



Pathogen-Mediated Tritrophic Interactions: Baculovirus-Challenged Caterpillars Induce Higher Plant Defenses than Healthy Caterpillars

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Received: 22 January 2019 / Revised: 6 May 2019 / Accepted: 13 May 2019
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Abstract

Although the tritrophic interactions of plants, insect herbivores and their natural enemies have been intensely studied for several decades, the roles of entomopathogens in their indirect modulation of plant-insect relationships is still unclear. Here, we employed a sublethal dose of a baculovirus with a relatively broad host range (AcMNPV) to explore if feeding by baculovirus-challenged *Helicoverpa zea* caterpillars induces direct defenses in the tomato plant. We examined induction of plant defenses following feeding by *H. zea*, including tomato plants fed on by healthy caterpillars, AcMNPV-challenged caterpillars, or undamaged controls, and subsequently compared the transcript levels of defense related proteins (i.e., trypsin proteinase inhibitors, peroxidase and polyphenol oxidase) and other defense genes (i.e., proteinase inhibitor II and cysteine proteinase inhibitor) from these plants, in addition to comparing caterpillar relative growth rates. As a result, AcMNPV-challenged caterpillars induced the highest plant anti-herbivore defenses. We examined several elicitors and effectors in the secretions of these caterpillars (i.e., glucose oxidase, phospholipase C, and ATPase hydrolysis), which surprisingly did not differ between treatments. Hence, we suggest that the greater induction of plant defenses by the virus-challenged caterpillars may be due to differences in the amount of these secretions deposited during feeding or to some other unknown factor(s).

Keywords Plant defense · Induce defense · Immune responses · Saliva · Oral secretions · Ventral eversible gland · Herbivore perception

Introduction

Plants have evolved complex suites of defenses (physical and chemical) against insect herbivores, which can be constitutive and/or induced by assaults from herbivores or pathogens. Induced defenses are regulated by three endogenous phytohormones, jasmonic acid (JA), salicylic acid (SA) and

ethylene (ET) (Bostock 2005). Induced resistance depends on rapid recognition of the attacker. Plants can perceive an array of cues from insect herbivores, including feeding damage (Heil 2009), oral secretions (Acevedo et al. 2015; Schmelz 2015), insect footsteps (Peiffer and Felton 2009), ovipositional cues (Hilker and Fatouros 2015), insect pheromones (Helms et al. 2013), and vibrational cues associated with chewing (Appel and Cocco 2014). Microbes associated with herbivores can present further cues that can alter perception of herbivores by plants (Acevedo et al. 2017; Chung et al. 2013; Chaudhary et al. 2014; Tan et al. 2018). While herbivore-associated microbes may impact the herbivore's physiological and ecological traits by facilitating digestion, detoxifying toxic dietary components, or fixing nitrogen (Clark et al. 2010; Dillon and Dillon 2004; Warnecke et al. 2007), these microbes may directly or indirectly manipulate plant defenses or alter plant quality (Shikano et al. 2017a).

To date much of the research on tritrophic interactions among plants, microbes and insect herbivores has focused on how plant traits and/or defenses influence microbes and pathogens associated with insects (reviewed in Cory and Hoover 2006; Shikano 2017). Conversely, emerging evidence

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10886-019-01077-1>) contains supplementary material, which is available to authorized users.

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indicates that herbivore-associated microbes can influence plant traits. For instance, bacteria in the oral secretions of the Colorado potato beetle were shown to suppress induced defenses in tomato and potato (Chung et al. 2013, 2017). Similarly, bacteria in the regurgitant of the fall armyworm *Spodoptera frugiperda* suppress defenses in tomato (Acevedo et al. 2017). In some cases, proteins from endosymbiotic bacteria are released in saliva, such as GroEL, a bacterial chaperonin, produced by the endosymbiont *Buchnera aphidicola* of the potato aphid *Macrosiphum euphorbiae* (Chaudhary et al. 2014). GroEL activates pattern-triggered immunity in plants resulting in reduced performance of the aphid (Chaudhary et al. 2014). Herbivore-associated bacteria may indirectly alter oral secretions or saliva by activating immune-related proteins. In recent studies, *Helicoverpa zea* (tomato fruitworm) gut-associated bacteria such as *Enterobacter ludwigii* indirectly manipulated tomato and maize plant antiherbivore responses by activating salivary glucose oxidase (GOX), which induced polyphenol oxidase (PPO) activity and proteinase inhibitors against chewing herbivores (Wang et al. 2017, 2018).

While most of the examples of herbivore associated microbes impacting induced plant defenses are with bacteria, Tan et al. (2018) recently showed that a polydnavirus injected by the parasitoid *Microplitis croceipes* during oviposition in *H. zea* downregulated GOX, which in turn reduced induction of defenses in tomato by parasitized caterpillars. The most well-studied plant-insect-virus system is between plants, caterpillars and baculoviruses (Shikano 2017). Baculoviruses are a family of lethal insect-specific viruses, most of which infect the larval stage of lepidopterans. Herbivore-induced plant defenses are well-known to influence the abilities of baculoviruses to infect, kill and replicate in their hosts (Ali et al. 1998; Ali et al. 1999; Elder et al. 2013; Felton et al. 1987; Felton and Duffey 1991; Hoover et al. 1998, 2000; Keating et al. 1989; Shikano et al. 2017c, 2017d). These viruses can modify the defenses/immunity of their host insects, depending on the insect-virus system. In *H. zea* challenged with *Autographa californica* multiple nucleocapsid nucleopolyhedrovirus (AcMNPV), the virus can activate the insect immune system by increasing total haemocyte numbers and phenoloxidase and/or flavin adenine dinucleotide (FAD)-glucose dehydrogenase activity in the haemolymph of AcMNPV-infected caterpillars (Trudeau et al. 2001; Pan et al. 2019b).

