Salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and their interactions mediate plant responses to pathogen and herbivore attack. JA-SA and JA-ET cross-signaling are well studied, but little is known about SA-ET cross-signaling in plant-herbivore interactions. When the specialist herbivore tobacco hornworm (*Manduca sexta*) attacks *Nicotiana attenuata*, rapid and transient JA and ET bursts are elicited without significantly altering wound-induced SA levels. In contrast, attack from the generalist beet armyworm (*Spodoptera exigua*) results in comparatively lower JA and ET bursts, but amplified SA bursts. These phytohormone responses are mimicked when the species’ larval oral secretions (OS Se and OS Ms) are added to puncture wounds. Fatty acid-amino acid conjugates elicit the JA and ET bursts, but not the SA burst. OS Se had enhanced glucose oxidase activity (but not β-glucosidase activity), which was sufficient to elicit the SA burst and attenuate the JA and ET levels. It is known that SA antagonizes JA; glucose oxidase activity and associated hydrogen peroxide also antagonizes the ET burst. We examined the OS Ma-elicited SA burst in plants impaired in their ability to elicit JA (antisense [as]-irf3 and ET (inverted repeat [ir]-aco) bursts and perceive ET (35s-etr1b) after fatty acid-amino acid conjugate elicitation, which revealed that both ET and JA bursts antagonize the SA burst. Treating wild-type plants with ethephone and 1-methylcyclopropane confirmed these results and demonstrated the central role of the ET burst in suppressing the OS Ma-elicited SA burst. By suppressing the SA burst, the ET burst likely facilitates unfettered JA-mediated defense activation in response to herbivores that otherwise would elicit SA.

Plants are continuously challenged by a variety of biotic agents that attack in different ways, over different spatial scales, and with different consequences for a plant’s Darwinian fitness. To survive, plants recognize and respond differently to different attackers deploying chemical or morphological defenses that kill, starve, poison, repel, and trap their attackers or attract the natural enemies of these attackers. Each attacker, depending on its natural history, evolves different counterresponses to these plant defenses, which, in turn, increases the need for a plant to recognize different attackers and tailor specific responses.

How plants cope with these demands is the subject of intensive research, and it is clear that three phytohormones and their interactions play a central role: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET; Reymond and Farmer, 1998; Thomma et al., 2001; Glazebrook, 2005; De Vos et al., 2006). SA is known to play a central role in defense against biotrophic pathogens by containing their spread with a preventive cell suicide, known as the hypersensitive response. Additionally, SA elicits a long-lasting, induced resistance response to a broad range of invading pathogens, known as systemic acquired resistance (Ross, 1961; Ryals et al., 1996; van Loon et al., 1998; Potlakayala et al., 2007). JA, on the other hand, plays a key role as an elicitor of defense responses to necrotrophic pathogens by initiating SA-independent induced systemic resistance (Pieterson et al., 1998; Vijayan et al., 1998) and to herbivores (Halitschke and Baldwin, 2005; De Vos et al., 2006). There is also considerable evidence for dose-dependent antagonism of SA on JA-mediated herbivore defenses (Doherty et al., 1988; Péna-Cortes et al., 1993; Doares et al., 1995; Baldwin et al., 1997), as well as pathogen defenses (Stout et al., 1999; Gupta et al., 2000; De Vos et al., 2006; Mur et al., 2006). This important form of cross-talk has been described in a large number of Arabidopsis (*Arabidopsis thaliana*) accessions (Koornneef and Pieterson, 2008). Less is known about JA’s effects on SA signaling, but in JA- and coronatine-insensitive mutants, SA-mediated gene expression and defenses are typically enhanced (Kloek et al., 2001; Li et al., 2004) and JA treatment suppresses SA-dependent pathogen-related (PR) protein expression (Niki et al., 1998).

The gaseous hormone ET, in addition to its central role in many physiological processes such as fruit ripening and senescence, modulates defense responses, particularly those mediated by the JA cascade, rather than eliciting defense responses on its own (for review, see von Dahl and Baldwin, 2007). For example, the ET burst elicited by herbivore attack enhances the production of JA-elicted proteinase in-
hbitors in tomato (*Solanum lycopersicum*; O'Donnell et al., 1996), but suppresses JA-elicited nicotine production in the native tobacco *Nicotiana attenuata* (Kahl et al., 2000; Winz and Baldwin, 2001). Whereas both ET-JA and JA-SA interactions are important for various pathogen responses, the ET-SA interaction is not as well studied. Recently, Leon-Reyes et al. (2009) demonstrated that ET modulated the role that NPR1 plays in JA-SA cross-talk in Arabidopsis.

