Phytohormone elicitation in maize by oral secretions of specialist *Mythimna separata* and generalist *Spodoptera litura*

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**ABSTRACT**

The innate machineries for regulation of plant defense responses against herbivores are strikingly based on stress response-associated phytohormones. However, the dynamics of the effects of distinct types of chewing herbivores’ oral secretion (OS) on phytohormone regulation are not well known. We explored the phytohormone regulation in maize leaves in response to OS from larvae of a specialist herbivore (*Mythimna separata*) and a generalist herbivore (*Spodoptera litura*). In response to mechanical damage with application of those OSs, maize leaves rapidly upregulated the endogenous levels of oxylipins and abscisic acid (ABA) in quantitatively similar manners. Moreover, it appeared that bacteria in OS were responsible for the coordination of ABA levels. Our findings suggest that, during predation by the specialist and generalist lepidopteran herbivores, their OSs similarly upregulate phytohormone levels and the presence of their oral bacterial community makes a minor impact on maize defense responses.

**Introduction**

Plants respond to herbivory by reacting to an array of molecules that are present in herbivores’ oral secretions (OS) and are able to induce defense responses. The OS from lepidopteran larvae and other herbivorous arthropods contains an array of elicitors, including fatty acid-amino acid conjugates, peptides, oligosaccharides, etc. (Arimura 2021). Moreover, other oral factors, including oral bacteria, concomitantly serve to coordinate induced plant defense responses (Mason et al. 2019).

Phytohormone signaling is importantly responsible for the cellular intermediate signaling in response to herbivory, including herbivore OS factors (Schmelz et al. 2009; Erb et al. 2012). Representative, oxylipins, including jasmonates (jasmonic acid [JA] and jasmonoyl-L-isoleucine [JA-Ile]), are responsible for the defense responses to multiple herbivore species (Eckardt 2008; Zhang et al. 2017), and ethylene works synergistically with both wound stimuli and OS elicitors during herbivory (Schmelz et al. 2009; Arimura 2021). Salicylic acid (SA) is well known to play a master role in the signal transduction for a broad-spectrum of immune responses (Ding and Ding 2020), and is even involved in the defense responses to herbivory, especially when sucking arthropods such as spider mites and aphids attack (Arimura et al. 2009). Likewise, abscisic acid (ABA) is well known to be involved in environmental stress responses and growth/development in higher plants (Chen et al. 2020), but ABA is also known to serve as a positive regulator against lepidopteran larvae in *Arabidopsis thaliana* (Bodenhausen and Raymond 2007). The concomitant effects from ABA signaling to JA signaling are in concert with defense-signaling cross-talk, whereby SA signaling is antagonistic to JA signaling, eventually leading to adjustments of the downstream defense systems in higher plants (Erb et al. 2012).

On the other hand, some larvae can partner with oral bacteria that help to disrupt these plant defense responses. For example, it has been shown that OS bacteria of the Colorado potato beetle (*Leptinotarsa decemlineata*) can suppress defense responses of the tomato host plants, which is achieved by the activation of SA signaling, thereby resulting in the suppression of JA signaling for anti-herbivore defenses in tomato (Chung et al. 2013). More recently, similar effects of bacteria in OS from larvae of *Spodoptera litura*, a generalist herbivore, have been elucidated in defense responses of *A. thaliana* host plants (Yamasaki et al. 2021). The OS bacteria, including *Staphylococcus epidermidis* (an anaerobic staphylococcus), appear to have a positive role in SA and ABA signaling but a negative role in oxylipin signaling, thereby leading to a benefit for the predator by the presence of OS bacteria (Yamasaki et al. 2021). However, as such innate functions of oral bacteria are likely to be variable and specific depending on the compatibility between plant and herbivore species (Mason et al. 2019), more comprehensive insights based on multiple studies and a specific model case are required.

In the current study, to understand the nature of the OS and OS bacteria of distinct herbivore species on the plant defense response of a single host plant, we explored the effects of OSs collected from the generalist *S. litura* as well as the specialist *Mythimna separata* on the regulation of phytohormones in maize, a model monocot plant.

**Materials and methods**

**Plants and insects**

Maize (*Zea mays* L. cv. Royal Dent) plants were grown in plastic pots for 10–14 days in climate-controlled rooms at...
24 ± 1°C with a photoperiod of 16 h (80 μE m⁻² s⁻¹). M. separata Walker and S. litura (Lepidoptera: Noctuidae) used in this study were incubated in climate-controlled rooms at 24 ± 1°C with a photoperiod of 16 h (6:00-22:00), as previously reported in Rim et al. (2019) and Yamasaki et al. (2021).

