Lipase Activity in Insect Oral Secretions Mediates Defense Responses in Arabidopsis[1][C][W][OA]

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How plants perceive herbivory is not yet well understood. We investigated early responses of the model plant Arabidopsis (*Arabidopsis thaliana*) to attack from the generalist grasshopper herbivore, *Schistocerca gregaria* (Caelifera). When compared with wounding alone, *S. gregaria* attack and the application of grasshopper oral secretions (GS) to puncture wounds elicited a rapid accumulation of various oxylipins, including 13-hydroperoxy octadecatrienoic acid, 12-oxo-phytodienoic acid (OPDA), jasmonic acid, and jasmonic acid-isoleucine. Additionally, GS increased cytosolic calcium levels, mitogen-activated protein kinase (MPK3 and MPK6) activity, and ethylene emission but not the accumulation of hydrogen peroxide. Although GS contain caeliferin A16:0, a putative elicitor of caeliferan herbivores, treatment with pure, synthetic caeliferin A16:0 did not induce any of the observed responses. With mutant plants, we demonstrate that the observed changes in oxylipin levels are independent of MPK3 and MPK6 activity but that MPK6 is important for the GS-induced ethylene release. Biochemical and pharmacological analyses revealed that the lipase activity of GS plays a central role in the GS-induced accumulation of oxylipins, especially OPDA, which could be fully mimicked by treating puncture wounds only with a lipase from *Rhizopus arrhizus*. GS elicitation increased the levels of OPDA-responsive transcripts. Because the oral secretions of most insects used to study herbivory-induced responses in Arabidopsis rapidly elicit similar accumulations of OPDA, we suggest that lipids containing OPDA (arabidopsides) play an important role in the activation of herbivory-induced responses.

Insect herbivores represent one of the major factors limiting plant growth and fitness. Plants, therefore, have evolved sophisticated strategies to detect and resist insect herbivory. Plants respond to specific compounds that indicate the presence of herbivores; these are classified as herbivory-associated molecular patterns (HAMPs; Felton and Tumlinson, 2008; Mithöfer and Boland, 2008). Well-known HAMPS are fatty acid derivatives present in herbivore saliva, such as fatty acid-amino acid conjugates, bruchins, and caeliferins, or benzyl cyanides present in oviposition fluids (Hilker and Meiners, 2006). Plants differ in their sensitivity to HAMPs, which is consistent with expectations of specific HAMP recognition systems (Schmelz et al., 2009). The amphiphilic nature of some elicitors can also destabilize plant membranes, leading to the activation of downstream responses (Maffei et al., 2004; Maischak et al., 2007). Plants can also sense the herbivory-mediated degradation or digestion products of plant material such as pectin, oligogalacturonide fragments, oligosaccharides, or fragments of the chloroplastic ATP synthase (Doares et al., 1995; Creelman and Mullet, 1997; Bergey et al., 1999; Schmelz et al., 2006).

Herbivore attack is transduced into specific defense responses by a sophisticated signaling system (for review, see Wu and Baldwin, 2010). This complex system, which consists of evolutionarily conserved defense pathways, is activated in various plant species by the perception of herbivory and HAMPS. One of these conserved responses is the rapid activation of the jasmonate (JA) biosynthetic pathway by wounding (for review, see Howe, 2004) or HAMP detection (Schmelz et al., 2009; for review, see Wu and Baldwin, 2010). JA biosynthesis starts in the chloroplast with the release of α-linolenic acid, which is converted to 13-hydroperoxy octadecatrienoic acid (13-HPOT) by 13-lipoxygenases and subsequently into cyclopentenone 12-oxo-phytodienoic acid (OPDA) by the activity of allene oxide synthase and allene oxide cyclase. After transfer to the peroxisome and the reduction of OPDA by OPDA reductase 3 (OPR3), followed by three cycles of decarboxylation, JA is formed (for review, see Wasternack, 2007). JA is subsequently conjugated with Ile to form jasmonoyl-Ile (JA-Ile) by JA:amino acid synthetase. JA-Ile is bound by the CORONATINE-INSENSITIVE (COI1)-JA-zim domain protein complex, leading to the degradation of negative transcriptional regulators and thus the activation of signal transduction (Thines et al., 2007; Chini et al., 2009; Sheard et al., 2010).

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Until now, it was not completely elucidated in Arabidopsis (*Arabidopsis thaliana*) which lipases are involved in the supply of the precursors for JA biosynthesis. In addition to DONGLE and DEFECTIVE IN ANTHER DEHISCENCE1, several other phospholipase A family lipases are known to participate in the formation of JA. However, no mutants defective in one or several of these lipases are completely compromised in JA formation under unelicited conditions or after wounding (Ishiguro et al., 2001; Hyun et al., 2008; Ellinger et al., 2010). It is also still unclear which role these lipases play in the formation of JA after herbivory.

In Arabidopsis, OPDA levels increase after wounding and herbivore attack (Reymond et al., 2000, 2004). OPDA is recognized not only as a precursor of JA biosynthesis but also as a signal in its own right. OPDA induces COI1-dependent and -independent transcriptional regulation (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008), changes in intracellular calcium levels (Walter et al., 2007), and alterations of the cellular redox status (Böttcher and Pollmann, 2009). Additionally, OPDA was shown to affect the growth of insect herbivores (Dabrowska et al., 2009). In Arabidopsis, OPDA is esterified in galactolipids, called arabidopsides (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2006; Böttcher and Weiler, 2007; Kourtchenko et al., 2007).

Another important phytohormone that is regulated by wounding and herbivory is ethylene (ET). ET interacts with JA responses (for review, see Adie et al., 2007) and tunes herbivory-related local and systemic responses (Rojo et al., 1999; Kahl et al., 2000). Aside from their inducibility, little is known about the regulation of herbivory-induced JA and ET biosynthesis. In tomato (*Solanum lycopersicum*) and in two tobacco species, *Nicotiana tabacum* and *Nicotiana attenuata*, mitogen-activated protein kinases (MAPKs) are known to be important regulators of wound- and herbivory-induced JA and ET levels (Kandoth et al., 2007; Seo et al., 2007; Wu et al., 2007). However, how MAPKs function in herbivory-induced JA and ET biosynthesis in the model plant Arabidopsis is not well understood, although indirect evidence points to the involvement of a MAPK phosphatase (Schweighofer et al., 2007). In addition to MAPK activation, Ca^{2+} signaling and reactive oxygen species (ROS) are induced by wounding and herbivory (Orozco-Cárdenas and Ryan, 1999; Maffei et al., 2004, 2006; Sagi et al., 2004; Leitner et al., 2005) and mediate herbivory-induced gene expression (Kanchiswamy et al., 2010).

It was recently claimed that the application of caeliferin A16:0 to wounds elicits JA and ET biosynthesis in Arabidopsis (Schmelz et al., 2009), suggesting that this HAMP may play a central role in grasshopper oral secretion (GS)-elicited responses. Here, we use the well-established genetic and molecular tools available for Arabidopsis to examine the regulatory machinery of GS-induced defense signaling. We show that, unlike wounding alone, applying GS to wounded Arabidopsis leaves highly promotes OPDA, JA, JA-Ile, and ET levels, MAPK activity (MPK3 and MPK6), and cytosolic calcium levels ($[Ca^{2+}]_{cyt}$). We show that the observed responses are independent of caeliferin A16:0 and that lipase activity in herbivore-derived oral secretions (OS) mediates the accumulation of oxylipin levels in Arabidopsis leaves. Our results suggest a new function for arabidopsides in the elicitation of herbivore-induced defense responses.

RESULTS

Grasshopper-Induced Defense Responses in Arabidopsis

The signal transduction machinery in Arabidopsis that is induced by wounding or herbivore feeding is well known (Reymond et al., 2000, 2004; Stintzi et al., 2001; Taki et al., 2005; Ehlting et al., 2008), but little is known about the influence of OS on Arabidopsis wound-induced defense responses. Recent studies indicate that herbivore-derived signals influence wound-induced transcript and phytohormone levels (Reymond et al., 2000, 2004; Schmelz et al., 2009).

We found that feeding of the locust *Schistocerca gregaria* induced JA accumulation and ET release in Arabidopsis (Supplemental Fig. S1). To distinguish wound- and *S. gregaria*-induced responses and to standardize herbivory elicitation, we mechanically wounded leaves and immediately treated the wounds with either water (W+W) or GS (W+GS) to investigate GS-specific defense responses (Baldwin, 1990). In addition to testing the OS of *S. gregaria*, we also tested OS from a population of native grasshoppers (*Chorthippus* spp.) collected near the Max Planck Institute for Chemical Ecology in Jena, Germany, for the ability of their secretions to alter wound-induced oxylipin levels in Arabidopsis leaves. We found that the OS of these closely related species induced similar responses in Arabidopsis leaves, suggesting that common mechanisms may underlie their elicitation. Because native grasshoppers were only temporarily available and we had a continuous supply of *S. gregaria* nymphs, we continued our analyses using the latter (see "Materials and Methods").

The various oxylipins we profiled after W+W and W+GS treatments are known to be part of the JA biosynthesis pathway. For 13-HPOT, significant increases were observed from 5 to 15 min and 2 h after leaves were treated with W+GS compared with W+W treatments (Fig. 1A). OPDA levels in leaves after W+GS treatment were highly elevated in comparison with levels after W+W treatment (Fig. 1A; Supplemental Fig. S2). From 5 min to 2 h after W+GS treatment, OPDA levels increased 8- to 10-fold compared with W+W treatments. JA and JA-Ile levels were slightly elevated within the first 15 min after W+GS treatment, while 2 h after W+GS induction, JA levels had more than doubled; compared with JA levels in plants treated by W+W, JA-Ile levels increased more than 1.5-fold (Fig.

