown in Chapter 2. Contrastingly, the JA antagonistic SA pathway was induced by FAW frass in maize which led to reduced growth of the fungal pathogen *C. heterostrophus*.

Another important conclusion from the study was that digestion of frass proteins abolished the frass-mediated defenses in plants. Therefore, I fractionated the FAW frass proteins and isolated the plant chitinase Pr4 from the protein fraction that induced pathogen defenses in maize. Plant chitinases have been shown to accumulate in response to pathogen attack as well by herbviory (Kasprzewska, 2003). Pr4 application on the plants reduced herbivore defenses in maize and induced pathogen defenses. Plants treated with Pr4 were more palatable to the herbivore and they grew faster on them, while the fungal pathogen *C. heterostrophus* grew much slower on Pr4 treated plants. These results puts Pr4 in company of other proteins such as apyrases and glucose oxidase that are deposited by the herbivore on the host plant and suppress the plant’s herbivore defenses (Musser et al., 2002; Wu et al., 2012). This is also the
first evidence of an effector molecule isolated from the frass of a chewing herbivore that can also be classified as a DAMP (Damage Associated Molecular Pattern) (Heil, 2009). Several instances are known where the insects may detoxify the plant toxins after ingestion, or may simply avoid ingestion of such toxins (De Moraes and Mescher, 2004; Zhu-Salzman et al., 2005). However, this is the first evidence where a plant protein induced in response to caterpillar herbivory accumulates in the caterpillar gut and is eventually deposited on the plant with the insect frass and acts as a DAMP.

Fractionation of proteins from the frass also revealed the presence of insect gut protein IIM that triggered herbivore induced wound response in maize. IIM is an insect gut protein which belongs to the peritrophin family of proteins that bind chitin microfibrils to form the peritrophic matrix (PM) present in most Lepidopteran insect larvae (Hegedus et al., 2009). Immuno-depletion of IIM from frass attenuated the wound-induced defenses and enhanced the pathogen defenses in maize. Therefore, IIM can act as one of the only herbivore-related chemical cues that can act as both an elicitor for herbivore-induced wound responses as well as suppressor of pathogen defenses in maize. The antagonism between these two defense pathways has been well known (Howe and Jander, 2008; Thaler et al., 2012). IIM’s role in simultaneous activation of one pathway and suppression of the other puts it in a class of its own since it implies it may have regulatory roles in both pathways. IIM is also highly conserved among most Lepidopterans and accentuates its importance as a possible universal elicitor molecule for most host-herbivore systems.

Finally, this study sheds light on one of the important paradigms of herbivore-mediated induction of host defenses in plants. Induction of plant defenses in response to such chemical cues (or elicitors) has been known to be specific to host-herbivore systems. Frass mediated induction of plant defenses is no exception. In the last chapter of this study I show that frass mediated defenses are different for each host-herbivore system. Tomato fruit worm caterpillars that feed on the tomato leaves induce a sustained herbivore defense when applied to tomato leaves, however, frass from the same herbivore fed on the tomato fruits when injected in green fruits, suppressed herbivore defenses and induced pathogen defenses. Since, caterpillar frass contains molecules from the insect, the plant and the microbes that may be growing on the
frass and the endosymbionts of the insect, the cues in caterpillar frass are likely to change. Notably, FAW frass induced a much stronger and sustained herbivore defense response in rice unlike the trend that I observed in maize. Therefore, frass-mediated induction of defenses in plants is not only a function of the composition of the frass, but also the tissue on which it is deposited, the insect and the plant species involved in that particular host-herbivore system.

In conclusion, it can be said that this study is only a tip of the iceberg when it comes to understanding how frass in different host-herbivore systems manipulate plant defenses. Frass is the most abundant cue of herbivory that a herbivore can leave on the plant, therefore a much better understanding of the mechanism of frass-induced plant defenses is necessary to control pests on crops. For maize alone, 18% of total crop production is lost to herbivores each year (Oerke, 2006). Conventional pesticide applications are costly and harmful for the environment. There is also growing evidence of resistance to transgenic Bt crops and more sustainable and efficient pest management techniques need to be developed soon (Gassmann et al., 2011; Gassmann et al., 2014). Plants have been shown to have trans-generational memory when it comes to pest tolerance (Rasmann et al., 2012). Treatment of crop plants with caterpillar frass and the elicitors identified from could be a cheap, sustainable pest management technique of the future.
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