It is well established that a plant’s response to herbivore attack frequently differs from that of even careful simulations of the mechanical wounding that herbivore feeding causes (Baldwin, 1988; Baldwin et al., 2001; see also Mithofener and Boland, 2008, for examples in which the timing of mechanical damage can mimic herbivore-specific responses). Insect-specific elicitors, found in insect oral secretions (OS) or oviposition fluids, are frequently responsible for the specificity of the responses. In lepidopteran OS, several types of elicitors have been identified. A Glc oxidase (GOX) was isolated from *Helicoverpa zea* OS; GOX suppresses plant defense responses (Eichenseer et al., 1999; Musser et al., 2005). β-Glucosidase from the OS of white cabbage butterfly (*Pieris brassicae*) larvae elicits the production of volatiles from cabbage plants; these volatiles attract parasitic wasps (*Cotesia glomerata*) to feeding larvae. The volatiles released are comparable to those released by the treatment of leaves with β-glucosidase from almonds, and the parasitic wasps did not discriminate between cabbage leaves treated with almond β-glucosidase and leaves treated with larval OS (Mattiaci et al., 1995). In addition to these proteins, peptides called inceptins from the fall armyworm’s OS, proteolytic postigestive products of the host plant’s chloroplastic ATP synthase γ-subunit (Schmelz et al., 2006, 2007), as well as fatty acid-amino acid conjugates (FACs) composed of either linolenic acid or linoleic acid and their derivatives and an amino acid moiety, Gln or Glu (Alborn et al., 1997; Pohner et al., 1999; Spitterell and Boland, 2003; Maffei et al., 2004), are established elicitors in lepidopteran OS. Volicitin, a hydroxyl FAC (N-17-hydroxylinolenoyl-1-L-Gln) that was the first FAC identified in beetle armyworm (*Spodoptera exigua*) OS, induces volatile release in maize (*Zea mays*) seedlings (Alborn et al., 1997). Since then, different forms of FACs have been found in other insect species (Pohner et al., 1999; Halitschke et al., 2001; Mori et al., 2001; Spitterell and Boland, 2003). The effects of applying the two most abundant FACs in the OS of the specialist herbivore of *N. attenuata*, tobacco hornworm (*Manduca sexta*; Lepidoptera, Sphingidae), have been intensively studied. When synthetic FACs are applied to wounded *N. attenuata* leaves, they simulate most of the responses elicited by attack from tobacco hornworm larvae, including the activation of mitogen-activated protein kinase activity and the elicited changes in transcription, proteome, and defensive secondary metabolites (Halitschke et al., 2001; Halitschke and Baldwin, 2003; Giri et al., 2006; Wu et al., 2007).

Less is known about the actual amounts of these elicitors that come in contact with leaf tissues during the feeding process. Peiffer and Felton (2005) showed that *H. zea* larvae secrete microgram amounts of GOX onto the leaf (e.g. 2.47 µg on tobacco [*Nicotiana tabacum*] leaves). Peiffer and Felton (2009) recently estimated the amount of total regurgitate that is applied to leaves during larval feeding; frequently <10 nL was applied. Given that tobacco hornworm regurgitate is able to elicit a full JA burst with all of the associated responses when it is diluted 1/1,000 (Schittko et al., 2000), the small quantities of OS that are transferred to leaves during feeding are sufficient to elicit herbivore recognition responses in plants.

Here we compare the ET, SA, and JA responses in *N. attenuata* plants in response to attack from the two most common Lepidopteran herbivores feeding on *N. attenuata* plants in the plant’s native habitat: the specialist tobacco hornworm and the non-native generalist beet armyworm (Steppuhn et al., 2004). As described previously, feeding by tobacco hornworm larvae elicits prominent ET and JA bursts (Halitschke et al., 2004; von Dahl et al., 2007), and we report here that feeding by beet armyworm larvae results in smaller JA and ET bursts, and a prominent SA burst, which is lacking from the response to tobacco hornworm attack. These contrasting phytohormone responses can be mimicked by applying the larvae’s specific OS to mechanical wounds, and we identify GOX activity and its associated hydrogen peroxide (*H₂O₂*) production as the elicitor in OSs, responsible for the differences in phytohormone responses. To further understand the role of phytohormone cross-talk, we examined the phytohormone responses in *OSₐₑ*-elicited *N. attenuata* plants impaired in their ability to elicit JA (antisense [as]-lox3) and ET (inverted repeat [ir]-aco) bursts and to perceive the ET burst (35s-etr1b) and discovered that both ET and JA bursts antagonize the SA burst. We propose that, by suppressing the SA burst, the ET burst facilitates unfettered JA-mediated defense activation when plants are attacked by herbivores that also elicit SA signaling.