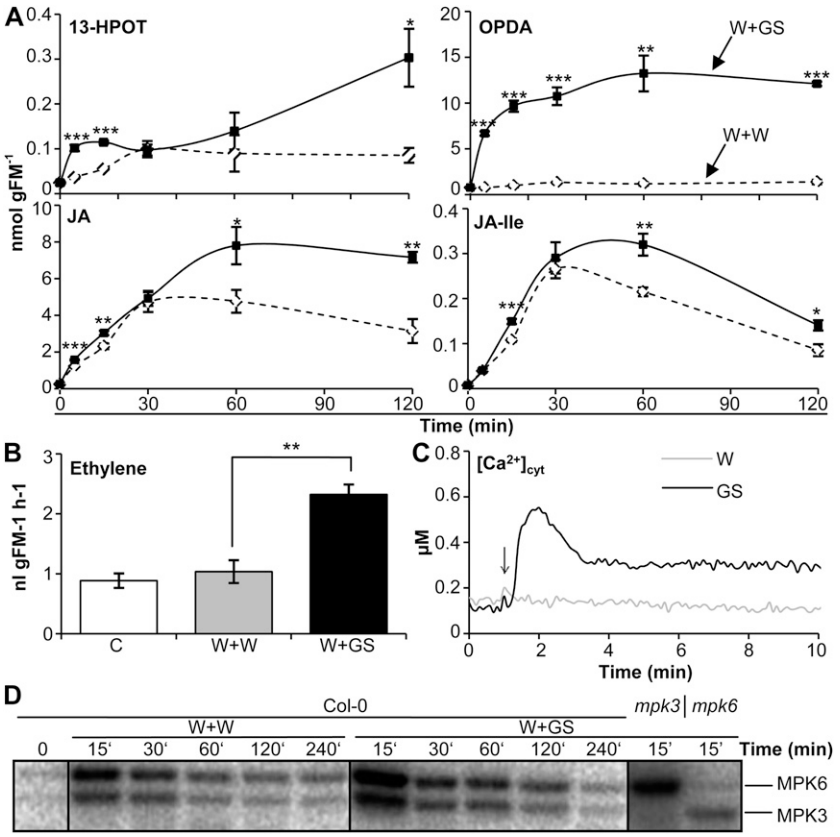

Figure 1. Responses induced by *S. gregaria* GS in Arabidopsis (Col-0). A, Mean levels ± SE ($n \geq 3$) of 13-HPOT, free OPDA, JA, and JA-Ile. Leaves were wounded and either water (W+W) or GS (W+GS) was applied, or leaves remained untreated (time point 0). Samples were harvested at the indicated time points. B, ET emissions ± SE ($n \geq 3$). Leaves were W+W or W+GS treated or untreated (C). C, $[Ca^{2+}]_{cyt}$. Leaves were treated with water (W) or GS. The application time point is indicated by the arrow. The graph shows representative curves. D, MPK3 and MPK6 activities. Leaves of Col-0 as well as *mpk3* and *mpk6* mutant plants were W+W or W+GS treated. Treated samples as well as untreated control samples were harvested at the indicated time points. Asterisks indicate significant differences between W+W and W+GS treatments at the same time point (independent-samples *t* test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). FM, Fresh mass.

1A). These data show that the application of GS to wounded leaves amplifies the levels of various oxylipins more than does wounding alone.

Another herbivory-induced phytohormone is ET (Kahl et al., 2000). Comparisons showed that W+GS-treated plants released more than 2-fold the amount of ET in the first 5 h after treatment than did W+W-treated plants (Fig. 1B). Similar changes in oxylipin and ET levels were observed in Arabidopsis accessions Columbia (Col-0), Landsberg *erecta*, and Wassilewskija (Supplemental Fig. S3).

Changes in $[Ca^{2+}]_{cyt}$ after herbivore feeding were reported recently in lima bean (*Phaseolus lunatus*; Maffei et al., 2004). Therefore, these were measured after the application of GS (Fig. 1C). GS induced an increase in $[Ca^{2+}]_{cyt}$ within a few seconds. The $[Ca^{2+}]_{cyt}$ rose from around 0.15 μM before treatment to around 0.6 μM within less than 1 min after GS application and then decreased again. After 1 to 2 min, $[Ca^{2+}]_{cyt}$ stabilized; the baseline was higher than before treatment (Fig. 1C).

It is known that MAPKs can be activated by HAMP recognition (Wu et al., 2007). Our data show that two MAPKs, MPK3 and MPK6, were more active after treatment by W+GS than by W+W (Fig. 1D). The most pronounced difference was observed 15 min after treatment. Taken together, these data clearly show that GS are potent elicitors of well-described defense responses in Arabidopsis.

GS-Induced Changes Are Not Dependent on Caeliferin A16:0

Caeliferins were reported to be active elicitors in *Schistocerca americana* OS in maize (*Zea mays*; Alborn et al., 2007). Additionally, caeliferin A16:0 was shown to elicit JA and ET levels in Arabidopsis (Schmelz et al., 2009). We also detected caeliferin A16:0 in the GS of *S. gregaria* (Supplemental Fig. S4, C and D). To test whether caeliferin A16:0 was responsible for the observed effects of GS on wounded leaves, authentic synthetic caeliferin A16:0 was tested for its ability to induce OPDA, JA, JA-Ile (Fig. 2A), and ET (Fig. 2B) as well as changes in $[Ca^{2+}]_{cyt}$ (Fig. 2C). Surprisingly, our attempts to induce defense responses by applying caeliferin A16:0 to Arabidopsis leaves were unsuccessful (Fig. 2). We increased the caeliferin concentration up to 2 mM without being able to induce any of the described responses. In contrast, applying GS significantly induced OPDA levels (Fig. 2A; *t* test, $P < 0.001$, GS versus buffer [B_1]) and JA accumulation (Fig. 2A; *t* test, $P < 0.05$, GS versus B_1) as well as the release of ET (Fig. 2B; *t* test, $P < 0.05$, GS versus B_1). The differences in JA and JA-Ile accumulation between treatments with B_1 or GS were less pronounced when compared with water as a control, most likely due to the slight activity of the phosphate buffer itself. The changes in $[Ca^{2+}]_{cyt}$ after GS application were also not mimicked by the application of caeliferin A16:0, which did not change $[Ca^{2+}]_{cyt}$ (Fig. 2C).

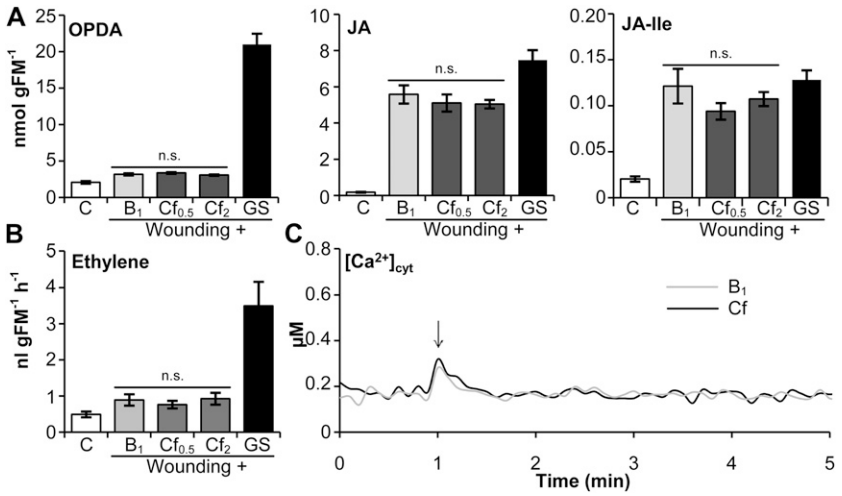

Figure 2. Caeliferin A16:0 does not mediate responses induced by *S. gregaria* GS in Arabidopsis (Col-0). A, Mean levels ± SE ($n \geq 4$) of free OPDA, JA, and JA-Ile. Leaves were wounded and 50 mM sodium phosphate buffer, pH 8 (B_1), 0.5 mM caeliferin A16:0 ($Cf_{0.5}$), 2 mM caeliferin A16:0 (Cf_2), or GS was applied, or leaves remained untreated (C). Samples were harvested after 2 h. B, ET emissions ± SE ($n = 4$). Either leaves were wounded and B_1, $Cf_{0.5}$, Cf_2, or GS was applied, or leaves were kept untreated (C). C, $[Ca^{2+}]_{cyt}$. Leaves were treated with B_1 or caeliferin A16:0 (Cf). The application time point is indicated by the arrow. The graph shows representative curves. For statistical analysis, see text (independent-samples *t* test: n.s. = no significant difference [$P > 0.05$]) FM, Fresh mass.

MPK3/6 Function in GS-Induced Responses in Arabidopsis

The homologs of Arabidopsis MPK6 and MPK3 in tomato and *N. attenuata* are known to regulate herbivory-induced oxylipin and ET accumulation (Kandoth et al., 2007; Wu et al., 2007; Kallenbach et al., 2010). We used *mpk3* and *mpk6* mutant plants to analyze their influence on these GS-induced phytohormones. Increased levels of 13-HPOT, OPDA, JA, and JA-Ile were observed in leaves 120 min after W+W and W+GS treatment (Fig. 3A). In contrast to their homologs in *N. tabacum* and *N. attenuata*, *mpk3* and *mpk6* mutant plants did not exhibit significant reductions in their levels of 13-HPOT, OPDA, JA, and JA-Ile levels after elicitation when compared with wild-type plants. Interestingly, MPK6-impaired plants showed even slightly elevated JA and OPDA levels (Fig. 3A; *t* test, $P < 0.05$, wild-type versus *mpk6* plants after W+GS treatment).

SIPK is known to participate in HAMP-induced ET release in *N. attenuata*, and MPK3 and MPK6 are important for pathogen-induced ET release in Arabidopsis (Wu et al., 2007; Han et al., 2010). We tested whether MPK6, the homolog of SIPK, is necessary for W+GS-induced ET release in Arabidopsis. After W+GS treatment, ET emissions in *mpk6* plants were reduced about 30% compared with emissions in wild-type plants (Col-0; Fig. 3B; *t* test, $P < 0.01$, wild-type versus *mpk6* plants after W+GS treatment). In contrast, the *mpk3* mutant plants showed a slight but not significant tendency to release more ET after W+GS treatment (Fig. 3B). W+W-induced ET levels in these mutants were not impaired. These data demonstrate that GS-induced oxylipin accumulation is not mediated by MPK3 and MPK6 alone in Arabidopsis; however, GS-induced ET levels are partially MPK6 dependent.

