The SA-JA antagonism in *Nicotiana attenuata*: tuned by Ethylene?

Diploma Thesis

submitted by

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Abbreviations

ACC	1-aminocyclopropane- 1 -carboxylate
ET	Ethylene
ETP	Ethephon
FAC	Fatty acid-amino acid conjugates
ISR	induced systemic resistance
JA	Jasmonic acid
1-MCP	1-methylcyclopropene
NPR1	Non-expressor of *PR-1*
OS	*Manduca sexta* Oral secretion
SA	Salicylic acid
SAR	systemic acquired resistance
w + w	wounding plus water
w + OS	wounding + Oral secretion
WT	wild type

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1. Abstract

1.1 English Abstract

The phytohormones, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), mediate plant responses to pathogen and herbivore attack through a complex network of regulatory interactions. JA/SA and JA/ET cross-signaling are known to tailor defense responses, in contrast SA/ET interactions are not well-studied, particularly in responses to herbivore attack. When *Nicotiana attenuata* is attacked by *Manduca sexta*, larval oral secretions (OS) elicit rapid and transient JA and ET bursts which are greater than those elicited by wounding. When *N. attenuata* plants growing in native populations in Utah were OS-elicited, OS elicitation also found to results in bursts in both free and total SA. These results were confirmed with greenhouse-grown plants. Treating wild type (WT) plants separately with ethephon and 1-methylcyclopropene (1-MCP), an ET releaser and ET receptor antagonist, decreased and increased, respectively, the OS-elicited SA burst. When transformed *N. attenuata* plants, silenced in OS-elicited ET production (ir-*aco*) or perception (35s-*etr1a*) were OS-elicited, the OS- and wound-elicited SA bursts were amplified in both genotypes, demonstrating that the ability to produce or perceive ET suppresses the SA response. Fatty acid-amino acid conjugates (FACs) in OS are the elicitors of the JA and ET bursts but not the SA burst. SA/ET cross-communication likely tunes responses to different herbivores: attack from *M. sexta,* a specialist, elicits a larger ET and a smaller SA burst than does attack from *Spodoptera exigua* larvae, a generalist.

We propose that by suppressing the SA burst, the ET burst reduces SA/JA antagonism and allows for the unfettered activation of JA-mediated defense responses.

1.2. Deutsche Zusammenfassung

Die Pflanzenhormone, Salizylsäure (SA), Jasmonsäure (JA) und Ethylen (ET) vermitteln gemeinsam Abwehrreaktionen von Pflanzen gegen Pathogene und Herbivoren. Die Interaktion der JA/SA und JA/ET Signalwege sind bekannt für ihre maßgeschneiderte Verteidigungsantwort, währenddessen SA/ET Interaktionen im Miteinander und/oder Gegeneinander von Pflanze und Herbivor bis jetzt noch nicht tiefgreifend erforscht wurden. Beim Befall von *Nicotiana attenuata* mit *Manduca sexta*, lösen orale Sekrete (OS) der Raupe schnelle und transiente JA und ET Ausbrüche aus, welche die Mengen, ausgelöst durch Verwundung allein, weit übertreffen. *N. attenuata* Pflanzen, die in natürlichen Populationen in Utah gewachsen sind, zeigen nicht nur eine Zunahme des Hormongehaltes von JA, sondern auch sowohl von freier als auch konjugierter SA, nach Auslösung mit OS auf. Diese Daten konnten von im Gewächshaus generierten Daten bestätigt werden. Weder Fettsäure-Aminosäure Konjugate, welche als Auslöser der JA und ET Produktion gelten, noch ß-Glucosidase Aktivität in OS konnten für die Anhebung des SA Gehaltes verantwortlich gezeichnet werden. Die Steigerung der ET Emission durch die Behandlung von Wildtyppflanzen (WT) mit entweder dem ET Präkursor 1-Aminocyclopropan-1-Carboxylat (ACC) oder dem ET Auslöser Ethephon, resultierte in keinem Effekt zu den produzierten SA Mengen bzw. darin, diese zu verringern. Die Inhibierung der ET Wahrnehmung durch Vorbehandlung von WT Pflanzen mit dem ET-Rezeptor-Antagonisten 1-Methylcyclopropan, oder durch den Einsatz von transformierten 35s-etr1a Pflanzen,

wies eine drastische Erhöhung der SA Mengen nach OS Behandlung auf, was die antagonistische Rolle von ET auf die SA Produktion demonstriert. Die Kommunikation der SA/ET Signalwege scheint massgeblich an der Abstimmung der Reaktion der Pflanze auf verschiedene Herbivoren beteiligt zu sein: bei Befall mit *M. Sexta* - einem Spezialisten, im Gegensatz zu *Spodoptera exigua* - einem Generalisten, werden größere Mengen an ET und geringere Mengen an SA produziert. Schlussfolgernd kann gesagt werden, daß durch die Inhibierung des SA Gehaltes, die ET Produktion im Zusammenspiel mit NPR1 stattfindet, um eine uneingeschränkte JA-vermittelte Verteidigung zu ermöglichen, auch wenn Pflanzen von Herbivoren attackiert werden, die ebenso SA Signalwege initiieren.

2. Introduction

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

How plants cope with these demands is 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; 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 (HR). SA, which functions as a signal in the HR, in turn elicits a long-lasting, induced resistance mechanism against a broad range of invading pathogens, known as systemic acquired resistance (SAR) (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 against necrotrophic pathogens by initiating the SA-independent induced systemic resistance (ISR) (Pieterse et al., 1998; Vijayan et al., 1998) and against herbivores (Halitschke and Baldwin, 2005). 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). Little is known about JA's effects on SA signaling, but in JA- and coronatine-insensitive (*coi*) mutants, SA-mediated gene expression and defenses are greatly 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 (reviewed in von Dahl and Baldwin, 2007). For example, the ET burst elicited by herbivore attack enhances the production of JA-elicited proteinase inhibitors in tomato (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). In all examples studied to date, ET tunes JA responses but little is known, about JA to influence ET production or the responses it mediates. While both ET/JA and JA/SA interactions are important for various pathogen responses, the ET/SA interactions are not well-studied in plant responses to herbivory.

One of the best-studied model systems for plant-insect interactions is the relationship between the native tobacco species *N. attenuata* Torr. ex. Wats. and its specialized herbivore, the tobacco hornworm *Manduca sexta* (Lepidoptera, Sphingidae). *N. attenuata*, a species native to the Great Basin Desert/USA, also known as Wild Tobacco or Coyote Tobacco (Goodspeed 1954) is a member of the *Solanaceae*, one of the most fascinating families in the plant kingdom, with about 2500 species.

