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Herbivore Oral Secreted Bacteria Trigger Distinct Defense Responses in Preferred and Non-Preferred Host Plants

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Abstract Insect symbiotic bacteria affect host physiology and mediate plant-insect interactions, yet there are few clear examples of symbiotic bacteria regulating defense responses in different host plants. We hypothesized that plants would induce distinct defense responses to herbivore- associated bacteria. We evaluated whether preferred hosts (horsenettle) or non-preferred hosts (tomato) respond similarly to oral secretions (OS) from the false potato beetle (FPB, *Leptinotarsa juncta*), and whether the induced defense triggered by OS was due to the presence of symbiotic bacteria in OS. Both horsenettle and tomato damaged by antibiotic (AB) treated larvae showed higher polyphenol oxidase (PPO) activity than those damaged by non-AB treated larvae. In addition, application of OS from AB treated larvae induced higher PPO activity compared with OS from non-AB treated larvae or water treatment. False potato beetles harbor bacteria that may provide abundant cues that can be recognized by plants and thus mediate corresponding defense responses. Among all tested bacterial isolates, the genera *Pantoea*, *Acinetobacter*, *Enterobacter*, and *Serratia* were found to suppress PPO activity in tomato, while only *Pantoea* sp. among these four isolates was observed to suppress PPO activity in horsenettle. The distinct PPO suppression caused by symbiotic bacteria in different plants was similar to the pattern of induced defense-related gene expression. *Pantoea* inoculated FPB suppressed JA-responsive genes and triggered a SA-responsive gene in both tomato and horsenettle. However, *Enterobacter* inoculated FPB eliminated JA-regulated gene expression and elevated SA-regulated gene expression in tomato, but did not show evident effects on the expression levels of horsenettle defense-related genes. These results indicate that suppression of plant defenses by the bacteria found in the oral secretions of herbivores may be a more widespread phenomenon than previously indicated.

Keywords Symbiotic bacteria · Oral secretions · Plant defense · False potato beetle · Horsenettle · Tomato · Induced resistance

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Introduction

Symbiotic bacteria present in many herbivore insects influence the host's nutrition, detoxification of plant toxins, defense against predators or parasites, reproduction, and other physiological and ecological traits of insects (Gündüz and Douglas 2009; Clark et al. 2010; Oliver et al. 2010; Kohl and Dearing 2012). In recent years, a growing number of reports have shown that insect associated symbionts also benefit their hosts through manipulating plant physiology or plant defenses. For example, *Wolbachia* increased leaf miner

(*Phyllonorycter blancardella*) fitness by eliciting a green-island phenotype in apple leaves through regulating phytohormone cytokinin levels (Kaiser et al. 2010; Giron et al. 2013). In maize, endosymbiont *Wolbachia* in the larvae of western corn rootworms (*Diabrotica virgifera virgifera*) inhibited maize defense gene expression (Barr et al. 2010). However, another study was unable to replicate this result and found that *Wolbachia* was not able to suppress maize defense responses (Robert et al. 2013). In another two examples that had similar findings, the oral secretions of insects contain suppressing bacteria that trigger the SA signaling pathway and hence benefit the insects by suppressing JA-responsive anti-herbivore defenses (Chung et al. 2013b; Su et al. 2015). However, currently most of studies are focusing on the effect of insect symbionts on mediating induced defenses of single host-plants, and the effect of insect associated symbionts on defense responses in different host plants has received scant attention and remains poorly understood.

Some insect symbionts come into direct contact with plant cells (Sugio et al. 2015). Coleoptera, the most speciose group of insects, are unique in that they generally do not possess salivary glands, and their oral secretions (OS) are thus limited to regurgitant arising from their gut (Miller 1961). During herbivory, microbial symbionts in OS from beetles are directly secreted onto plant wounded sites (Chung et al. 2013b). Plants defend themselves against abiotic and biotic attack via the rapid and accurate perception of attack-derived cues, using a variety of induced defense mechanisms against their attackers (Acevedo et al. 2015). Symbiotic bacteria in the OS of the larvae of the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) mediate tomato induced defenses to benefit the growth of the larvae. Three microbial symbionts belonging to the genera *Stenotrophomonas*, *Pseudomonas*, and *Enterobacter* isolated from CPB regurgitant are responsible for suppression of induced defenses in tomato. The plant responds as if the threat is microbial instead of responding to a chewing herbivore. The bacteria in the insect's OS elicit salicylic acid (SA)-regulated defenses, and suppress jasmonic acid (JA)-related genes, so plants are unable to fully activate their JA mediated resistance against the herbivore (Chung et al. 2013b). It was hypothesized that this may be a common strategy for beetles to counter plant defenses by producing OS that contain suppressing bacteria (Acevedo et al. 2015; Chung and Felton 2011; Chung et al. 2013a, b). However, this hypothesis has not been extensively tested.

In this article, we chose to test this hypothesis by using two related Solanaceae plants: horsenettle (*Solanum carolinense*) and tomato (*Solanum lycopersicum*). Tomato is one of the best studied models of induced defenses (Cooper and Goggin 2005; Fowler et al. 2009; Green and Ryan 1972; Li et al. 2002; Pautot et al. 1993; Peiffer et al. 2009; Zebelo et al. 2014), whereas in the non-model plant horsenettle, there is considerably less information about defense signaling (Kariyat et al. 2012; Portman et al. 2015). The false potato beetle (*Leptinotarsa juncta*, FPB), which is closely related to the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*), is found primarily in the mid-Atlantic and southeastern regions of the United States (Jacques 1988; Jr and Fasulo 2015). False potato beetle has been reported as a specialist on solanaceous weeds such as horsenettle, but also may feed on other solanaceous plants, such as ground cherry, husk tomato, and nightshade (Jr and Fasulo 2015; Wise 2007).

To test whether defense responses of horsenettle and tomato are specific to certain orally secreted bacteria, we isolated bacteria from the OS of FPB larvae, and we directly applied each isolate onto wounded plants. To reveal the distinct responses of horsenettle and tomato to a specific bacterial symbiont, we evaluated whether the signaling pathways of these two plants were differentially regulated. We quantified the expression of several defensive marker genes in mechanically wounded horsenettle and tomato plants treated with the two bacterial isolates. Plants respond to attack by microbes or herbivores by activating specific phytohormone signaling pathways (Erb et al. 2012; Petek et al. 2014). Most plant associated microbes and a majority of phloem feeding insects induce salicylic acid (SA) pathway related defenses, whereas chewing herbivores mainly activate jasmonic acid (JA) and (or) ethylene (ET) pathway defenses (Pieterse and Dicke 2007; Zarate et al. 2006). In tomato, JA accumulation is upstream of the activities of defensive enzymes such as polyphenol oxidases (PPOs) (Felton et al. 1989). The induction of PPO activity could reflect not only JA-responsive defenses, but also may indicate SA-responsive defenses due to the cross-talk between JA/ET and SA signaling pathways (Pieterse et al. 2012; Thaler et al. 2012). Following up on previous results, our results demonstrate that specific microbes in OSs collected from FPB larvae manipulate varied defense responses in different plants.

