Plant Biotechnology 35, 237–242 (2018)
DOI: 10.5511/plantbiotechnology.18.0528a

Original Paper

The effects of Lepidopteran oral secretion on plant wounds: A case study on the interaction between *Spodoptera litura* and *Arabidopsis thaliana*

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Received April 18, 2018; accepted May 28, 2018 (Edited by H. Kouchi)

Abstract This paper is about the cellular responses of plants to chewing insect attacks. We deployed a recently developed experimental system to monitor the responsiveness of *Arabidopsis thaliana* (Arabidopsis) to the application of oral secretion (OS) from Lepidopteran generalist herbivore *Spodoptera litura* (*S. litura*). Oral secretion from *S. litura* contains gut regurgitant and saliva. We identified significant differences in the wound closure morphologies (e.g., dried and sealed tissue) between mechanically damaged leaves with and without an application of *S. litura* OS at the site-of-injury. Experimental controls were mechanically wounded leaves. Wounds were walled off by visible vertical cross sections. Cell death was restricted to the immediate areas of the wounds. In contrast, mechanically damaged leaves treated with *S. litura* OS did not display a clear sealing pattern due to an absence of a defined vertical cross section at the wound site. Notably, OS treated leaves exhibited a wider area of visible premature senescence (the declining of chlorophyll content caused by death of chloroplasts) around the injury than controls. More pronounced senescence was also observed around the injury in *S. litura* OS treated wounds than in controls. Heat inactivated *S. litura* OS elicited a similar response to non-heat inactivated samples. The causal compound is heat stable and thus not a protein. Our results suggest that *S. litura* OS: (1) inhibited wound recovery responses in leaves; (2) promoted senescence around injured areas. The function of senescence may be to relocate nutritional resources to support plant survival when attacked.

Key words: insect-plant interaction, oral secretion, plant wound, senescence.

Introduction

Lepidopteran (moth and butterfly) larvae are significant agricultural pests. Their caterpillars feed by chewing and ingesting plant tissue. To defend themselves, plants employ stable defense mechanisms such as thick cuticles and thorns. They also have inducible defense mechanisms that are mediated primarily by phytohormone Jasmonic Acid (JA) (Stahl et al. 2018).

Physical rupturing and subsequent loss of cell compartmentation occurs when insects mechanically breach the outer protective layers of plant tissue. Open wound sites result in a material loss of water. Additionally, these openings provide easy access for pathogens like fungi and bacteria into plant tissue (Mengiste et al. 2003).

Wound recovery processes take place after recognizing endogenous molecules derived from damaged cells. In most dicot plants including Arabidopsis, cell death occurs in areas adjacent to the wound. Physical sealing of the plant wound is followed by the accumulation of lignin, callose, and other phenolic compounds at the wound site (Denness et al. 2011; Jacobs et al. 2003; McCabe 2013). While JA triggers these wound closing procedures, damage caused by sharp objects or even robotic mechanical worms (Mec Worms) do not account for the intensity and specificity of plant defence mechanisms caused by herbivore insects (Felton 2008; Mithöfer et al. 2005).

Plant tissue first comes into contact with *S. litura OS* after being punctured mechanically by chewing insects. *S. litura* OS is comprised of insect regurgitant and saliva as well as various microorganisms (Wang et al. 2016). Regurgitant from larvae digestive systems contains insect-derived substances like plant materials at various stages of digestion (Felton 2008).

Utilizing an imaging technique developed by Betsuyaku et al. (2018), we were able to monitor plant

Abbreviations: Arabidopsis, *Arabidopsis thaliana*; JA, jasmonic acid; *NLS*, *NUCLEAR LOCALIZATION SIGNAL*; OS, oral secretion; *S. litura*, *Spodoptera litura*; *VSP1*, *VEGETATIVE STORAGE PROTEIN1*; YFP, YELLOW FLUORESCENT PROTEIN.

