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Gut-Associated Bacteria of *Helicoverpa zea* Indirectly Trigger Plant Defenses in Maize

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Abstract

Insect-associated microbes can contribute to the physiological and ecological functions of insects. Despite a few examples in beetles and piercing-sucking insects, the varied mechanisms of how insect-associated bacteria mediate plant-insect interactions are still not fully understood. The polyphagous herbivore *Helicoverpa zea* is a major agricultural pest that harbors certain microbes in their digestive systems. *Enterobacter ludwigii* is one of the gut-associated bacteria identified from field-collected caterpillars, and it has been shown to indirectly induce defenses in the dicot plant tomato by triggering the biosynthesis of salivary elicitors, but there are no clear mechanisms to show how gut microbes alter these salivary cues and how a different host plant responds to these inducible elicitors. Here, we conducted a series of assays to determine whether infection with *E. ludwigii* affects *H. zea* larval growth, immunity, and salivary responses and thus influences induced defenses of maize to herbivory. Inoculating lab-reared caterpillars with *E. ludwigii*, did not significantly affect the growth of caterpillars, but two immunity-related genes *glucose oxidase* (*GOX)* and *lysozyme* (*LYZ*) were more highly expressed in both salivary glands and midguts compared with $MgCl_2$ solution-treated caterpillars. Oral elicitors were evaluated for their role in triggering maize-specific defense responses. Our results show that saliva and its main component protein glucose oxidase (GOX) from *E. ludwigii*-inoculated caterpillars played a role in inducing maize anti-herbivore responses. These findings provide a novel concept that introducing bacteria to an herbivore may be an important approach to pest control through alteration of insect immune responses and thus indirect induction of plant resistance.

Keywords Gut bacteria · Induced defense · Symbiosis · Microbiome · Immunity · Saliva · Glucose oxidase

Introduction

Plants have developed sophisticated mechanisms for recognizing insect-derived cues and triggering defenses to avoid being consumed by insect herbivores. In plants damaged by chewing insects, jasmonic acid (JA) signaling mediated defense responses are induced. The responses can be modified by feeding cues present in insect saliva, regurgitant and frass (Alborn et al. 1997; Musser et al. 2002; Acevedo et al. 2015; Ray et al. 2016). The saliva of lepidopteran larvae is mainly produced by the labial glands and released through the spinneret (Felton et al. 2014). Induction of plant defenses to such chemical cues in saliva is known to be specific to host-herbivore systems. For example, glucose oxidase (GOX), one of the most abundant proteins in insect saliva, suppresses direct defenses in tobacco, while inducing defenses in tomato (Musser et al. 2002; Tian et al. 2012). Moreover, insect saliva contains a blend of various molecules that differentially affect plant defense responses. For example, GOX from *Helicoverpa zea* saliva induces direct defenses in tomato by eliciting JA and anti-herbivore related genes such as proteinase inhibitor 2 (*Pin*2), while adenosine triphosphatases (ATPases) suppress these genes (Tian et al. 2012; Wu et al. 2012). Additionally, the composition of some elicitors or effectors such as GOX in saliva are highly variable among different lepidopteran caterpillars (Louis et al. 2013). Moreover, the food (host plants and artificial diet) that caterpillars

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consume affects the protein composition of the labial salivary glands qualitatively and quantitatively (Peiffer and Felton 2005; Bede et al. 2006).

Oral regurgitant, which is different from saliva produced by salivary glands, arises from the foregut of insects and contains fatty acid amino acid conjugates (FACs) and insect and plant-derived proteins and microbes that could modulate plant defense responses (Chung et al. 2013; Schmelz 2015). Although caterpillar regurgitant may contain herbivore-derived cues, the frequency of regurgitation varies greatly depending on the host plants and herbivore species (Peiffer and Felton 2009). The complexity of induced plant defenses in a response to 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.

A comparatively large number of studies have been conducted to identify elicitors or effectors in saliva and regurgitant, but the role of insect gut-associated microbes on the regulation of plant defenses has received considerably less attention. The insect-gut microbial community plays an important role in the host's nutrition and digestion, development, detoxification, reproduction and other physiological and ecological traits of insects (Moran et al. 2005; Akman and Douglas 2009; Clark et al. 2010; Kohl and Dearing 2012). In Lepidoptera larvae, bacterial communities differed depending on the environment where they had been reared. For example, larvae originating from a field population did not share any OTUs (operational taxonomic units) with a laboratory population that mainly fed on artificial diets (Staudacher et al. 2016; Wang et al. 2017). A recent paper shows that the microbes in caterpillar guts are at low-density compared with other insects and vertebrates assayed using the same methods (Hammer et al. 2017), nevertheless there are multiple examples of how gut microbes of caterpillars can mediate plant-insect interactions (Mason et al. 2014; Acevedo et al. 2017; Wang et al. 2017).

In our previous work, we identified gut-associated bacteria found in the oral secretions of field-collected *H. zea* (Wang et al. 2017) and found that multiple bacteria including *Enterobacter ludwigii*, indirectly modulate tomato plant defenses through the induction of the salivary protein glucose oxidase (GOX) (Wang et al. 2017). In this current study, we isolated the bacterium *E. ludwigii* from field-collected *H. zea,* and investigated its effects on herbivore-induced defenses in maize. Furthermore, this study also examines the effects of gut bacteria on host fitness and immune responses, and how these changes affect plant responses to herbivory.

Methods and Materials

Plants and Insects Maize plants (*Zea mays* cv. B73 inbred line) were grown in sterilized Hagerstown loam soil until they reached mid-whorl (V5-V6) development stage. Plants were grown in glasshouse conditions with 14:10 h of light: dark at the Pennsylvania State University, University Park. They were watered as needed and fertilized once with three grams of Osmocote plus (15–9-12, Scotts, 3 Marysville, OH).