**RESULTS**

**Generalist and Specialist Herbivores Elicit Different Patterns of SA, JA, and ET Accumulation**

The specialist herbivore tobacco hornworm and the non-native generalist herbivore *S. exigua* are the two most common lepidopteran herbivores feeding on *N. attenuata* plants in their native habitats (Steppuhn et al., 2004). We compared SA, JA, and ET accumulation patterns in *N. attenuata* plants attacked by these two herbivores. The measures of total SA were consistently 4- to 5-fold higher than the measures of free SA in all experiments, but the kinetics of total SA mirrored those of free SA (Rayapuram and Baldwin, 2007; C. Diezel and I.T. Baldwin, unpublished data), so we
report here only the free SA levels. Beet armyworm attack elicited significantly larger SA levels, up to 6-fold compared to unattacked plants, than did attack from tobacco hornworm larvae (ANOVA; $F_{2,14} = 2.602; P < 0.05$; Fig. 1A). In contrast, JA values induced by tobacco hornworm attack largely exceeded those measured in response to beet armyworm attack (ANOVA; $F_{2,15} = 13.009; P < 0.05$; Fig. 1B). The herbivore-induced ET burst followed the same pattern. Tobacco hornworm attack resulted in 3-fold increases in ET emission compared to unattacked plants, whereas beet armyworm attack did not significantly alter the emission of this phytohormone (ANOVA; $F_{2,7} = 15.183; P < 0.05$; Fig. 1C).

Because differences in feeding behavior (timing and biomechanics of leaf damage) could be responsible for the different SA, JA, and ET responses, we used a standardized mechanical wounding treatment to determine whether OS elicitation could account for the differences in plant responses. Treatment of standardized puncture wounds produced by a fabric pattern wheel with OS from the two herbivore species recapitulated the differences in phytohormone signaling elicited by herbivore attack (Fig. 2). Elicitation with OS from tobacco hornworm (OS$_{M_6}$) or beet armyworm (OS$_{Se}$) larvae qualitatively reproduced the phytohormone patterns observed during active feeding, but the absolute values of the JA and ET bursts were different from those observed during insect feeding, which is likely due to the differences in the time of leaf harvesting and the differences in the wounding event between the OS elicitation and real herbivore feeding. SA levels increased up to 3-fold in plants elicited with OS$_{Se}$ compared to in plants wounded and treated with water or OS$_{M_6}$ (ANOVA; $F_{2,6} = 9.663; P < 0.05$ Fig. 2A). JA levels, on the other hand, increased up to 4-fold in plants treated with OS$_{M_6}$ compared to in water-treated control plants, whereas OS$_{Se}$-treated plants accumulated JA to levels that did not differ from levels in wound- and water-treated plants (ANOVA; $F_{2,6} = 56.608; P < 0.05$; Fig. 2B). ET emissions from plants treated with OS$_{M_6}$ were significantly higher than those in water- and OS$_{Se}$-elicited plants (ANOVA; $F_{2,6} = 15.523; P < 0.05$; Fig. 2C).

**OS Components and Their Role in Eliciting SA via H$_2$O$_2$ Production**

Different classes of OS-derived elicitors are known to regulate different phytohormone responses. In *N. attenuata*, FACs found in tobacco hornworm OS are able to mimic most of the known OS$_{M_6}$-elicited changes in transcripts, proteins, and metabolites (Halitschke et al., 2003; Voelckel and Baldwin, 2004; Giri et al., 2006; von Dahl et al., 2007; Pandey et al., 2008; Gaquerel et al., 2009). Notably, the two most abundant FACs—N-linolenoyl-l-Gln (C18:3-Gln) and N-linolenoyl-l-Glu (C18:3-Glu)—found in tobacco hornworm OS are necessary and sufficient to elicit the OS-specific JA (Halitschke et al., 2003) and ET (von Dahl et al., 2007) bursts. Levels of these FACs were significantly lower in plants treated with OS$_{Se}$ compared to those treated with OS$_{M_6}$ (Fig. 3), which is consistent with the reduced JA and ET bursts after OS$_{Se}$ elicitation. In contrast, adding C18:3-Gln and C18:3-Glu to wounds in wild-type leaves did not significantly elicit SA accumulation compared to adding these compounds to the leaves of the detergent-treated control (ANOVA; $F_{2,6} = 2.717; P = 0.87$; Supplemental Fig. S1).

$\beta$-Glucosidase is another insect-derived elicitor of plant defense responses (Mattiacci et al., 1995). This hydrolytic enzyme has been postulated to target SA-glucoside conjugates to release free SA; however, treating puncture wounds with commercial almon$\beta$-glucosidase at concentrations similar to those found in OS$_{Se}$ did not significantly increase SA levels compared to treating puncture wounds with water (ANOVA; $F_{2,11} = 0.204; P = 0.82$; Supplemental Fig. S2).

Labial salivary gland extracts treated with active GOX from OS$_{Se}$ have been shown to increase SA-
mediated PR-1a protein levels in cultivated tobacco plants (Musser et al., 2005). We measured GOX activity in the two OS and found that GOX activity was 2.5-fold lower in OSMs compared to in OSSe (Student’s t test; P ≤ 0.001; Supplemental Fig. S3). Moreover, treating leaf puncture wounds with a solution containing GOX and Glc at concentrations similar to those measured in OSSe significantly increased free SA levels (ANOVA; F_{4,15} = 2.269; P < 0.05; Fig. 4A). No significant increases in SA levels were observed when leaves were treated with GOX or Glc separately, demonstrating that it was GOX activity, and likely elevated H2O2 levels, that were responsible for the SA burst. Boiling OSSe to denature its proteins inactivated its ability to elicit SA accumulation (Fig. 4B). Interestingly, boiled OSSe elicited higher JA levels than did unboiled OSSe or even OSMs (ANOVA; F_{4,11} = 27.435; P < 0.05; Fig. 4C), suggesting that it is the GOX activity of OSSe that suppresses the JA burst when wounds encounter OSSe during beet armyworm attack. In contrast, boiling OSMs, which contains low GOX activity levels (Supplemental Fig. S2), did not alter the JA-eliciting activity of OSMs.