This article can be found at http://www.jspcmb.jp/
Published online August 22, 2018

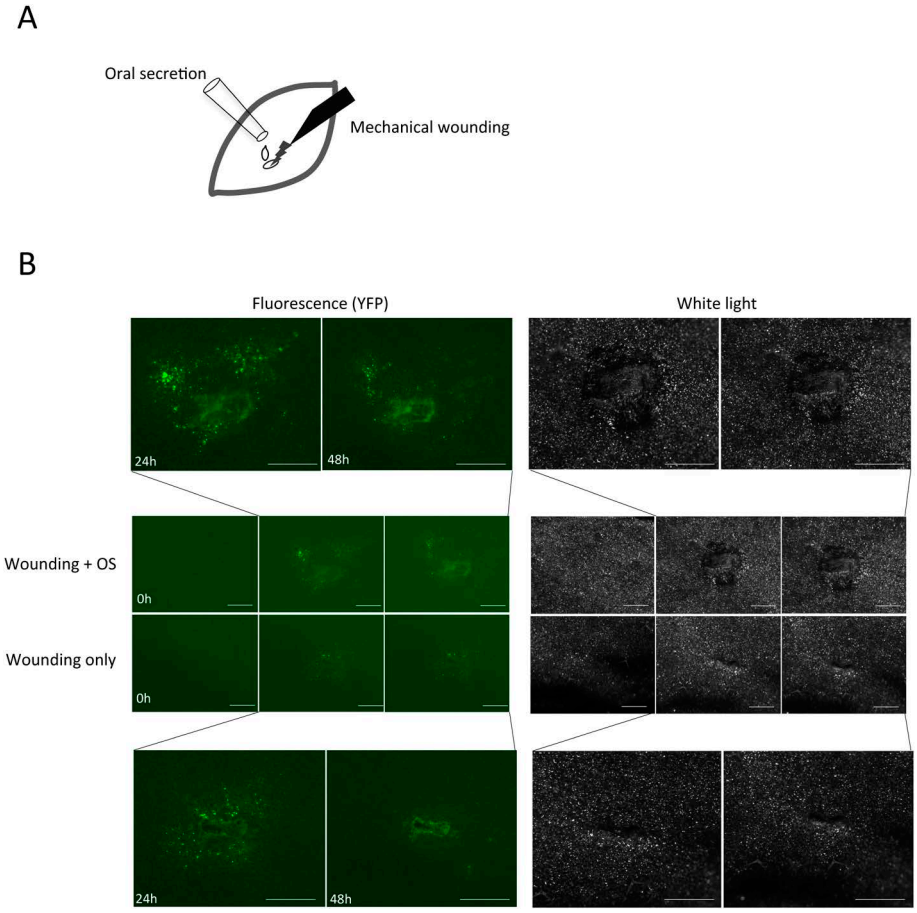

Figure 1. Activation of the JA pathway around wounded areas treated with and without OS. A, Illustration of the experimental procedure. B, Left panel, YFP signal from transgenic Arabidopsis plants harboring *VSP-YFP*; right panel, black and white pictures were taken in parallel as a reference. Scale bar: 1 mm.

responses to leaf damage from *S. litura*. We hypothesized that *S. litura* OS treated wounds recover differently than those treated with water alone.

Using OS from the generalist chewing insect *S. litura* and Arabidopsis as the host plant, we found evidence that suggested: (1) OS modulates protective responses to wounding in plants; (2) OS triggers premature senescence which we define as yellowing of leaf tissue linked to declining chlorophyll content caused via the loss of chloroplasts in mesophyll cells. This prompts reallocation of nutritional resources which originated from degradation of chlorophyll machineries (Guo and Gan 2005; Lim et al. 2007).

Materials and methods

Plant materials and treatment

Plant materials, growth conditions, as well as undetached leaf imaging techniques were set up using a reporter line. As described by Betsuyaku et al. (2018), this reporter line consisted of the promoter region of the *VEGETATIVE STORAGE PROTEIN 1 (VSP1)* which is fused to *YELLOW FLUORESCENT PROTEIN (YFP)* with a *NUCLEAR LOCALIZATION SIGNAL (NLS)*.

Autofluorescence and wound-site observations were performed using Columbia-0 Arabidopsis plants. These plants were grown in standard conditions of 16 h light/8 h dark cycles at 22°C. The blade of a 23G syringe was used to wound leaves. 0.5 μl of OS was applied to the wounds. 0.5 μl of water was applied to the control leaves.