ROS Are Not Induced by GS in Arabidopsis

The production of ROS in response to herbivory is reported for *Medicago truncatula*, lima bean, and *N. attenuata* (Leitner et al., 2005; Maffei et al., 2006; Diezel et al., 2009). To assess whether hydrogen peroxide (H_2O_2) is induced by GS, 3,3-diaminobenzidine (DAB) staining was performed (Fig. 4A). The intensity of the dark brown color correlates with the abundance of a DAB-derived polymer produced in the presence of H_2O_2. For this reason, the color can be correlated with the amount of H_2O_2. H_2O_2 accumulation was examined at 1, 2, 4, 6, and 8 h after W+W and W+GS treatments. DAB staining was detected directly at the wound sites and increased with time (Fig. 4A). In leaves treated with W+W or with W+GS, there was no difference in DAB staining intensities.

Herbivory-derived H_2O_2 was shown to reduce JA accumulation and ET emissions in *N. attenuata* (Diezel et al., 2009). The *respiratory burst oxidase homolog* (*rboh*) mutants are deficient in NADPH oxidases, which are proposed to be responsible for wound- and herbivory-induced H_2O_2 production (Orozco-Cárdenas and Ryan, 1999; Orozco-Cárdenas et al., 2001; Sagi et al., 2004). For that reason, the responses of *rbohd* mutant plants and wild-type plants, both types treated with W+GS, were compared. Mutant plants were examined with respect to their accumulation of H_2O_2 (Fig. 4B), JA (Fig. 4C), and ET (Fig. 4D). The H_2O_2 accumulation after W+GS was strongly reduced in the *rbohd* mutant. The *rbohd* mutant plants tended to accumulate more JA after W+GS treatment. Interestingly, ET emissions after W+GS were more than 50% higher in the mutants than in the wild-type plants (Fig. 4D; ANOVA, $P < 0.01$, W+GS-treated wild type versus the mutant line). These results demonstrate that although H_2O_2 is not particularly induced by GS, wound-induced H_2O_2 levels negatively affect GS-induced responses.

GS-Induced Oxylipin Responses Are Mediated by Insect Lipases

To narrow the spectrum of possible effectors, fractionated GS was used for induction experiments. Less than 10 kD is depleted of large compounds like proteins

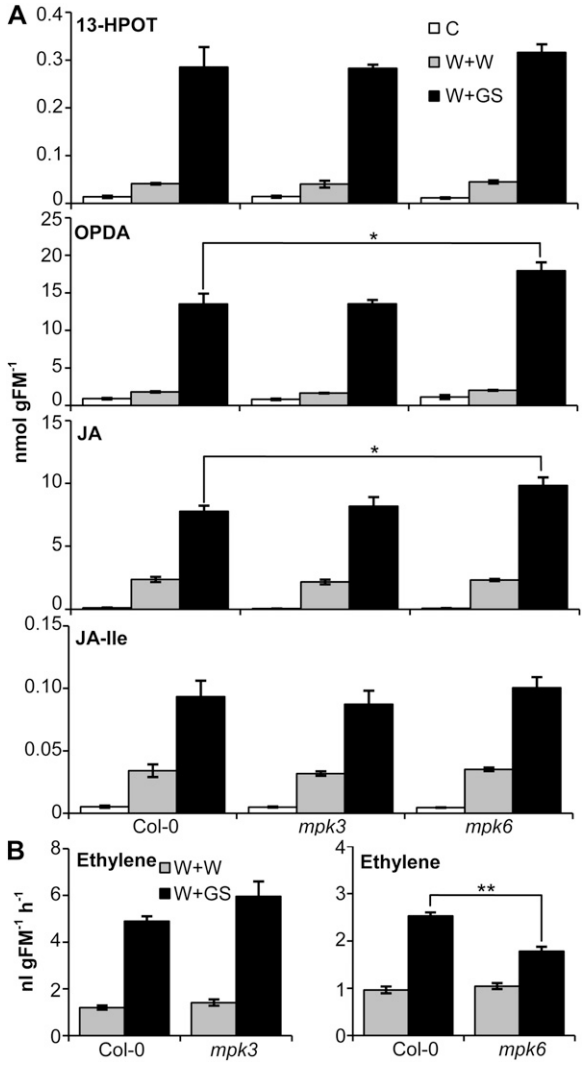

Figure 3. Role of MPK3 and MPK6 in responses induced by *S. gregaria* GS in Arabidopsis. A, Mean levels ± SE ($n = 5$) of 13-HPOT, free OPDA, JA, and JA-Ile. Leaves of Col-0 plants as well as *mpk3* and *mpk6* mutants were wounded and water (W+W) or GS (W+GS) was applied, or leaves remained untreated (C). Samples were harvested 2 h after treatment. B, ET emissions ± SE ($n \geq 3$). Leaves of Col-0 plants as well as *mpk3* and *mpk6* mutant plants were W+W or W+GS treated. Asterisks indicate significant differences between wild-type and mutant plants for the same treatment (independent-samples *t* test: * $P < 0.05$, ** $P < 0.01$). FM, Fresh mass.

(data not shown). The application of the less than-10-kD fraction to wounded leaves did not significantly increase the accumulation of OPDA and JA (Fig. 5A). In contrast, the greater than-10-kD fraction induced a strong increase in these phytohormones, similar to GS (Fig. 5A; *t* test, $P < 0.05$, greater than-10-kD GS fraction or GS application versus water application). This indicates that the OPDA and JA accumulation is likely mediated by large molecules. The application of both the greater than-10-kD GS fraction and GS to wounded leaves significantly induced JA-Ile accumulation (Fig. 5A; *t* test, $P < 0.05$, greater than-10-kD GS fraction or GS application versus water application). Interestingly, the JA-Ile levels are somewhat induced by the less than-10-kD GS fraction as well (Fig. 5A). Additionally, GS was boiled or treated with isopropanol in combination with heat to inhibit enzyme activities. The treated GS induced no increase in OPDA levels, in contrast to untreated GS (Fig. 5B; *t* test, $P < 0.01$, W+GS versus W+W), indicating that the elicitor of OPDA accumulation is heat sensitive. We next tested if the high release of OPDA observed in GS-treated leaves depends on enzymatic activity in the leaves. Importantly, GS but not boiled or heat- and isopropanol-treated GS were able to increase levels of free OPDA from heat- and isopropanol-treated leaf tissue (Fig. 5C; *t* test, $P < 0.01$, GS application versus water application). For boiled leaf tissue, similar results were obtained (data not shown). These data imply that enzymatic activity in GS but not leaf tissue is mediating the GS-induced accumulation of OPDA in Arabidopsis.

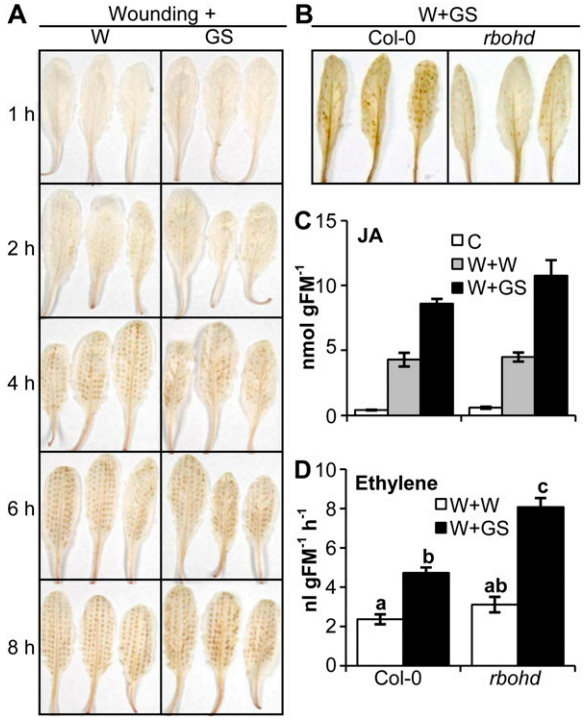

Figure 4. Role of wound-induced H_2O_2 for JA and ET levels in Arabidopsis induced by *S. gregaria* GS. A, Representative H_2O_2 accumulation. Leaves of Col-0 plants preincubated with DAB were wounded and water (W+W) or GS (W+GS) was applied. At the indicated time points, the reaction was stopped. B, Representative H_2O_2 accumulation. Leaves of Col-0 plants as well as *rbohd* mutant plants preincubated with DAB were W+GS treated. After 4 h, the reaction was stopped. C, Mean levels ± SE ($n \geq 3$) of JA. Leaves of Col-0 plants as well as *rbohd* mutant plants were either W+W or W+GS treated, or leaves remained untreated (C). Samples were harvested after 2 h. D, ET emissions ± SE ($n \geq 3$). Leaves of Col-0 plants as well as *rbohd* mutant plants were treated with W+W or W+GS. Different letters indicate significant differences among treatments (ANOVA: $P < 0.05$, Tukey's honestly significant difference test). FM, Fresh mass. [See online article for color version of this figure.]