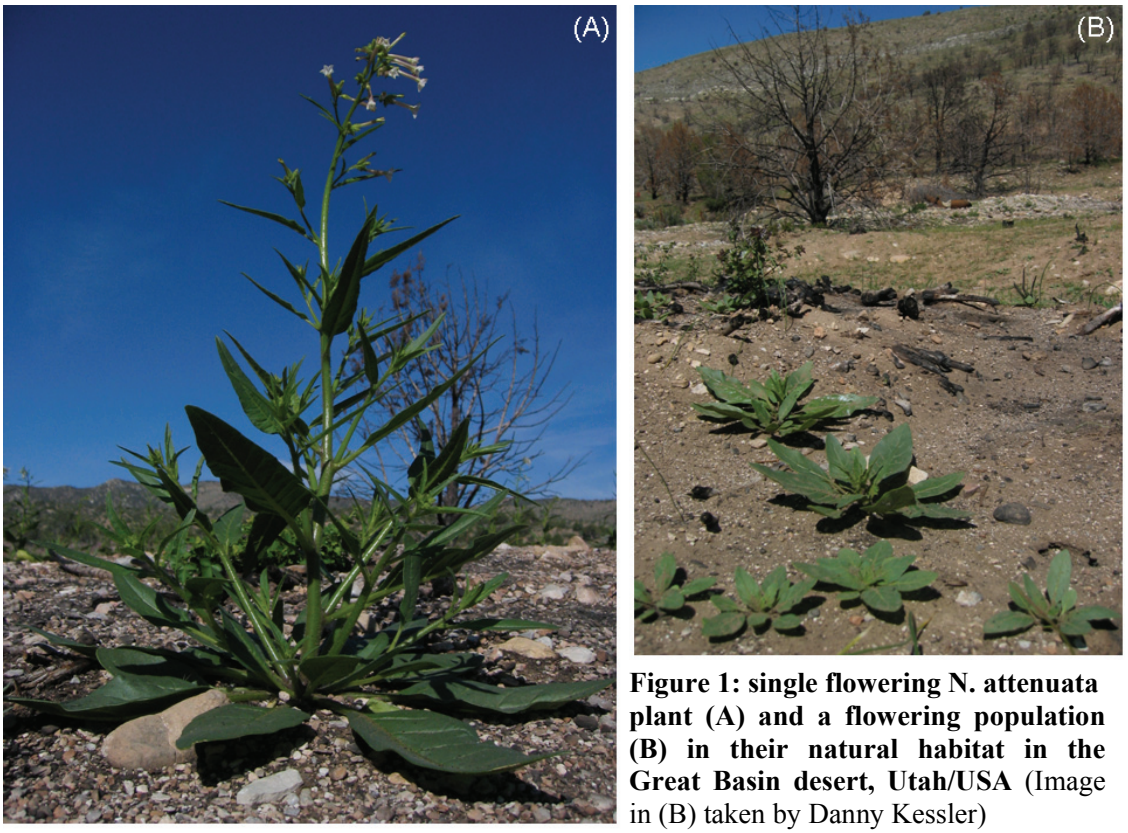


Figure 1: single flowering N. attenuata plant (A) and a flowering population (B) in their natural habitat in the Great Basin desert, Utah/USA (Image in (B) taken by Danny Kessler)

The family is also known as the nightshade family and includes many plants which are used both for their nutritional and pharmacological properties including Datura (*Datura spec.*), eggplant (*Solanum melongena*), deadly nightshade (*Atropa belladonna*), chilli pepper (*Capsicum annuum*) potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*) and many others. Seeds of *N. attenuata*, a primarily selfing annual, germinate into the immediate post-fire environment in response to smoke-derived signals (Baldwin, 1994; Preston, 2000; Baldwin et al., 2005).

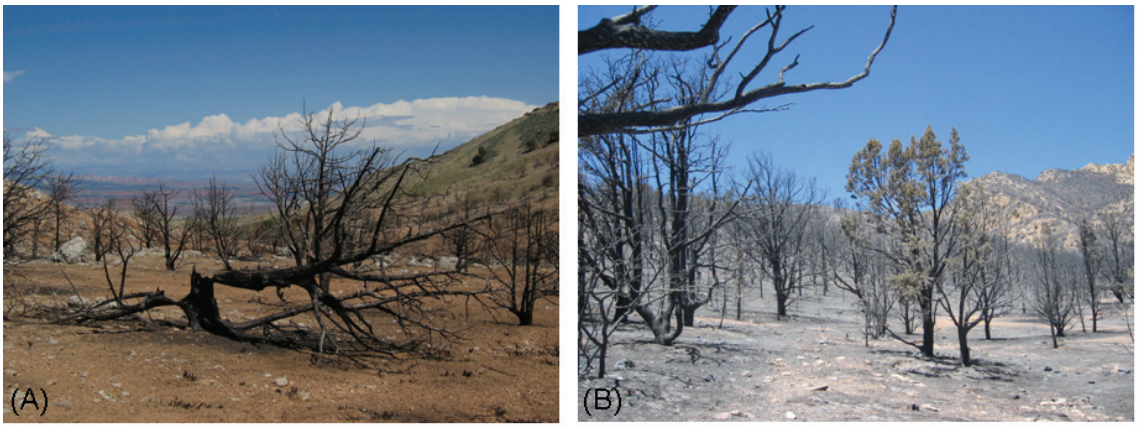


Figure 2: typical post-fire environment in which *N. attenuata* occurs as a pioneering plant
Images taken by Danny Kessler (A) and Rayko Halitschke (B)

M. sexta larvae prefer to feed on the foliage of *N. attenuata*, but various other members of the solanaceaeous group can also serve as hosts. They have a short life cycle, lasting about 30 to 50 days, in which the larvae stage lasts on average 20 days. At the end of the larval stadium, which means after reaching the fifth larval instar, the caterpillar burrows underground and pupates. In most habitats, *M. sexta* has on average two generations per year. *M. sexta* as a specialist has evolved nicotine detoxification mechanisms and also accumulates a considerable amount of nicotine in its haemolymph, which causes grave mortality to its parasitoids (Barbosa et al., 1991). To thwart this adaptation, *N. attenuata* has evolved mechanisms to fine-tune its defense signaling, presumably via ET.

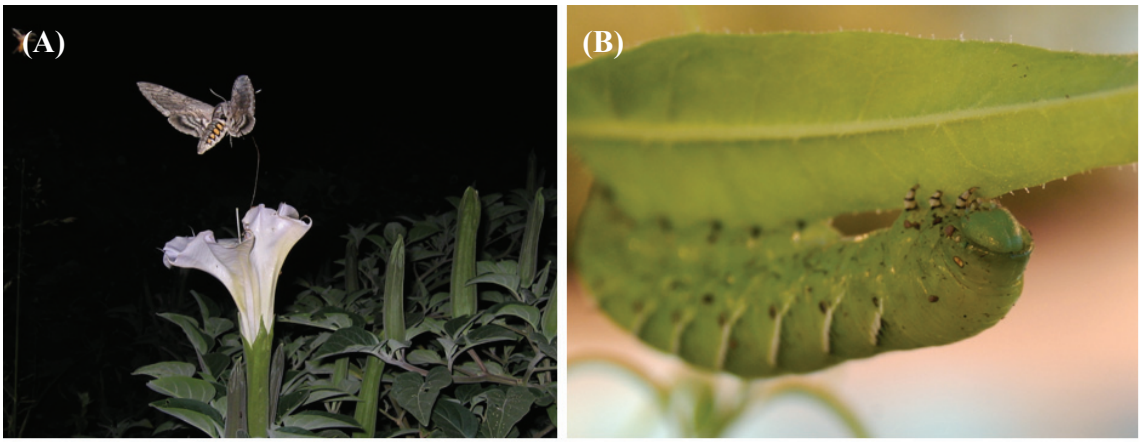


Figure 3: *Manduca quinquemaculata* moth sucking nectar on *Datura wrightii* flowers and larvae of its closely related species *M. sexta* feeding on *N. attenuata*. Images are taken by Danny Kessler (A) and Georgios Wenetiadis (B)

Here we provide evidence that the dramatic ET burst, which is elicited when larvae of the tobacco hornworm *M. sexta* attack their host plant *N. attenuata* (Kahl et al., 2000; von Dahl et al., 2007), plays a central role in mediating the JA/SA antagonism. The effectiveness of *N. attenuata*'s JA-elicited defenses against herbivore attack and the relevance of JA signaling in the plant's native habitat have been well established (Baldwin, 1998; Kessler et al., 2004; Paschold et al., 2007). Given the importance of JA-mediated defenses against *M. sexta* attack, we were surprised to find that OS-elicited *N. attenuata* plants growing in native populations exhibited both the expected JA burst and a more delayed SA burst, which could potentially antagonize the JA-dependent responses.