Microscope setup

Chlorophyll autofluorescence and YFP were detected by using Texas Red and YFP filters. We used a DFC7000T camera controlled by LasX software (Leica Microsystems) to acquire color pictures and autofluorescent images. Two weeks after the initial treatment, we used Z-stack to acquire images for control and OS treated leaves.

Quantification techniques

ImageJ (National Institute of Health) was used to measure: a) the open area of plant wounds; and b) leaf senescence. The area of senescence was calculated by subtracting open space—where there are no cells—from the area of autofluorescent loss.

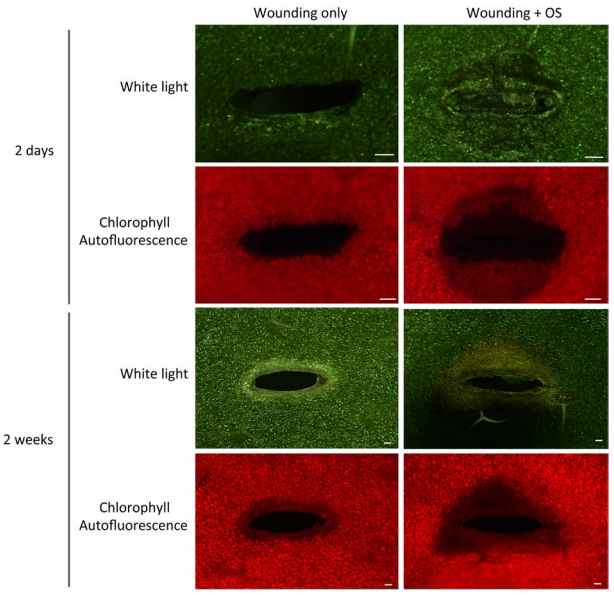

Figure 2. Morphology of plant wounds as seen under white light and the chlorophyll autofluorescence Pictures were taken at 2 days (top) and 2 weeks (bottom) after wounding. Leaves were wounded by piercing them with a syringe. 0.5 μl of OS (right) or water (left) was applied to the damaged tissue. Scale bar: 100 μm.

S. litura rearing and preparation of regurgitant

S. litura was reared using a standard artificial diet of Silkmate 2M. Rearing conditions were 25±1°C, 60±10% RH, 16 h light/8 h dark cycle. We followed the methodology of Shiojiri et al. (2000) to obtain oral secretion by gently agitating and squeezing 2 to 4 instar larvae using plastic tips for micropipettes. To exclude possible involvement of chemicals from the artificial diet, larvae were fed with wild-type Arabidopsis for 24 h before sampling their OS.

Oral secretions were stored at −80°C until use. For heat inactivation, 5 μl of OS was boiled at 96°C for 2 min.

Results

Activation of Jasmonic Acid (JA) Pathway in Response to S. litura Oral Secretion (OS)

We applied the method Betsuyaku et al. (2018) developed to observe the JA pathway in undetached leaves. This technique was suited to observe artificially induced stress from simulated insect chewing. Specifically, this method enabled us to observe the promoter activity of the JA pathway specific marker gene *VEGETATIVE STORAGE PROTEIN1* (*VSP1*). The *VSP1* promoter was fused to *YELLOW FLUORESCENT PROTEIN (YFP)* with a *NUCLEAR LOCALIZATION SIGNAL (NLS)*.

To facilitate this interaction, we used undetached Arabidopsis leaves fixed under a fluorescent stereomicroscope. The leaves were then injured with a syringe to simulate mechanical wounding stress as a control. Oral secretion from *S. litura* larvae was then applied to the artificial wounds to simulate herbivore insect stress (Figure 1A).

Both *S. litura* OS and control samples exhibited a clear YFP signal. However, *S. litura* OS treated leaves displayed more pronounced YFP signals than controls (Figure 1B). This observation confirms previous findings that herbivore stress activates more intense JA defense responses than by wounding alone (Shinya et al. 2016).

Morphology of leaf wounds

In Figure 1, there was a slight yet noticeable difference in the wound closure patterns between *S. litura* OS treated leaves and control leaves within 24 h from wounding.