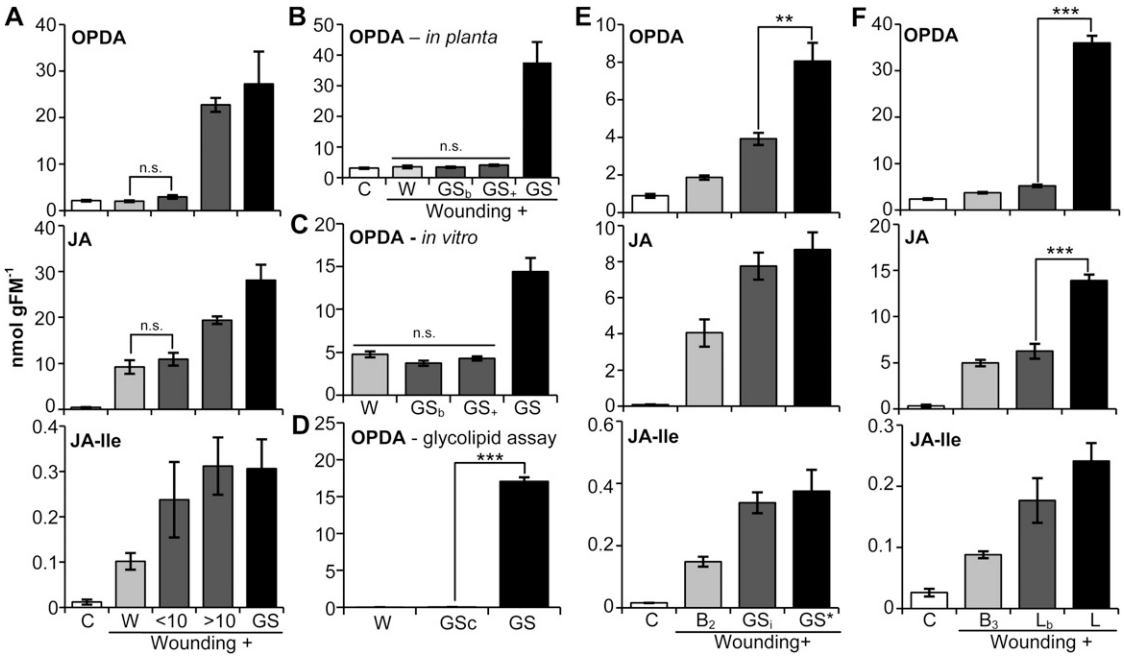

Figure 5. Lipase-dependent oxylipin accumulation in Arabidopsis (Col-0). A, Mean levels ± SE ($n = 4$) of free OPDA, JA, and JA-Ile. Leaves were wounded and water (W), the less than-10-kD *S. gregaria* GS fraction (<10), the greater than-10-kD GS fraction (>10), or GS was applied, or leaves remained untreated (C). Samples were harvested 2 h after treatment. B, In planta OPDA accumulation ± SE ($n = 4$). Wounded leaves were treated with W, boiled GS (GS_b), isopropanol- and heat-treated GS (GS_+), or GS. Samples were harvested after 2 h. C, In vitro OPDA release ± SE ($n \geq 3$). Isopropanol- and heat-treated plant material was incubated with W, GS_b, GS_+, or GS for 10 min. D, OPDA release from glycolipids ± SE ($n \geq 3$). Glycolipid extract from Arabidopsis (Col-0) was incubated with W or GS or GS was added without incubation time (GSc). E, Mean levels ± SE ($n = 5$) of free OPDA, JA, and JA-Ile. Leaves were wounded and either 2.5% (v/v) ethanol (B_2) with 1 mM orlistat-treated GS (GS_i) or with 2.5% (v/v) ethanol-incubated GS (GS*) was applied, or leaves remained untreated (C). Samples were harvested 2 h after treatment. F, Mean levels ± SE ($n \geq 3$) of free OPDA, JA, and JA-Ile. The wounded leaves were treated with 0.1 M Tris-HCl, pH 7.5 (B_3), boiled fungal lipase (L_b), or fungal lipase (L). Samples were harvested after 2 h. Asterisks indicate significant differences between the indicated treatments (independent-samples *t* test: n.s. = no significant difference [$P > 0.05$], ** $P < 0.01$, *** $P < 0.001$). FM, Fresh mass.

Compared with other plant species, Arabidopsis possesses high levels of esterified OPDA bound to lipids; these are referred to as arabidopsides (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2006; Böttcher and Weiler, 2007; Kourtchenko et al., 2007). Our data indicate that enzymatic activity in GS leads to the high accumulation of OPDA after contact with wounded leaf tissues. To test if lipase activity of GS can release OPDA out of arabidopsides, we performed lipase activity assays using glycolipid extracts of Arabidopsis (Col-0) leaves. Figure 5D shows that glycolipids without GS or glycolipids and GS together but without incubation time yielded only marginal amounts of free OPDA, whereas glycolipids and GS incubated together yielded high levels of free OPDA (Fig. 5D; *t* test, $P < 0.001$, GS without incubation time versus GS). These data demonstrate that lipase activity in GS can hydrolyze OPDA out of glycolipids in Arabidopsis.

To analyze if the GS proteome contains lipase-like proteins, we used two-dimensional PAGE (2-D PAGE) and de novo sequencing of protein spots. As expected for a digestive fluid, two of the protein spots we identified had high homology to insect lipases (lipase, Q177T4, *Aedes aegypti*; similar to lipase, XM_001948948.1, *Acyrthosiphon pisum*; Supplemental Table S1). To determine if lipase activity in GS accounts for OPDA accumulation in wounded Arabidopsis leaves, we incubated GS with the lipase inhibitor orlistat prior to treatment (Fig. 5E). Compared with untreated GS, the orlistat-treated GS induced 50% less OPDA in wounded Arabidopsis leaves (Fig. 5E; *t* test, $P < 0.01$, W+GS_i versus W+GS* [see Fig. 5 legend for definitions]), whereas orlistat added to GS directly before application induced no changes (Supplemental Fig. S5; *t* test, $P = 0.740$, $GS_{(i)}$ versus GS*). Because results from orlistat addition to the GS with and without incubation time differed significantly (Supplemental Fig. S5; *t* test, $P < 0.05$, $GS_{(i)}$ versus GS_i), it can be assumed that orlistat-induced changes are mediated by altered GS properties and not by the interaction between orlistat and leaf tissue. However, other than reduced OPDA levels, only slight decreases in JA and JA-Ile levels were observed after lipase inhibitor treatment of the GS.

To test whether an externally added lipase not only contributes to but could also be sufficient to induce the accumulation of JAs on wounded leaves, plants were treated with a lipase solution (Fig. 5F; Supplemental Fig. S6). The sn1-specific lipase from the fungus *Rhizopus arrhizus* was shown to release OPDA from arabidopsides (Stelmach et al., 2001). The application of *R. arrhizus* lipase solution (L) to wounded leaves (W+L) highly increased the levels of 13-HPOT, OPDA, JA, and JA-Ile in comparison with the application of the corresponding buffer ($W+B_3$) 2 h after treatment (Fig. 5F; Supplemental Fig. S6; *t* test, $P < 0.01$, W+L versus $W+B_3$). Levels of OPDA and JA were significantly reduced after application of the heat-treated lipase solution (L_b) than after application of untreated lipase solution (Fig. 5F; *t* test, $P < 0.001$, $W+L_b$ versus W+L). The heat-treated lipase solution probably maintained some residual activity leading to slightly, but nevertheless significantly elevated, levels of OPDA (Fig. 5F; *t* test, $P < 0.01$, $W+L_b$ versus $W+B_3$); JA-Ile levels tended to be higher in leaves treated with $W+L_b$ than in leaves treated with $W+B_3$ (Fig. 5F; *t* test, $P \leq 0.07$, $W+L_b$ versus $W+B_3$). These data provide evidence for an important role of lipase activity in GS-mediated oxylipin accumulation in Arabidopsis leaves. To test whether GS and L are only able to induce responses on the wounding side, GS and L were applied to the surface of unwounded leaves. Supplemental Figure S7 shows that GS was not able to induce JA and JA-Ile levels when only applied to the leaf surface (*t* test, $P > 0.9$, GS versus W), whereas OPDA levels were marginally but significantly increased (*t* test, $P < 0.01$, GS versus W). The L, which is supposed to possess higher lipase activity than GS, also slightly induced OPDA levels as well as JA and JA-Ile levels (Supplemental Fig. S7; *t* test, $P < 0.05$, L versus B_3). GS- andL-mediated changes in the OPDA levels were nearly nine times higher in combination with wounding when compared with application to unwounded leaf surfaces. Also, L-mediated changes in JA and JA-Ile levels were approximately five times higher when applied to wounded leaves compared with unwounded leaves. It is possible that the accumulation of oxylipins in response to GS and L application to the unwounded leaf surface is a result of small wounding events produced by the treatment or harvesting procedure. These data demonstrate that GS-mediated accumulation of oxylipins requires contact of GS with wounded leaf tissue.

Lipase-Independent Defense Elicitations

We tested if lipase-independent elicitors in GS mediate defense responses in Arabidopsis. Application of the less than-10-kD fraction of GS, which was shown to not induce lipase-mediated responses (Fig. 5A), induced similar $[Ca^{2+}]_{cyt}$ when compared with the greater than-10-kD fraction (Fig. 6A). Additionally, GS-induced $[Ca^{2+}]_{cyt}$ changes could not be abolished by treating the GS with heat before use (Fig. 6A). However, $[Ca^{2+}]_{cyt}$ measurements after the application of *R. arrhizus* lipase revealed that this lipase is also sufficient to induce changes in $[Ca^{2+}]_{cyt}$ (Fig. 6A). Regardless, the signature of the $[Ca^{2+}]_{cyt}$ after fungal lipase treatment showed a different pattern than after GS treatment. The first peak, which is normally present after GS application, was missing, suggesting that lipase activity is not the main elicitor of $[Ca^{2+}]_{cyt}$. There are some hints that the amphiphilic nature of some elicitors can change $[Ca^{2+}]_{cyt}$ (Maffei et al., 2004; Maischak et al., 2007). However, the $[Ca^{2+}]_{cyt}$ signature after Triton X-100 treatments showed a steady increase of $[Ca^{2+}]_{cyt}$ over