**Collection of non-sterilized OS (OS⁺) and bacteria-free OS (OS⁻)**

In order to prepare non-sterilized larvae grown on plants, maize plants were grown in plastic pots (1 plant per pot) for about 10 days in the non-sterilized condition in a climate-controlled room (see above), and used for the diet of larvae hatched on the filter paper. The larvae were grown on the potted plants until they developed to the third instar stage, and then OS (OS⁺) was first collected.

To prepare bacteria-free M. separata and S. litura larvae grown on fresh maize plants, fresh S. litura eggs (about 2–3 egg masses) were washed twice, first with 5 mL of 1% NaClO briefly and secondly with 5 mL of 1% NaClO for 5 min, on sterile filter paper (diam. 90 mm) in a petri dish. The eggs were immediately washed three times with 5 mL of distilled water and then dried. The petri dish was kept under sterile conditions for about 1–2 days in a climate-controlled room at 24 ± 1°C with a photoperiod of 16 h, until the eggs hatched. The hatched larvae (about 10 larvae) were transferred onto approximately 2-week-old bacteria-free plants (2-3 plants) in a sterilized plastic container (0.3 L), containing MS medium supplemented with 0.5% sucrose and 1.5% agar. The OS (OS⁺) was first collected from third instar larvae (about a week after hatching) using a glass capillary tube (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany).

The larvae were then transferred onto freshly prepared non-sterilized or sterilized plants and reused for OS collection once a day until the larvae had grown to the fifth instar stage. To ensure that materials were bacteria-free, we excluded all materials when any bacterial or fungal contamination was encountered. We confirmed the absence of bacteria based on polymerase chain reaction using a pair of primers and a generalist herbivore (S. litura) similarly induced the absence of bacteria based on polymerase chain reaction using a pair of internal transcribed spacer-specific primers, according to the method described in Yamasaki et al. (2021).

**Plant treatment**

Mechanical damage (MD) was performed by rolling a stainless steel tracing wheel (Clover Mfg. Co., Ltd., Osaka, Japan) on the surface of a leaf of the potted plants. Forty MD spots were applied per plant leaf (2-mm intervals between MD spots). The OS⁺ or OS⁻ solution diluted with an equal volume of DW, or DW serving as a control, was immediately applied to the MD spots (about 0.5 μL per spot). The treated plants were incubated at 24 ± 1°C with a light intensity of 80 μE m⁻² s⁻¹.

**Hormone analysis**

Maize leaves (60-100 mg fresh weight) were harvested and immediately frozen in liquid nitrogen. Using 2 mL screw cap microtubes (Sarstedt, Tokyo, Japan), samples were homogenized in FastPrep®-24 (MP Biochemicals, Santa Ana, CA, USA) using five 2.3 mm zirconia beads and 1 mL ethyl acetate solvent spiked with deuterated internal standards (10 ng d3-JA, 5 ng d3-JA-Ile, 10 ng d6-ABA, and 20 ng d4-SA). The hormone analysis was performed according to the method described in Tzin et al. (2017), with minor modifications. As labeled 12-oxo-phytodienoic acid (OPDA) was not available for quantification, the amount of OPDA was quantified based on the d3-JA standard and an empirically determined conversion factor (mOPDA = 1.19 × mJA) previously reported in Schäfer et al. (2011).

**Statistics and reproducibility**

We performed one-way ANOVA with post hoc Tukey’s HSD using the program (http://astatsa.com/OneWay_Anova_with_TukeyHSD/) for comparing multiple samples. The sample sizes and number of replicates for all of the sets of assays and analyses are indicated in the legends of the corresponding figures.

**Results**

To assess the effects of M. separata OS and S. litura OS on phytohormone regulation, the time course of endogenous phytohormone levels in maize leaves after mechanical damage (MD) with application of OSs collected from larvae of M. separata or S. litura were evaluated (Figure 1). Application of M. separata OS or S. litura OS to mechanically damaged leaf sites increased the levels of oxylipins (JA, its active form [JA-Ile], and its immediate precursor [OPDA]) and ABA rapidly after 0.5 h and also thereafter at 2 and 4 h after the onset of MD + the respective OS, in comparison to the levels with MD + distilled water serving as mock treatment. SA levels were, however, affected neither by MD + M. separata OS nor MD + S. litura OS for up to 4 h.