Because baculovirus infection influences immune responses and protein synthesis in its host (Ikeda et al. 2013), we hypothesized that induced immune responses in AcMNPV-infected *H. zea* may be concurrently associated with altered expression of other proteins, such as those in the salivary labial glands and digestive system. Baculovirus-mediated changes in salivary proteins could influence the plant's perception of the caterpillars, and ultimately the

defenses expressed by the plant. Thus, we examined whether tomato plant (*Solanum lycopersicum*) responses to feeding by AcMNPV-infected *H. zea* differ from plant responses to healthy *H. zea*. We assessed both changes in the composition of proteins in the insects' saliva and defensive responses in tomato plant foliage associated with AcMNPV-infection.

Methods and Materials

Plants Tomato (*Solanum lycopersicum*, cv. *Better Boy*) plants were grown in a temperature-controlled greenhouse with supplemental lighting with a photoperiod of 16 L:8D at Pennsylvania State University (University Park, PA, USA), and fertilized with 3 g of Osmocote plus (15–9–12, Scotts, Marysville, OH, USA) 10 days after all seeds germinated. Four-leaf-stage plants were used in all experiments.

Insects *Helicoverpa zea* eggs were obtained from Frontier Agricultural Sciences (Newark, DE, USA). Subsequent generations were maintained in the laboratory on an artificial wheat-germ based diet at 25 °C and 16 L:8D photoperiod. Moths were provided with 10% sugar solution and eggs were collected every 24 h for preparing experimental caterpillars.

Baculovirus Preparation The wild-type AcMNPV (strain C6) occlusion bodies (OBs) were obtained from Dr. Robert Harrison (ARS, USDA). The OBs were propagated in *Trichoplusia ni* caterpillars and semi-purified by multiple centrifugations to remove debris. The virus concentration was estimated by counting OBs using an improved Neubauer brightline haemocytometer (0.1 mm deep; Hausser Scientific, Horsham, PA, USA) at 400 × magnification. A suspension containing a LD₁₅ dose (520 OBs) of AcMNPV OBs was used in all experiments. This dose was selected because it produced 15% mortality in a dose response bioassay (Fig. S1) and our goal was to examine effects of baculovirus infection in this system at a sublethal dose.

AcMNPV is known to have a broad host range and has been commercialized as microbial insecticides (Loopex® (Andermatt Biocontrol AG, Switzerland) and Loopex FC® (Sylvar Technologies Inc., Canada)) for use against the cabbage looper (*Trichoplusia ni* L.) on vegetables. Both formulations produce long-term control of pest populations. *H. zea* shares several host plants with cabbage loopers (e.g., tomato); therefore, *H. zea* caterpillars are likely to be exposed to AcMNPV in field situations where applications occur to control cabbage loopers. Because *H. zea* is only a semi-permissive host to AcMNPV, susceptibility of this pest to AcMNPV is low (Fig. S1).

AcMNPV Inoculation Newly molted fourth instar caterpillars were orally microinjected with 520 OBs (LD₁₅) suspended in

1 μ l of 60% glycerol using a syringe fitted with a 32-gauge stainless steel blunt tip needle (Popper & Sons, New Hyde Park, NY) mounted on a Pax-100 microapplicator (Burkhard Scientific, Uxbridge, UK). Healthy caterpillars that received 60% glycerol containing no OBs served as the control. Immediately after AcMNPV inoculation, caterpillars were placed individually in 30 ml plastic cups and fed *ad libitum* on artificial diet. We found that immune responses of *H. zea* caterpillars challenged with AcMNPV (LD₁₅) showed induced responses at 72 and 96 h post-inoculation (hpi) (Pan et al. 2019b). Therefore, we used caterpillars at 72 and 96 hpi to explore responses of plants to feeding-damage from AcMNPV-challenged (i.e., immune-challenged) *H. zea*.

Plant Treatments To induce anti-herbivore plant defenses, one healthy *H. zea* or AcMNPV-challenged *H. zea* (72 or 96 hpi) was placed in a clip-cage (diameter: 2 cm) on the terminal leaflet of the youngest fully expanded leaf of a four-leaf stage plant. There were no significant differences between the weights of healthy and AcMNPV-challenged *H. zea* that were placed on the plants (data not shown). Undamaged plants received an empty clip-cage. All *H. zea* consumed the entire area inside of the clip-cages within 3 h, all *H. zea* caterpillars and clip-cages were immediately removed from damaged and undamaged leaflets. The remaining leaflet tissue (i.e., tissue outside of the clip-cage area) was used for experiments described below.

Leaf Tissue Collection For RNA extractions, a leaf tissue sample (~100 mg fresh weight) from each damaged and undamaged leaflet was collected 24 h post *H. zea*-feeding damage (i.e., feeding inside clip cage). Equal portions of three leaflets were pooled to produce one sample to extract total RNA and assess the relative expressions of JA marker genes in each plant treatment; 4–8 samples were collected from each plant treatment. From separate plants, a single leaf tissue sample (~50 mg fresh weight) was collected from each plant to perform plant enzyme activity assays from damaged and undamaged leaflets 48 h post the *H. zea* feeding damage. All leaf tissue samples were frozen at –80 °C until RNA extraction or enzyme assays. Leaflet tissue that was left-over on the plant after samples were collected for enzyme activity assays were immediately used to assess the plants' resistance to *H. zea* feeding by measuring the growth rate of a subsequent naïve cohort of *H. zea* on the leaf tissue. The number of samples collected for each measure of plant defense is listed in the experiments described below.