GOX catalyzes the oxidation of D-Glc, resulting in the concomitant production of D-glucuronic acid and H2O2. Consistent with this mechanism, we found that H2O2 concentrations were higher in OSSe than in OSMs. Moreover, we measured H2O2 concentrations in boiled OSMs and OSSe and found that boiling reduced H2O2 levels in OSSe to levels found in OSMs. When GOX or Glc alone was added to OSMs, there was also no increase in H2O2 levels, suggesting that both Glc and GOX are at levels too low in OSMs to significantly increase H2O2 levels (ANOVA; F_{5,12} = 30.406; P < 0.05; Fig. 5). Supplementing the OS of both species with Glc and GOX resulted in similarly high levels of H2O2 in the OS (ANOVA; F_{2,6} = 2.645; P > 0.05; Supplemental Fig. S4), demonstrating that H2O2 scavenging is not the explanation for the low H2O2 levels in OSMs.

**JA Suppresses the FAC-Specific SA Burst**

JA and SA have long been thought of as antagonists even though more subtlety in their interplay has been recently described (Mur et al., 2006). To determine whether the OSMs- and FAC-elicited JA bursts antagonize SA accumulation during tobacco hornworm feeding, we used plants genetically silenced for lipoygenase3 (as-lox3), a key JA biosynthetic gene (Fig. 6).

As previously reported (Halitschke and Baldwin, 2003), FAC-elicited as-lox3 plants accumulated 4-fold less JA than did similarly treated wild-type plants (ANOVA; F_{4,15} = 6.162; P < 0.05; Fig. 6B). Concomitantly, SA levels in FAC-elicited as-lox3 plants were significantly elevated, which was not observed in wild-type plants (ANOVA; F_{5,24} = 3.118; P < 0.05;
Fig. 6A). These results demonstrate that while FACs do not elicit an SA burst in wild-type plants, they do in plants with silenced JA signaling.

ET after the OS were boiled (ANOVA; $F_{5,17} = 15.391$; $P < 0.05$; Fig. 7A), which suggested that GOX activity antagonizes ET production. Wound-induced levels of ET were not statistically altered by treating leaves with GOX and Glc. Rather, the FAC-elicited ET burst was reduced by more than one-third when GOX and Glc were supplemented with the FAC solution, which is consistent with the hypothesis that GOX activity is a negative regulator of ET production in N. attenuata (ANOVA; $F_{4,14} = 18.616$; $P < 0.05$; Fig. 7B).

ET Suppresses the OSMs-Elicited SA Burst

To further explore the role of ET signaling in modulating the SA burst, we measured the OSMs-elicited SA bursts in transgenic N. attenuata plants rendered ET insensitive by the ectopic expression of the mutant etr1-1 receptor of Arabidopsis (35s-etr1b), as well as in plants impaired in their ability to produce ET (ir-aco). OSMs elicitation resulted in a 3-fold higher SA burst in these two genetic backgrounds compared to untransformed plants (Fig. 8A). Treating wounded wild-type leaves of N. attenuata with ethephone, an ET releaser, reduced the SA burst to 30% of that elicited by wounding and water treatments (ANOVA; $F_{2,10} = 4.891$; $P < 0.05$; Fig. 8B). Moreover, the OSMs-elicited SA burst increased by 25% when wild-type plants were rendered ET insensitive by a prior overnight exposure to 1-methylcyclopropane (1-MCP), an ET receptor antagonist (ANOVA; $F_{4,19} = 8.388$; $P < 0.05$).

GOX Suppresses the FAC-Specific ET Burst

We next examined the effect of GOX activity on ET emission. Boiling OSMs did not alter the well-described FAC-elicited ET burst, which is not surprising given that boiling does not denature the FACs that elicit the ET burst (von Dahl et al., 2007). However, supplementing OSMs with GOX decreased ET emissions by 25% compared to supplementing with OSMs alone. In addition, plants treated with OSMs elicited 33% more ET than those treated with OSMs + GOX (ANOVA; $F_{5,17} = 15.391$; $P < 0.05$).
Nonwounded plants and plants that were wounded following a 1-MCP treatment did not differ in their SA levels from 1-MCP-free plants (Supplemental Fig. S5).