In order to observe wound closure patterns with greater clarity, we punctured leaves through their vertical sections using a syringe. Close examination of wound-sites without S. *litura* OS treatment showed dried and sealed tissue along the vertical cross section of the wounded area (Figure 2, left).

The sealing of vertical cross sections were visible two days after being punctured in control leaves. In OS treated samples, there were no sealed vertical cross sections apparent in the damaged tissue (Figure 2, top).

In OS treated leaves, we found that wounds exhibited a thin and loose covering of a yet to be determined substance, possibly dried *S. litura* OS, along the epidermal surface. This loose covering was temporary and not visible after two weeks.

We quantified the degree of tissue sealing by measuring the open area of plant wounds walled off by sealed vertical tissue (Figure 3A). Wounded tissue in controls displayed an open space surrounded by dry, sealed tissue. In *S. litura* OS treated leaves, minimal open space was observed at the puncture site after two days (Figure 3B).

After two weeks, there were no significant differences in the sizes of the open areas between treated leaf wounds and untreated controls. This suggested that *S. litura* OS delayed transversal sealing processes (Figure 3A). Nonetheless, the tissue surrounding *S. litura* OS treated wounds has a larger necrotic region than in controls (Figure 2, bottom).

Loss of chlorophyll is enhanced in S. litura OS treated wounds

We visualized senescence and cell viability by monitoring the autofluorescence of chlorophyll. Two days after wounding, a loss in the autofluorescence was visible in cells immediately adjacent to the disrupted tissue in controls. In *S. litura* OS treated leaves, the surrounding area of the wound exhibited a more pronounced loss of chlorophyll (Figure 3C).

After two weeks, the levels of chlorophyll continued to decline in control leaves. However, this decline was restricted to a few layers of cells around the disrupted tissue. In *S. litura* OS treated leaves, we observed

a decrease in chlorophyll in a larger area and in a less restrictive manner (Figure 2 bottom; 3C).

Leaf shrinkage

Leaves with large necrotic lesions exhibited mild to severe shrinkage (Figure 4B). Up to 60% of leaves treated with OS showed a severe shrunken morphology. None of the control samples displayed a similar degree of shrinkage (Figure 4A).

Characterizing the effective compound in S. litura OS

We applied heat inactivated OS to plant wounds to characterize its active compounds. Heat inactivated *S. litura* OS demonstrated a similar response to non-heat inactivated OS samples (Figure 3B, C). Our findings indicated that the compound responsible for triggering these morphological effects was heat stable.

After a two week interval, heat inactivated *S. litura* OS triggered cell death and leaf curling at comparable rates to unheated samples (Figures 3A, C, 4A).

Discussion

We propose two hypotheses to explain the effects of *S. litura* OS on open plant wounds:

Hypothesis A: Benefit for plants

A well-documented plant response to OS from Lepidopteran insects is the emission of Herbivore Induced Plant Volatile (HIPV) which attracts natural enemies such as parasitoids. The site of tissue damage is the primary source of HIPV emissions (Turlings and Tumlinson 1992). By delaying the sealing of leaf wounds, *S. litura* OS may facilitate HIPV emissions and indirectly contribute to plant defense.

Fatty acid conjugates (FACs) trigger HIPV emissions in *S. litura* and other Lepidopteran species (Yoshinaga et al. 2014). It is possible that FACs also play a role in inhibiting wound sealing due to their heat stability.

Hypothesis B: OS suppresses plant sealing responses and benefits S. litura

The suppression of plant sealing mechanisms may benefit the growth of *S. litura* larvae. There are at least three possibilities for how this might occur:

a) Plant wound sealing is accompanied by depositing phenolic compounds that accumulate in the region immediately adjacent to where cell death and sealing occurs (Cui et al. 2013; Jacobs et al. 2003). Unsealed tissue at wound site may facilitate ingestion by neonates whose mandibles are not yet fully developed.