Plant Resistance to Caterpillar Feeding The caterpillars' midgut is alkaline and relies on serine proteases for digestion (Felton and Duffey 1991), thus the accumulation of serine proteinase inhibitors in plant tissue could affect the growth of feeding caterpillars. A 4-d old caterpillar was fed with the

leaf tissue collected from a leaflet that was previously undamaged or damaged by a healthy or virus-inoculated caterpillar. Each 4-d old caterpillar was fed one leaflet sample for 5 d in a plastic cup (30 ml) lined with 1% agar to maintain leaf moisture. The final weight of each caterpillar was measured and the relative growth rate (RGR) was calculated as follows: $RGR = (\text{Final weight} - \text{initial weight}) / (\text{mean weight} \times \text{feeding days})$ (Waldbauer 1968).

The experiment was conducted once using plants damaged by caterpillars that were 72 h post AcMNPV-inoculation. A total of 12, 14 and 19 caterpillars were fed leaflet tissue collected from undamaged, healthy *H. zea*-damaged, and AcMNPV-challenged (72 hpi) *H. zea*-damaged plants, respectively. The experiment was repeated three times using plants damaged by *H. zea* that were 96 h post AcMNPV-inoculation. In trials 1 and 2, each leaflet sample was supplied to a single naïve *H. zea* to determine RGR. In trial 3, each leaflet was equally divided into two pieces to feed two naïve *H. zea* held in separate cups. The numbers of naïve *H. zea* larvae fed undamaged leaflets, healthy *H. zea*-damaged leaflets, and AcMNPV-challenged (96 hpi) *H. zea*-damaged leaflets, respectively, in each trial were as follows: 10, 13 and 13 in trial 1; 18, 27 and 27 in trial 2; 17, 32 and 42 in trial 3.

Feeding Choice Tests We conducted three combinations of two-way choice tests: (1) undamaged leaflet vs. leaflet damaged by healthy *H. zea* ($n = 29$), (2) undamaged leaflet vs. leaflet damaged by AcMNPV-challenged (96 hpi) caterpillars ($n = 33$), and (3) leaflet damaged by healthy *H. zea* vs. leaflet damaged by AcMNPV-challenged (96 hpi) *H. zea* ($n = 35$). A single leaf disk was cut from each leaflet using a cork borer (diameter: 2 cm). One leaf disk was placed 6 cm away from the other leaf disk in a petri dish (diameter: 10 cm) to provide a two-way choice. The dish was lined with 1% agar on the bottom to maintain leaf moisture. One 2-d old *H. zea* larva was placed in the middle of the two leaf disks and maintained at 25 °C and 16:8 (L:D) for 5 d. After 5 d of feeding, the ingested leaf area was measured by digitally scanning the remaining leaf disk and ImageJ was used to calculate the consumed leaf area. This experiment was conducted once.

Plant Defense Protein Assays Plant trypsin proteinase inhibitors (TPIs) are an important group of jasmonate and herbivore-induced defense proteins (Zavala et al. 2004), which could inhibit insect digestion and disrupt nutrient absorption (Felton 2005). The activity of TPI was measured according to Chung and Felton (2011) with minor modifications. Each leaf sample was ground in liquid nitrogen and immediately homogenized in 1.25 ml 0.046 M tris buffer (0.0115 M CaCl₂, pH 8.1) containing 5% insoluble polyvinylpyrrolidone (PVPP). The homogenate was fully mixed, then maintained on ice for 10 min to recover, and subsequently centrifuged at 11,000 g for

10 min at 4 °C. Then 10 µl of supernatant was mixed with 10 µl of working trypsin solution that contained 1 mg of bovine trypsin (Sigma, T1426) in 1 ml 1 mM HCl and 80 µl 0.046 M tris buffer. The mixture was incubated for 10 min at room temperature; 100 µl of a substrate consisting of 2 mM N α -p-Tosyl-L-arginine methyl ester hydrochloride (TAME; Sigma, T4626) in 0.046 M tris buffer was then added to the mixture. The change in absorbance was measured with a Spectra Max 190 microplate reader (Molecular Devices, Silicon Valley, CA, USA) at 247 nm for 5 min. The protein concentration in leaflet tissue was quantified using the Bradford assay (Bradford 1976) and a dilution series of bovine serum albumin (BSA) was used as a standard curve. The percentage of TPI was calculated as follows: (TPI (%) = (1 – slope of the sample/slope of the working trypsin) × 100); the activity of TPI (%) was normalized as per mg of protein content.

Peroxidase (POD) and polyphenol oxidase (PPO) are important anti-herbivore defense enzymes in tomato (Felton and Duffey 1991). Activities of POD and PPO were measured following methods described by Pan et al. (2019a). Leaflet samples for TPI, POD and PPO were collected from the same leaflets used to assess plant resistance to *H. zea* feeding (i.e., *H. zea* RGR measurements).

RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Collected leaf tissue (~100 mg) was ground using a GenoGrinder 2000 (Spex SamplePrep, Metuchen, NJ, USA), and total RNA was extracted by following a Trizol protocol as previously described (Chung et al. 2013). Complementary DNA (cDNA) was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) with 1 µg of total RNA. The qRT-PCR reaction was performed according to Peiffer et al. (2009). Specific primers (Table S1) for proteinase inhibitor II (*Pin2*) and cysteine proteinase inhibitor (*CysPI*) were used as JA marker genes to evaluate the levels of JA-inducible proteins in damaged or undamaged tomato plants according to Wang et al. (2017). The house-keeping gene ubiquitin (*Ubi*) was employed to normalize cycle threshold (C_T) values (Rotenberg et al. 2006). The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) was used to determine relative levels of *Pin2* and *CysPI* defense transcripts.

Salivary Elicitors Labial (salivary) glands were dissected (Peiffer and Felton 2005) from healthy and AcMNPV-challenged (96 hpi) *H. zea* to assay for potential elicitors/ effectors (i.e., GOX, PLC and ATPase). The activities of glucose oxidase (GOX) (Eichenseer et al. 1999) and phospholipase C (PLC) (Le Chevalier et al. 2015) were measured according to published methods. One labial gland sample

consisted of a pair of labial glands that were dissected from a single caterpillar and homogenized. We employed 17–18 sample replicates from healthy or AcMNPV-challenged (96 hpi) *H. zea* to assay GOX and PLC activities.