Defense responses of plants start upon the introduction of elicitors contained in herbivore saliva vectored during feeding (Roda et al., 2004). Two classes of OS-derived elicitors have been isolated from lepidopteran larvae: enzymes like ß-glucosidase and

fatty acid-amino acid conjugates (FACs). A detailed study by Halitschke et al. (2003) showed that the two major FACs found in *M. sexta* OS are able to induce 86% of the transcriptional responses shown in *N. attenuata* after herbivore attack. They demonstrated that this transcriptional remodeling is connected to the induction of JA. Although, JA and ET signaling is activated when FACs are introduced into wounds during feeding (Halitschke et al., 2001; von Dahl et al., 2007) the elicited SA burst neither depends on the presence of FACs nor on the activity of β-glucosidase that could release SA from its sugar conjugates (Malamy et al., 1992). However, ET insensitive plants pre-treated with the ET receptor antagonist, 1-methylcyclopropane (1-MCP), or ectopically expressing a mutant ET receptor accumulated dramatically increased SA levels after OS elicitation, suggesting ET-dependent suppression of the SA burst during herbivore attack.

Besides the specialist herbivore *M. sexta* the generalist *Spodoptera exigua* (Lepidoptera, Noctuidae) also uses *N. attenuata* as a host species. *S. exigua* is native to Southeast Asia but first discovered in North America about 1876. It annually re-invades the southern half of the United States where *N. attenuata* is growing naturally, as the weather warms, because the herbivore would die off during the cold period. Overwintering is generally limited to Arizona, Florida, and Texas. The eggs are laid in numbers of about 80 per nest and the caterpillar burrows into the soil below the plant where it pupates without a cocoon. The life cycle can be completed in as few as 24 days (undergoing five instars), and six generations have been reared during five months of summer in Florida (Wilson 1934).

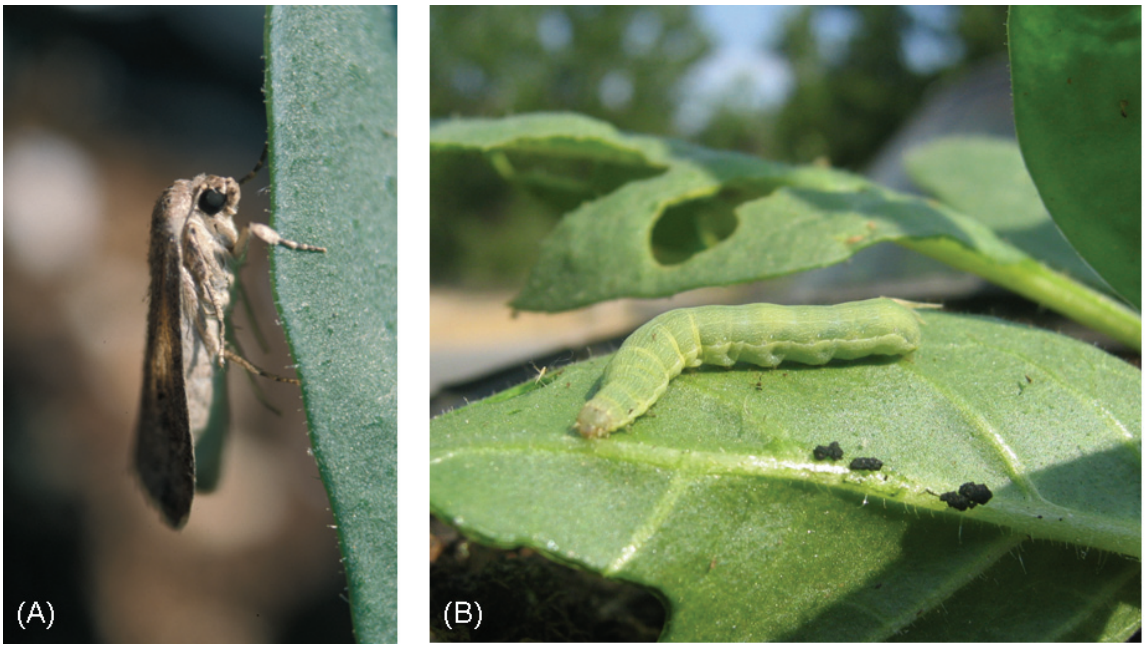


Figure 4: *Spodoptera exigua* moth (A) and its larvae (B) on *N. attenuata*.
Images are taken by Danny Kessler

The observation of the opposing regulation of SA and ET accumulation during the attack of two different herbivores was striking: feeding of the specialist *M. sexta* larvae elicited a larger ET and a smaller SA burst in comparison to the hormone levels in response to feeding of the generalist *S. exigua* larvae.

3. Materials and Methods

3.1 Plant growth:

Wild type *Nicotiana attenuata* Torr. Ex. Watson. (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae) plants were obtained from an inbred line in its 22th generation that originated from seeds collected on the DI ranch in Utah in 1998. Seeds of WT and genetically transformed 35s-*etr1b* plants (A-03 538-1), ectopically expressing the *Arabidopsis thaliana* mutant ethylene receptor *etr1-1* under the control of a CaMV 35S promoter (von Dahl et al., 2007) were germinated on Gamborg's B5 medium (Krügel et al., 2002). In short: seeds were sterilized and incubated in 0.1 M gibberellic acid (GA_3) and 1:50 diluted liquid smoke (v/v) (House of Herbes, Passaic, NY, USA) before germination on Gamborg's B5 at a 26°C/16 h 155 μm/s/m^2 light: 24°C/8 h dark cycle (Percival, Perry Iowa, USA). When seedlings entered an age of 10 d, they were planted singly into Teku pots (Waalwijk, The Nederlands) and transferred into 1-L pots after an additional 10 d. Plants were grown in the greenhouse with a day/night ratio of 16 (26-28ºC)/8 (22-24ºC) h under supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600 W Na lights (Philips, Turnhout, Belgium).

3.2. Insect rearing and feeding experiments:

Manduca sexta (Tobacco hornworm) eggs, purchased from Carolina Biological Supply (USA) 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. For collection of OS, larvae were reared on *N. attenuata* leaves until the 3rd instar. *Spodoptera exigua* (Beet armyworm) larvae hatched from eggs supplied by the Plant Protection Centre of Bayer AG (Monheim, Germany) were cultured on artificial diet consisting of 300 g/L agar, 400 g/L bean flour, 3 g/L sodium ascorbate, 3 g/L ethyl p-hydroxybenzoate, and 1 g/L formaldehyde with a photoperiod of 14-16h photophase at 22°C to 24°C until reaching the third instar.

Caterpillar herbivory bioassays were performed in clip-cages. One 3rd instar *S. exigua* or two 1st instar *M. sexta* larvae per plant were allowed to feed on +1 leaves for three days.

3.3. Experimental Setup and plant treatment:

Plants were selected in natural populations in the southwestern of the USA at the time they were about to elongate. Two groups with three plants each were chosen for one treatment. These three plants grew within 20 cm of each other. Every six-plant group were separated from the next six-plant group by 5 meters. One group was wounded six times with a pattern wheel on the second fully developed leaf and the other group was wounded the same way and treated with 20 µL *M. sexta* oral secretion

(diluted 1:1 with H_2O), which was applied immediately to the wound. Control plants remained untreated. At defined time points the treated leaves were harvested.

In the glasshouse, the three youngest fully expanded leaves (position +1, +2 and +3) were mechanically wounded with a pattern wheel by producing four rows of punctured holes on each side of the mid-vein. Fresh wounds were treated with 20 µL of *M. sexta* oral secretion (OS) (diluted 1:1 with H_2O), 1-aminocyclopropane-1-carboxylic acid (ACC, Sigma); in 5mM MES (6mg/mL), ethephone (6 mg/mL 5 mM MES) or 5 mM MES. Control plants remained untreated.