**DISCUSSION**

In response to herbivore and pathogen attack, plants activate not one, but many, signal cascades; these in turn recruit a suite of defenses. The specificity of the defense response elicited against a particular attacker is in part tailored by the cross-communication among these signal transduction pathways (Glazebrook, 2005; Koornneef and Pieterse, 2008; Leon-Reyes et al., 2009). Biotic attackers, to borrow from Shakespeare, “come not [as] single spies, but in battalions,” and many herbivores function as Trojan horses, vectoring and inoculating plants with pathogens during feeding. Given the high probability that many homopteran insects, such as aphids and whiteflies, transmit disease-causing viruses and pathogens, it is not surprising that plants activate SA signaling in response to their attack (Zarate et al., 2007). Moreover, there is the distinct possibility that certain herbivores deliberately elicit SA responses in plants to suppress effective JA-dependent defenses (Moran et al., 2002; Kaloshian and Walling, 2005; Pegadaraju et al., 2005; De Vos et al., 2006).

Here, we show that when beet armyworm larvae attack wild-type plants, they elicit a 5-fold larger SA burst than when tobacco hornworm larvae attack (Fig. 1A). The response to beet armyworm attack also differs from the response to tobacco hornworm attack in that no ET and lower JA bursts are elicited (Fig. 1, B and C). Remarkably, these differences in phytohormone responses are faithfully mimicked when puncture wounds in leaves are treated with either OSMs or OSSe (Fig. 2).
GOX has been implicated as a potential key mechanism used by caterpillars to counteract induced plant defenses by interfering with JA-dependent signaling (Musser et al., 2002). GOX activity and its reaction product, \( \text{H}_2\text{O}_2 \), were 2.5-fold higher in OS Se in comparison to OS Ms (Supplemental Figs. S3 and S5). When Glc and GOX were applied to puncture wounds in OS Se- and OS Ms-treated plants, OS Se contained only trace quantities of volicitin in their OS and large quantities of linolenic acid- and linoleic acid-Gln conjugates (Roda et al., 2004), and because the addition of GOX and Glc (but neither alone) to boiled OS Se or OS Ms produces similarly elevated \( \text{H}_2\text{O}_2 \) levels (Supplemental Fig. S4), we conclude that the amount of active GOX found in OS Se is sufficient to account for the SA burst either directly or via the production of \( \text{H}_2\text{O}_2 \). Interestingly, because GOX and Glc-supplemented FAC solutions elicited attenuated ET and JA bursts compared to pure FAC solutions (Fig. 7B), the GOX activity of OS Se can also account for the lower JA bursts and the lack of ET bursts elicited by OS Se. In summary, we propose that the elevated GOX activity of OS Se is sufficient to account for observed changes in the classical FAC-elicited phytohormone response, but additional work needs to be done to completely exclude a potential role of the different FAC profiles of the OS.

Whereas the elevated GOX activity of OS Se can account for all of the observed differences in elicited phytohormone responses, the different FAC profiles found in the two OSs (Fig. 3) may also play a role in tailoring the phytohormone responses. The two most abundant FACs in OS Ms (linolenic acid- and linoleic acid-Gln conjugates) occurred at low concentrations in OS Se and the two other highly abundant OS Ms FACs (linolenic acid- and linoleic acid-Glu conjugates) were not detected. In addition, one FAC (palmitoyl acid-Gln conjugate) was more abundant in OS Se than in OS Ms. These differences in the FAC profiles may contribute to the different ET bursts observed in OS Se- and OS Ms-treated plants. OS Se contained only trace quantities of volicitin (8% of total conjugates), which is consistent with the results of Pohnert et al. (1999); they found that beet armyworm larvae reared on lima bean (Phaseolus lunatus) produced only minor amounts of volicitin in their OS and large quantities of \( N \)-palmitoyl-\( L \)-Gln, \( N \)-linoleoyl-\( L \)-Gln, and \( N \)-linolenoyl-\( L \)-Gln. These results suggest that the FAC composition of OS Se is highly diet dependent. Our results also differ from those of Halitschke et al. (2001), which did not detect volicitin in OS Se but, given that volicitin is a minor constituent compared to \( N \)-linolenoyl-\( L \)-Glu, \( N \)-linoleoyl-\( L \)-Glu, \( N \)-palmitoyl-\( L \)-Glu, and \( N \)-linolenoyl-\( L \)-Gln, we propose that it plays a minor role. It is important to emphasize that, whereas the two most abundant FACs of OS Ms are not elicitors of the SA burst in wild-type plants, they are effective elicitors in JA-deficient plants (Fig. 6A). This difference underscores that it is not the elicitor alone that determines the phytohormone response, but the interactions of the elicitor with the plants’ endogenous signal interactions that determine which phytohormone accumulates.