ATPase activity was evaluated using the ENLITEN® ATP Assay System Bioluminescence Detection Kit (Promega, FF2000). A total of 7 replicate samples were assayed for each treatment group, with each replicate containing 3 pairs of homogenized labial glands.

The diversity and abundance of protein species in the labial glands of healthy and AcMNPV-challenged (96 hpi) *H. zea* was compared by protein gel electrophoresis using a 12% Tris-glycine gel and Coomassie blue stain. A total of 4–5 replicate samples were assayed for each treatment group, with each replicate containing 3 pairs of homogenized labial glands.

Application of Macerated Labial (Salivary) Glands, Regurgitant and Ventral Eversible Gland (VEG) Secretions to Wounded Tomato Leaves As *H. zea* caterpillars sporadically deposit regurgitant on tomato foliage during feeding (Peiffer and Felton 2009), we examined the effects of deposited regurgitant on tomato defense responses. The ventral eversible gland (VEG) is another important secretory gland located on the surface of the ventral thorax in most noctuid caterpillars (Felton 2008). VEG secretions are known to trigger plant defense responses (Zebelo and Maffei 2012). Labial gland (containing saliva), regurgitant and VEG secretions were collected from healthy and AcMNPV-challenged (96 hpi) *H. zea*. A pair of labial glands was dissected from each treated caterpillar and homogenized with 20 µl of 0.1 M PBS buffer (pH 7.0) to produce one sample. Regurgitant was collected using a pipette to harvest 10 µl regurgitant from the mouthparts of one caterpillar, harvested regurgitant was mixed with 10 µl of 0.1 M PBS buffer (pH 7.0) and set up as one sample in each caterpillar treatment. VEG secretions were collected from the VEG gland of each caterpillar treatment via capillary action using a pipette. VEG secretions collected from 10 caterpillars were combined as one sample and suspended in 20 µl of 0.1 M PBS buffer (pH 7.0). All labial glands and regurgitant were collected within 2 h before being applied on the plants. Samples were maintained on ice until application on plants. All samples of VEG secretions were collected within 6 h and stored in –80 °C before application on plants.

The terminal leaflet of the top fully expanded leaf of a four-leaf stage plant was damaged using a pipette tip to punch a hole on the midvein. Then 20 µl of each sample type from each caterpillar treatment (macerated labial gland, $n = 18$; regurgitant, $n = 18$; VEG secretion, $n = 18$ –20) was applied to the mechanically induced wound. Twenty µl of PBS was applied to a wounded leaflet to serve as the positive control ($n = 8$ –9), and undamaged plants were used the negative controls ($n = 6$ –7).

Statistical Analysis Differences in TPI activities and caterpillar RGR were compared using general linear model (GLM) with plant induction treatment (undamaged, damaged by healthy caterpillars, and damaged by AcMNPV-challenged (96 hpi) caterpillars), trials and their interaction as factors. The relationship between plant TPI activities and caterpillar RGR was evaluated using linear regression analysis. One-way analysis of variance (ANOVA) was used to compare means between plant treatments. ANOVA was used to assess differences in mean TPI, POD and PPO activities, relative expression levels of *Pin2* and *CysPI* and caterpillar RGR between plant induction treatments (undamaged, damaged by healthy or AcMNPV-challenged (72 or 96 hpi) caterpillars). Post-hoc comparisons among treatments were made using Dunnett's C or LSD post hoc tests, depending on whether variances were equal. Student's t test was used to compare the leaf area consumed in choice tests, GOX activity, PLC activity, and ATPase activity.

Results

Induction of Plant Defenses by AcMNPV-Challenged *H. zea* at 72 Hpi

H. zea RGR differed among all treatments. Caterpillars that fed on undamaged plant tissue grew significantly faster than those fed on plant tissue damaged by healthy or AcMNPV-challenged (72 hpi) caterpillars; however, they grew at a similar rate after feeding on plant tissue damaged by healthy or

AcMNPV-challenged (72 hpi) caterpillars (Fig. 1a: $F_{2,42} = 11.07$, $P < 0.001$). The activities of TPI, PPO and POD induced by healthy and AcMNPV-challenged caterpillars did not differ significantly, despite significant differences in levels of TPI between undamaged and damaged leaflets ($F_{2,38} = 33.55$, $P < 0.001$; Fig. 1b), POD ($F_{2,47} = 5.75$, $P = 0.006$; Fig. 1c) and PPO ($F_{2,49} = 12.44$, $P < 0.001$; Fig. 1d).

Induction of Plant Defenses by AcMNPV-Challenged *H. zea* at 96 Hpi

Plant Resistance to Caterpillar Feeding Four-days old *H. zea* caterpillars fed for 5 d with previously damaged or undamaged leaves grew at different rates (Induction treatment by trial: $F_{4,193} = 2.56$, $P = 0.04$; Fig. 2a). The 4-d old caterpillars fed leaf disks from undamaged leaflets grew fastest, followed by caterpillars fed leaf disks from leaflets damaged by healthy *H. zea*. Insects grew the slowest when fed leaf disks from leaflets damaged by AcMNPV-challenged (96 hpi) *H. zea* (Induction treatment: $F_{2,196} = 63.39$, $P < 0.001$).

Plant Defense Protein Activities Induction of TPI activity differed among the three treatments (Induction treatment by trial: $F_{4,153} = 5.47$, $P < 0.001$; Fig. 2b). Leaflets damaged by AcMNPV-challenged (96 hpi) *H. zea* had the highest TPI activity, followed by those damaged by healthy *H. zea*, while TPI activity was the lowest in undamaged leaflets (Induction treatment: $F_{2,156} = 62.37$, $P < 0.001$). This trend was consistent across trials even though the total levels of TPI activity varied among the three trials (Trial: $F_{2,156} = 51.64$, $P < 0.001$).