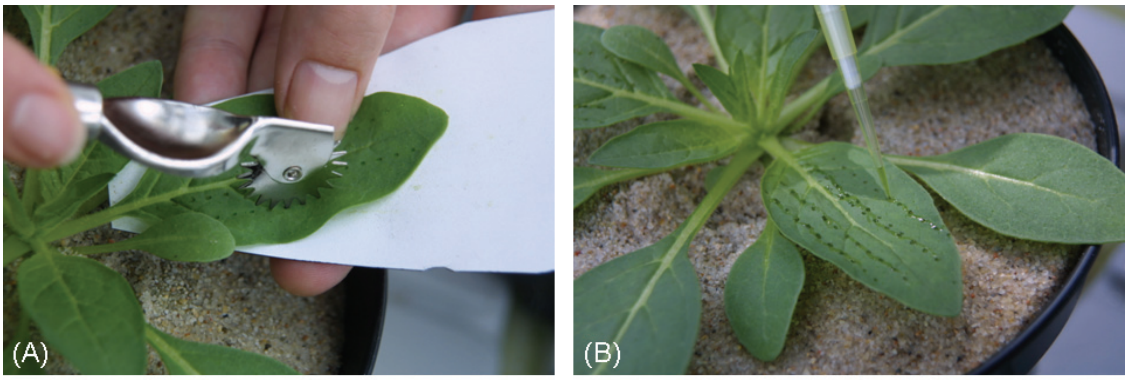


Figure 5: A 25 day old *N. attenuata* plant beeing treated for phytohormone analysis with a fabric pattern wheel, by rolling over the leaf surface on both sides parallel to the mid-vein (A) and adding OS immediately onto the punctured wounds (B).
Images taken by Danny Kessler

To inhibit ethylene perception plants were exposed to 1-methylcyclopropane (1-MCP). Six plants were placed into a 20-L container and exposed to 1-MCP for 8 h during the dark cycle. Following Kahl et al., (2000), 500 mg of Ethylblock (0.43% 1-MCP, van der Sprong, Roelofarendsveen, Netherlands) was weighed into a vial and 10 mL of an alkaline solution (0.75% KOH + NaOH) was added to release 1-MCP. Fatty

acid/amino acid conjugates (FACs) were applied to fresh wounds in 20 µL of 0.05% Triton-X-100 (Sigma) at a concentration of 50ng μL^{-1} (0.12 mM) *N*-linolenoyl-L-Gln (18:3-Gln, 1) and 138ng μL^{-1} (0.34 mM) *N*-linolenoyl-L-Glu (18:3-Glu, 3).

3.4 Analysis:

3.4.1. JA and SA quantification

Free SA and JA was extracted from 200-300 mg of fresh plant leaf material that was homogenized in FastPrep tubes containing 900 mg FastPrep matrix (BIO 101, Vista, CA, USA) and extracted in 1 mL of ethylacetate spiked with 200 ng (or 100ng for SA quantification in herbivore experiment) of D_4-SA and $^{13}C_2$-JA, as internal standards. Samples were homogenized twice by reciprocal shaking at 6.5 m s^{-1} for 45 seconds and centrifuged at 13,000 rpm for 20 min at 4°C. Supernatants from 2 extraction steps were pooled and evaporated until dryness. The dried residue was dissolved in 500 µL of 70% of methanol, vortexed and centrifuged. Subsequent 200 µL of the supernatant were transferred in HPLC vials and analyzed by HPLC-MS/MS.

Conjugated SA was extracted as described previously (Malamy et al., 1992). The first 2 extraction steps were conducted in the same way as in the procedure for free SA extraction with the exception that the extraction buffer was 90% MeOH, which was spiked with 100 ng of internal standard. For the second extraction step, pure MeOH was used. The supernatants were pooled and evaporate to dryness at 30°C in an Eppendorf

Concentrator. The dried extracts were dissolved in 0.5 mL H_2O and vortexed for 1 min. After addition of 0.5 mL 0.2 M acetate buffer (pH 4.5) containing 0.1 mg/mL ß-glucosidase (22 units/mg, Sigma), the samples were incubated at 37°C for 3 h and centrifuged (5min at 13,000 rpm). Finally, 200 µL of the supernatants were transferred to HPLC vials for quantification by HPLC-MS/MS.

3.4.2. HPLC-MS/MS

SA and JA measurements were conducted on a Varian 1200 Triple-Quadrupole-LC-MS system (Varian, Palo Alto, CA, USA). 15 µL of each sample were injected onto a ProntoSIL column (C18; 5 µm, 50 × 2 mm, Bischoff, Germany) attached to a precolumn (C18, 4x2mm, Phenomenex, USA). 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 rate of [time/flow (mL/min): 0:00/0.4; 1:00/0.4; 1:30/0.2; 10:00/0.2; 10:30/0.4; 12:30/0.4; 15:00/0.4]. The compounds were detected in the ESI negative mode. Molecular ions (M-H) with *m/z* 137 and 141 generated from endogenous SA and internal standard, respectively, were fragmented under 15V collision energy. The ratios of ion intensities of their respective daughter ions, *m/z* 93 and 97, were used to quantify endogenous SA.

For endogenous JA and its internal standard, molecular ions (M-H) with *m/z* 209 and 211, respectively, were fragmented under 12V collision energy. The ratios of ion

intensities of their respective daughter ions, *m/z* 59 and 61, were used for JA quantification.

3.4.3. β-glucosidase assay:

The presence of β-glucosidase activity was determined, following the procedure described in Mattiacci et al., (1995), in SA-inducing and non-SA-inducing *M. sexta* OS. OS samples were assayed in triplicates. The incubation mixture, which contained 5 mM 4-nitrophenyl β-D-glucopyranoside (Sigma, Steinheim, Germany) in 1 mL 0.1 M Tris buffer and 125 µL of OS solution (diluted 1:1 with H_2O), was briefly vortexed and incubated in a water bath for 2h at 30 °C. The reaction was stopped by immersing the incubation tubes in boiling water for 10 min. All tubes were centrifuged at 10,000 x g for 10 min after incubation and the absorbance of the supernatant was measured in an Ultrospec 3000 (Pharmacia Biotech) spectrophotometer. The concentration of *p*-nitrophenol, the reaction product, was determined at 400 nm by using a molar extinction coefficient of 18,130. One unit was defined as the amount of enzyme hydrolyzing 1 µmol of substrate per min at 30 °C.

3.4.4. Ethylene measurements:

Ethylene emissions were measured continuously and non-invasively in real-time with a photoacoustic spectrometer (INVIVO, Adelzhausen, Germany) as described in von Dahl et al., (2007) for "stop-flow" measurements. The youngest fully expanded leaves of slightly elongated plants were subject to feeding by 3 neonate *M. sexta* larvae or one 3rd instar *S.exigu*a 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. The cuvettes were flushed by a flow of purified air at 130 to 150 mL min^{-1} which had previously passed through a liquid N_2 cooling trap to remove CO_2 and H_2O.

3.4.5. Statistical analysis:

All statistical analyses were performed with StatView Version 5.0 (SAS Institute Inc. Copyright© 1992-1998) software. Prior to statistical analysis the data was tested for homogeneity of variances and data presented in Figs. S1, 1, 3C and 4 were log transformed. Statistical analysis was performed using ANOVA or the Student's t-test as indicated.