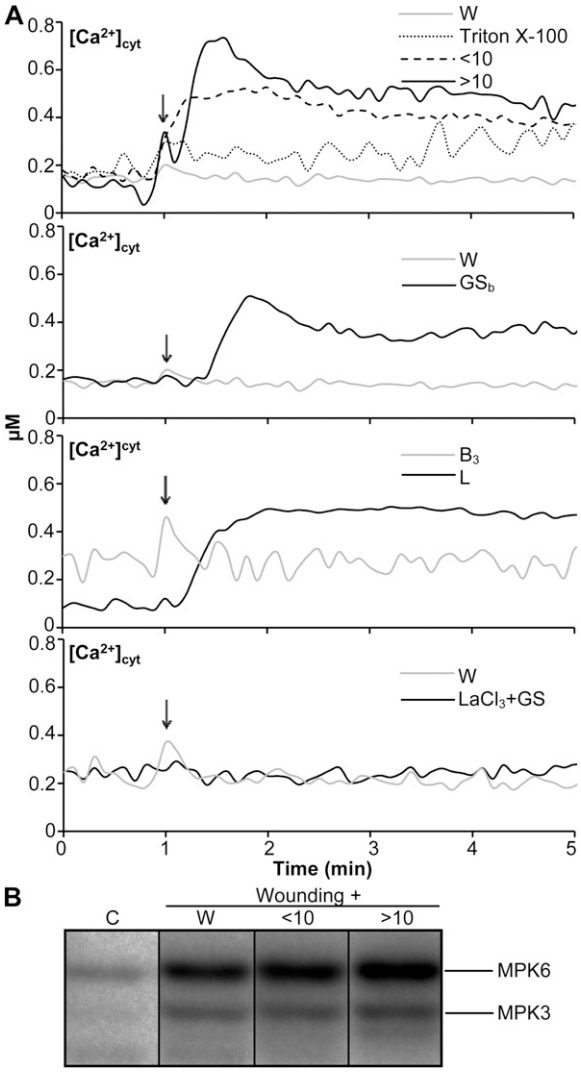

Figure 6. Lipase-independent defense responses in Arabidopsis. A, $[Ca^{2+}]_{cyt}$. Leaves were treated with water (W), Triton X-100, the less than-10-kD GS fraction (<10), the greater than-10-kD GS fraction (>10), boiled GS (GS_b), *R. arrhizus* lipase (L), or the corresponding buffer (B_3). Additionally, leaves were preincubated in 1 mM $LaCl_3$ for 1 h and then treated with GS ($LaCl_3$+GS). The test substance application is indicated by the arrows. The graphs show representative curves. B, MPK3 and MPK6 activities. Leaves of Col-0 plants were wounded and water, the less than-10-kD GS fraction, or the greater than-10-kD GS fraction was applied.

time and lacked the early peak, demonstrating that detergent effects alone likely do not elicit the immediate GS-induced $[Ca^{2+}]_{cyt}$ increase. Furthermore, the GS-induced $[Ca^{2+}]_{cyt}$ changes could be abolished by pretreatment with the Ca^{2+} channel inhibitor $LaCl_3$, suggesting that an increase in $[Ca^{2+}]_{cyt}$ is mediated through Ca^{2+} channel-driven Ca^{2+} import (Fig. 6A).

Wounding and application of the less than-10-kD GS fraction induced higher MAPK activity when compared with W+W treatments, although the relative increase is less pronounced when compared with application of the greater than-10-kD fraction (Fig. 6B). These data demonstrate that, in parallel to GS inherent lipase activity, other unknown elicitors of GS also induced defense responses in Arabidopsis leaves. Possible elicitors could be fragments of plant cells, other defense-inducing enzymes, or herbivore-derived elicitors (for review, see Heil, 2009). Future research will focus on the analyses of additional elicitors in GS.

GS-Induced Changes in OPDA-Regulated Transcripts

OPDA was shown to specifically change the transcript abundance of various genes in Arabidopsis (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008). After finding high levels of OPDA induced by GS, we tested if these correlate with the activation of OPDA-related gene expression. We analyzed the expression of two marker genes, glutathione *S*-transferase 1 (*GST1*) and *OPR1* (Fig. 7), which were shown to be regulated by OPDA (Stintzi et al., 2001). *GST1* showed a 2.5- to 3-fold increase in transcript abundance 4 h after treatment with W+GS compared with W+W. After 7 h, transcript levels in W+GS-treated leaves were more than 4.5-fold higher than those in W+W-treated leaves. *OPR1* showed a 1.5- to 2.5-fold increase in transcript abundance after GS application at 4 and 7 h after treatments, respectively. Interestingly, we observed that only the OPDA-inducing greater than-10-kD GS fraction was able to increase the *GST1* and *OPR1* transcript abundance, whereas the less than-10-kD fraction, which was not able to induce OPDA levels, did not mediate similar inductions (Fig. 7). These data show that the induction of OPDA-mediated gene expression is part of the Arabidopsis response to GS.

High OPDA Accumulation upon Herbivory Is a Common Response in Arabidopsis

Most studies analyzing herbivory-induced responses in Arabidopsis elicit plants with lepidopteran insects (Reymond et al., 2000, 2004; Stotz et al., 2000; De Vos et al., 2005; Mewis et al., 2006; Ehlting et al., 2008). To test if lepidopteran insect species show similar oxylipin patterns, we applied the OS of *Heliothis virescence*, *Spodoptera littoralis*, *Spodoptera frugiperda*, *Manduca sexta*, *Pieris rapae*, and *Heliothis subflexa* to wounded Arabidopsis leaves (Fig. 8). The data clearly show that all OS are able to quickly induce elevated levels of OPDA in the wounded leaves of Arabidopsis. Additionally, JA and JA-Ile levels were elevated after treatment with wounding and OS application compared with W+W. Unlike JA levels, JA-Ile levels varied according to the application of the different OS. Additionally, the data show that the food provided to the insects had only a minor influence on the oxylipin-eliciting properties of the tested OS. These results demonstrate that the high OPDA release upon herbivory is induced by unrelated insect herbivore species; in other words, OPDA accumulation appears to be a general response of Arabidopsis plants to herbivory.

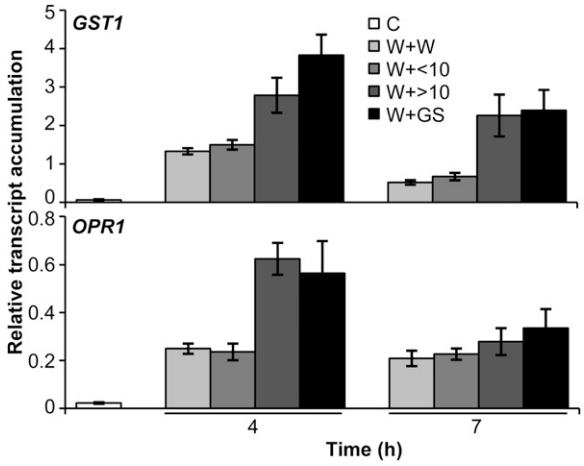

Figure 7. *S. gregaria* GS induce OPDA-mediated gene expression. Relative abundance ± SE (n = 5) of *GST1* and *OPR1* transcripts is shown. Leaves of Arabidopsis plants (Wassilewskija) were wounded and water (W+W), the less than-10-kD GS fraction (W+<10), the greater than-10-kD GS fraction (W+>10), or GS (W+GS) was applied, or leaves remained untreated (C). Samples were harvested at the indicated time points. After RNA preparation and cDNA synthesis, the relative transcript abundance in correlation with the eIF4A1 transcript levels was determined by qPCR.

DISCUSSION

Plants have evolved sophisticated mechanisms to distinguish herbivory from mechanical wounding. Insect herbivore attack differentially regulates phytohormone levels, transcriptional responses, and defense metabolites in Arabidopsis plants, and its effect differs from that of mechanical wounding alone (Reymond et al., 2000, 2004; Stintzi et al., 2001; Mewis et al., 2006; Ehlting et al., 2008; Schmelz et al., 2009). However, when compared with what we know about other plant species, our knowledge about the molecular events leading to the recognition of herbivory and transduction to specific responses in the model plant Arabidopsis is limited. Here, we show that grasshopper elicitation differentially affects early wound-induced molecular events, such as oxylipin release, ET accumulation, MAPK activity, and intracellular Ca^{2+} levels, in Arabidopsis (Fig. 1). Our data show, to our knowledge for the first time, that lipase

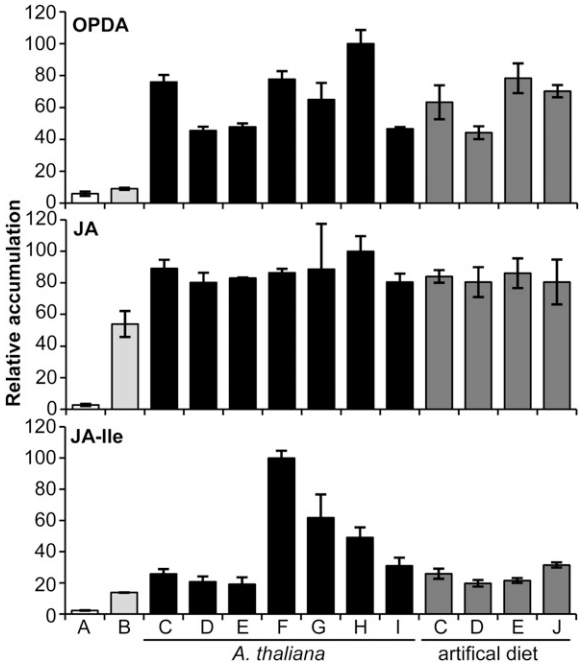

Figure 8. Oral secretions of various insects induce OPDA, JA, and JA-Ile accumulation in Arabidopsis (Col-0). Relative abundance ± SE (n = 4) of OPDA, JA, and JA-Ile is shown. Leaves were wounded and treated with water (B) or regurgitate from *S. gregaria* (C), *H. virescence* (D), *S. littoralis* (E), *S. frugiperda* (F), *M. sexta* (G), a population of native grasshoppers (*Chorthippus* spp.; H), *P. rapae* (I), or *H. subflexa* (J), or leaves remained untreated (A). Insects were fed either Arabidopsis or artificial diet as indicated. Samples were harvested after 2 h.

activity in insect OS can directly elicit changes in oxylipins, especially OPDA. Thus, we provide a new mechanism for how insect feeding can alter the levels of herbivory-induced defense metabolites.