Salivary GOX is commonly found among different caterpillar species, but its activity is highly variable. For example, high activity is reported in salivary homogenates of bean armyworm caterpillars and the \( \text{Mamestra configurata} \) (Bertha armyworm), but not in the true armyworm (\( \text{Pseudaletia unipuncta} \)), or the specialist alfalfa butterfly (\( \text{Colias eurytheme} \); Merkx-Jaques and Bede, 2004). Salivary GOX activity originates predominantly from the labial glands, with...
<11% of total activity detected in mandibular glands and only minor amounts from the hemolymph (Eichenseer et al., 1999). *H. zea* larvae reared on different host plants produce varying amounts of GOX in their labial glands (Peiffer and Felton, 2005). GOX has been shown to play important antimicrobial roles in *H. zea* (Musser et al., 2005) and honeybees are known to secrete GOX into honey, which suppresses bacterial growth (White and Subers, 1963). The antimicrobial role of GOX in insects may have facilitated their amplification and use in eliciting plant pathogen defenses, perhaps as a means of countering JA-mediated defenses.

Recently, Leon-Reyes et al. (2009) showed that in Arabidopsis, ET modulates NPR1’s mediation of SA-JA cross-signaling. Whereas the NPR1 homolog of *N. attenuata* plays the opposite role in mediating SA signaling than it plays in Arabidopsis (Rayapuram and Baldwin, 2007), we show here that ET signaling plays a similar role to the role it plays in Arabidopsis: that of suppressing the SA response. Rayapuram and Baldwin (2007) showed that silencing Na-NPR1 did not affect the amount of ET released after Bertha armyworm attack or after OS$_{Ms}$ elicitation in the leaves of ir-npr1 glasshouse-grown plants and that silencing Na-NPR1 reduced JA levels, but increased SA in field-grown OS$_{Ms}$-elicited plants, which reveals JA-SA, but not ET-SA antagonism. We show that in OS$_{Ms}$-elicited transgenic 35s-etr1b and ir-aco plants, which are impaired in ET perception or production, as well as in OS$_{Ms}$-elicited wild-type plants pretreated with a competitive inhibitor of ET receptors, 1-MCP, SA accumulates up to 3-fold more than in OS$_{Ms}$-elicited wild-type plants (Fig. 8; Supplemental Fig. 5). Both 35s-etr1b plants and 1-MCP-treated wild-type plants are impaired in their ability to perceive ET and are known to synthesize more ET in response to OS$_{Ms}$ elicitation than do wild-type plants, whereas ir-aco plants are impaired in the production of the elicited ET burst (von Dahl et al., 2007). These results suggest that residual GOX activity of OS$_{Ms}$ (Supplemental Fig. S3) is sufficient to elicit an SA burst as long as the ET burst that is normally elicited by OS$_{Ms}$ is either impaired or not perceived. Little is known about GOX activity in tobacco hornworm larval OS. GOX-derived H$_2$O$_2$ plays only a minor role in the elicitation of *Manduca* spp.-specific plant responses and genes coding enzymes of the JA-signaling cascade are down-regulated in response to H$_2$O$_2$ treatment (Halitschke and Baldwin, 2003). Together with the low levels of GOX activity in OS$_{Ms}$ reported here, these results underscore that tobacco hornworm attack elicits predominately JA-based signaling, which is in turn mediated by the FACs in larval OS.

Together, these observations suggest that SA-ET as well as SA-JA cross-signaling is responsible for tuning the responses of *N. attenuata* plants to the FAC- and GOX-based elicitors of their lepidopteran herbivores (Fig. 9). These differences in the elicited response profiles of the plant can be useful for either the plant or the herbivore, depending on their strategies. The results presented here are consistent with the hypothesis that generalist herbivores, such as beet armyworm, may enhance their fitness by activating the SA pathway concomitantly with the JA pathway so as to weaken JA-mediated resistance (Stotz et al., 2002; Cipollini et al., 2004; Zarate et al., 2007). However, much additional work is required to understand the consequences of this signaling cross-talk for *N. attenuata*’s defense physiology. These results also underscore the value of using transgenic plants impaired in specific parts of their signaling cascades to understand the complex signaling interactions between herbivores and their hosts.
MATERIALS AND METHODS

Plant Material and Growing Conditions

Wild-type Nicotiana attenuata Torr. ex S. Watson plants were from an inbred line in its twenty-second generation that originated from seeds collected on the DE ranch in Utah in 1988. Seeds of wild-type and genetically transformed plants were germinated on Gamborg’s B5 medium (Duchefa) as described previously (Krügel et al., 2002). The transformed plants used were 35s-etrlb plants (A-03 538–1), ecotopically expressing the Arabidopsis (Arabidopsis thaliana) mutant ET receptor etr-1 under the control of a cauliflower mosaic virus 35S constitutive promoter (von Dahl et al., 2007), as well as the transformed as-etr1b (Halitschke and Baldwin, 2003) and the etr-1c line, silenced in the expression of the ET biosynthesis gene ACO (A03-321-10 as described in von Dahl et al., 2007). Briefly, seeds were sterilized and incubated in 0.1 M GA3 (www.carl-rot.de) and 1.5 diluted liquid smoke (v/v) (House of Herbs) before germination on Gamborg’s B5 medium at a 26°C/16 h 155 μm s−1 light; 24/8 h dark cycle (Perclon). Plants were grown in the glasshouse with a day/night cycle of 16 h (26°C–28°C)/8 h (22°C–24°C) h under supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600-W sodium lights (Philips).