4. Results

4.1. OS-elicitation of SA and JA bursts in *N. attenuata* plants growing in nature

Five groups of three similarly sized and minimally damaged *N. attenuata* plants of unknown genetic diversity growing in a native population in a one-year-old burn in southwest Utah had their leaves wounded with a pattern wheel and the wounds were immediately treated with either water (W) or *M. sexta* oral secretions (OS), or were left unwounded (time point zero). The concentrations of both free SA and total SA increased after 180 min in both W and OS-treated plants in comparison to untreated control plants (Fig. 6 (A), Fig. 7). OS elicitation amplified the W-elicited increase in free SA levels two-fold above controls. Changes in total SA amounts mirrored those of the free SA levels, but were three-fold higher (Fig.7). JA levels responded as has been demonstrated for glasshouse-grown *N. attenuata* plants (Ziegler et al., 2001): OS elicitation amplified the W-induced increase 3.5-fold with maximum values attained 60 min after elicitation (Fig. 6 (B)). These results demonstrate that neither strict genotypic nor environmental control are necessary to detect OS-elicited SA and JA bursts, as these responses are readily detected in relatively undamaged plants of unknown genetic origin growing in a native population.

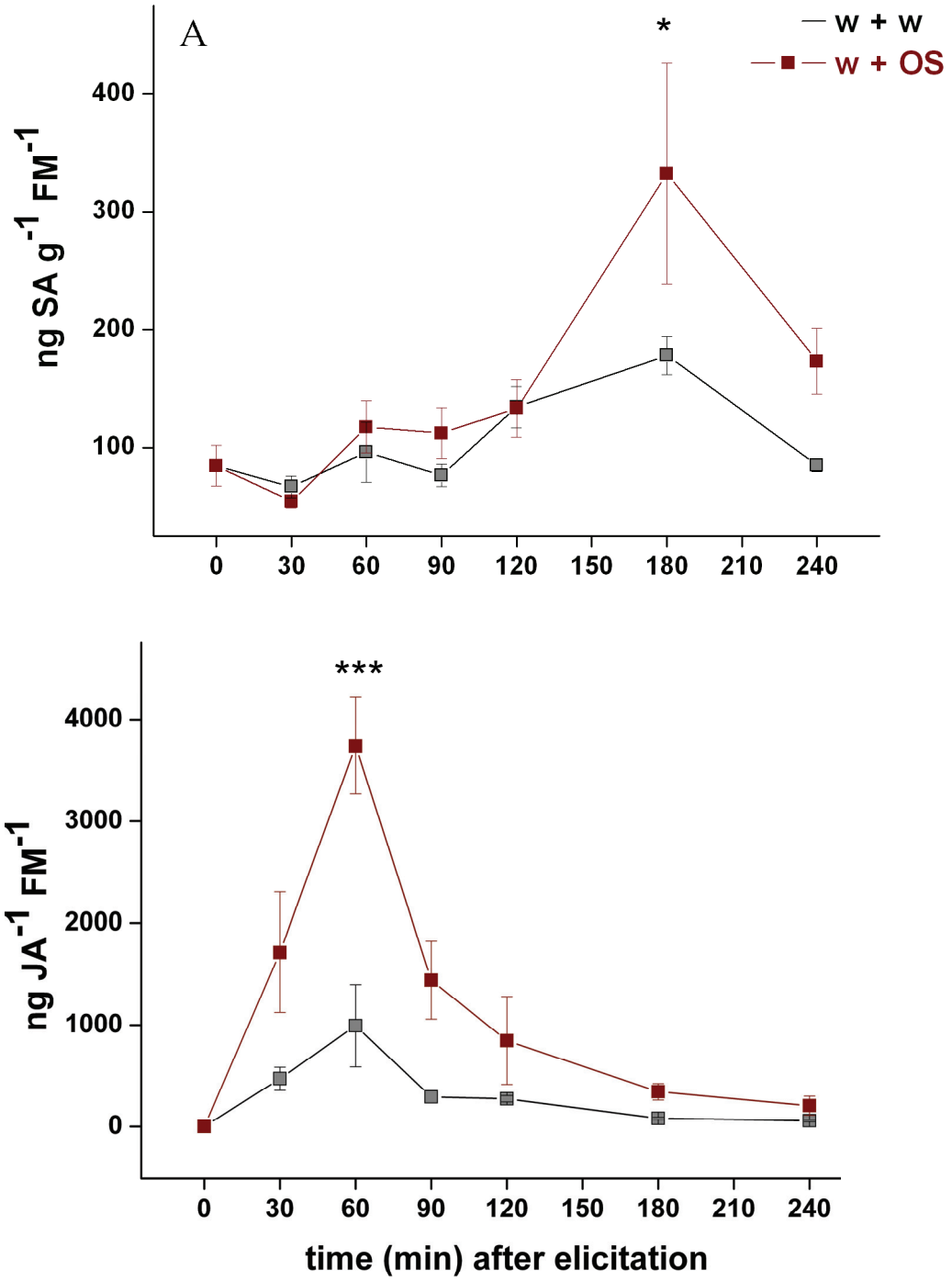


Figure 6. ***OS-elicited SA and JA accumulation in natural-grown*** **N. attenuata** ***plants***
Mean ± SE (n = 5) free SA (A) and JA (B) levels per fresh mass (FM) in *N. attenuata* plants growing in a native population in SW Utah. Fully mature leaves were wounded with a fabric pattern wheel and the wounds were either treated with water (w + w, black line) or *M. sexta* oral secretions (w + OS, red line). Control plants (0 min) were left unwounded. Asterisks indicate significant differences among treatments at the indicated time points (ANOVA, $F_{12,51} = 5.327$, * = $P < 0.05$; *** = $P < 0.001$).

4.2. OS- and FAC-elicited SA levels in glasshouse-grown plants

Free and total SA levels of glasshouse-grown *N. attenuata* plants elicited with OS, W or left untreated as controls changed similarly to the pattern observed in field-grown plants. Maximum values were attained 120 min after elicitation (Fig. 7) and OS treatment resulted in significantly higher SA levels in comparison to W-elicited SA levels (Student's *t*-test; $P = 0.0231$).

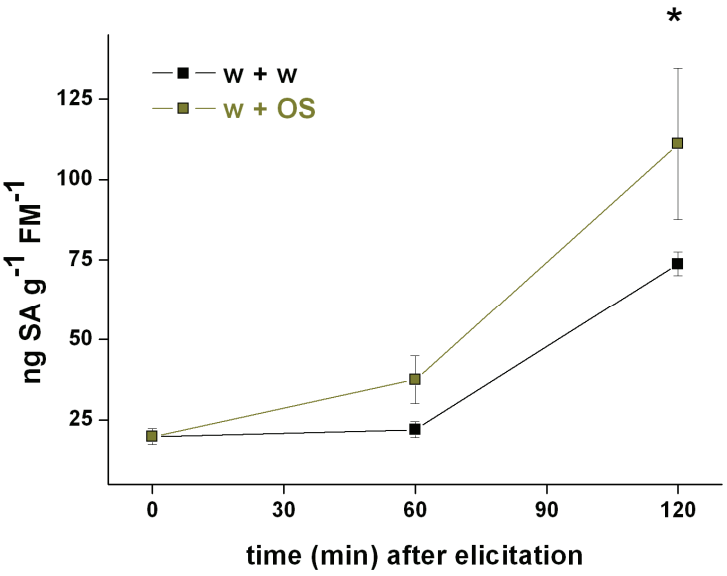


Figure 7: *OS-elicited SA accumulation in glasshouse grown* N. attenuata *plants*
Mean ± SE (n = 5) free SA levels of wounded *N. attenuata* plants 60 and 120 min after immediately treating wounds with *M. sexta* oral secretions (w + OS; green line) or water (w + w; black line). SA levels of untreated plants are shown at 0 min. Asterisks indicate significant differences to the controls at the individual time point (ANOVA, * = $P < 0.05$).