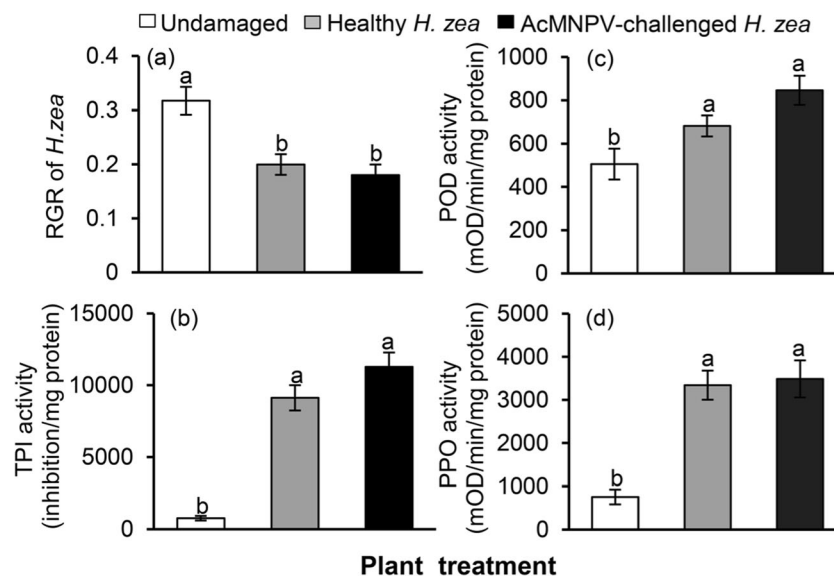


Fig. 1 Tomato plant defenses induced by *Helicoverpa zea* caterpillars 72 h after inoculation with virus (AcMNPV). **a** The mean (\pm SE) relative growth rate (RGR) of 4-d old caterpillars that fed for 5 d on undamaged tomato leaflets or leaflets that were previously damaged by healthy or AcMNPV-challenged (72 hpi) *H. zea*. The mean (\pm SE)

activities of **b** trypsin proteinase inhibitors (TPI), **c** peroxidases (POD) and **d** polyphenol oxidases (PPO) in undamaged tomato leaflets or leaflets that were previously damaged by healthy or AcMNPV-challenged (72 hpi) *H. zea*. Bars with different letters represent a significant difference between treatments ($P < 0.05$)

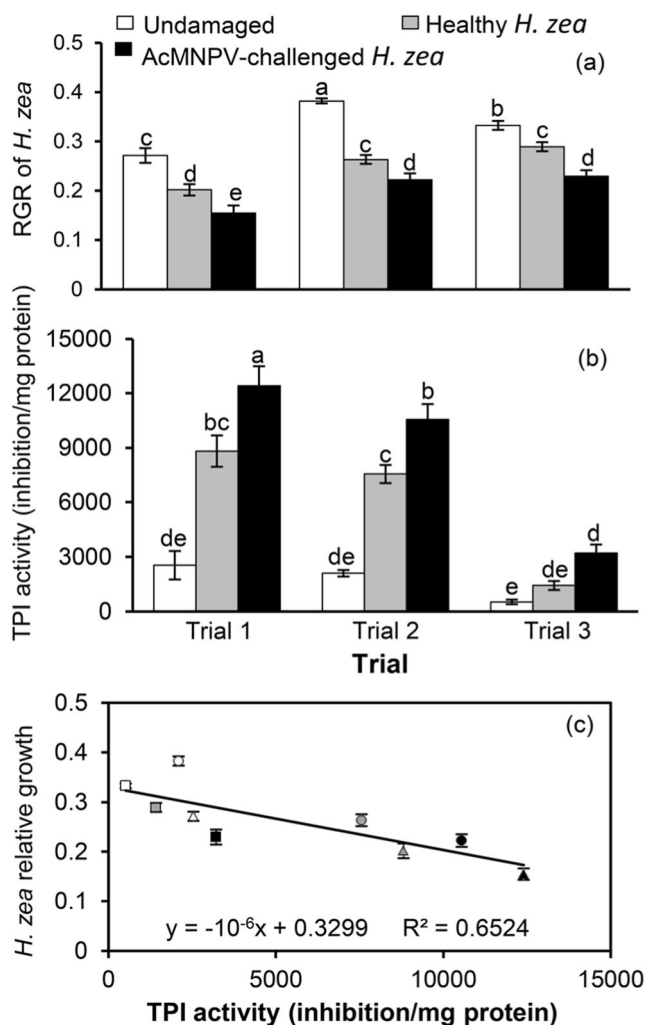


Fig. 2 Tomato plant defenses induced by *Helicoverpa zea* caterpillars 96 h after inoculation with virus (AcMNPV). **a** The mean (\pm SE) relative growth rate (RGR) of 4-d old *H. zea* that fed for 5 d on undamaged tomato leaflets or leaflets that were previously damaged by healthy or AcMNPV-challenged (96 hpi) *H. zea*. **b** The mean (\pm SE) activities of trypsin proteinase inhibitors (TPI) in undamaged tomato leaflets or leaflets that were previously damaged by healthy or AcMNPV-challenged (96 hpi) *H. zea*. Bars with different letters represent a significant difference between treatments ($P < 0.05$). **c** Relationship between the levels of TPI activity in the leaflets and the relative growth rate of *H. zea* ($P < 0.05$). Leaflets were undamaged or had been damaged by healthy or AcMNPV-challenged (96 hpi) *H. zea*. Different symbols represent different trials (trial 1, triangle; trial 2, circle; trial 3, square)

Higher TPI activity was associated with slower caterpillar RGR ($F_{1,7} = 13.14$, $P = 0.008$, $R^2 = 0.65$; Fig. 2c).