b) The *bos1* mutant, which was originally identified as being susceptible to necrotic fungal pathogen *Botrytis cinerea*, exhibited the characteristic of "runaway" cell death at wound sites (Cui et al. 2013; Mengiste et al. 2003). The pattern of *bos1* mutant cell death includes the dilution of phenolic compounds that seal plant wounds. There is no direct evidence that the cell death we observed is identical to that of *bos1*. Nor do we have direct evidence that secondary metabolites are diluted in wounded leaves. However, given that many secondary metabolic compounds contain anti-herbivore effects, it is reasonable to speculate that the dilution of these secondary metabolic compounds may help facilitate the growth of *S. litura* larvae (Fürstenberg-Hägg et al. 2013).

c) OS from chewing insects suppresses the expression

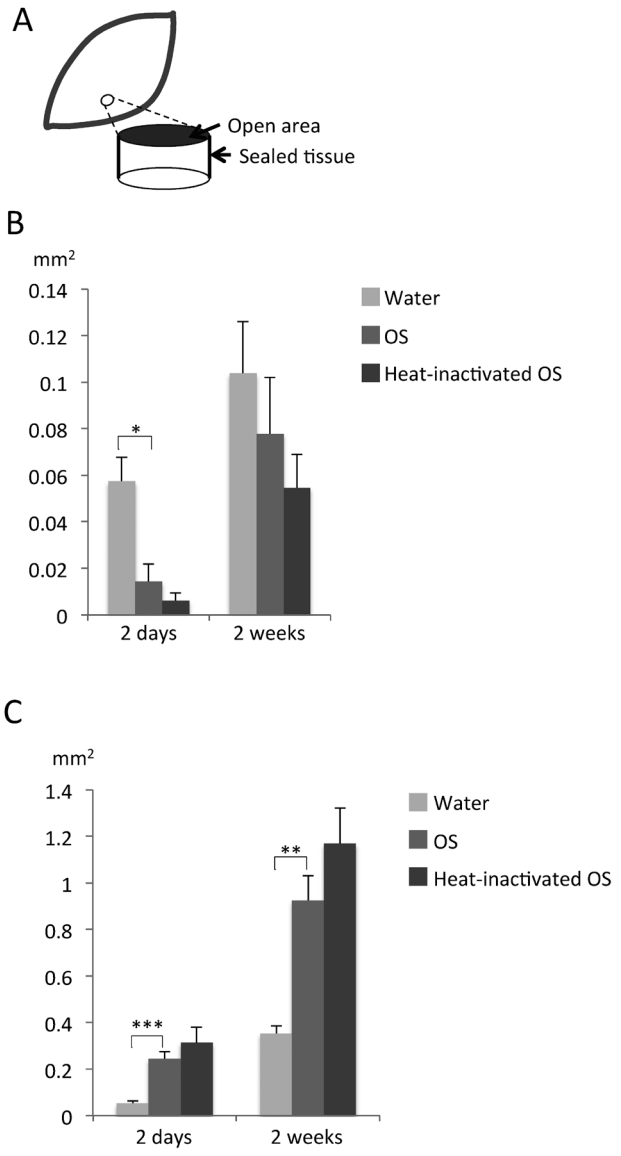

Figure 3. Quantification of open areas and loss of chlorophyll. A, Illustration of an open area surrounded by sealed tissue. B, Open areas measured at 2-day (left) and 2-week (right) intervals after wounding. C, Area of chlorophyll depletion. Note that the open areas of wound sites were subtracted from loss of chlorophyll area to estimate the area of chlorophyll depletion. Bars show SE ($n \geqq 7$). Asterisks indicate a significant differences between water control and OS treatments, based on Student's *t*-test (* $p<0.01$; ** $p<0.001$; *** $p<0.0001$).