Previous work by Halitschke et al., 2003 demonstrated that fatty acid/amino acid conjugates (FACs) found in OS are necessary and sufficient for the elicitation of both the JA (ANOVA, $F_{2,13} = 68.984$, $P < 0.05$; Fig. 8A) and ethylene bursts (von Dahl et al., 2007). To determine if FACs were also responsible for the elicitation of the SA burst, we tested the two most abundant FACs found in OS. Treatment of puncture wounds with these FACs at concentrations found in *M. sexta* OS did not significantly increase

free SA levels above those found when puncture wounds were treated with water or the detergent-containing buffer (ANOVA, $F_{2,14} = 2.717$, $P = 0.87$; Fig 8B).

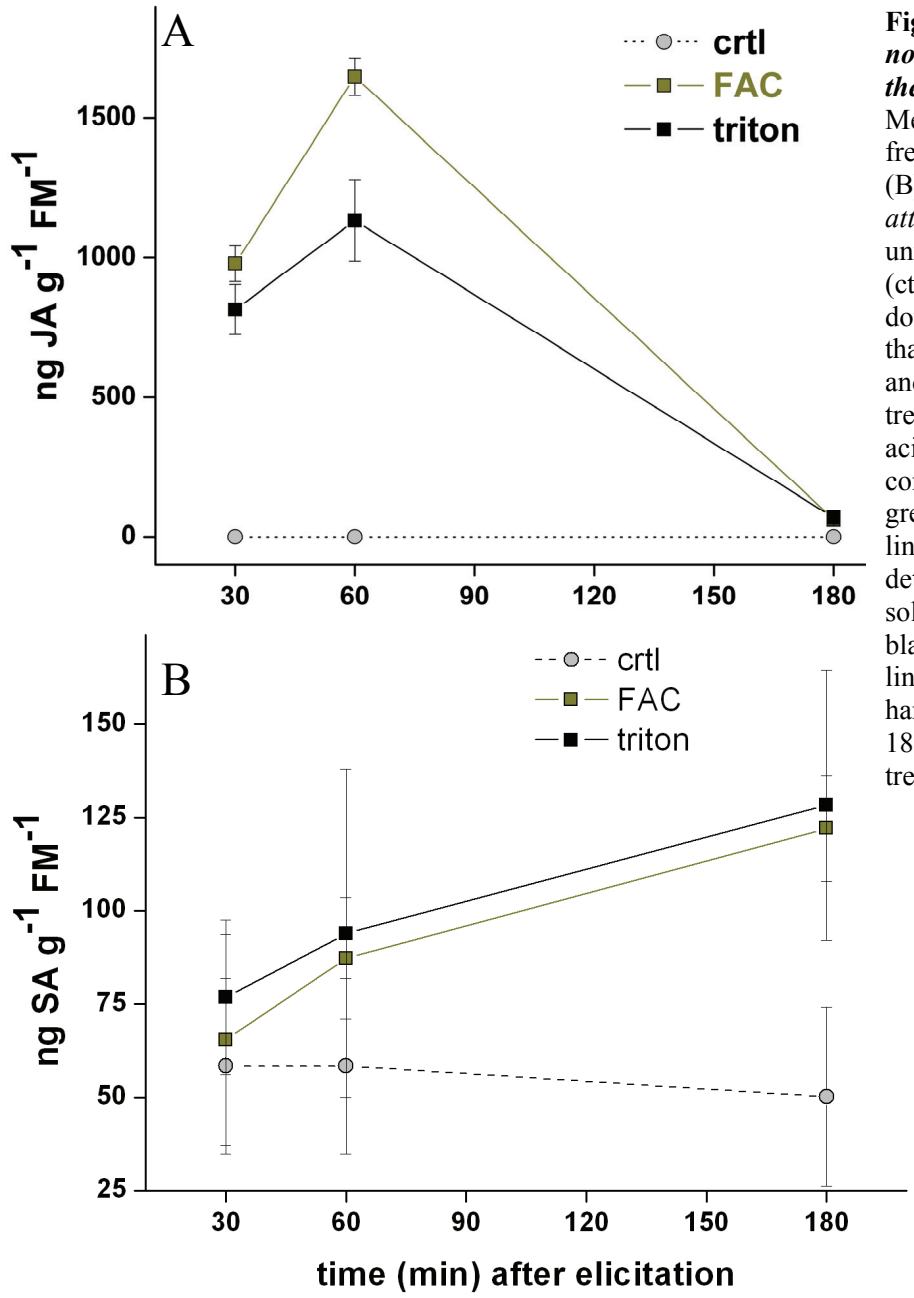


Figure 8. ***FACs are not the elicitors of the SA burst.*** Mean ± SE (n = 5) free JA (A) and SA (B) levels in *N. attenuata* leaves of untreated plants (ctrl; open circle, dotted line) or plants that were wounded and subsequently treated with fatty acid amino acid conjugates (FAC; green squares, green line) or with the detergent containing solution (triton; black squares, black line). Leaves were harvested 30, 60 and 180 min after the treatments.

In addition to FACs, at least one more class of elicitors of indirect defense responses is known to occur in the OS of herbivores: lytic enzymes such as β-glucosidase (Mattiacci et al., 1995). The ability of OS-treatment to amplify the W-elicited SA burst varied from one OS sample to another (Fig. 2B).