Field colonies of *Helicoverpa zea* larvae were collected from 'Providence' sweet corn (*Z. mays*) at the Russell E. Larson Agricultural Research Center located at Rock Springs, Pennsylvania, USA. Laboratory-reared *H. zea* were obtained from Frontier Agricultural Sciences (Newark, DE, USA), and reared on an artificial diet (which included the antibiotics streptomycin and aureomycin) in a growth chamber with a 16-h photoperiod at 27 °C under laboratory conditions (Peiffer and Felton 2005). Laboratory-reared colonies were used for all experiments, unless stated otherwise.

Effect of the Inoculation of *E. ludwigii* to Lab-Reared Caterpillars on Maize Induced Defenses *E. ludwigii* were isolated from field collected *H. zea* and identified as previously described (Wang et al. 2017). The liquid cultures of *E. ludwigii* (NCBI Accession: KX398658) were stored at −80 °C in 20% sterile glycerol until use. For inoculation, *E. ludwigii* was grown in 2xYT media (16 g Trypton, 10 g yeast extract and 5 g NaCl per liter) overnight in a rotary shaker at 200 rpm and 27 °C. The bacterial cells were centrifuged at 5000 g for 10 min and suspended in sterile 10 mM $MgCl_2$ solution before pipetting 50 μl of cell suspension onto a 10 mg cube of artificial diet (made without any antibiotics). Day-two 6th instar *H. zea* larvae were inoculated when they consumed the diet cube spiked with *E. ludwigii* or $MgCl_2$ solution. The inoculated larvae were confined on the terminal leaflets of the third leaf (counting from the top down) of V5-V6 stage maize plants using clip cages (diameter: 3 cm) to standardize the amount of damage. Empty clip cages were placed on the undamaged control plants. The cage and larva were removed after the larva consumed the entire confined area (about 4 h). One hundred mg of leaf tissue around the damaged sites was harvested 24 h later and stored at −80 °C for RNA extraction. For insect bioassay, the maize leaves damaged by *H. zea* larvae were detached and placed in a 1 oz. plastic cup containing 2% agar to maintain moisture. Newly hatched *H. zea* larvae were reared on artificial diet for 2 days before being introduced into the cups. Thirty insects were used for each treatment in the bioassay. Insects were maintained at room temperature with a 16:8 h L: D photoperiod. Larval weights were measured after the larvae were kept feeding on damaged leaves for 5 days.

Detection of *E. ludwigii* on Plant Leaves during Caterpillar Feeding To verify the possibility that gut bacteria come into contact with maize plant wounds during caterpillar feeding, *E. ludwigii* inoculated *H. zea* caterpillars were allowed to feed on maize plants for 60 min. Then, the tissue around the feeding site was harvested with alcohol-sterilized scissors and forceps,

placed into a sterile centrifuge tube with 2 ml 2xYT liquid media and incubated overnight at 27 °C in a rotary shaker at 200 rpm. Plant leaves treated with wounding and *E. ludwigii* were used as the positive control. The presence of *E. ludwigii* in the bacteria culture was detected by PCR using specific primers developed by previous work (Wang et al. 2017). The PCR conditions had an initial denaturation step of 5 min. at 95 °C, followed by 30 cycles of 95 °C for 1 min, 62 °C for 30 s, and 72 °C for 1.5 min and a final extension step of 7 min at 72 °C. The PCR reaction contained 0.4 μM of each primer, 12.5 μl of the GoTaq Green Master Mix (Promega), 2 μl of the overnight liquid-grown bacteria previously diluted 1:5 in sterile water, and 8.5 μl of water for a total volume of 25 μl.

Application of Regurgitant from *E. ludwigii*-Inoculated *H. zea* Larvae to Wounded Maize Plants Regurgitant were collected from day-two 6th instar larvae using a pipette to gently squeeze the larva until the regurgitant was expelled from the mouthpart. After wounding maize plants with a wounding tool (Ray et al. 2015), 20 μl of regurgitant mixed with 0.1 M phosphate-buffered saline (PBS; pH 7.0) was applied to each plant. Leaf tissue surrounding the wounding sites was collected at 24 h after regurgitant application and defense gene expression was then examined by qRT-PCR as described below.

Application of Saliva from *E. ludwigii*-Inoculated *H. zea* Larvae to Wounded Maize Plants *H. zea* saliva was collected as previously described (Tian et al. 2012) from 2-d-old 6th-instar caterpillars that had been inoculated with *E. ludwigii* or MgCl2 as described above. Fresh saliva was collected, diluted in PBS and used on the same day. The maize leaves were wounded, and immediately, the saliva collected from *E. ludwigii* inoculated caterpillars ($n = 10$) diluted in 20 μl of PBS was applied to the wound site. Maize leaves were harvested 24 h after treatment, frozen in liquid nitrogen, and stored at −80 °C for RNA extraction and qRT-PCR examination of defense gene expression.

Ablation of the Spinneret to Prevent Salivation Day-one 6th instar *H. zea* larvae were placed on ice until flaccid. The larvae were immobilized with a hair clip and the spinnerets cauterized with a heat pen (Electron Microscopy Sciences, Hatfield, PA, U.S.A.) as previously described (Peiffer and Felton 2005). The cauterized larvae were placed back onto the diet overnight to recover. The ablated larvae were examined to determine if the ablation successfully stopped caterpillar salivation. Both ablated and intact larvae were used to determine the effect of saliva on maize induced defenses.

Effect of *E. ludwigii* on Glucose Oxidase (GOX) Secretion onto Maize Leaves To detect the release of GOX onto maize leaves by *H. zea* larvae inoculated with *E. ludwigii*, a western blot method using a specific antibody for GOX was used. *H. zea* larvae were caged (diameter of the cage: 3 cm) on leaves and allowed to feed for 4 h. Fifty mg of leaf tissue was then harvested from around the feeding site of each damaged leaf. For each replicate of each treatment, three leaf tissues from three plants of each treatment were placed in a 2-mL tube with 0.5 mL of 0.065 M Tris-HCl (pH 6.8) with 0.7% SDS. Tubes were vortexed for 20–30 s. Then the mixture was transferred to a 10 kDa MWCO Micron centrifugal filter device (Millipore Corporation, Bedford, MA, USA) and centrifuged at 14,000 g for 60 min. Twenty-five μL of SDS sample buffer was added to recover the concentrated proteins. Samples were then separated, blotted and detected as previously described (Peiffer and Felton 2005). To obtain a standard curve, a known amount of purified GOX was loaded onto another gel using the same method. The two blots were scanned and analyzed using Sigmascan Pro 5.0 (SPSS Science, Chicago, IL). The contrast of the two gels was adjusted to the same background pixel intensity and then average band intensity was measured. We created a standard curve to determine the amount of GOX recovered from the damaged leaves by plotting a graph of the average band intensity vs. the concentration of GOX.