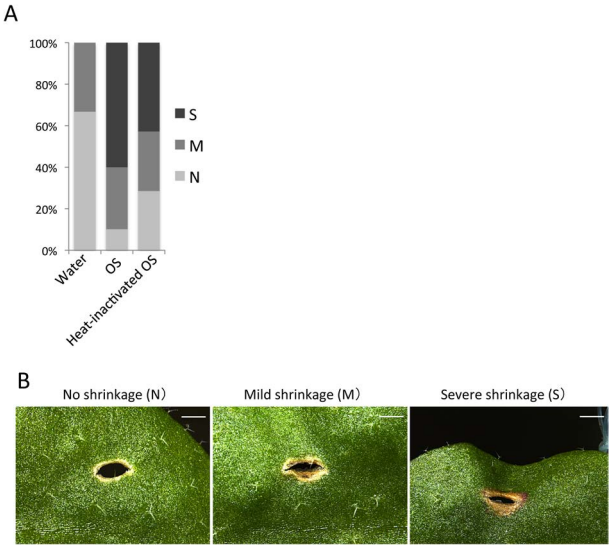

Figure 4. OS application and leaf shrinkage. A, Degree of leaf shrinkage after: OS; heat-inactivated OS; and water application on wounds. B, examples of: no shrinkage (N); mild shrinkage (M); and severe shrinkage (S) of leaves. Scale bar: 1 mm.

of wound inducible genes and inhibits plant defense mechanisms (Consales et al. 2012; Reymond et al. 2000; Savchenko and Dehesh 2013). This suppression results in increased larval growth and decreased HIPV emissions. As HIPVs attract natural enemies such as parasitoids, reducing its production benefits larvae (Consales et al. 2012).

Resource reallocation resulting from chewing insect attacks

Unrestricted cell senescence in *S. litura* OS treated wounds leads to rapid chlorosis. This disrupts the development of flat leaves.

S. litura wounds may appear to deplete the nutrients within leaves. Premature leaf senescence is often associated with herbivore stress as the insects are benefiting from the nutrition found within the host plant (Sandström et al. 2000; Steinbauer et al. 2014). Counterintuitively, our research suggests that leaf senescence may help limit pest infestation and increase plant survival. We deduced this by:

a) Hyper-senescence phenotype resulted in the inhibition of aphid growth (sucking insects) in Arabidopsis (Pegadaraju et al. 2005).

b) Kempema et al. (2007) demonstrated that expressions of senescence associated genes, such as *SENESCENCE ASSOCIATED GENE 13 (SAG13)*, *SAG12*, and *SAG21*, are upregulated in Arabidopsis plants when attacked by silverleaf whiteflies.

c) Systemic tissue of rice plants treated with the salivary gland extract of the brown planthopper, a chewing insect, shifted their global expression pattern towards nutrient remobilization. Of the genes whose expression were upregulated in response to salivary gland extract, up to 58% of them had secondary functions associated with senescence (Petrova and Smith 2014).

Senescence syndrome, which is a decline in photosynthesis, degradation of macromolecules, mobilization of nutrients, and ultimate cell death, is accompanied by upregulation of SAG genes (Guo and Gan 2005). A key area of future research will be to identify SAG genes whose expression is up or down regulated in response to *S. litura* OS application.

Conclusion

Our work demonstrated that (1) oral secretion from *S. litura* inhibits wound sealing; (2) the perception of *S. litura* oral secretion triggers senescence around the damaged leaf tissue. Based upon these observations, we hypothesize that *S. litura* induces reallocation of resources at and around tissue damage. The combination of these responses may contribute to the survival of plants when they are attacked by Lepidopteran insects.

In the future, control of tissue sealing and senescence procedures at wound sites by generating transgenic plants may be possible. Enhanced regulation of plant defense mechanisms would likely help plants become more resistant to Lepidopteran attacks.

Acknowledgements

We are grateful to the members of the Faculty of Life and Environmental Sciences at the University of Tsukuba for their encouragement. Special thanks are due to Prof. Yooichi Kainoh for his guidance, Aki Sugita for her research assistance and Hideko Ito for administrative support. Lastly, we thank my postdoctoral supervisor Prof. Nam Hai-Chua at the Rockefeller University for his ongoing mentorship and inspiration.

This research was supported, in part, by the Canon Foundation Grant "Pursuit of Ideal", Tomizawa Jun-ichi & Keiko Fund of Molecular Biology Society of Japan for Young Scientists, Tobe Maki Foundation, the Urakami Foundation for Food and Culture Promotion. It was also supported by the Japan Science and Technology Agency [PRESTO117665 & ERATOJPMJER1502], and the Japan Society for the Promotion of Science [Grants-in-Aid for Research Activity Start-up (22880008) and for Young Scientists (B) (23780040)].

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