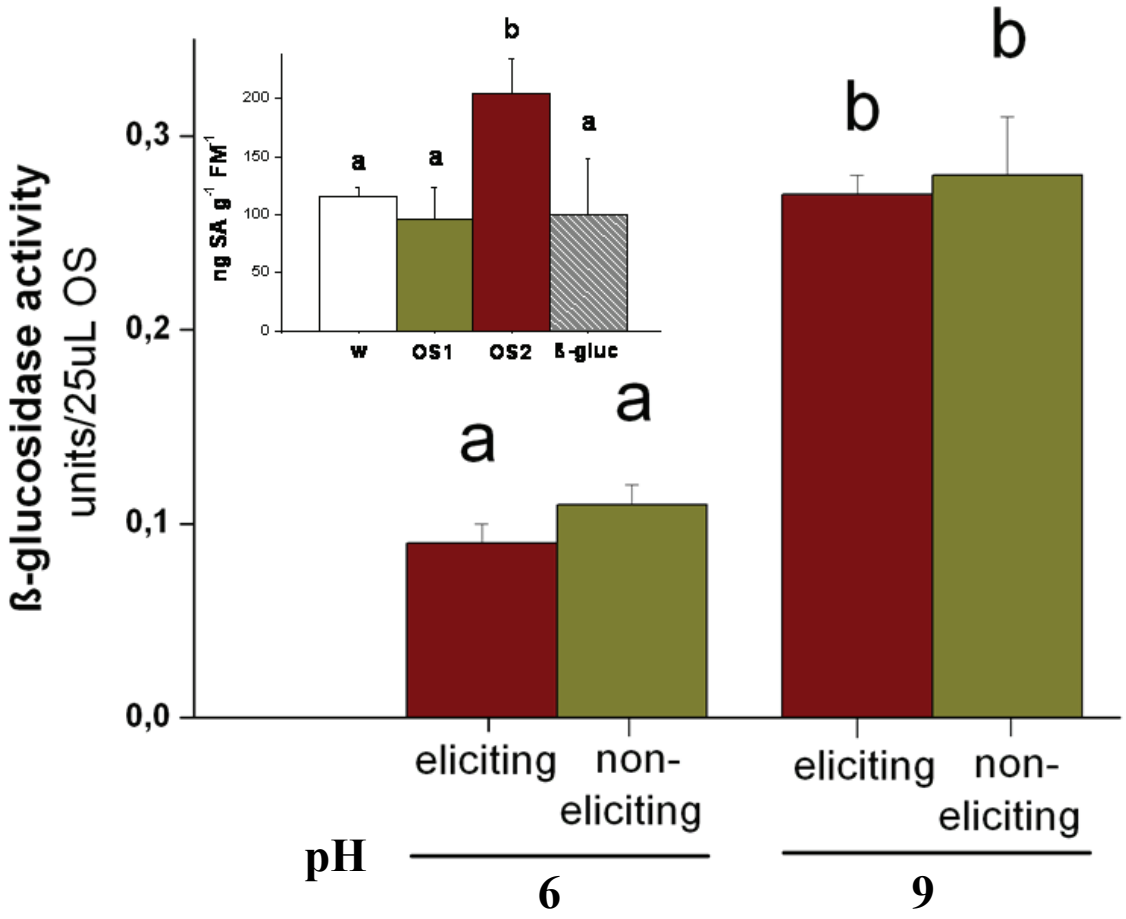


Figure 9: ***ß-glucosidase is not the elicitor of the SA burst.***
Mean ± SE β-glucosidase activity (n = 3) in SA-eliciting (red bars) and non-SA-eliciting (green bars) *M. sexta* oral secretions (OS) at pH 6 and 9 (ANOVA, $F_{3,7} = 93.856$, $P < 0.001$). **Inset:** Mean ± SE (n = 5) SA levels in *N. attenuata* WT plants 180 min after wounding and treatment of the wounds with water (w, white bar), non-SA-eliciting OS (OS1, green bar), SA-eliciting OS (OS2, red bar) or β-glucosidase at concentrations present in *M. sexta* OS (β-gluc, striped , grey bar). Different letters indicate significant differences among treatments.

To determine if β-glucosidases present in *M. sexta* OS could account for this variability in SA-eliciting ability, we measured β-glucosidase activity in SA-inducing as well as in non-SA-inducing OS. Comparable β-glucosidases activity in both OS samples was detected. Moreover, β-glucosidases activity did vary between measurements made at pH 6 and pH 9, the range of midgut pHs found in Lepidopteran species (Fig. 9).

4.3. Ethylene suppresses the OS-elicited SA burst

When wounded leaves of WT *N. attenuata* plants were treated with ethephone (ETP), an ET releaser, SA levels decreased by 30% in comparison to those in wounded leaves that were wounded and treated only with water (Fig. 10A). After the addition of ACC, a precursor of ET, to wounded leaves, SA levels did not differ from the SA levels measured after water or ethephone addition to wounds (ANOVA, $F_{3,14} = 6.659$, $P < 0,05$; Fig. 10A). In contrast, OS-elicited SA levels increased by 25% when WT plants were rendered ethylene insensitive by a preceding overnight exposure to 1-MCP an ethylene receptor antagonist (Fig. 10B).

To further explore the role of ethylene signaling in the regulation of the SA burst, transgenic *N. attenuata* plants rendered ET insensitive by the ectopic expression of the mutant etr1-1 receptor of Arabidopsis (35s-*etr1b*) were used. OS-elicited 35s-*etr1b* plants accumulated significantly higher free SA levels (nearly three-fold) compared to OS-elicited WT plants (Fig. 10C).

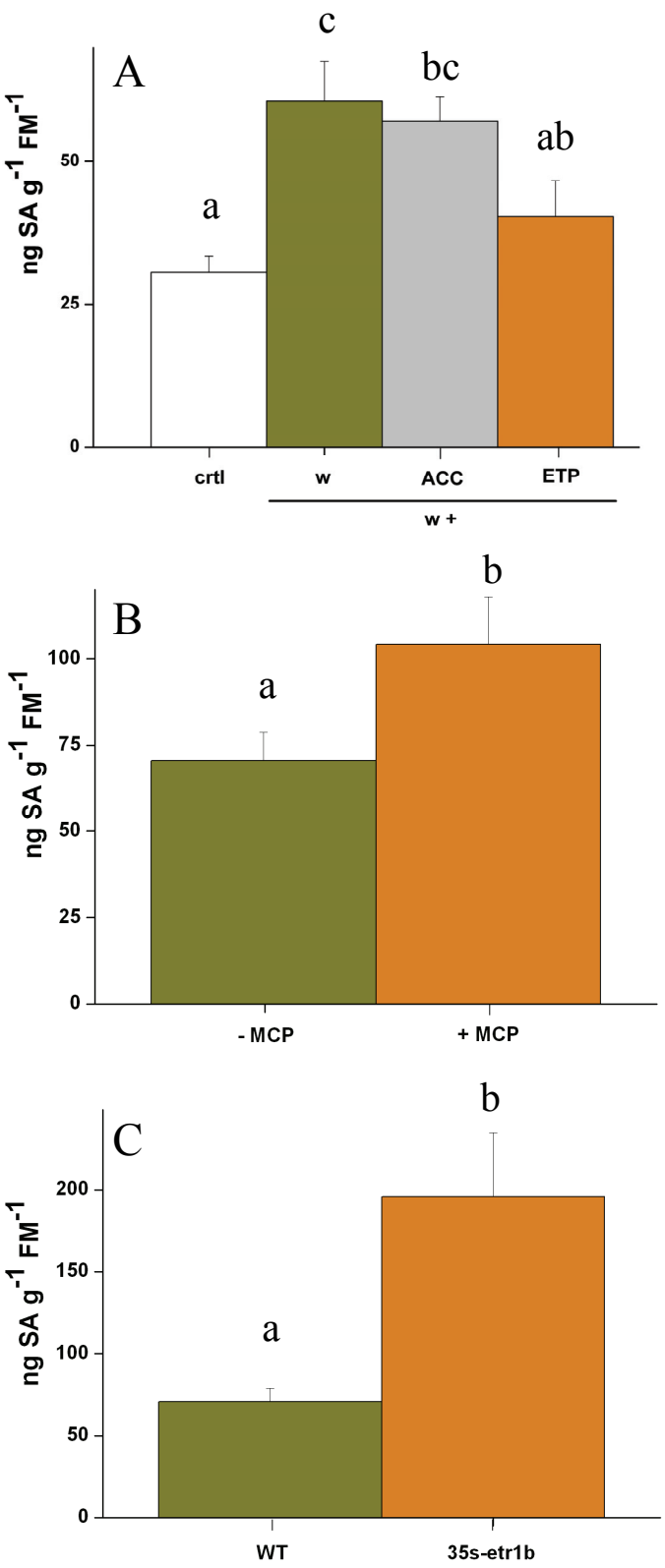


Figure 10: ***Ethylene suppresses OS-elicited SA accumulation.***
Mean ± SE (n = 5) free SA levels in: **A)** wild type (WT) *N. attenuata* plants 120 min after wounding and treating the wounds (+ w) with water (w, green bar), 1-ACC (ACC, grey bar) or ethephone (ETP, orange bar). Control plants remained untreated (crtl, white bar; ANOVA, $P < 0.05$); **B)** 120 min after OS elicitation of WT plants that were previously exposed to 1-MCP (+ MCP, orange bar) to block their ET receptors and control unexposed plants (- MCP, green bar); and **C)** 120 min after OS elicitation of WT (green bar) and ET-insensitive 35s-*etr1a* (orange bar) plants. Different letters indicate significant differences between treatments of the respective panels (Student's *t*-test; $P < 0.05$).

Together these results suggested that ethylene negatively regulates the SA burst, as increased ethylene levels suppressed SA accumulation and rendering plants ethylene insensitive, amplified the SA levels.

4.4 Differential elicitation of SA and ET by a generalist and specialist herbivore.

The two most abundant Lepidopteran herbivores found on *N. attenuata* plants growing in their native habitats are the specialist, *M. sexta*, and the generalist *S. exigua* (Steppuhn et al., 2004). These two species were chosen to compare the plants' SA/ET responses. Larvae number and instars were adjusted to ensure that damage amounts were comparable between treatments. During the 22h feeding period, three neonate *M. sexta* larvae removed 0.235 cm^2 ± 0.04 leaf area while one third instar *S. exigua* larvae removed 0.353 cm^2 ± 0.083 (Student's *t*-test, $P = 0.212$).