Leaflets damaged by healthy and AcMNPV-challenged caterpillars did not induce different activity levels of POD and PPO, although damage by caterpillar feeding induced significantly higher levels of POD ($F_{2,44} = 7.32$, $P = 0.002$; Fig. 3a) and PPO activities ($F_{2,42} = 13.56$, $P < 0.001$; Fig. 3b).

Feeding Choice Tests Two-days old *H. zea* ingested more undamaged leaflet tissue than leaflet tissue previously

damaged by healthy *H. zea* ($t_{1,56} = 2.90$, $P = 0.005$; Fig. 4) and AcMNPV-challenged (96 hpi) *H. zea* ($t_{1,64} = 2.18$, $P = 0.033$). Interestingly, the 2-d old *H. zea* ingested more leaf tissue damaged by healthy *H. zea* than leaf tissue damaged by AcMNPV-challenged caterpillars ($t_{1,68} = 3.60$, $P = 0.001$).

Plant Defense Gene Expression Plants damaged by AcMNPV-challenged (96 hpi) caterpillars induced higher levels of the defense related genes *Pin2* ($F_{2,16} = 18.51$, $P = 0.0001$; Fig. 5a) and *CysPI* ($F_{2,15} = 21.93$, $P < 0.001$; Fig. 5b) compared to plants damaged by healthy *H. zea* and undamaged plants. Healthy *H. zea* induced roughly 39-fold higher *Pin2* and 7-fold higher *CysPI* expression levels compared to undamaged plants. Plants damaged by AcMNPV-challenged caterpillars produced approximately 2.5-fold higher *Pin2* transcripts and 2-fold higher *CysPI* transcripts than plants damaged by healthy *H. zea*.

Salivary Elicitors Inoculation of AcMNPV did not alter the activities of GOX ($t_{1,33} = 0.15$, $P = 0.98$; Fig. 6a), PLC ($t_{1,33} = 0.27$, $P = 0.98$; Fig. 6b) or ATPase ($t_{1,7.012} = 0.57$, $P = 0.590$; Fig. 6c) in *H. zea* labial glands (i.e., saliva) at 96 hpi. The protein profiles in labial glands of healthy and AcMNPV-challenged *H. zea* were both analyzed by SDS-PAGE and stained with Coomassie blue. Although scores of proteins in each lane were separated into several bands, we did not find a unique pattern, indicating that protein diversity and abundance in the labial glands were similar between treatments (Fig. 7).

Compared to undamaged leaflets, TPI activities were significantly higher in wounded leaflets treated with macerated labial glands ($F_{3,48} = 10.43$, $P < 0.001$; Fig. 8a), regurgitant ($F_{3,48} = 4.57$, $P = 0.007$; Fig. 8b) and VEG secretions ($F_{3,48} = 8.15$, $P < 0.001$; Fig. 8c). However, there were no significant differences in induction between wounded leaflets treated with elicitors from AcMNPV-challenged (96 hpi) and healthy *H. zea*. This suggests that any potential qualitative differences in saliva, regurgitant or VEG secretions between healthy and AcMNPV-challenged *H. zea* were not responsible for the differences in TPI activities under these conditions.

Discussion

The third trophic level plays a key role in mediating the interactions of many insect herbivores with their host plants (Price et al. 1980). Early research focused primarily on the bottom-up effects of plant traits on the third trophic level, but emerging evidence indicates that the third trophic level (especially gut bacteria) can play an important top-down role in the expression of plant traits such as induced defenses (Shikano et al. 2017a). In addition to bacteria, other herbivore-associated microbes may exert top-down effects on plant defense traits. The parasitoid *M. croceipes* can release polydnavirus into *H. zea* caterpillars during oviposition. Tan

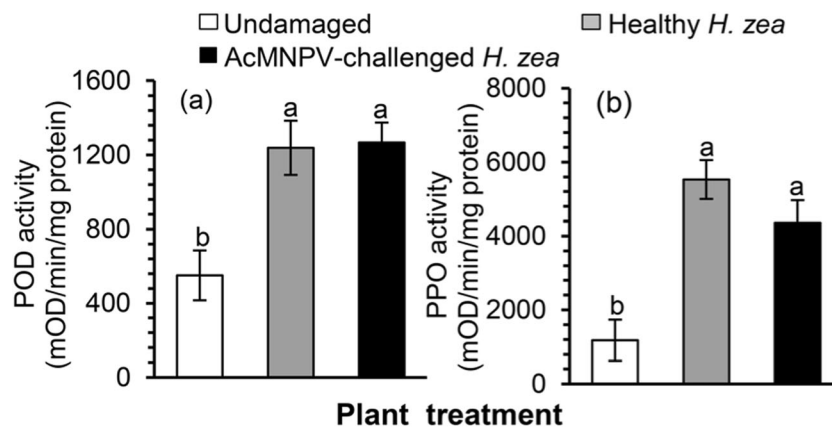


Fig. 3 Tomato plant defenses induced by *Helicoverpa zea* caterpillars 96 h after inoculation with virus (AcMNPV). The mean (\pm SE) activities of **a** peroxidases (POD) and **b** polyphenol oxidases (PPO) in

undamaged tomato leaflets or leaflets that were previously damaged by healthy or AcMNPV-challenged (96 hpi) *H. zea*. Bars with different letters represent a significant difference between treatments ($P < 0.05$)

et al. (2018) demonstrated that the polydnavirus indirectly suppressed tomato plant defenses by decreasing salivary GOX expression, which is an important elicitor of plant defenses. The lower levels of induced defenses improved the weight gain of the host caterpillars and increased survival of the parasitoids (Tan et al. 2018). Similarly, polydnavirus (calyx fluid preparation) and venom injected into *Pieris brassicae* caterpillars by the parasitoid *Cotesia glomerata* suppressed salivary glucose dehydrogenase and β -glucosidase precursors implicated as elicitors of plant defenses (Cusumano et al. 2018). Because many braconid and ichneumonid parasitoids harbor polydnaviruses, this may represent a widespread ecological phenomenon in plant-insect herbivore interactions.