Application of Commercialized Glucose Oxidase (GOX) to Wounded Maize Plants Maize plants were mechanically damaged using a wounding tool. Twenty μl of solution containing either 4 ng/μl or 12 ng/μl fungal GOX (; Sigma-Aldrich) was applied to the wounded sites of maize leaves. GOX was applied to the plants in amounts based on the quantification of GOX secreted by $MgCl_2$ solution or *E. ludwigii* inoculated *H. zea* caterpillars on maize plants. Unwounded plants were used as the controls.

Effect of *E. ludwigii* Inoculation on *H. zea* Fitness In the first bioassay, neonate *H. zea* larvae were placed on artificial diet for two days, and then transferred onto detached maize leaves that were treated with *E. ludwigii* inoculated caterpillars or GOX after 24 h of treatments. Larval mass was measured after 5 days. *E. ludwigii* was grown individually in 2xYT medium at 27 °C overnight and diluted with 2xYT medium to obtain an optical density (OD) of 0.1 at a wavelength of 600 nm. The bacterial cells were centrifuged at 5000 g for 10 min and re-suspended in sterile 10 mM $MgCl_2$ solution, with the volume equal to half of the volume of medium. Two hundred microliters of each bacterial suspension or $MgCl_2$ solution was added to a 0.5-g non-antibiotic artificial diet cube (c.0.5*0.5*0.5 cm^3). Similar mass 3rd-instar caterpillars were selected and allowed to feed on the spiked diet. The diet was changed every other day in order to keep it fresh. The caterpillars were weighed after 5 days of feeding and their relative growth rate was calculated (Hoffmann and Poorter 2002; Mohan et al. 2008). The caterpillars were maintained on diet until pupation. The pupation time after treatment and mass of pupa were measured.

RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR To isolate the *H. zea* labial glands and midgut, larvae were placed on ice and chilled until flaccid. Then, the larvae were fastened ventral side up on a dissecting pan with two steel pins punctured through both ends. *H. zea* tissues were collected and washed with distilled water separately. The *H. zea* dissected tissues were frozen with liquid nitrogen immediately and kept in −80 °C freezer before use. Prior to the extraction of *H. zea* tissues, each five pairs of glands and three-midgut samples were extracted in 100 μl TRIzol solution by homogenizing the tissues with hand-held pestle in a micro-centrifuge tube kept on ice. Then, the remaining 1.9 ml TRIzol solution was added into each homogenized sample. One hundred mg of maize leaf tissue frozen in liquid nitrogen was homogenized in a GenoGrinder2000 (OPS Diagnostics, USA) and total RNA was extracted using the TRIzol reagent (Life Technologies, USA) following a modified instruction (Acevedo et al. 2017). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with FastStart Universal SYBR Green Master Mix (Roche Applied Science, USA). The specific primers used for the reference and target genes (*actin* and *MPI*, respectively) were the same as described previously (Ray et al. 2015). The synthesized cDNA was further diluted 1:10 for qRT-PCR. Primers used for qRT-PCR assays of relative expression are shown in Table S1. The Ct values of each sample were normalized using *actin* and relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). RNA extraction, cDNA synthesis, and qRT-PCR were performed for each biological replicate separately.

Statistical Analysis The normal distribution and homogeneity of data sets were verified to meet the assumptions of analysis of variance (ANOVA). Data were analyzed by one-way ANOVA followed by Fisher's least significant difference (LSD) test using MINITAB (Minitab Inc., State College, PA, USA) or by an unpaired t-test using GRAPHPAD PRISM 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

Maize Defense Response to both Field-Collected and Lab-Reared *H. zea* Caterpillars To test the effect of *H. zea* caterpillar gut bacteria on JA regulated plant defense in maize, we allowed either field-collected or lab-reared caterpillars to feed on maize plants. Field-collected caterpillars induced significantly higher transcript accumulation of the JA-regulated gene *maize proteinase inhibitor* (*MPI*) compared to maize plants damaged by lab-reared larvae at 24 h (Fig. 1; $F = 10.5$; $P < 0.001$).

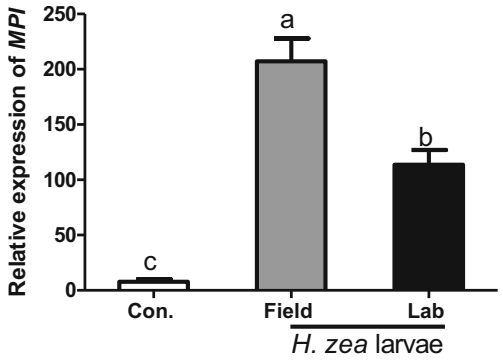

Fig. 1 *Maize protease inhibitor* (*MPI*) gene expression in maize plants damaged by field-collected *Helicoverpa zea* larvae and lab-reared larvae. Values are means ± SEM. Different letters represent significant differences. *MPI* gene expression was measured 24 h after insect feeding. Controls (Con.) were undamaged plants (ANOVA $F_{(2,10)} = 61.85$, $P < 0.001$, $n = 4–5$; Fisher's test)

Maize Defense Response Triggered by *E. ludwigii*-Inoculated Caterpillars Affected Caterpillar Growth To investigate whether gut-associated bacteria affect defense responses in maize plants, we measured the expression of selected JA and SA signaling pathway-related marker genes in maize plants damaged by caterpillars that had been inoculated with bacteria. *E. ludwigii* isolated from field-collected *H. zea* larvae increased GOX activities in labial glands of *H. zea* larvae as previously reported (Wang et al. 2017). Maize plants damaged by *E. ludwigii*-inoculated lab-reared larvae had significantly higher expression levels of the JA-responsive *MPI* compared to plants damaged by $MgCl_2$ solution-inoculated caterpillars, while the expression level of SA-responsive pathogenesis-related gene 5 (*PR5*) was suppressed in maize plants damaged by *E. ludwigii*-inoculated larvae (Fig.2a, b). In addition, the weight of larvae fed on detached maize leaves previously damaged by *E. ludwigii*-inoculated *H. zea* larvae was significantly lower than that of larvae fed on maize leaves damaged by $MgCl_2$ solution-inoculated larvae (Fig. 2c).