Oxylipin Accumulation Induced by GS-Inherent Lipase Activity

Arabidopsis contains OPDA esterified to membrane lipids, classified as arabidopsides (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2006; Böttcher and Weiler, 2007; Kourtchenko et al., 2007). Arabidopsides were described to possess senescence-promoting (Hisamatsu et al., 2006), growth-inhibiting (Nakajyo et al., 2006), antimicrobial (Andersson et al., 2006), and antifungal (Stelmach et al., 2001; Kourtchenko et al., 2007) properties. It was also proposed that arabidopsides function as storage molecules for OPDA and serve as substrates for JA biosynthesis (Kourtchenko et al., 2007). It was already shown that arabidopside levels are highly induced by wounding (Stelmach et al., 2001; Buseman et al., 2006; Böttcher and Weiler, 2007). Dabrowska et al. (2009) proposed that OPDA has a direct negative influence on herbivores; accordingly, arabidopsides may act as direct defense compounds against herbivores. Previous work showed that damage from feeding *P. rapae* in contrast to wounding elicits higher levels of free OPDA than of JA in Arabidopsis (Reymond et al., 2000, 2004; Stintzi et al., 2001). The same was observed after damage from grasshopper feeding (Supplemental Fig. S1A). Our data show that damage from grasshopper feeding and treatment with GS on wounded leaves quickly elicits levels of free OPDA in Arabidopsis (Fig. 1A; Supplemental Fig. S1A) and that this increase depends on lipase activity in GS but not in the plant (Fig. 5). High OPDA levels are found mainly at the site of wounding (Supplemental Fig. S8), and the identification of lipase-like proteins in GS further supports our conclusion that insect lipases are among the main elicitors of OPDA released in the wounded tissues of Arabidopsis. Since we found that the OS of all insects we tested elicit high levels of free OPDA in Arabidopsis leaves (Fig. 8), we hypothesize that OPDA release mediated by insect lipases might be a general response to herbivory in Arabidopsis. Accordingly, OPDA accumulation induced by the OS from the lepidopteran *M. sexta* could also be significantly reduced by pretreatment with the lipase inhibitor orlistat (Supplemental Fig. S9). Elicitations with the purified insect lipases that mediate these reactions will help to further clarify the role of these lipases in herbivory-induced OPDA release. Our data add an additional mechanism to those already known from the literature on how plants recognize the herbivory-mediated degradation of their metabolites (for review, see Heil, 2009).

OPDA was shown to elicit the transcript levels of many JA-responsive and non-JA-responsive genes in Arabidopsis in a COI1-dependent or COI1-independent manner (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008). We demonstrate that two of these genes (*OPR1* and *GST1*) were significantly induced by the OPDA-releasing fraction of GS (Fig. 7), which indicates that in Arabidopsis, OPDA-related signaling is induced by grasshopper feeding. Although the function and mechanism of OPDA signaling in defense responses to herbivores are not fully understood, we hypothesize that OPDA-mediated responses in Arabidopsis might help distinguish mechanical wounding from herbivore attack. In our proposed model, lipases from insect OS release OPDA from arabidopsides in damaged leaf tissue, leading to the activation of OPDA-related signaling (Fig. 9). It is tempting to speculate that high levels of the signaling molecule OPDA in arabidopsides might have evolved as a way of rapidly detecting herbivory in Arabidopsis and related species. Stumpe et al. (2010) showed that OPDA, but not JA, is present in the moss *Physcomitrella patens*, indicating the presence of an evolutionarily conserved function of OPDA in plants. Future studies will investigate whether OPDA orchestrates herbivory-induced defense responses in Arabidopsis.

Our data show that W+GS treatment induced higher levels of JA and JA-Ile than did W+W treatment. OPDA, released by insect feeding, might enter surrounding intact cells to serve as a precursor for JA

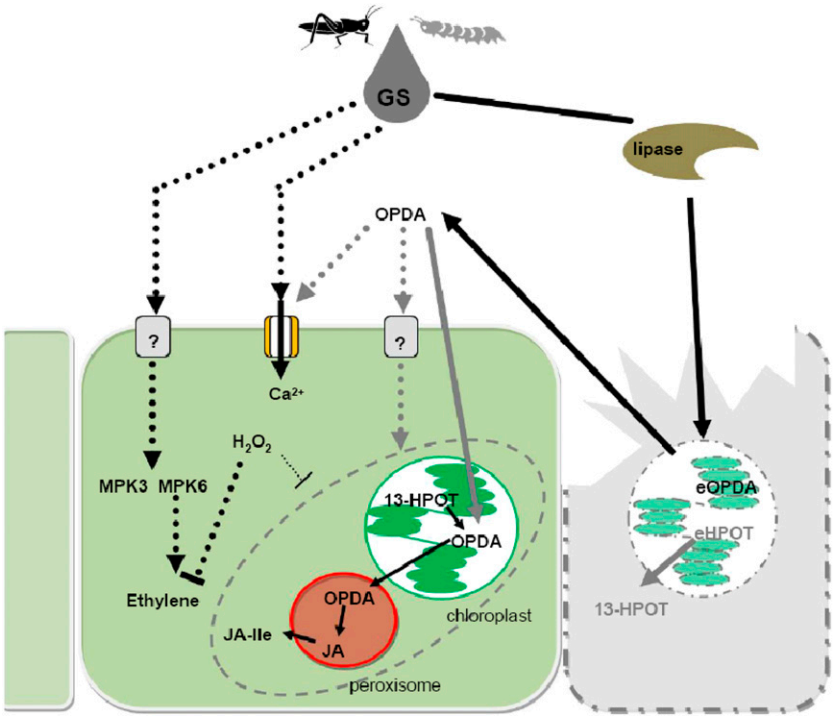

Figure 9. Summary of *S. gregaria* GS-induced responses in Arabidopsis. The model shows herbivore-damaged cells (gray) and intact cells (green). GS lipase activity releases esterified OPDA from lipids (eOPDA) and possibly also 13-HPOT (eHPOT). OPDA may directly elicit signaling events or supply substrates for JA biosynthesis in adjacent cells. GS activates MPK3 and MPK6 through an unknown mechanism. MPK6 activity positively affects ET release. GS triggers a Ca^{2+} influx in the cytosol mediated by Ca^{2+} channels. The released OPDA might contribute to the observed Ca^{2+} response. Wound-induced H_2O_2 levels slightly suppress JA and ET accumulation. Signaling events are indicated by dotted arrows, and fluxes and metabolic processes are indicated by solid arrows. Hypothetical events are shaded gray instead of black.

biosynthesis (Miersch and Wasternack, 2000). It could also be possible that extracellular OPDA serves only as a signal to induce responses that include changes in $[Ca^{2+}]$ (Walter et al., 2007) or gene expression (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008); these then may elevate levels of JA and JA-Ile biosynthesis. The JA levels were not as much affected by pretreatment of GS with orlistat than the OPDA levels, perhaps because the released OPDA is still sufficient for JA biosynthesis (Fig. 5D). Despite higher levels of JAs, slightly higher levels of both esterified and free OPDA were observed after W+GS treatment in comparison with W+W treatment (Supplemental Fig. S2), suggesting elevated de novo biosynthesis of OPDA. It is tempting to speculate that wound-induced accumulation of esterified OPDA is involved in priming plant defense responses against herbivores. Interestingly, treatment with either GS or fungal lipase was sufficient to elevate levels of the potential OPDA precursor 13-HPOT (Fig. 1A; Supplemental Fig. S6). The presence of membrane-bound 13-HPOT in Arabidopsis was proposed already (Stenzel et al., 2003; Andersson et al., 2006). It remains unclear whether the measured increase in 13-HPOT is derived from de novo synthesis by radical- or lipoxygenase-dependent mechanisms or is released from lipid-bound forms. Since 13-HPOT participates in various biochemical pathways, such as the biosynthesis of JAs and of phytoprostanes, and was found to have signaling activity such as inducing changes in gene expression (Stenzel et al., 2003; Mueller, 2004; Mueller and Berger, 2009), we propose that its accumulation after GS induction is important for herbivory-induced signaling in Arabidopsis.

Lipase Activity-Independent Defense Responses

In addition to lipase activity, our results indicate the activity of other important effectors in GS. Even if the nature of the elicitor remains unknown, we show that the less than-10-kD GS fraction possesses the ability to induce changes in the $[Ca^{2+}]_{cyt}$ (Fig. 6A). Boiling GS was not able to abolish the $[Ca^{2+}]_{cyt}$ changes after GS application (Fig. 6A), and the application of fungal lipase solution induced $[Ca^{2+}]_{cyt}$ signatures other than the GS (Fig. 6A). Nevertheless, as OPDA, JA, and JA-Ile were shown to induce $[Ca^{2+}]$ changes in tobacco cells (Walter et al., 2007) and JA and JA-Ile in Arabidopsis (Sun et al., 2010), it seems likely that the relative increases in oxylipins could influence intracellular Ca^{2+} levels after W+GS treatment. Recently, the detergent effects of insect OS have been suggested to influence plant responses to herbivory (Maischak et al., 2007). We show that the observed $[Ca^{2+}]_{cyt}$ changes have a signature that differs from that of a detergent (Fig. 6A), one that can be completely blocked by the Ca^{2+} channel inhibitor $LaCl_3$ (Bush, 1995; Fig. 6A); this indicates that the observed changes are mediated by Ca^{2+} channels and likely not by detergent-like compounds in the GS. $[Ca^{2+}]_{cyt}$ changes are among the earliest herbivory-induced signals (Maffei et al., 2007) and therefore may influence later herbivory-induced responses. Ca^{2+}-regulated protein kinases have long been hypothesized to play a role in ET emission and recently were shown to influence herbivory-induced transcriptional responses in Arabidopsis (Tatsuki and Mori, 2001; Kanchiswamy et al., 2010), demonstrating that Ca^{2+} plays an important role in herbivory-induced

responses in Arabidopsis. Further experiments with Arabidopsis plants altered in Ca^{2+}-dependent signaling mediators are needed to elucidate the role of this signaling molecule in herbivory-induced responses.