Next, to assess the effects of oral bacteria in M. separata OS or S. litura OS on phytohormone regulation, we treated plants with bacteria-free OS collected from each of these species of larvae grown on sterilized maize (OS⁺) or with non-sterilized OS collected from larvae grown on maize in the normal growth conditions (OS⁻). Application of S. litura OS with MD slightly increased OPDA levels after 2 h, in comparison to the levels with MD + S. litura OS⁻ (Figure 2). In contrast, application of M. separata OS⁻ or S. litura OS⁻ with MD decreased ABA levels, in comparison to the levels with MD + the respective OS⁻ (Figure 2).

**Discussion**

It was shown previously that maize plants increase their JA (and ethylene) levels in response to various OS elicitors, including volicitin [N-(17-hydroxylinolenoyl)-L-glutamine] from a lepidopteran and caeliferin A16:0 from Schistocerca americana (Schmelz et al. 2009). Here, we added new evidence that OSs from a specialist herbivore (M. separata) and a generalist herbivore (S. litura) similarly induced increases of oxylipins (JA, JA-Ile, and OPDA) and ABA in maize (Figure 1). This regulation is somehow different from that in A. thaliana plants treated with S. litura OS, in which not only oxylipins and ABA but also SA was upregulated (Yamasaki et al. 2021). Likewise, given the fact that in rice leaves treated with OS collected from Spodoptera mauritia or Mythimna loreyi, oxylipins and ethylene, but not ABA or SA, were upregulated (Fukumoto
et al. 2013; Mujiono et al. 2020), we conclude that regulation of the oral factor-induced phytohormones largely depends on the compatibility between plant and herbivore species.

Indeed, we expected that oral bacteria in both *M. separata* OS and *S. litura* OS would strikingly contribute to the coordination of the phytohormone regulation in maize; however, they exhibited only minor roles in the induction of ABA levels by both *M. separata* OS and *S. litura* OS and the induction of OPDA levels by *S. litura* OS (Figure 2). These observations were not expected, because it has been shown that

**Figure 1.** Time course of endogenous levels of phytohormones. Leaf samples were harvested 0, 0.5, 1, 2 and 4 h after mechanical damage (MD) with application of distilled water (DW) or oral secretion (OS) collected from larvae of *Mythimna separata* or *Spodoptera litura*, and endogenous levels of phytohormones (12-oxo-phytodienoic acid [OPDA], jasmonic acid [JA], jasmonoyl-L-isoleucine [JA-Ile]), salicylic acid [SA], and abscisic acid [ABA]) were analyzed. Data represent the mean and standard error (n = 6). Means indicated by different small letters are significantly different among data at the indicated time points, based on an ANOVA with post-hoc Tukey’s HSD (P < 0.05). ns, not significant.

**Figure 2.** Phytohormone levels in maize leaves in response to non-sterilized oral secretion (OS+) or bacteria-free oral secretion (OS-). Leaf samples were harvested 2 h after mechanical damage (MD) with application of distilled water (DW), OS+ or OS- collected from larvae of *Mythimna separata* or *Spodoptera litura*, and endogenous levels of phytohormones (12-oxo-phytodienoic acid [OPDA], jasmonic acid [JA], jasmonoyl-L-isoleucine [JA-Ile]), salicylic acid [SA], and abscisic acid [ABA]) were analyzed. Untreated leaves served as a control. Data represent the mean and standard error (n = 6). Means indicated by different small letters are significantly different, based on an ANOVA with post-hoc Tukey’s HSD (P < 0.05). ns, not significant.
transcripts of the JA-inducible proteinase inhibitor gene were certainly upregulated in maize leaves by bacteria isolated from *S. frugiperda* (Acevedo et al. 2017). Moving forward, considering the variable effects of oral bacteria in *S. litura* OS between maize (this study) and *A. thaliana* (in which ABA and SA are upregulated but oxylipins are downregulated, as described by Yamasaki et al. (2021)), the effects of oral bacteria are very likely to depend on the compatibility between the bacteria and the plant species. Importantly for further study, we need to identify the oral bacterial community responsible for ABA/OPDA regulation and clarify their effect on the fitness of the herbivores.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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