***E. ludwigii*-Inoculated *H. zea* Caterpillars Did Not Routinely Secrete Detectable *E. ludwigii* onto Maize Plants during Feeding** To determine whether *H. zea* caterpillar deposited gut-associated bacteria while feeding on maize plants, we used the specific primer sequence ENTITS3 (Table S1). This primer pair is designed based on the ITS region of *E. ludwigii* and is highly specific and sensitive when used in combination with the universal primer EC5 (Wang et al. 2017). Using this specific primer pair, we detected the existence of *E. ludwigii* in the maize leaves treated with *E. ludwigii*. However, we observed a detectable amount of *E. ludwigii* in only two samples of maize leaves damaged by *E. ludwigii*- inoculated caterpillars while there were no significant bands for the other three samples (Fig. S1). This result combined with previously published work (Peiffer and Felton 2009) demonstrated that

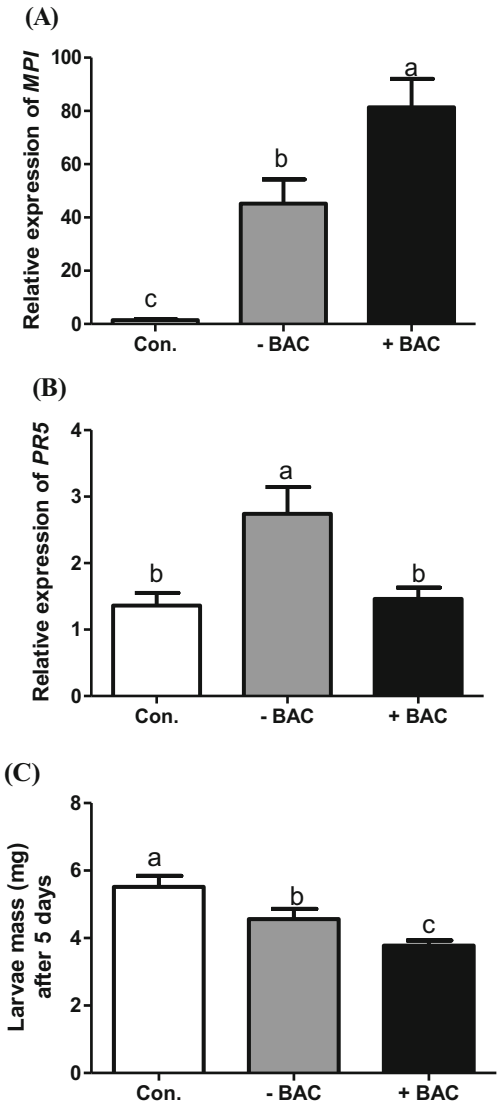

Fig. 2 *Enterobacter ludwigii* inoculated lab-reared larvae triggered higher jasmonic acid (JA) related defense responses in maize plants. Plant tissues for JA-responsive maize protease inhibitor (*MPI*) gene expression (**a**) and salicylic acid (SA)-responsive pathogenesis-related gene 5 (*PR5*) (**b**) examination were harvested 24 h after damage by *Helicoverpa zea* larvae inoculated with *E. ludwigii* (+BAC) or $MgCl_2$ solution (-BAC), and controls were undamaged plants (ANOVA *MPI*, $F_{(2,\ 15)} = 24.46$, $P < 0.001$, $n = 6$; *PR5*, $F_{(2,\ 10)} = 6.562$, $P = 0.0151$, $n =$ 3–5; Fisher's test). (C) Day-two *H. zea* neonates were fed on excised leaflets damaged by *H. zea* larvae inoculated with *E. ludwigii* (+BAC) or $MgCl_2$ solution (-BAC) and larval mass was measured after 5 d, and controls (Con.) were *H. zea* neonates fed on undamaged plants (ANOVA $F_{(2,\ 87)} = 10.5$; $P < 0.001$, $n = 30$; Fisher's test)

natural *H. zea* larvae do not routinely secrete detectable amounts of regurgitant onto maize leaves while feeding.

Direct Application of *E. ludwigii* Suppressed JA-Responsive MPI Expression To determine if direct application of *E. ludwigii* onto maize impacted its induced defenses, we applied 20 μl of *E. ludwigii* to mechanically wounded maize leaves. We found maize JA-responsive *MPI* was significantly suppressed by *E. ludwigii* compared to 2xYT media treatments, while *E. ludwigii* highly triggered maize SA-responsive *PR5* expression compared to maize plants treated with 2xYT media (Fig. S2).

Application of Regurgitant Collected from *E. ludwigii*-Inoculated *H. zea* Triggered JA-Responsive Defense in Maize Plants Although *H. zea* larvae infrequently regurgitate when feeding (Peiffer and Felton 2009), we cannot rule out the possibility that application of regurgitant to wounded leaves could induce the expression of herbivore defense genes. Regurgitant was collected from *E. ludwigii* or $MgCl_2$-inoculated *H. zea*, and 20 μl of the regurgitant was applied to wounded sites per plant (V5-V6 stage) for 24 h. The data showed that the expression of *MPI* in maize plants treated with regurgitant collected from *E. ludwigii*-inoculated *H. zea* was higher compared to plants treated by regurgitant collected from $MgCl_2$-inoculated *H. zea* (Fig. 3).