Methods and Materials

Plant and Insect Materials Horesenettle (*Solanum carolinense*) seeds were collected from farmland of the Pennsylvania State University Agriculture Experiment Station located in Rock Springs, PA, USA. Tomato seeds (*Solanum lycopersicum* cv. Better Boy) were obtained commercially (Harris Seeds, Rochester, NY, USA). All plants were grown in Pro-mix potting soil under greenhouse conditions with a photoperiod of 16 L:8D. Plants were fertilized once with 3 g of Osmocote plus (15–9-12, Scotts) and watered every day until they reached the four-leaf stage for all the experiments as described previously (Peiffer and Felton 2005).

Adult FPBs were collected from the field at Rock Springs, and the laboratory colony has been raised on horsenettle plants grown in a greenhouse since 2014. Eggs of FPB were collected

from horsenettle leaves. After the eggs hatched, the larvae were reared on detached horsenettle leaves until pupation under conditions of 16 L:8D at 25 ± 2 °C. Field FPB larvae colonies were directly collected from the field at Rock Springs during the summer of 2015. Laboratory and field colonies were maintained separately. Laboratory maintained FPB colonies were used for all experiment, unless stated otherwise.

Antibiotics Treatment In order to reduce the microbes present in FPB OS and to test the effects of microbes in OS on plant induced responses, larvae were treated with antibiotic cocktails. Preparation of antibiotic (AB) solutions and AB treatments of larvae were as described by Chung et al. (2013b). Generally, AB solutions were prepared in 50 ml of MilliQ water and contained three anti-bacterial agents: 0.01 g neomycin sulfate (MP Biomedicals), 0.05 g aureomycin (BioServ), and 0.003 g streptomycin (Sigma). Detached leaves were treated with 200 μl of AB and placed in a chemical hood until dry. One larva was placed in a 1 oz. cup containing one AB-treated leaflet on top of a layer of 1 % agar to maintain leaf moisture. For untreated larvae, leaves received 200 μl of water without AB. Leaves were changed daily in order to keep them fresh. Larvae that consumed two complete leaves over a 2-day period were used for the herbivore treatment.

Herbivore Treatment To estimate plant-induced responses after herbivory, one third-instar untreated or AB-treated larvae confined to a clip cage was placed on the third leaf counted from the top of the plant (Musser et al. 2006). An empty clip cage was placed on each control plant. When the larva consumed the confined area, the larva and the cage were removed. After 24 h, approximately 100 mg of leaf tissue were harvested from damaged leaves for RNA extraction; 48 h later, 50 mg of leaf tissue were harvested to measure PPO activity. Detached leaf samples were placed in liquid nitrogen and stored at −80 °C until used.

Polyphenol Oxidase (PPO) Activity Harvested leaf tissue was pulverized with a metal milling ball in a 2 ml tube using GenoGrinder 2000 (Spex SamplePrep, Metuchen, NJ, USA) at 1200 strokes per min for 1.5 min for tomato or 2 min for horsenettle (Kim et al. 2012). Samples were extracted with 1.25 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 5 % insoluble polyvinylpolypyrrolidone and centrifuged at 11,000 x g for 10 min at 4 °C. Five μl of supernatant were mixed with 200 μl substrate containing 3 mM caffeic acid in 0.1 M potassium phosphate buffer and the change in absorbance was measured at 450 nm for 5 min (Felton et al. 1989). After centrifugation, the supernatants of horsenettle samples were transferred into a new tube and placed on ice for 5 min. PPO activity was expressed as change in absorbance/min/mg of protein. The amount of protein in each sample was quantified by Bradford assay using bovine serum albumin as a standard (Bradford 1976).

Isolation of Bacteria in OS and Application to Wounded Plants To isolate bacteria in OS from FPB larvae, OS from 4th instars fed on untreated leaves were collected with a pipette tip. Fresh, crude OS was diluted with sterile water and cultured on 2xYT agar plates at 27 °C for 24 h. Twenty one colonies were randomly selected and subcultured on 2xYT agar plates. Single colonies of each subculture were grown in 3 ml of 2xYT liquid media overnight in a rotary shaker at 200 rpm and 27 °C. The liquid cultures were stored at −80 °C in 20 % sterile glycerol until used (Chung et al. 2013b).

To determine the effects of bacteria isolated from OS of FPB larvae on plant defenses, we applied individual isolates to wounded plants. Each plant leaf was mechanically wounded using a pipette tip to punch a hole in the mid-vein. Each isolate was grown in 5 ml of 2xYT liquid media at 27 °C overnight. Twenty μl of a single isolate (OD_{600} = 0.1) and 2xYT media were applied to the wounded sites. Plants were harvested for RNA extraction (24 h later) or PPO activity (48 h later).

Quantification of Regurgitation on Horsenettle and Tomato Leaves during FPB Larvae Feeding Leaf petioles were put in 50 μl of fluorescent dye (Alexa Fluor 488, Invitrogen, Carlsbad, CA, USA) solution (0.2 mg/ml in water) and placed in a plastic box with wet paper towels until the dye solution was absorbed completely. Peiffer and Felton (2009) found that 10 μg of fluorescent dye (same concentration we used in our experiment) has no obvious effects on *Helicoverpa zea* growth. As a negative control, 50 μl of water were used instead of dye. One third-instar larva was fed on a leaf containing the dye overnight. Then larvae were transferred to plants, and after feeding for a couple of hours, the damaged sections of each leaf were mounted on a glass slide. The slides were analyzed for a fluorescence signal using an Olympus FV1000 Laser Scanning Confocal Microscope at the Penn State Microscopy and Cytometry Facility-University Park, PA, USA (Chuang et al. 2014; Chung et al. 2013b; Peiffer and Felton 2009).

Application of OS to Wounded Plants To examine the effects of microbes in OS on plant defenses, OS were collected from AB-treated or untreated larvae and diluted 1:4 *v*/v with sterile water. Plants were mechanically wounded by using a pipette tip (diam: 5 mm) to remove a hole on the mid-vein, and 20 μl of diluted OS from each treatment group of larvae were applied. Wounded control plants received 20 μl of water to the wounds.