The less than-10-kD fraction of GS still induced JA-Ile levels (Fig. 5A). W+W- and W+GS-induced JA-Ile is present in several times lower quantities than wound-induced JA, indicating that the JA levels are not limiting for JA-Ile biosynthesis. It could be possible that the less than-10-kD fraction of GS is still inducing the biosynthetic steps involved in the conversion of JA to JA-Ile. Therefore, lipase-independent pathways might be involved in regulating JA-Ile accumulation after W+GS treatment. It might be possible that Ca^{2+}-dependent signaling is involved in JA-Ile biosynthesis, since $[Ca^{2+}]_{cyt}$ is still induced by the less than-10-kD fraction of GS.

A response that is partially independent of the lipase activity-containing fraction is the activation of MPK3 and MPK6, which was observed after wounding and the application of the less than-10-kD GS fraction (Fig. 6B). Future research will focus on the GS-derived elicitors of $[Ca^{2+}]_{cyt}$ increase and MAPK activation and their roles in herbivory-induced downstream responses.

MAPKs Partially Contribute to GS-Induced Phytohormone Levels

From tomato and *N. attenuata*, it is known that the herbivory-induced accumulation of JA and its precursors is mediated by MAPKs (MPK1, MPK2, and MPK3 for tomato, SIPK and WIPK for *N. attenuata*; Kandoth et al., 2007; Kallenbach et al., 2010). We tested whether their homologs in Arabidopsis, MPK3 and MPK6, regulate GS-induced changes in oxylipin contents, as both MAPKs were activated rapidly after GS treatment (Fig. 1D). To our surprise, neither *mpk3* nor *mpk6* mutants showed reduced W+GS-induced oxylipin levels (Fig. 3A). Therefore, the function of these two MAPKs in herbivory-induced oxylipin elicitation might not be evolutionarily conserved. However, we found that MPK6 mediates ET accumulation after GS treatment (Fig. 3B), which is in line with the role of the MPK6 homolog SIPK in herbivory-induced ET emissions in *N. attenuata* (Wu et al., 2007). The tendency to higher ET emissions in the *mpk3* plants (Fig. 3B) could be due to higher MPK6 activity after W+GS treatment, since both MPK3 and MPK6 can be activated partially by the same MAPK kinases (Andreasson and Ellis, 2010). In the absence of MPK3, higher MPK6 activity and ET release could be due to enhanced MPK6 phosphorylation by upstream MAPK kinases. Even in the *mpk6* mutants, W+GS treatment increased the emission of ET to higher levels than did W+W treatment, indicating that other signaling pathways are involved in the regulation of the GS-dependent ET release. MPK3 was shown to be necessary for the ET release in *mpk6* mutant seedlings treated with the necrotrophic fungus *Botrytis cinerea* (Han et al., 2010). Using plants silenced in both MPK3 and MPK6 will help to further identify the possibly overlapping roles of both MAPKs in the regulation of ET after herbivory in Arabidopsis (Han et al., 2010). Interestingly, the OPDA and JA levels were 33% and 26% increased in *mpk6* plants (Fig. 3A; *t* test, $P < 0.05$, wild-type versus *mpk6* plants after W+GS treatment). If *mpk6* plants have altered steady-state levels of OPDA-containing lipids or if structural or developmental properties of *mpk6* plants lead to higher oxylipin levels after W+GS treatments are interesting questions for future research. Also, the cross talk with the phytohormone ET (for review, see Adie et al., 2007), which is emitted in lower amounts after W+GS treatment in *mpk6* mutant plants compared with wild-type plants (Fig. 3B), might influence oxylipin levels.

GS-Induced Defense Responses Are Altered by Wound-Induced H_2O_2

H_2O_2 production after wounding and herbivory was reported to alter wound- and herbivory-induced plant responses (Orozco-Cárdenas and Ryan, 1999; Sagi et al., 2004; Maffei et al., 2006; Diezel et al., 2009). Additionally, it was reported that H_2O_2 production is regulated by MAPK and Ca^{2+} signaling; both are induced by GS treatments (Keller et al., 1998; Sagi and Fluhr, 2001; Asai et al., 2008). We, too, found H_2O_2 to be induced by wounding; however, DAB staining revealed no effect of GS treatment on wounded leaves. Nevertheless, DAB staining is not sensitive enough to record subtle changes in H_2O_2 production, which might still be differentially induced when W+W is compared with W+GS treatments. By using *rbohd* mutant plants with highly reduced wound-induced H_2O_2 production, we demonstrate that wound-induced H_2O_2 is a negative regulator of W+GS-induced JA and ET levels. These data are consistent with a previous report from Diezel et al. (2009) showing that herbivory-induced H_2O_2 negatively influences JA and ET in *N. attenuata*. Future work is needed to understand the role of wound-induced H_2O_2 in mediating herbivory-induced responses in Arabidopsis.

Caeliferin A16:0 Is Not Sufficient to Induce GS-Mediated Defense Responses

Recently, herbivore-derived elicitor screening in various plant species revealed that caeliferin A16:0 induced JA accumulation and ET release in Arabidopsis (Schmelz et al., 2009). However, we were unable to induce any of the GS-induced responses using synthetic caeliferin A16:0, although we were able to detect this compound in the OS of *S. gregaria*. We cannot rule out the possibility that different chemical compositions of test substances, plant treatments, or plant growth conditions may have affected the plant's sensitivity to caeliferin A16:0.

CONCLUSION

Our data highlight a previously unknown mechanism for how herbivory leads to increased levels of

oxylipins in plants. We demonstrate that lipase activity in the OS of grasshoppers leads to increased levels of OPDA and OPDA-related signaling in Arabidopsis. The finding that similar increases in OPDA levels are induced by various insects in Arabidopsis suggests a common modus operandi and indicates the role arabidopsides may play in herbivore perception. Lipase-independent grasshopper-induced activation of $[Ca^{2+}]_{cyt}$ levels and MAPK activation demonstrate parallel induction of complex signaling cascades by herbivores (Fig. 9).

MATERIALS AND METHODS

Plant Material

Experiments were carried out with Arabidopsis (*Arabidopsis thaliana*) ecotypes Col-0, Landsberg *erecta*, and Wassilewskija as well as the mutants *mpk3* (provided by Brian Ellis, Michael Smith Laboratories, Vancouver, Canada), *mpk6* (provided by Justin Lee, Leibniz-Institut für Pflanzenbiochemie, Halle, Germany), and *rbohd* (provided by Miguel Angel Torres, Universidad Politécnica de Madrid, Spain).

Plant Growth

Arabidopsis plants were grown on substrate consisting of 80% Fruhstorfer Nullerde, 10% vermiculite, and 10% sand, fertilized with Triabon (1 g L^{-1}) and Osmocote Exact Mini (1 g L^{-1}), and treated with *Steinernema feltiae*. The controlled-environment chamber provided 10 h of light per day, a temperature of 21°C, and 60% humidity. The light intensity was 190 to 220 μmol m^{-2} s^{-1}.

Insects

Manduca sexta larvae were obtained from in-house colonies. Larval regurgitate was collected from third-instar larvae. *Spodoptera frugiperda*, *Spodoptera littoralis*, *Heliothis subflexa*, and *Heliothis virescence* were cultivated on an agar-based diet (Bergomaz and Boppre, 1986) or Arabidopsis. *Schistocerca gregaria* were obtained from Bugs International and b.t.b.e. Insektenzucht GmbH and were fed Arabidopsis. *Pieris rapae* were provided by Marcel Dicke (Wageningen University, The Netherlands) and were fed Arabidopsis. Additionally, the grasshopper population of the lawn at the Beutenberg Campus in Jena, next to the Max Planck Institute for Chemical Ecology, served as a source for grasshoppers (*Chorthippus* spp.) termed "native grasshoppers."

Elicitors and Test Substances

Synthetic caeliferin A16:0 was purchased from ChemPep and dissolved in 50 mM Na_2HPO_4 buffer (pH 8) stock solution as described previously (Schmelz et al., 2009). Structure and exact mass were verified by NMR (Supplemental Fig. S10) and high-resolution liquid chromatography-mass spectrometry (Supplemental Fig. S4, A and B). *Rhizopus arrhizus* lipase (Sigma-Aldrich) was dissolved in 100 mM Tris-HCl buffer (pH 7.5) to a concentration of 0.5 units μL^{-1}. OS (including GS) were collected according to Turlings et al. (1993) with the modifications of Alborn et al. (2003). For fractionation, GS were filtered using a 10-kD cutoff-column (Viraspin 500; GE Healthcare). The flow-through fractions were termed as less than 10 kD, and the supernatants were termed as greater than 10 kD. After fractionation, flow through and supernatant were adjusted to the original volume with aqua bidest. To inhibit enzyme activity, the GS and leaf tissue were incubated with 5 volumes of isopropanol at 80°C for 10 min (Heller and Steinberg, 1972; Yadav et al., 1998; Sana et al., 2004). Before use, the GS isopropanol solution was completely evaporated under N_2 and the pellet was dissolved in the original volume of water. For heat inhibition, GS and fungal lipase solution were boiled for 10 min. It is possible that other substances than enzymes also are affected by the two methods mentioned before. For in vitro treatment, 100 mg of enzyme-inhibited plant tissue was incubated with 5 μL of test substance for 10 min at room temperature. For lipase inhibition tests, GS and *M. sexta* OS were incubated with 1 mM tetrahydrolipstatin (orlistat; Cayman Chemicals; dissolved in ethanol) for 1 h at 37°C. The water, GS, and *M. sexta* OS controls were treated with the same amount of ethanol and incubated for 1 h at 37°C. As an additional control, 1 mM orlistat was added to the accordingly treated GS directly before application. GS and *M. sexta* OS for lipase inhibitor treatments were diluted 1:10 before treatment. GS, GS fractions, and heat-treated GS were used in a 1:5 dilution.