***E. ludwigii*-Inoculated *H. zea* Saliva Elicited JA-Responsive Defense in Maize Plants** To test whether saliva of *E. ludwigii* inoculated *H. zea* larvae causes distinct defense responses, we collected saliva directly from *E. ludwigii* or $MgCl_2$ solution-inoculated caterpillars and applied it onto maize plants. Application of saliva collected from *E. ludwigii*-inoculated *H. zea* significantly increased the expression of both JA-responsive *MPI* (Fig.4a). The spinnerets of caterpillars were ablated to examine the effects of saliva on the regulation of maize induced defenses. The ablation technique does not alter the rates of regurgitation (Peiffer and Felton 2009). For maize plants damaged by *E. ludwigii*-inoculated *H.zea* larvae, ablation of the spinneret significantly reduced *MPI* expression compared with plants damaged by *E. ludwigii*-inoculated larvae with intact spinnerets. The expression of *MPI* demonstrated an insignificant decline in maize plants damaged by $MgCl_2$ solution-inoculated larvae after the spinnerets were ablated. There were no significant differences in *MPI* expression of maize plants damaged by $MgCl_2$

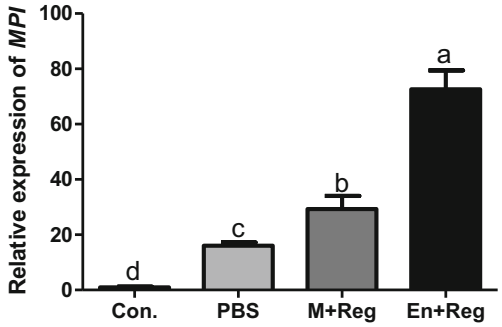

Fig. 3 Regurgitant collected from *Enterobacter ludwigii* inoculated caterpillars elicited higher levels of maize protease inhibitor (*MPI*) gene expression. Plant tissues for gene expression measurement were harvested 24 h after treatment. Undamaged plants were used as the control. *Zm-actin* gene was used as the reference gene for maize plants. Different letters represent significant differences (ANOVA $F_{(3,18)} =$ 50.63, $P < 0.001$, n = 4–6; Fisher's test)

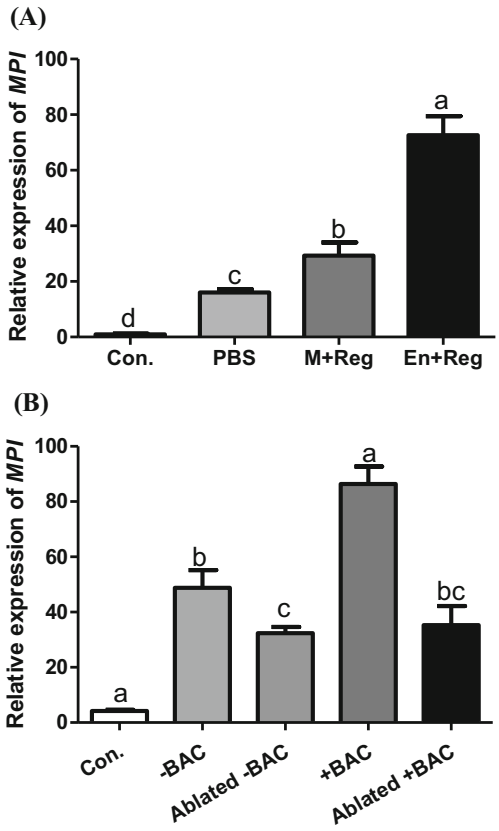

Fig. 4 *Enterobacter ludwigii* inoculated *Helicoverpa zea* larvae saliva induced higher maize protease inhibitor (*MPI*) gene expression. (A) Maize plants were treated with mechanical wounding and saliva. The saliva collected from 10 caterpillars combined with 20 μL of 0.1 M phosphate-buffered saline (PBS; pH = 7.0) was applied to the mechanically wounded site. Plant tissues for *MPI* gene expression measurement were harvested 24 h after treatment. Controls (Con.) were undamaged plants. Different letters represent significant differences (ANOVA, $F_{(3,\ 16)} = 23.22$; $P < 0.001$, $n = 5$; Fisher's test). (B) Maize plants were damaged by $MgCl_2$ solution (-BAC) or *Enterobacter ludwigii* (+BAC) inoculated *Helicoverpa zea* with intact and ablated spinnerets. Plant tissues for *MPI* gene expression measurement were harvested 24 h after treatment. Controls (Con.) were undamaged plants. Different letters represent significant differences (ANOVA $F_{(4,\ 25)} = 33.64$; $P < 0.001$, n = 6; Fisher's test)

solution or *E. ludwigii*-inoculated larvae when the spinnerets of *H. zea* larvae were ablated (Fig. 4b). These results indicate that induced defense responses in maize plants by bacteria-inoculated larvae are due to changes in saliva and not regurgitant.

***E. ludwigii* Triggers Immunity and Antibacterial Defense-Related Genes Expression in both *H. zea* Salivary Gland and Midgut** As described in a previous study, *E. ludwigii*--inoculated caterpillars had significantly higher GOX activity in the labial glands than that of $MgCl_2$ solution inoculated larvae (Wang et al. 2017). To confirm this finding, we examined the relative expression of *GOX* gene in both the labial glands and midgut of *E. ludwigii*-inoculated caterpillars In both tissues from *E. ludwigii*-inoculated caterpillars, the expression of *GOX* gene was significantly higher compared to $MgCl_2$ solution inoculated larvae (Fig. 5). Lysozyme is another abundant protein in saliva that shows antibacterial function, and has also been found in various tissues of Lepidoptera insects, such as salivary glands and midgut (Liu et al. 2004; Lemos and Terra 1991). The transcript level of *LYZ* gene was much higher in both the labial glands and midgut of *E. ludwigii*-inoculated caterpillars than that of $MgCl_2$ solution inoculated larvae (Fig. S3).

The Inoculation of *E. ludwigii* Influences Caterpillars to Secrete GOX onto Maize Plants To determine if the inoculation of *E. ludwigii* affects the secretion of GOX from the salivary glands of *H. zea* larvae, we looked for GOX proteins on wounded sites of maize leaves using a specific GOX antibody. Western blots showed that significantly more GOX protein was detected on maize leaves damaged by *E. ludwigii*-inoculated *H. zea* larvae compared to plants damaged by $MgCl_2$ solution-inoculated larvae (Fig. 6).