DNA Extraction, PCR, 16S rRNA Gene Sequencing to Taxonomically Classify Bacterial Isolates To identify bacteria to the genus level, cells from a single cultured bacterial

colony were collected with a pipette tip and suspended in 10 μl of sterile water. Cells were lysed by heating the bacterial suspension at 95 °C for 10 min. DNA released from the cells was used to amplify the 16S ribosomal RNA (rRNA) gene by using the polymerase chain reaction (PCR). Universal 16S rRNA primers 530F and 1392R were used. The reaction mixture consisted of 2 μl of suspension, 12.5 μl of GoTaq Green Master Mix (Promega), 1 μl of 10 μM forward/reverse primers, and 8.5 μl of water. The PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min 30 s, and finally 72 °C for 7 min (Chung et al. 2013b). To eliminate unincorporated primers and dNTPs, enzymatic digestion was performed on 5 μl of the PCR products by adding 2 μl of EXOSAP-IT (USB Corporation). The mixture was incubated at 37 °C for 15 min, followed by 80 °C for 15 min. Two μl of the resulting products were sequenced with primer 530F at the Penn State Genomics Core Facility. To identify the suppressing bacteria to genus level, 16S rRNA sequences were analyzed by the Ribosomal Database Project Naïve Bayesian rRNA Classifier (Chung et al. 2013b; Wang et al. 2007).

RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) RNA extraction was conducted as previously described (Louis et al. 2013). One μg of RNA was used as template to synthesize cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Complementary DNA diluted 1:10 with water was used as template for qRT-PCR. Primers used for qRT-PCR assays to assess relative expression are shown in Table S1. All qRT-PCR reactions used FastStart Universal SYBR Green Master Mix (Roche) with the 7500 Fast Real-Time PCR System (Applied Biosystems) following the protocol: 95 °C for 10 min, then 40 cycles of 15 s at 95 °C, and 60 s at 60 °C. The tomato ubiquitin gene was used as a reference to normalize CT values. Relative quantification of gene expression was calculated relative to undamaged controls using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001; Rotenberg et al. 2006).

Re-Inoculation of the Isolated Bacteria to Larvae To determine whether larvae with the suppressing bacteria could have the same effect on plant induced defense as the suppressing bacteria directly applied on plants, we reintroduced the bacteria to AB-treated larvae. Each isolate was grown individually in 2xYT media at 27 °C overnight and diluted with 2xYT (OD_{600} = 0.1, 10^9 CFU/ml). Measuring OD of bacteria at the wavelength of 600 nm is a common method for estimating the concentration of bacterial cells. The bacterial cells were pelleted by centrifugation at 5000 x *g* for 10 min and re-suspended in sterile suspension buffer (10 mM $MgCl_2$). Detached leaves were treated with 200 μl of each bacterial suspension or buffer and placed in a chemical hood until the suspension dried (ca. 1.5–2 h). Larvae were allowed to feed on leaves that were treated or untreated with AB for 2 d as described above; then each larva received leaves that were inoculated with the bacterial isolates or suspension buffer for 2 d, receiving freshly prepared leaves daily. The four treatments included: undamaged plant (Con.); AB-treated larvae that received suspension buffer (buffer) only; AB-treated larvae inoculated with *Pantoea*; and AB-treated larvae inoculated *Enterobacter*.

Specific Primer Design and Detection of Bacteria from OS Deposited on Plants To make specific primers for isolated bacteria, cells were collected and lysed as described above. DNA released from the cells of *Pantoea* and *Enterobacter* was used to amplify the internal transcribed spacer (ITS) region between the 16S and 23S genes by using PCR. The universal primers used were GS4 forward (5′- GGCTTGGA TCACCTCCTT-3′) and EC5cmp reverse (5′- TGCC AGGGCATCCACCG-3′) (Gitaitis et al. 2002; Gurtler and Stanisich 1996). The PCR and conditions were the same as described. PCR Products were treated with EXOSAP-IT as described and sequenced with the GS4 primer (1 μM) at the Penn State Genomics Core Facility. The resulting DNA sequence from the ITS region was used to develop specific primers. The specific PCR conditions for *Pantoea* and *Enterobacter* are shown in Supplementary Method S1. The selected potential primer pairs were tested with pure bacteria cells and OS of FPB larvae. PCR products were sequenced at the Penn State Genomics Core Facility to confirm specificity and sensitivity.

To determine whether FPB secreted the same bacteria on different host-plants, FPB larvae were placed on the third leaf from the top of both tomato and horsenettle plants with clip cages sterilized with 70 % ethanol. Undamaged control plants were treated with an empty clip cage. After the larva consumed the confined area, the larva and the cage were removed. The larval feeding sites on the leaves were detached by scissors, which were sterilized with 70 % ethanol for harvesting each tissue. The collected leaf tissues were suspended in 2.5 ml tube with 1 ml liquid 2xYT media. Suspensions were cultured overnight in a rotary shaker at 200 rpm at 27 °C. Ten μl of the cultured suspension were heated at 95 °C for 10 min. DNA released from the bacterial cells was amplified with specific primers. Gel electrophoresis was conducted to test the PCR products. The PCR products were sequenced and verified.

Statistical Analyses The normal distribution and homogeneity of variance were verified. A $\log_{10}$-transformation was performed to meet the assumption of analysis of variance (ANOVA) where required. Data were analyzed by using One-way ANOVA followed by Fisher's Least Significant Difference (LSD) test using Minitab (Minitab Inc., State College, PA, USA) or unpaired *t*-test using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Graphpad Prism 5 was used for all graph drawing.

Results

Symbiotic Microbes in Oral Secretions (OS) from FPB Larvae Suppressed both Preferred and Non-Preferred Host Plant Defenses Because laboratory colonies of FPB were maintained in a comparatively more sterile condition than field colonies, we compared the effect of OS collected between the two colonies from two different locations on plant induced defenses. On culturing OS collected from both laboratory and field colonies, we found there was no significant difference in quantities of bacterial colonies in OS of both colonies (Fig. S1) ($t = 0.271$; $P = 0.805$). Polyphenol oxidase activity was treated as a rapid and effective assay to study plant induced defenses regulated by the JA signaling pathway. We treated horsenettle (the preferred host) leaves with AB or non-AB-treated FPB from both the laboratory and the field. After culturing OS collected FPB larvae treated with AB, we were not able to observe an evident amount of bacteria on 2xYT agar plates (data not shown). After 48 h, higher PPO activity was measured for both field ($F = 16.25$; $P < 0.001$) and laboratory ($F = 31.62$; $P < 0.001$) collected insect colonies treated with AB, than for untreated larvae or the undamaged horsenettle plants (Figs.1a, b). This suggests that laboratory maintained FPB larvae still possess sufficient symbiotic microbes that suppressed plant-induced defenses similar to the field collected colonies. Thus, we used laboratory maintained FPB colonies for all following experiments.