Here, we demonstrated an interesting phenomenon involving the third trophic level; the entomopathogen AcMNPV can indirectly induce plant anti-herbivore defenses. Tomato plants damaged by AcMNPV-challenged *H. zea* caterpillars expressed higher amounts of defense-related proteins than those damaged by healthy *H. zea*. Baculoviruses have been identified in hundreds of species of caterpillars and in some

cases can induce epizootics with very high infection rates (Cory and Myers 2003). In one case, virtually all the *Spodoptera exempta* caterpillars collected in the field tested positive for the *S. exempta* nucleopolyhedrovirus (*SpexNPV*) DNA and 60% of these insects showed transcriptionally active virus (Vilaplana et al. 2010). The *SpexNPV* baculovirus occurs at exceptionally high levels in field populations of caterpillars and persistent infections exist without obvious symptoms. Because of the widespread occurrence of baculoviruses in caterpillar species, our observations that baculoviruses can alter the induction of plant defenses may not be an uncommon phenomenon.

In the polydnavirus studies cited above the suppression of plant defenses may enhance the fitness of the parasitoid and virus via the improved growth of their caterpillar hosts. In this baculovirus system, higher levels of induced defenses may alter virus fitness in different ways. In a previous study, induction of tomato foliage by *H. zea* feeding did not alter the infectivity of HzSNPV (Hoover et al. 1998), but in another

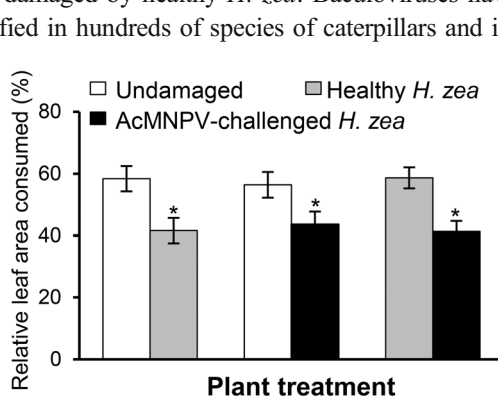


Fig. 4 Relative proportions of tomato leaf disks consumed during the choice experiments. Leaflets were undamaged or had been damaged by healthy or virus (AcMNPV)-challenged (96 hpi) *Helicoverpa zea* caterpillars. Bars with an asterisk indicate significant differences ($P < 0.05$)

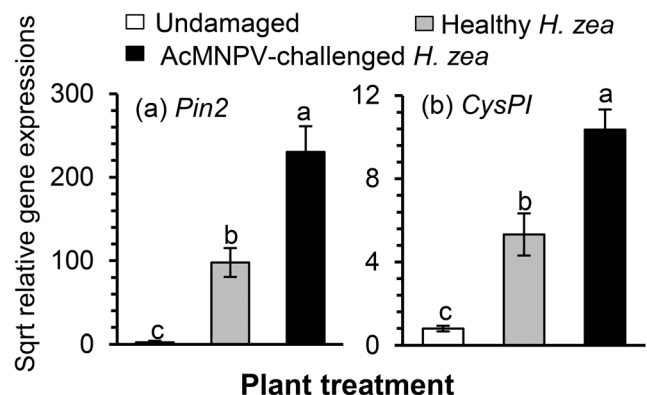


Fig. 5 Relative expressions of tomato plant defense genes in undamaged tomato plants or plants fed on by healthy or virus (AcMNPV)-challenged (96 hpi) *Helicoverpa zea* caterpillars: **a** proteinase inhibitor II (*Pin2*) and **b** cysteine proteinase inhibitor (*CysPI*). Bars with different letters represent significant difference between treatments ($P < 0.05$)

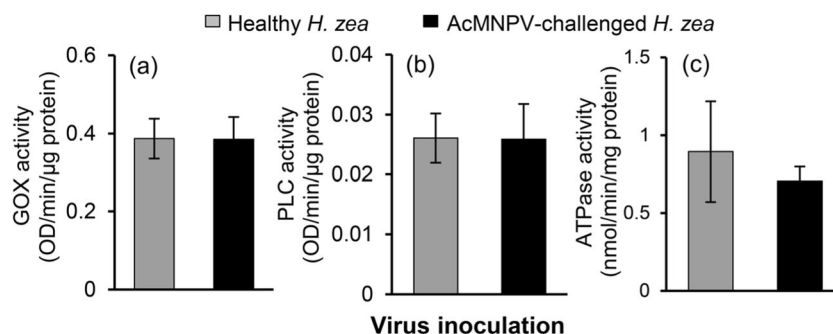


Fig. 6 The mean (\pm SE) activities of salivary effectors/elicitors **a** glucose oxidase (GOX), **b** phospholipase C (PLC) and **c** ATPase hydrolysis (ATPase) in the labial glands of healthy and virus (AcMNPV)-challenged

(96 hpi) *Helicoverpa zea* caterpillars. There were no significant differences between treatment groups ($P > 0.05$)

case, induced tomato foliage enhanced the infectivity of HzSNPV to *H. zea* (Ali et al. 1998). The main difference in the studies is that in the latter paper the insects were smaller and reared throughout the larval period on plants. Induced defenses can alter caterpillar susceptibility and viral transmission rates. Reduced growth rate of fall armyworms on induced soybean foliage prolonged their period of susceptibility to *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) (Shikano et al. 2018). In other words, feeding on induced plants delayed developmental resistance (age-dependent susceptibility to infection) of fall armyworms to the virus. In another system, induced defenses in red oaks had a strong effect on gypsy moth (*Lymantria dispar*) mortality by *L. dispar* nucleopolyhedrovirus (LdMNPV), but the effects were virus density dependent: at lower virus density, average infection rates were lower on induced foliage, but at higher virus density, they were higher on induced foliage (Elder et al. 2013). Because of reduced caterpillar growth on induced plants, viral progeny production (occlusion bodies) could be reduced in these caterpillars, which was the case in SfMNPV-infected fall armyworms fed induced soybean foliage (Shikano et al. 2017c). Changes in viral progeny production would affect virus transmission. Overall, it remains to be determined whether the increases in plant defenses induced

by virus infected caterpillars in our study benefits the virus or the caterpillar.