GOX Had Dose-Dependent Effects on JA-Regulated Defenses in Maize against Herbivory GOX has been identified as the most abundant protein in the saliva of *H. zea* larvae (Tian et al. 2012). Application of commercial fungal GOX that has similar substrate specificity as that of caterpillar saliva significantly induced JA-responsive *MPI* expression in maize. Maize treated

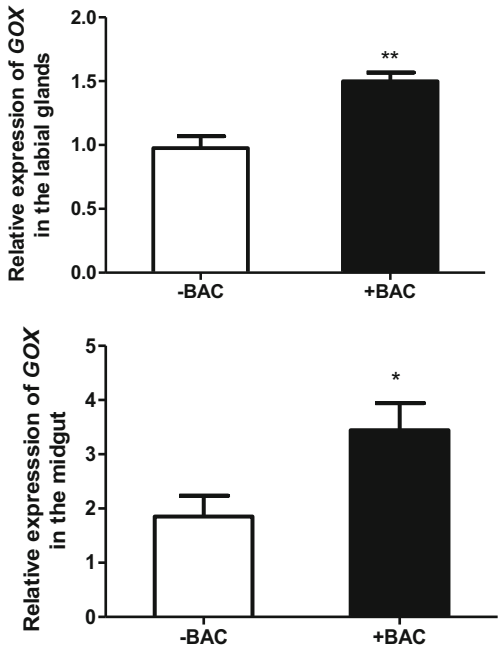

Fig. 5 Inoculation of *Enterobacter ludwigii* to caterpillars induced glucose oxidase (*GOX*) genes expression in both labial glands and midgut. Gene expression was measured 48 h after inoculation. The *actin* gene was used as the reference gene for *Helicoverpa zea* larvae. +BAC, caterpillars inoculated with *E. ludwigii*; -BAC, caterpillars inoculated with $MgCl_2$ solution. The asterisk indicates a significant difference (unpaired *t-test*; $t_{(7)} = 4.635$; $P = 0.0024$, n = 4–5; $t_{(8)} = 2.518$; $P = 0.0359 < 0.05$, n = 5)

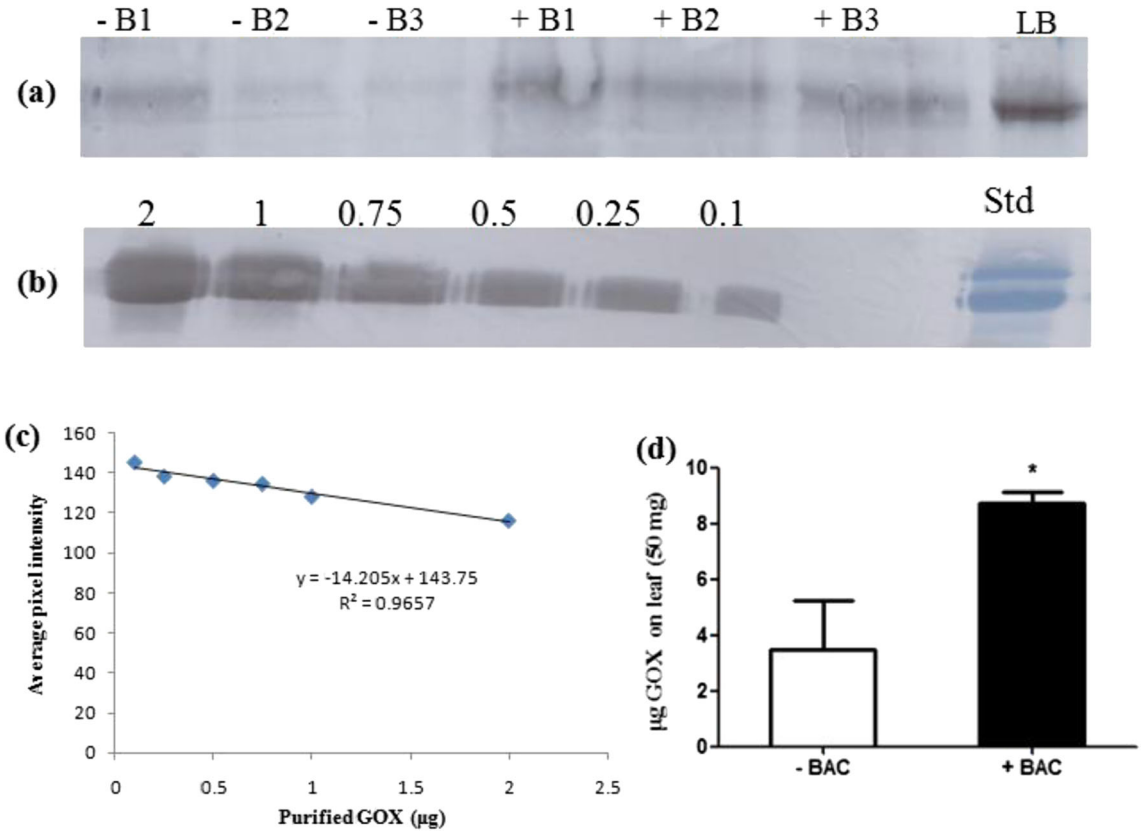

Fig. 6 *Helicoverpa zea* inoculated with *Enterobacter ludwigii* secreted more glucose oxidase (GOX) proteins onto feeding sites of maize leaves. (**a**) GOX proteins were washed off maize leaves damaged by *E. ludwigii*-inoculated larvae (+B1–3), and maize leaves fed on by $MgCl_2$ solution-inoculated larvae (-B1–3); (**b**) Western blots of purified GOX. A pair of homogenized *H. zea* labial glands (LB) on the right side of the gel was used as a positive control. (**c**) Standard curve of concentration of purified GOX vs average pixel intensity of the blot. (**d**) The amounts of GOX secreted on maize plants by larvae inoculated with $MgCl_2$ solution (-BAC) or *E. ludwigii* (+BAC) were quantified according to the standard curve. Values are mean ± SE (n = 3). The asterisk indicates a significant difference (unpaired *t-test*; $t(4) = 2.942$; $P = 0.0423 < 0.05$). M, standard protein marker