To investigate the effect of symbiotic microbes in FPB larvae OS on tomato (the non-preferred host), tomato plants were damaged by AB or non-AB-treated larvae similar to the treatments described above on horsenettle. Tomato leaves damaged by AB treated larvae showed higher PPO activities than leaves damaged by untreated larvae (Fig. 1c) ($F = 15.72$; $P < 0.001$). In summary, these results demonstrate that the microbes in OSs from FPB larvae suppressed induced defenses of both preferred and non-preferred plants.

Oral Secretions Produced during FPB Feeding on Different Plants The amount of regurgitant produced by FPB may vary depending on the host plants. For example, *H. zea* were more likely to regurgitate when feeding on corn compared to other hosts such as tomato (Peiffer and Felton 2009). Hence, we measured the OS of FPB larvae during herbivory on horsenettle and tomato. We first fed larvae with a piece of leaflet dyed with fluorescent solution, and then larvae were allowed to feed on the leaves of horsenettle and tomato for a couple of hours until the wounding sites were evident. The FPB fed leaves were examined for regurgitant using fluorescence microscopy. We found that larvae secreted 2.2 pl/μm regurgitant on horsenettle and 1.7 pl/μm regurgitant on tomato secreted, but there was no significant difference (Fig. S2) ($t = 0.903$; $P = 0.39$). These results demonstrate that FPB larvae secreted similar amount of OS when feeding on different host plants.

Oral Secretions from FPB Larvae Suppressed both Tomato and Horsenettle Induced Defenses Since similar amounts of OS were detected on tomato and horsenettle plants. We determined whether its application to wounded leaves could have similar effects on plant induced responses. To further verify our hypothesis, 20 μl of OS collected from AB-treated or untreated larvae were applied to mechanically wounded plants. Both wounded tomato ($F = 10.85$; $P < 0.001$) and horsenettle ($F = 7.047$; $P = 0.002$) plants treated with OS collected from untreated larvae showed lower PPO activities than those treated with water or OS from AB treated larvae (Figs. 2a, b). These results were similar to plants damaged by AB or non-AB treated larvae. Polyphenol oxidase is one of JA-regulated enzymes that serves an anti-nutritive role by disrupting the normal digestive processes of herbivores (Bosch et al. 2014; Constabel 1995; Felton 2005). Taken together, these results indicate that the microbes in OS from FPB larvae suppressed both the preferred and non-preferred plant induced defenses.

Suppression of Induced Defenses by Individual Symbiotic Bacterial Isolates from FPB OS Varies in Different Plants To examine if microbial symbionts present in OS affect induced defenses, we measured PPO activities in mechanically wounded tomato and horsenettle to which individual bacterial isolates cultured from OS from untreated FPB larvae were applied. Among the 21 bacterial isolates (F1-F21), four significantly suppressed PPO activities in tomato compared with 2xYT media treatment (Figs. 3a, b, c). We classified these isolates that decreased PPO activities in tomato to be members of the genera *Pantoea* ($t = 2.4$; $P = 0.038$), *Acinetobacter* ($t = 2.88$; $P = 0.015$), *Enterobacter* ($t = 2.31$; $P = 0.042$), and *Serratia* ($t = 2.52$; $P = 0.029$) (Table S2). These four isolates were then applied to mechanically wounded horsenettle, and we found only one of the four isolates, *Pantoea* sp. suppressed PPO activities in horsenettle compared with 2xYT media treatment (Fig. 3d) ($t = 3.76$; $P = 0.004$). To reveal the mechanism of the suppression in plant defenses caused by bacterial isolates, we quantified expression levels of JA- and SA-regulated genes in both tomato and horsenettle leaves that were wounded and treated with *Pantoea* sp., which suppressed PPO activities in both tomato and horsenettle, or with *Enterobacter* sp., which only decreased PPO activities in tomato. We found that both *Pantoea* sp. and *Enterobacter* sp. decreased JA-responsive *CysPI* expression alongside increasing SA-regulated *Pr1* expression compared with W + 2xYT in tomato ($P < 0.05$; Figs. 4a, b), whereas only *Pantoea* sp. inhibited JA-regulated *Opr3* expression

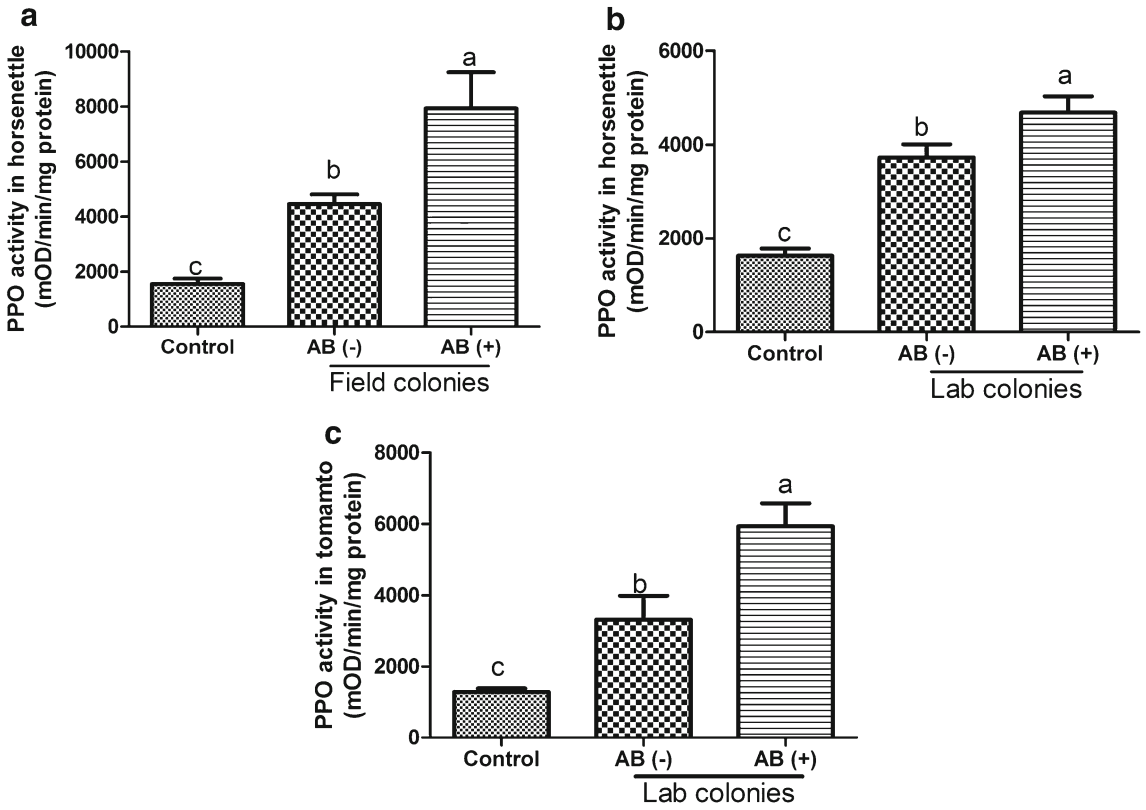

Fig. 1 Polyphenol oxidase (PPO) activities in horsenettle damaged by antibiotic (AB)-treated or untreated field collected false potato beetle (FPB) larvae (**a**) or laboratory-maintained FPB larvae (**b**), and PPO activities in tomato damaged by AB-treated or untreated lab-maintained FPB larvae (**c**). PPO activities were measured 48 h after treatment. Undamaged plants were used as the control. Values are means ± SE (N = 5–7). Different letters above the bars indicate significant differences (Fisher's $P < 0.05$, following ANOVA)