Leaf Treatments

To mimic herbivory-induced responses, four fully expanded leaves from 4- to 5-week-old rosette-stage plants were mechanically wounded by rolling a fabric pattern wheel three times on each side of the leaf, followed by the immediate application of 5 μL of the test substance. The test substance was distributed over the entire wounded leaf. At the indicated time intervals, the entire treated leaves without petioles were collected, immediately frozen in liquid nitrogen, and stored at −80°C.

For the natural feeding experiments, four starved *S. gregaria* nymphs were placed on each Arabidopsis plant in separate boxes (20 cm × 28 cm × 20 cm), and damaged leaves were harvested at the indicated time points.

Protein Extraction, Separation by 2-D Gel, and Analysis of Protein Spots

Proteins from GS were extracted with the phenolic extraction procedure described by Giri et al. (2006). A 2-D Quant kit (Amersham Bioscience) was used for protein quantification. 2-D gel electrophoresis, digestion, and analysis were done according to Giri et al. (2006). ProteinLynx Global Server Browser version 2.3 software (Waters) was used for baseline subtraction and smoothing, de novo peptide sequence identification, and database searches. The peptide fragment spectra were searched against the UniProt_TREMBL or UniProt_Swissprot database (parameters were as follows: peptide mass tolerance, 20 ppm; estimated calibration error, 0.005 D; carbamidomethylation of Cys and possible oxidation of Met). The BLAST search was performed internally using the MS-BLAST algorithm (Lit17) with a minimum of one peptide matching at an expect score of 100, with no-gaphspmax100-sort_by_totalscore -span1 advanced options and PAM30MS search matrix.

Phytohormone and Hydroperoxy Fatty Acid Analysis

Oxylipins (13-HPOT, OPDA, JA, and JA-Ile) were extracted and analyzed as described by Kallenbach et al. (2010). It is particularly important to do the extraction on ice to avoid enzymatic cleavage of OPDA-containing lipids during the extraction procedure. To quantify JA and JA-Ile, 200 ng of [9,10-^{2}H]dihydro-JA and 40 ng of [$^{13}C_6$]JA-Ile were added as internal standards to each sample. 13-HPOT was quantified using 15-hydroperoxy-eicosadienoic acid (10 ng per sample; Cayman Chemicals) as an internal standard, and OPDA was quantified based on the JA standard and an empirically determined conversion ($m_{OPDA} = 1.19 \times m_{JA}$). For the extraction and analysis of free and esterified OPDA, the published phytohormone extraction method was slightly modified. After ethyl acetate extraction and evaporation, the sample was redissolved in 1 mL of methanol, and 1 mL of 15% (m/v) KOH was added, incubated for 1 h at 60°C, and neutralized with 2 mL of 1 M citric acid. After reextraction with ethyl acetate, the ethyl acetate phase was evaporated and the pellet was resuspended in 70% methanol for analysis. The extraction and analysis of free OPDA by the normal phytohormone extraction method in parallel samples enabled the calculation of the esterified OPDA.

[9,10-^{2}H]Dihydro-JA was synthesized by saponification and deuteration of methyl JA (Sigma-Aldrich), [$^{13}C_6$]JA-Ile was synthesized out of [$^{13}C_6$]L-Ile (Cambridge Isotope Laboratories), and JA (Sigma-Aldrich) and methanol for mass spectrometry analysis were purchased by VWR International.

ET Release

To analyze herbivory-induced ET release, all fully expanded leaves were mechanically wounded by rolling a fabric pattern wheel twice on each side of the midrib, followed by the immediate application of 3 μL of the test substance to the wounding site, or plants were induced by damage from feeding *S. gregaria*. For feeding experiments, four starved *S. gregaria* nymphs were placed on the plants, each plant in a separate box (20 cm × 28 cm × 20 cm), for the indicated times. ET released was measured from detached plants that were allowed to accumulate ET in a 50-mL flask for 5 h with a photoacoustic spectrometer (INVIVO; https://www.invivo-gmbh.de) as described by von Dahl et al. (2007).

Glycolipid Assay

The extraction of lipids was done by the isopropanol-hexane method (Hara and Radin, 1978) with the modifications described by Bonaventure et al. (2003). The lipid extracts from 2 g of Arabidopsis plant tissue were evaporated under N_2 and dissolved in 2 mL of Cl_3CH:acetic acid (100:1 by volume) and loaded on a 750-mg silica gel 60 column preconditioned with Cl_3CH:acetic acid (100:1 by volume). After two washing steps with 6 mL of Cl_3CH:acetic acid (100:1 by volume) and 5 mL of Cl_3CH:acetone (80:20 by volume) to remove neutral lipids, the glycolipids were eluted by 5 mL of acetone and 5 mL of acetone:acetic acid (100:1 by volume). The glycolipid fractions were combined and equally divided to 15 aliquots. The presence of lipid-bound OPDA was tested by KOH ester hydrolysis as described above. The extracted glycolipid fraction contained more than 35 nmol lipid-bound OPDA g^{-1} fresh weight leaf material used for lipid extraction, whereas no free OPDA could be determined. The solvents were evaporated completely under N_2, and the aliquots were immediately used for lipase activity assays.

For the glycolipid assays, 35 μL of water or 1:5 diluted GS was added to each aliquot and incubated for 30 min at room temperature. After the addition of 50 μL of ethyl acetate, the samples were incubated for 10 min at room temperature. Samples were mixed several times during incubation. GS treatment without incubation was performed by using water-incubated samples and application of 35 μL of 1:5 diluted GS directly before extraction. Afterward, OPDA was extracted with the ethyl acetate-phytohormone extraction method described above.

Transcript Analysis

After treatments, the RNA from five biological replicates was extracted. For cDNA synthesis, RNA was reverse transcribed using oligo(dT) primer and ReverAid reverse transcriptase (Invitrogen). Quantitative (q)PCR was performed on a Stratagene Mx3005P qPCR machine using a SYBR Green-containing reaction mix (Eurogentec; qPCR Core kit for SYBR Green I No ROX). The Arabidopsis eIF4A1 elongation factor was used as a standard for normalizing the cDNA concentrations. The primer sequences are summarized in Supplemental Table S2.

MAPK Assay

Protein extraction was performed according to Wu et al. (2007), and the determination of kinase activity was performed as described by Zhang and Klessig (1997) using myelin basic protein as a substrate. After reaction and the washing steps, the gels were dried on a gel dryer (Bio-Rad), and the images were obtained with a FLA-3000 phosphor imager system. For each sample, three biological replicates were pooled.

$[Ca^{2+}]_{cyt}$ Measurements

For the experiments, transgenic Arabidopsis (Col-0) plants expressing apoaequorin in the cytosol (Knight et al., 1997) were grown on Hoagland medium with 1% agar. After 21 d, leaves were dissected and placed onto 96-well plates. The leaves were incubated in 150 μL of 5 μM coelenterazine in the dark overnight at 21°C. After the addition of 40 μL of test substance, the luminescence was quantified with a microplate luminometer (Luminoscan Ascent, version 2.4; Thermo Fisher Scientific). Prior to the treatment with the test substance, the background luminescence was determined for 1 min, and after the measurement, the samples were discharged by adding a 0.1 M $CaCl_2$ and 10% ethanol solution. The measurements were calibrated using the equation of Rentel and Knight (2004). $LaCl_3$ (1 mM) treatments were done 1 h before treatment.

Additional wounding proved not to be necessary, as the leaves were excised from the plant and therefore were already prewounded. Experiments with and without additional wounding by a needle showed similar results (data not shown). Since thigmomorphogenic stress, such as touching, can induce Ca^{2+} responses, we used no additional wounding prior to the application of test substances in the main experiments.

H_2O_2 Measurement

DAB staining was modified after Thordal-Christensen et al. (1997). For DAB staining, fully expanded leaves from rosette-stage plants were cut from the plant and petioles were exposed to a 1 mg mL^{-1} DAB solution at pH 3.8. After 4 h of incubation, the plants were subjected to the treatment. After the indicated incubation times, the leaves were subjected to DAB destaining solution (1:1:3 glycerol:acetic acid:ethanol) at 60°C.

Statistical Analysis

Data were analyzed with SPSS Statistics 17.0. Depending on the hypothesis that was being tested, either one-way ANOVA followed by Tukey's honestly significant difference test or independent-sample *t* test was used as indicated. Each experiment was repeated at least twice.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers X65052 (eIF4A1), Y11727 (GST1), BT000007 (MPK3), AY120737 (MPK6), BT020365 (OPR1), and BT002651 (RbohD).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Feeding damage from *S. gregaria* induces changes in phytohormone levels of Arabidopsis.

Supplemental Figure S2. *S. gregaria* GS induce changes in free and esterified OPDA levels.

Supplemental Figure S3. *S. gregaria* GS induce phytohormone changes in different Arabidopsis accessions.

Supplemental Figure S4. Mass spectrometry analysis of caeliferin A16:0.

Supplemental Figure S5. Lipase-dependent oxylipin accumulation in Arabidopsis (Col-0).

Supplemental Figure S6. Lipase activity induces the accumulation of 13-HPOT levels in Arabidopsis (Col-0) leaves.

Supplemental Figure S7. *S. gregaria* GS and fungal lipase solution (L)-induced oxylipin levels in Arabidopsis (Col-0).

Supplemental Figure S8. OPDA accumulates in close proximity to the wounded site.

Supplemental Figure S9. *M. sexta* OS induce lipase-dependent OPDA accumulation in Arabidopsis (Col-0).

Supplemental Figure S10. NMR analysis of synthetic caeliferin A16:0.

Supplemental Table S1. Sequences of lipase-like proteins in *S. gregaria* GS.

Supplemental Table S2. Primers used for qPCR.

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