with a dilute solution of GOX, at a similar concentration as the in the $MgCl_2$-inoculated caterpillar was comparable to that treated by PBS only. However, when the concentration of GOX was increased, *MPI* expression significantly increased compared to PBS- or dilute GOX-treated maize (Fig. 7a). Additionally, the weight of larvae fed on detached maize leaves previously treated with 240 ng of GOX was significantly lower compared to larvae fed on maize leaves treated with PBS, while the weight of larvae fed on leaves pretreated with 80 ng of GOX did not show distinct difference to the weight of larvae fed on maize leaves treated with PBS (Fig. 7b).

Caterpillar Fitness Was Not Affected by the Inoculation of *E. ludwigii* To examine if the observed changes in the salivary glands and midgut of *E. ludwigii*-inoculated caterpillars cause any life history consequences, we measured *H. zea* larval and pupal mass grown on *E. ludwigii*-inoculated diet. After feeding on *E. ludwigii*-inoculated diet, the RGR of caterpillars did not show any significant difference compared to those fed on the control diet (Table 1; $t = 0.6058$; $P = 0.5495$). Similarly, caterpillars grown on *E. ludwigii*-inoculated diet have the same pupal masses ($t = 0.5206$; $P = 0.6078$) as those caterpillars grown on $MgCl_2$ solution-inoculated diet. Moreover, the development time of *E. ludwigii*-inoculated larvae reached the pupation in approximately 2 weeks, which is similar to the pupation time of larvae fed on bacteria-free diet ($t = 1.787$; $P = 0.0877$). We did not observe any differences in mortality, failure to pupate or development into adults between *E. ludwigii*-inoculated and $MgCl_2$ solution-inoculated larvae.

Discussion

Many lines of evidence show that lab-reared insect colonies generally possess much lower diversity and abundance of gut bacteria that field colonies, probably due to the relatively sterile environment in laboratories (Xiang et al. 2006; Tang et al. 2012b; Wang et al. 2017). We treated maize with either field-collected or lab-reared *H. zea* larvae and found that plants damaged by field-collected caterpillars had much higher JA signaling pathway-regulated *MPI* gene expression than maize damaged by lab colonies (Fig.1). Previously we had found field-

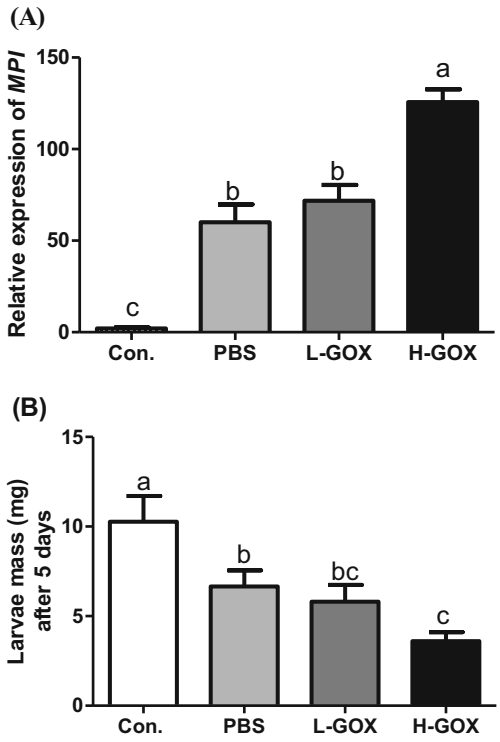

Fig. 7 Glucose oxidase (GOX) has dose-dependent effect on induced defense in maize plants against herbivory. (**a**) Maize protease inhibitor (*MPI*) in maize leaves, 24 h after wounding and application of 20 μL of phosphate buffered saline (PBS) and GOX (80 ng and 240 ng), and controls were undamaged plants (ANOVA $F_{(3,\ 20)} = 21.52$; $P < 0.001$; n = 6, Fisher's test) (**b**) Larval mass of day-two *Helicoverpa zea* neonates after 5 d on maize plants that were previously wounded and treated with 20 μL of PBS and two different concentration of GOX for 24 h, and controls were *H. zea* neonates fed on undamaged plants (ANOVA $F_{(3,\ 36)} = 7.693$; $P = 0.0004$; Fisher's test). L-GOX, low amount of GOX (80 ng); High amount of GOX (240 ng)

collected *H. zea* larvae triggered higher polyphenol oxidase activities in tomato plants compared with lab-reared larvae (Wang et al. 2017). Our results also agree with a previous study in which *MPI* expression showed a trend of induction when maize plants were treated with field-collected fall armyworm *Spodoptera frugiperda* caterpillars (Acevedo et al. 2017). These results all indicate that caterpillar gut-associated bacteria play a role in triggering plant induced defenses.

The gut-associated bacterium *E. ludwigii* isolated from field-collected *H. zea* larvae has been found to indirectly induce tomato defense responses by triggering a salivary elicitor

Table 1 The effect of *Enterobacter ludwigii* (+BAC) and $MgCl_2$ solution (-BAC) inoculated diet on larvae relative growth rate (RGR), pupal weight and pupation time

Treatment	Relative growth rate (RGR)	Pupal weight (g)	Pupation time (days)
-BAC	0.34 ± 0.0022	0.34 ± 0.0075	13 ± 0.53
+BAC	0.33 ± 0.0023	0.33 ± 0.0051	14 ± 0.45

(Wang et al. 2017). However, the effect of *H. zea* gut bacteria on mediating defense responses has not been extensively tested especially with caterpillars feed on host plants other than tomato. In this study we provide strong evidence that *E. ludwigii* found in field-collected *H. zea* larvae also triggered defense responses in maize through induction of immunity-related salivary elicitors. These results indicate that role of gut bacteria in mediating plant and insect interactions by altering host-insect immune responses may represent a more common phenomenon than previously recognized.