while inducing *Pr1* expression in horsenettle ($P < 0.05$; Figs. 4c, d). These results demonstrated the suppressing function of symbiotic microbes in OS from FPB varied in different plants.

Inoculation of AB-Treated Larvae with Symbiotic Bacteria Suppressed Plant Defense To further confirm that bacteria in OS from untreated larvae were responsible for manipulating induced defenses, we inoculated *Pantoea* sp. and *Enterobacter* sp. separately into AB-treated larvae. Inoculation of suppressing bacteria into AB-treated larvae restored the suppressing abilities we observed previously. Larvae inoculated with *Pantoea* sp. suppressed PPO activities in both tomato and horsenettle, whereas larvae inoculated with *Enterobacter* sp. only down-regulated PPO activities in tomato, but not in horsenettle ($P < 0.05$; Figs. 5a, b). The results were similar to plants that were treated with wounding and an individual bacterial isolate. These data indicate that plants' responses to insect symbiotic bacteria varied depending on the specific bacterial taxa present in OS.

Detection of FPB Orally Secreted Microbe during Insect Feeding on Different Plants To confirm that both *Pantoea* sp. and *Enterobacter* sp. were deposited on leaves by untreated FPB larvae, we designed specific primers for *Pantoea* sp., which could suppress both tomato and horsenettle induced defense, and *Enterobacter* sp., which could only inhibit tomato induced defense, by using the internal transcribed spacer (ITS) region of 16S–23S rDNA. The primer F11ITS (5′-GGTTAGG ACTCCGCCCTTTC-3′) developed from the ITS region of *Pantoea* sp. and F15ITS (5′-ACTTG CTGGCTGTGAGTGAA-3′) from *Enterobacter* sp. were highly specific and sensitive. When used in combination with the universal primer EC5cmp (5′-TGCCAGGGCATCCACCG-3′), we could distinguish *Pantoea* sp. and *Enterobacter* sp. from four different genera of bacteria isolated from FPB larvae OS (Fig. S3). Using these specific primers, we detected the presence of both *Pantoea* sp. and *Enterobacter* sp. in OS collected from non-AB treated larvae (Fig. 6a). Furthermore, we detected the presence of both *Pantoea* sp. (Fig. 6b) and *Enterobacter* sp. (Fig. 6c) on horsenettle and tomato leaves damaged by untreated larvae. These data demonstrate that AB treatment effectively eliminated both *Pantoea* sp. and *Enterobacter* sp. in AB-treated larvae and both *Pantoea* sp. and *Enterobacter* sp. were deposited upon tomato and horsenettle leaves by untreated larvae during herbivory.

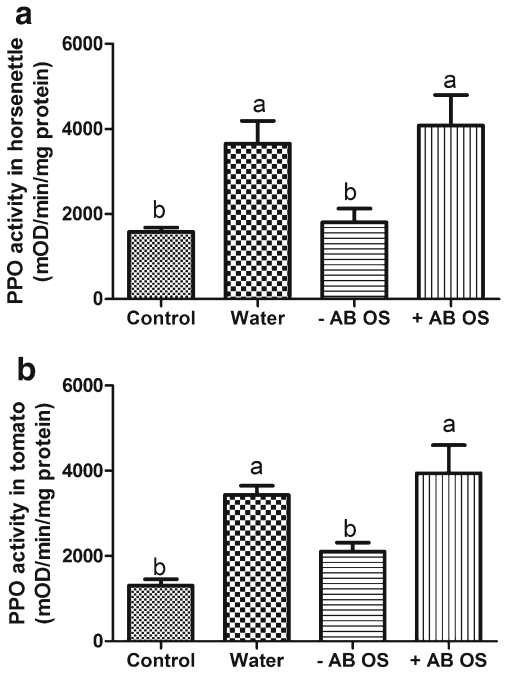

Fig. 2 Polyphenol oxidase (PPO) activities in mechanical wounded horsenettle (**a**) and tomato (**b**) treated with 20 μl of oral secretion (OS) from antibiotic (AB)-treated or untreated false potato beetle larvae. PPO activities were measured 48 h after treatment. Undamaged plants were used as the controls. Values are means ± SE ($N = 6$). Different letters above the bars indicate significant differences (Fisher's $P < 0.05$, following ANOVA)

Discussion

In the case of herbivores, microbial symbionts are known to influence their hosts including providing nutrition, digestion, and detoxifying toxins (Adams et al. 2013; Hansen and Moran 2014). In addition, insect symbionts have an important role in mediating plant defenses (Casteel and Hansen 2014; Chung et al. 2013b; Douglas 2013; Hansen and Moran 2014; Su et al. 2015; Sugio et al. 2015). Our findings provide further support that insect herbivores use symbiotic bacteria to cope with host defenses. Coleopteran species in particular frequently deposit copious amounts of oral secretion during herbivory (Chung and Felton 2011). As a closely related species of the Colorado potato beetle (Jr and Fasulo 2015), we observed that false potato beetle (FPB) also secreted substantial amounts of OS while feeding on host-plants (Fig. S2). Moreover, we verified the effectiveness of antibiotic treatment to FPB larvae. We confirmed that microbes in OS from non-AB treated FPB larvae suppressed anti-herbivore defense in both horsenettle and tomato (Figs. 2a, b). As some suppressing bacteria harbored in the beetle's OS are recognized by plants as a microbial attack, plants trigger an ineffective defense pathway against herbivores (Chung et al. 2013b). In the current study, we found that non-AB treated FPB larvae reduced JA-responsive defense-related enzyme activities in host-plants compared to AB treated FPB larvae. These results suggest it is a common "strategy" for beetles to eliminate plant induced defenses by depositing oral secretions possessed with suppressing bacteria during their herbivory.