Insect Rearing and Feeding Experiments

Tobacco hornworm (Manduca sexta) eggs, purchased from Carolina Biological Supply, were cultured in climate chambers until hatching. Freshly hatched larvae (neonates) were placed onto leaves growing at the +1 nodal position of individual plants in clip cages for feeding experiments. To increase survival rate, beet armyworm (Spodoptera exigua) larvae hatched from eggs supplied by the Plant Protection Centre of Bayer AG were reared on wild-type N. attenuata plants until they reached the second to third instar. One third-instar beet armyworm or two first-instar tobacco hornworm larvae per plant were allowed to feed for 3 d. The number of larvae placed on plants (the two to third-instar beet armyworm neonates and the one beet armyworm third-instar larvae) were selected because they produced comparable amounts of damage (data not shown).

For the collection of OS, larvae were reared on N. attenuata wild-type plants until the third to fifth instar. OS was collected on ice as described in Roda et al. (2004).

Plant Treatments

For experiments presented in Figure 3, the three youngest fully expanded leaves (positions +1, +2, and +3) were mechanically wounded with a pattern wheel to produce four puncture rows on each side of the midvein. Fresh wounds were immediately treated with 20 μL of tobacco hornworm OS (diluted 1:1 with water), ethephone (6 mg/mL in 5 mM MES buffer; www. riedeldehaeden.de), or 5 mM MES. Control plants remained untreated. To inhibit ET perception, plants were exposed to 1-MCP for 2 h and 8 h during the dark phase. Following Kahl et al. (2000), 500 mg of Ethylblock (0.43% 1-MCP; van der Sprong) was weighed into a vial and 10 mL of an alkaline solution (0.75% KOH + NaOH) was added to release 1-MCP.

For all other experiments, one leaf (position +1) was wounded and immediately treated with water, triton—the surfactant used for the dissolution of FACs, FACs (N-linolenoyl-L-Gln and N-linolenoyl-L-Glu at concentrations similar to those found in tobacco hornworm OS). Tobacco hornworm OS (OSdA), beet armyworm OS (OSbA), OS boiled during 10 min at 90°C (OSbB or OSb), OS with 0.01 μM Glc (OSbA, g or OSb, g) or Glc, OS with 0.5 units GOX (OSdA + GOX; http://www.sigmaaldrich.com), FACs with 0.01 μM Glc, and 0.5 units GOX (FAC + G + GOX).

Phytohormone Analysis

Free SA and JA were extracted by homogenizing 200 to 300 mg of leaf material in FastPrep tubes containing 900 mg of lysing matrix (BIO 101; Vista) and 1 mL of ethyl acetate spiked with 200 ng (100 ng during the analysis of caterpillar-attacked leaves) of D2-SA and D2-JA, as internal standards. Samples were homogenized twice by reciprocal shaking at 6.5 m s−1 for 45 s and centrifuged at 13,000 rpm for 20 min at 4°C. Supernatants from two extraction steps were pooled and evaporated until dry. The dried residue was dissolved in 500 μL of 70% of methanol, vortexed, and centrifuged.

SA and JA measurements were conducted on a liquid chromatography-tandem mass spectrometry system (Varian 1200). Fifteen microliters of each sample were injected onto a ProntoSL column (C18; 5 μm, 50 × 2 mm; Bischoff) attached to a precolumn (C18, 4 × 2 mm; Phenomenex). The mobile phase comprised solvent A (0.05% formic acid) and solvent B (0.05% formic acid in acetonitrile) used in a gradient mode (time/concentration [min/%] for B: 0.00/15; 1.30/15; 4.30/98; 12.30/98; 13.30/15; 15.00/15) with a flow of (time/flow [mL/min]: 0.08/0.4; 1.30/0.4; 10.00/0.12; 10.30/0.4; 12.30/ 0.4; 15.00/0.4). Compounds were detected in the electrospray ionization-negative mode. Molecular ions [M–H]− at m/z 137 and 209 and 213 generated from endogenous SA and JA and their internal standards, respectively, were fragmented under 15-V collision energy. The ratios of ion intensities of their respective daughter ions, m/z 93 and 97 and m/z 59 and 63, were used to quantify endogenous SA and JA, respectively.

FACs were measured continuously and non-invasively with a photoacoustic spectrometer (INVIVO) as described in von Dahl et al. (2007). The youngest fully expanded leaves of slightly elongated plants were subject to feeding by three neonate tobacco hornworm larvae or one third-instar beet armyworm larvae for 22 h. Leaves were excised at the onset of the experiment and transferred to 250-mL cuvettes and the headspace was allowed to accumulate over the entire feeding period or experiments requiring mechanical wounding and application of eliciting solutions, leaves were excised directly after treatment and transferred to 250-mL cuvettes, and the headspace allowed to accumulate over a 5-h time period. During measurements, cuvettes were flushed with a flow of pure air at 130 to 150 mL min−1, which had previously passed through a liquid N2 cooling trap to remove CO2 and water.