We found that *E. ludwigii*-inoculated caterpillars triggered significantly higher expression of immunity-related *GOX* and *LYZ* both in the labial glands and midgut of the caterpillar (Figs. 5 and S3). Insects largely depend on their immune system to combat invasions by other organisms (Zhu et al. 2015). The role of salivary proteins as antimicrobial agents is widely known in mammals and other organisms including insects (Rivera-Vega et al. 2017). For example, worker honey bees secrete GOX from the salivary glands onto larval food as an antibiotic (Ohashi et al. 1999). GOX oxidizes glucose to gluconic acid and hydrogen peroxide (H_2O_2) and it has been suggested that H_2O_2 can sterilize food contaminated by microbes and thus prevent the transmission of pathogens (Eichenseer et al. 1999; Musser et al. 2005; Kwakman et al. 2010). Moreover, the composition in the caterpillar saliva changes with exposure to bacteria. For example, the proteome of salivary glands of *Vanessa cardui* caterpillars significantly changed when caterpillars were challenged with a diet containing a peptidoglycan from *Bacillus subtilis* or *Escherichia coli*. The same change was seen at the protein level, with levels of specific chemosensory and odorant-binding proteins and proteins related to immunity in salivary glands were significantly affected by caterpillar diet (Celorio-Mancera et al. 2015). Similarly, we found that *E. ludwigii*-inoculated caterpillars secrete more than twice as much GOX during feeding on maize leaves compared with caterpillars inoculated with the control $MgCl_2$ solution (Fig. 6).

Plant defenses induced by feeding of lepidopteran larvae are generally modified by insect-derived cues found in the insect saliva and regurgitant (Acevedo et al. 2015). However, *H. zea* larvae do not routinely regurgitate during feeding, and the amount of *H. zea* regurgitant detected at the feeding site of maize leaf was calculated to be 1.02 nL, which is much less the amount (20 μL) of regurgitant we applied to wounded maize leaves (Peiffer and Felton 2009). It is possible that some of the regurgitant is re-ingested during feeding, so that caterpillars are eating their regurgitant together with plant tissues during feeding (Schittko et al. 2000). It is unlikely that caterpillars would selectively regurgitate on leaves that were ingested and yet leave the feeding sites largely free of regurgitant. *MPI* expression was significantly higher in maize treated with regurgitant collected from *E. ludwigii*-inoculated caterpillars than that collected from $MgCl_2$ solution treated caterpillars (Fig.3). Due to routine

method of collection, it is probable that the collected regurgitant may be contaminated with salivary proteins. In the case of saliva, it is possibly re-ingested during feeding, but sufficient and detectable amounts of salivary glucose oxidase from *H. zea* larvae are left on feeding margins and also deposited on other parts of the foliage (Peiffer and Felton 2005). Thus, differences in rates of GOX secretion likely explain the variation in responses of host plants to caterpillar salivation that we have observed.

Secretion and synthesis of GOX is highly dependent upon food resources, but also shows wide variation among different caterpillar species. For example in several studies using the host plant *Nicotiana attenuate,* GOX in *Manducase parata* OS was 0.094 U/mg protein, while in the OS of *Manduca sexta*, GOX is about 0.8 U/mg protein, and GOX in the OS of *Spodoptera exigua* was about 2.8 U/mg protein (Diezel et al. 2009; Qi et al. 2016). The GOX in the labial gland of *Helicoverpa armigera* was significantly higher than that in *Helicoverpa assulta*, and no glucose oxidase activity was detected in labial gland extracts of *Spodoptera litura* (Zong and Wang 2004). In addition, European corn borer (ECB) saliva has lower level of GOX activity compared to *H. zea* larvae saliva (Louis et al. 2013). Thus, the low level of GOX in ECB saliva or the lack of a GOX receptor or recognition mechanism in maize foliage was postulated as the reason ECB GOX did not activate downstream defense-related proteins, including *MPI* (Louis et al. 2013). However, we found *MPI* gene expression in maize leave was significantly triggered by the application of a high amount of GOX that is similar to the amount secreted by *E. ludwigii*-inoculated caterpillar onto maize plant (Fig. 7). Thus, we have identified GOX as a new herbivore elicitor for maize, which had previously been thought to be inactive in maize.

In addition to the increased levels of GOX contributing to the induction of maize defenses, we cannot rule out the contribution of other salivary proteins such as lysozyme. This glycosidase has been found in various tissues of Lepidoptera species, such midgut and salivary glands (Hultmark 1996; Tang et al. 2012a). Lysozyme has antibacterial activity associated with breaking down the bacterial cell wall peptidoglycan and lysing the bacterial cells (Liu et al. 2004). Thus, salivary lysozyme may provide the caterpillars with a pre-ingestive antibacterial factor (Liu et al. 2004). Bacterial cell wall fragments released by the action of lysozyme may also act as elicitors of plant defense, thus lysozyme may play an importance role in mediating plant induced defenses (Liu et al. 2004). Lipopolysaccharides (LPSs) or LPS-derived proteins are indispensable components of the cell surface of Gram-negative bacteria and can be recognized by plants to directly trigger some plant defense-related responses (Dow et al. 2000).

Our findings reported here and elsewhere (Chung et al. 2013; Wang et al. 2017; Acevedo et al. 2017; Wang et al. 2017) indicate that bacteria found in the guts of insect herbivores can significantly after the composition of herbivore oral cues (i.e., regurgitant, saliva) and consequently alter the expression of induced plant defenses. Because the gut bacterial community of these insects is strongly impacted by the environment including the particular host plant species (Chung et al. 2013; Hammer et al. 2017), there is considerably more phenotypic variability in the composition of herbivore oral secretions than previously recognized. There may be obvious costs and benefits to the herbivore resulting from such variability due to impacts on plant defenses, but the phenotypic plasticity in gut bacterial communities could be exploited for novel approaches to insect pest management. Inoculation of plants with bacteria such as *E. ludwigii* along with plant-growth promoting bacteria has the potential to enhance both plant resistance and growth.

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