Many lines of evidence show that laboratory maintained insect colonies generally possess lower diversity of gut bacteria than field collected colonies due to the relatively sterile environment and lower abundance in foods (Broderick et al. 2004; Xiang et al. 2006). By culturing OS collected from both field and laboratory maintained FPB, we found there was no significant difference in quantities of bacteria of the two colonies (Fig. S1). However, it remains to be determined if laboratory and field FPB larvae have different bacterial compositions. We therefore asked whether field and lab insect colonies treated with AB induced similar anti-herbivore responses. Antibiotic solution is efficient in eliminating the majority of bacteria in OS of FPB larvae (data not shown). Abundant bacteria on the wounded plants damaged by CPB larvae treated without AB were found by scanning electron microscopy, whereas very small amounts of bacteria were observed on leaves damaged by AB treated CPB (Chung et al. 2013b). Our results showed that there were no significant differences between field and laboratory FPB colonies in suppressing plant-induced defenses (Figs. 1a, b). This result indicates that laboratory maintained FPB still possessed sufficient microbes in their OS despite being maintained in the laboratory. Thus, we were able to investigate the role of beetle oral secreted bacteria by using laboratory-maintained FPB colonies.

The distinct nutrient content of the different plants may enable different bacteria to colonize different plants (Priya et al. 2012). Thus, we examined whether FPB larvae deposited the same bacteria on horsenettle and tomato plants. We verified the effectiveness of AB treatment on FPB larvae. As expected, we did not detect any *Pantoea* and *Enterobacter* in OS collected from AB treated FPB larvae, while we still observed the two bacteria in non-AB treated larvae (Fig. 6a). By using specific primers, the results of bacteria detection confirmed our assumption that FPB larvae secrete both *Pantoea* sp. and *Enterobacter* sp. onto horsenettle and tomato during feeding (Figs. 6b, c). The short period of host switching did not affect the presence of *Pantoea* sp. and *Enterobacter* sp. However, in our case we did not check the exact amounts of the two bacteria on different plants secreted by FPB larvae. It may be worthwhile to investigate this by utilizing molecular methods such as fluorescence *in situ* hybridization (FISH), or quantitative reverse transcriptase PCR (qRT-PCR) (Shinkai and Kobayashi 2007).

Here, we described insect associated symbionts that suppress plant-induced defenses that were plant species

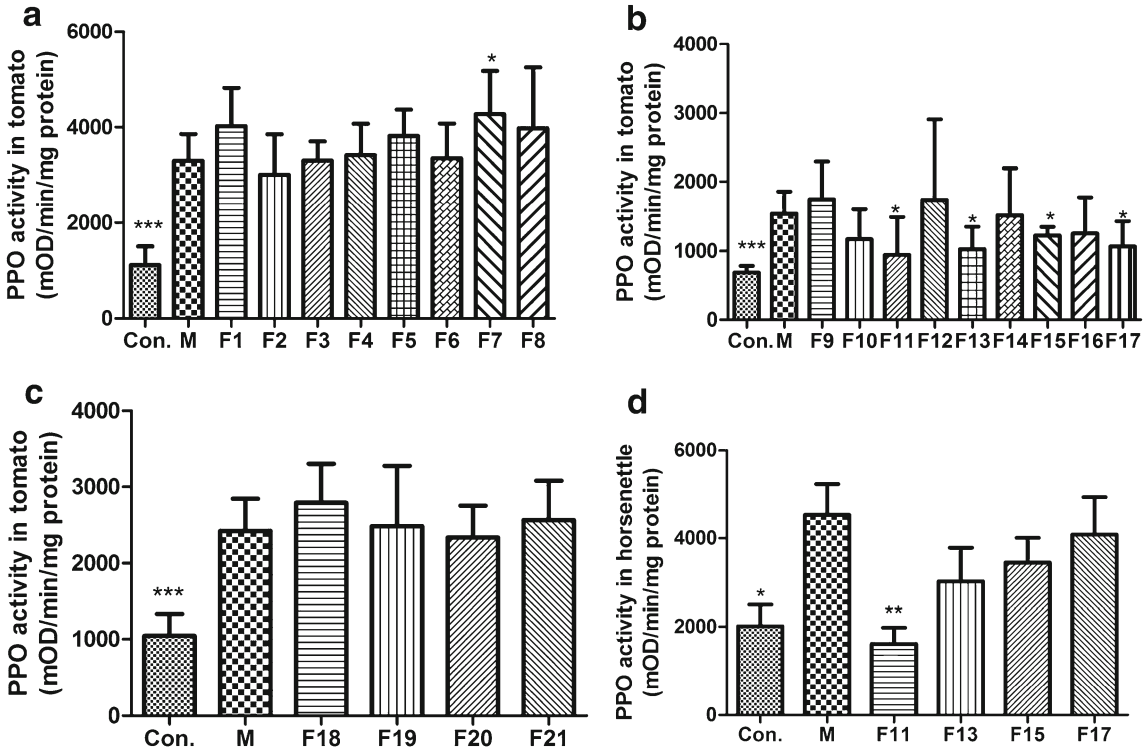

Fig. 3 Polyphenol oxidase (PPO) activities in plants that were treated with mechanical wounding and individual bacterial isolates cultured from oral secretions (OS) from untreated false potato beetle larvae. **a**, **b**, **c** Twenty μl of each isolate was applied to wounded tomato plants and **d** four bacterial isolates were applied to wounded horsenettle plants. PPO activities were measured 48 h after treatments. Values are means ± SE (N = 5–7). Asterisks indicate significant differences from wounding +2xYT media (M) treatment (unpaired t-test, $P < 0.05$). Con., undamaged plant; F11, wounding + *Pantoea* sp.; F13, wounding + *Acinetobacter* sp.; F15, wounding + *Enterobacter* sp.; F17, wounding + *Serratia* sp. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

dependent. Different bacteria appear to produce distinct effects on plants: both *Pantoea* sp. and *Enterobacter* sp. significantly suppressed JA-responsive anti-herbivore enzyme PPO activities in tomato, whereas only *Pantoea* sp. suppressed PPO activities in horsenettle (Figs. 3b, d). We obtained the same results by inoculating these bacteria separately into AB-treated larvae (Fig. 5). This led to our hypothesis that the ability of plants to perceive microbial attack may depend on interactions between specific bacterial taxa and the plant species. To confirm this hypothesis, we measured the relative expression levels of JA and SA pathway related genes. *Enterobacter* sp. induced *Pr1* gene expression in tomato, but not in horsenettle, whereas *Pantoea* sp. induced *Pr1* gene expression in both tomato and horsenettle (Fig. 4). These results indicate that the two different plants may possess distinct perception mechanisms towards the same microbe. This has been demonstrated in other systems. For example, a surface-exposed peptide of a cell wall transglutaminase in *Phytophthora* called Pep-13 plays a role as a MAMP (microbial-associated molecular pattern) to activate induced defenses in potato and parsley, but not in Arabidopsis (Brunner et al. 2002). In another study, the symbiont *Regiella insecticola* increased pea aphid performance on clover, but did not influence performance on fava bean (*Vicia faba*), on which most pea aphids perform well, although the mechanisms were not further investigated (Tsuchida et al. 2004). Thus, it would be worthwhile to study what components produced by FPB symbiotic bacteria cause the distinct suppression of defense responses in different plants.