The causal factor(s) for the increased induction of plant defenses by infected caterpillars is unknown. We cannot rule out differences in caterpillar feeding behavior, but this seems highly unlikely because infected and healthy

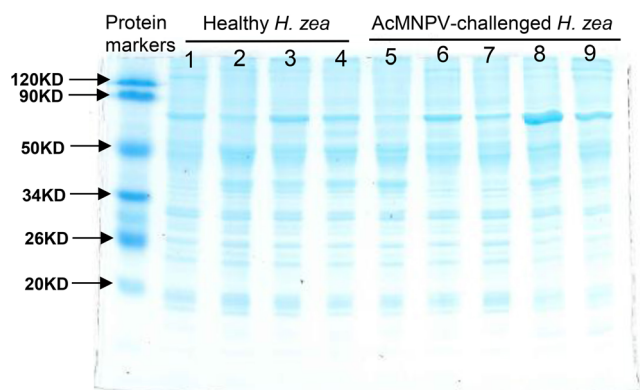


Fig. 7 Protein diversity in the salivary labial glands of healthy and virus (AcMNPV)-challenged *Helicoverpa zea* caterpillars. Proteins were separated using a 12% Tris-glycine gel and Coomassie blue stain

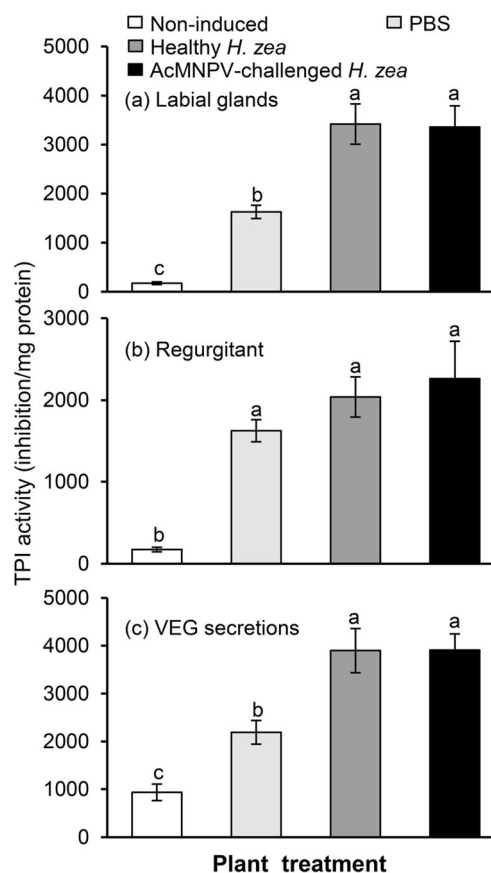


Fig. 8 Mean (\pm SE) trypsin proteinase inhibitors (TPI) activities in un-damaged tomato leaflets and leaflets that were mechanically wounded and treated with PBS or **a** macerated labial glands, **b** regurgitant, and **c** ventral eversible gland (VEG) secretions. Glands, regurgitant and secretions were collected from healthy or virus (AcMNPV)-challenged (96 hpi) *Helicoverpa zea* caterpillars. Bars with different letters represent significant differences between treatments ($P < 0.05$)

caterpillars were restricted within a feeding cage and given a limited amount of time (3 h) to consume equal amounts of foliage. We applied three separate types of secretions from caterpillars (saliva, regurgitant, and ventral eversible gland) to tomato leaves but observed no differences between secretions from infected or healthy caterpillars. This suggests that qualitative differences in the secretions do not explain the differences. This is further supported by data showing that the salivary effectors/elicitors GOX, PLC, and ATPase activities were comparable between treatments. We predict that AcMNPV may alter the rates of secretion, which could explain differences in induction of plant defenses. However, we do not have an accurate method to quantify the saliva or eversible gland secretions deposited during feeding; thus the mechanism(s) for increased plant defenses by infected caterpillars remains unknown.

Regardless of the mechanism(s) that influence this tritrophic interaction, which are likely to be complex, our study highlights that herbivore-associated microbes exert top-down effects on plant traits. These top-down effects are mediated through changes in the ability of infected caterpillars to induce higher levels of plant defenses than their healthy insect counterparts. Depending upon the type of microbes present (bacteria, polydnavirus or baculovirus), markedly different outcomes on plant phenotype may be observed. To fully understand how plants perceive herbivores, the potential roles of these herbivore-associated microbes need to be considered.

Acknowledgments This research was financially supported by the U.S. National Science Foundation (IOS-1645548) awarded to GWF, IS, and KH. QJP acknowledges financial support from China Scholarship Council (Grant 201506300111). IS acknowledges financial support from Natural Sciences and Engineering Research Council of Canada Postdoctoral Fellowship (NSERC PDF-488105-2016). The financial support from Northwest A&F University' Special Talent Fund to TXL is greatly appreciated. We appreciate all technical assistance and suggestions from M. Peiffer and F. McCullough. We thank Dr. D. Luthe (Department of Plant Science, Pennsylvania State University) for sharing her laboratory equipment.

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