FAC Analysis

Five microliters of OS were homogenized in 95 μL methanol spiked with 10 ng of N-cis-10-nonadecenoic acid—Gln (C19:1-Gln) used as internal standard. Extracts were then centrifuged to remove any particulate matter. Five-microliter aliquots of these solutions were analyzed by liquid chromatography-tandem mass spectrometry using the aforementioned LC settings. Identification of major FACs from tobacco hornworm OS was confirmed by comparison to authentic standards, as described in Halitschke et al. (2001). The structure identified as volicitin (N-17-hydroxynonthanoyl—Gln) produced a fragmentation pattern—a dehydrated ion at m/z 404, a doubly dehydrated ion at m/z 378, and a hydroxyl acid-derived ion at m/z 293—identical to that already published by Yoshinaga et al. (2007). Most abundant FACs were detected in the electrospray ionization-negative mode and quantified using parent-ion/daughter-ion selections designed, as follows, from previously published MS/MS spectra by Yoshinaga et al. (2007): 383/145 (N-palmitoyl —Gln, C16:0-Gln), 384/255 (N-palmitoyl—Glu, C16:0-Glu), 405/145 (N-linolenoyl—Gln, C18:3-Gln), 406/277 (N-linolenoyl—Glu, C18:3-Glu), 407/ 145 (N-linoleoyl—Gln, C18:2-Gln), 408/279 (N-linoleoyl—Glu, C18:2-Glu), 421/145 (volicitin). Collision energy was set to 18.5 V. Fragmentation profiles of FACs containing Gln typically included a common Gln-derived ion.

GOX Assay

GOX activity was measured in OS according to the procedure described in Kelley and Reddy (1988). Briefly, OS were collected from 30 second- to fourth-instar caterpillars. The reaction mixture contained 1.5-mL citrate sodium phosphate buffer (0.1 M; pH 4), 1 mL α-dianisidine (0.31 mM, http://www.sigmaaldrich.com), 0.3 μL α-Glc (1 μM), 0.1 mL horseradish peroxidase (60 units/mL, http://www.sigmaaldrich.com). The reaction mixture was incubated in an Ultraspec 3000 (Pharmacia Biotech; http://www.amershambiosciences.com) spectrophotometer for 5 min to establish an initial activity rate. Aliquots of 0.1 mL of diluted OS from tobacco hornworm or beet armyworm were then added to the reaction mixture and the A460 was recorded for 5 min. In parallel, similar incubations were performed by adding a 0.1-mL solution of Aspergillus niger GOX (0.15 units/mL, type VII; Sigma-Aldrich) used as standard. Resulting changes in absorbance were calculated from the initial linear portion of the curve. One activity unit was defined as the amount of enzyme oxidizing 1 μmol α-dianisidine per min at 37°C and pH 4.5.

H2O2 Assays

Concentrations of H2O2 in OS were determined using the procedure described in the Amplex red hydrogen peroxide/peroxidase assay kit (A22188; http://www.invitrogen.com). In the presence of peroxidase, the
amoeba red reagent (10-acetyl-3,7-dihydroxyphenoxazinone) reacts with H₂O₂ to produce resorufin, a red fluorescent oxidation product. Resorufin has an absorption maximum at 585 nm. For each measurement, 1 μL sample, 49 μL reaction buffer (0.05 mM sodium phosphate, pH 7.4), and 50 μL working solution (10 μM Amplex Red reagent and 0.2 units/mL horseradish peroxidase in reaction buffer) were combined in a 96-well microplate under exclusion of light. For the H₂O₂ standard curve, 50 μL of each standard (concentrations: 0.01 μM, 0.1 μM, 1 μM, and 2 μM) were added to the working solution. Measurements were conducted with a TECAN infinite M200 plate reader (http://www.tecan.de). Fluorescence was measured 30 min after incubation with an excitation of 540 nm and detected at 590 nm. Each measurement was corrected for background fluorescence by subtracting the value derived from a no-H₂O₂ control.

Statistical Analysis

Data were analyzed with Statview 5.0 (SAS Institute). Data were transformed if they did not meet the assumption of homoscedacity.

Supplemental Data

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

Supplemental Figure S1. FACS are the elicitors of the JA burst but not the elicitors of the SA burst in wild-type plants.

Supplemental Figure S2. β-Glucosidase is not the elictor of the SA burst.

Supplemental Figure S3. GOX activity in OS₈ and OS₉.

Supplemental Figure S4. Fresh OS of tobacco hornworm and beet armyworm do not quench H₂O₂ production when the OS of either species are supplemented with Glc or GOX.

Supplemental Figure S5. Unwounded plants and plants that were wounded following a 1-MCP treatment do not differ in their SA levels from those of 1-MCP-free plants, but 1-MCP treatment of OS₈-elicited plants resulted in significantly elevated SA levels.

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