Plants by utilizing their ability to efficiently regulate phytohormones, are defended against attackers. However, some insect herbivores take over and mediate plant signaling pathways for their own benefit. In the current study, we found that bacteria from FPB OS reduced defense-related enzyme activity and decreased the expression of JA-responsive anti-herbivore genes, but stimulated SA-responsive genes in FPB damaged host-plants. We argued that the suppression of plant induced defense was due to the existence of suppressing bacteria in FPB oral secretion. It has been well known that there is crosstalk between the JA and SA signaling pathways (Biere and Bennett 2013; Casteel and Hansen 2014; Stout et al. 2006; Thaler et al. 2012). Previous findings in our laboratory indicate that feeding by non-antibiotic treated CPB that possessed suppressing bacteria in OS on tomato suppress JA-responsive defenses by promoting SA signaling

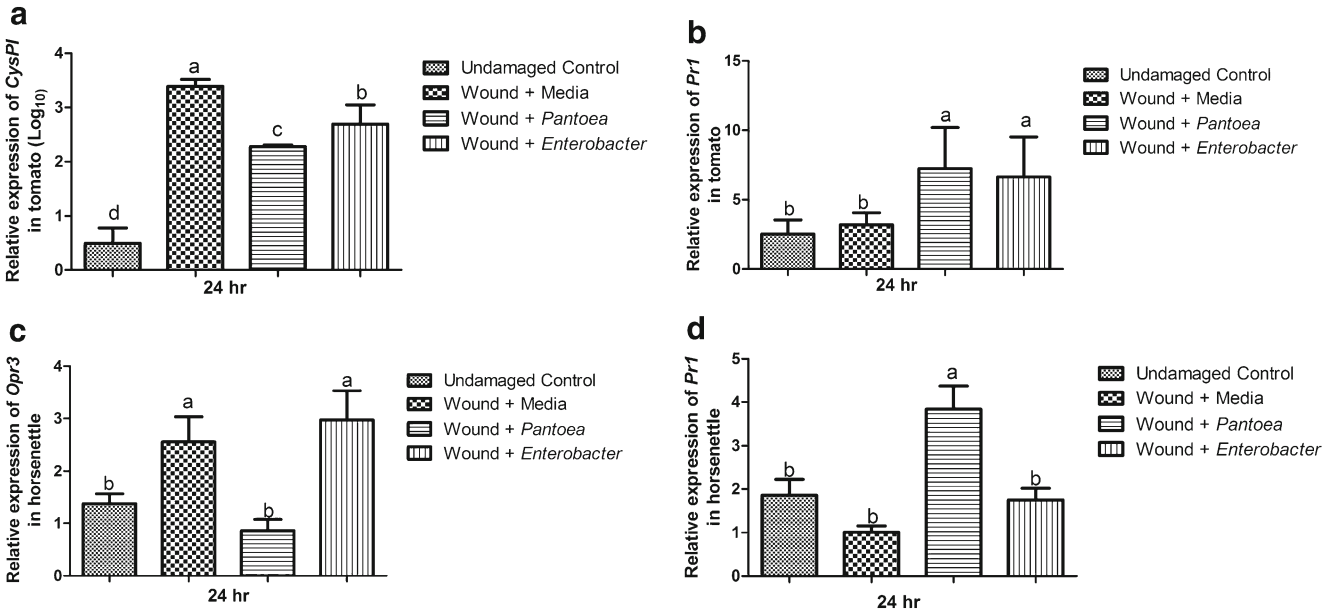

Fig. 4 Expression levels of jasmonic acid(JA)- and salicylic acid (SA)-regulated genes (normalized to the housekeeper gene *Ubiquitin*) in plants that were treated with mechanical wounding and two bacteria, *Pantoea* sp. or *Enterobacter* sp. Gene expression was measured 24 h after 20 μl of two bacterial isolates were applied to the wounded sites. **a** Relative expression of JA related gene cysteine proteinase inhibitor (*CysPI*) and **b** SA related gene *Pr1* in tomato leaves and **c** Relative expression of JA related gene 12-Oxophytodienoate reductase-3 (*Opr3*) and **d** SA related gene pathogenesis-related protein 1 (*Pr1*) in horsenettle leaves. Gene expression was measured 24 h after false potato beetle (FPB) larvae feeding. Undamaged plants were used as controls. Media is 2xYT media that was used to culture bacteria. Values are means ± SE, except the values of *CysPI* expression which are log transformed means ± SE (N = 4–5). Different letters represent significant differences (Fisher's $P < 0.05$, following ANOVA)

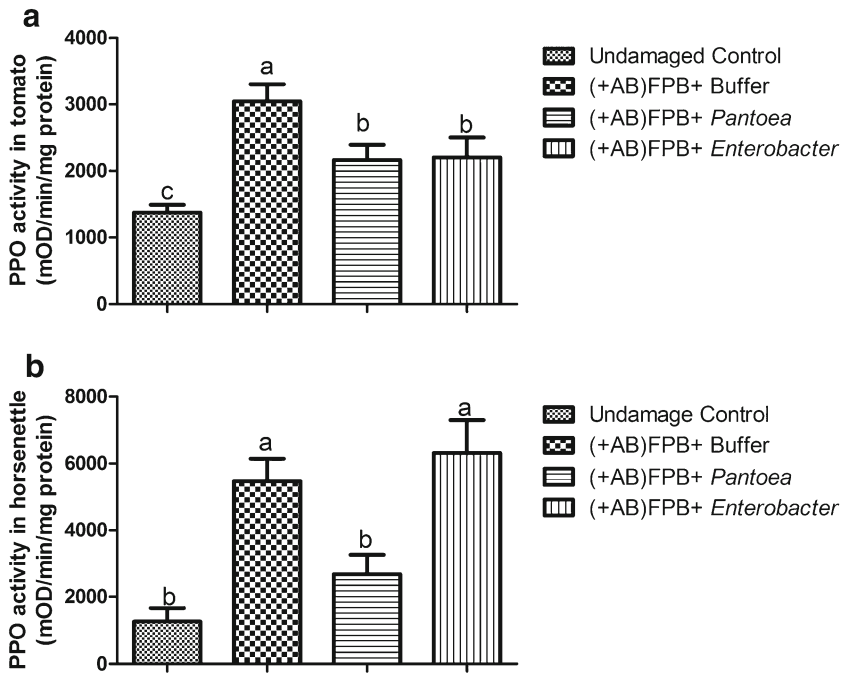

Fig. 5 Polyphenol oxidase (PPO) activities in plants damaged by antibiotic (AB)-treated or untreated larvae following inoculation the larvae with one of two bacterial isolates (*Pantoea* sp. or *Enterobacter* sp.) cultured from oral secretion (OS) of false potato beetle. Larvae were allowed to feed on AB-treated or untreated detached leaves for 2 d and then larvae were transferred to feed on leaves that were inoculated with suspension buffer or the bacteria isolates in suspension buffer for 2 d. PPO activities were measured 48 h after insect feeding. Undamaged plants were used as the controls. Buffer is 10 mM $MgCl_2$ sterile buffer that was used to re-suspend bacteria. Values are means ± SE (N = 6). Different letters represent significant differences (Fisher's $P < 0.05$, following ANOVA)

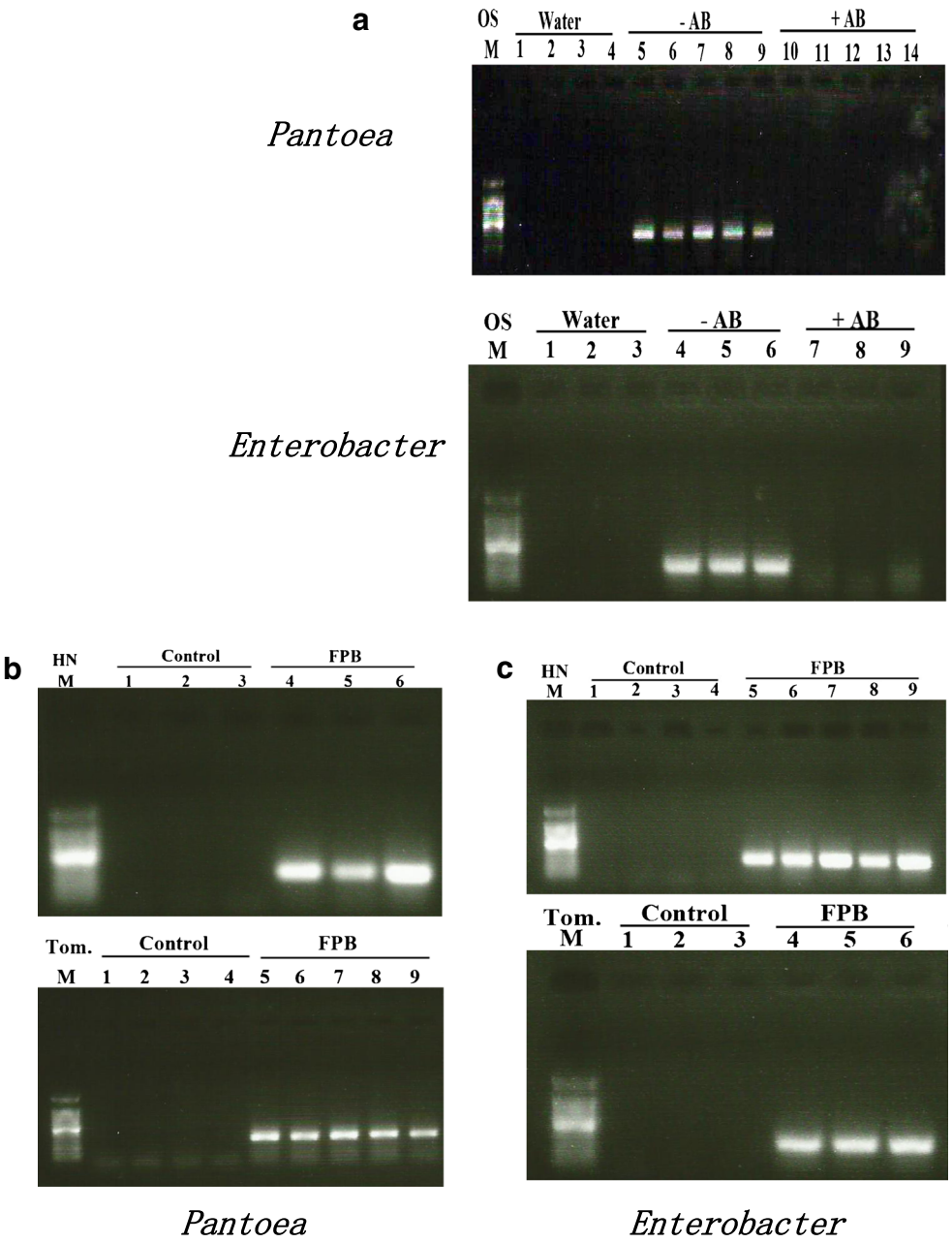

Fig. 6 Detection of *Pantoea* sp. and *Enterobacter* sp. in false potato beetle (FPB) larvae regurgitant and wounded sites of plants after damaged by FPB larvae. **a** Detection of *Pantoea* sp. and *Enterobacter* sp. in regurgitant of FPB larvae with or without antibiotic (AB) treatment. - AB, larvae feeding on water treated leaves; + AB, larvae feeding on antibiotic treated leaves. One μl of regurgitant was collected from each FPB lava 48 h after treatment and added to 10 μl sterile water. Sterile water was used as the control. Detection of the presence of *Pantoea* sp. **b** and *Enterobacter* sp. **c** on feeding sites of horsenettle and tomato after damaged by FPB larvae. Undamaged plants were used as the controls. Leaves were detached and cultured in 2xYT media overnight. The cultured mixtures were used as the templates to detect bacteria

(Chung et al. 2013b). In another case, whitefly infected with *Hamiltonella defense*, one of facultative symbionts, suppressed plant induced defenses and improved whitefly performance on tomato. In addition, the authors found that the symbiont-mediated suppression of JA-regulated defenses depends on the SA signaling pathway (Su et al. 2015). These examples demonstrate a novel ploy for insect herbivores to avoid plant defenses and benefit themselves by utilizing associated microbial symbionts. However, it still needs to be investigated whether other metabolites produced by bacteria in OS of FPB larvae suppress plant defenses, or whether there are other MAMPs from these bacteria that elicit the SA-signaling pathway to affect JA-responsive defenses.

In summary, the findings support the supposition that the perception of herbivory by plants involves not only mechanical injury to the plant and the presence of herbivore-derived elicitors released during feeding, but also the presence of microbes associated with the herbivore (Acevedo et al. 2015). However, different microbes in insects may have species-specific effects on different host-plants. Our work indicates that specific MAMPs associated with the herbivores' microbiota are perceived by plants during herbivory and thus may alter the outcome of plant responses.

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