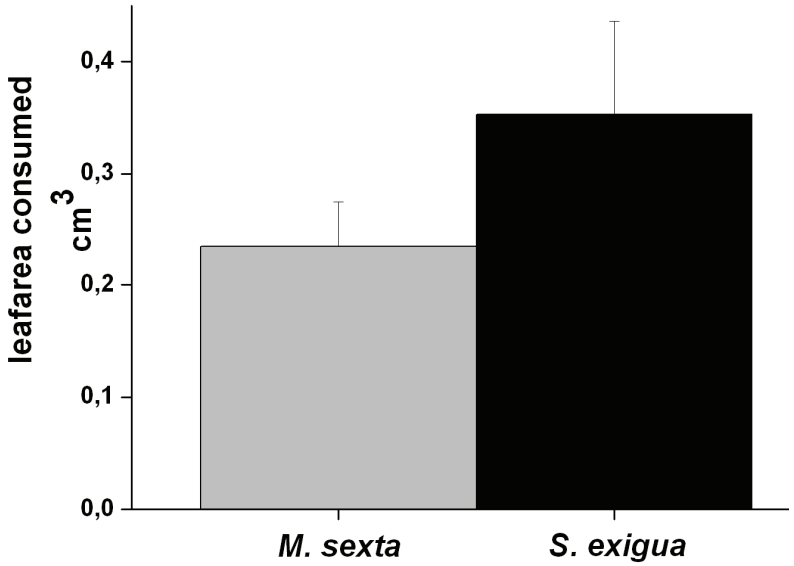


Figure 11: ***leafarea consumed by the N. attenuatas most abundant lepidopteran herbivores***
Mean ± SE (n = 8) leafarea consumed by *M. sexta* (grey bar) and *S. exigua* (black bar) after a 22h feeding period. ($P = 0.212$)

S. exigua attack significantly increased SA levels in WT plants in comparison to plants attacked by *M. sexta* larvae (ANOVA, $F_{2,14} = 2.602$, $P < 0.05$). Free SA levels of control plants increased six-fold after attack of *S. exigua* (Fig. 12A). ET emissions

showed the opposite pattern. ET emission of *N. attenuata* plants increased up to three-fold in response to *M. sexta* larvae attack, whereas only a slight increase was measured in plants attacked by *S. exigua* larvae (Fig. 12B). This antagonistic pattern of SA and ET levels in response to two different Lepidopteran species is consistent with the hypothesis that ET is a negative regulator of SA accumulation.

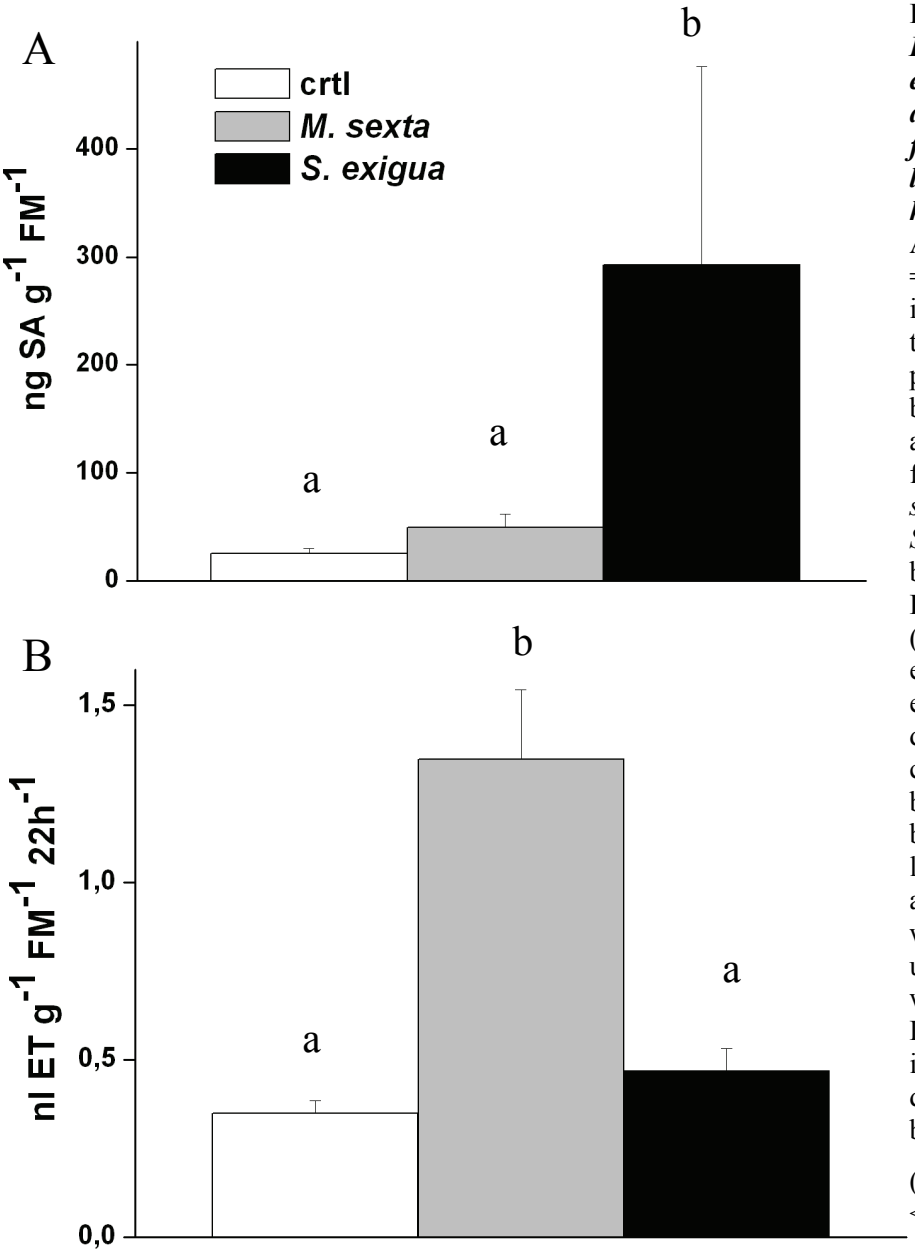


Figure 12: ***Differential elicitation of SA and ET by attack from two lepidopteran herbivores.***
A) Mean ± SE (n = 8) free SA levels in untreated wild type *N. attenuata* plants (ctrl, white bar) and 1 day after continuous feeding by *M. sexta* (grey bar) or *S. exigua* (black bar) larvae.
B) Mean ± SE (n = 3) ethylene emission of excised leaves during 22 h of continuous feeding by *M. sexta* (grey bar) or *S. exigua* larvae (black bar) and of leaves that were left undamaged (crtl, white bar). Different letters indicate significant differences between

(ANOVA, $P < 0.05$).

5. Discussion:

In response to herbivore and pathogen attack, plants activate not one but many signal cascades that recruit a suite of defenses. The specificity of the defense response elicited against a particular attacker is in part tailored by the cross-communication between these signal transduction pathways (Feys and Parker, 2000; Glazebrook, 2001; Thomma et al., 2001; Heidel and Baldwin, 2004). Biotic attackers, to borrow from Shakespeare, "not as single spies, but in battalions come" 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 (Moran and Thompson, 2001; Moran et al., 2002; Kaloshian and Walling, 2005; Pegadaraju et al., 2005; De Vos et al., 2006).

Insects, on the other hand may exploit aspects of the plant's signal cascade architecture to their advantage. The SA/JA antagonism, for example, may benefit silverleaf whiteflies that activate SA signaling when they attack their host plant, which in turn suppresses JA-regulated defenses that are more effective than SA-regulated defenses in deterring whiteflies (Zarate et al., 2007). Similar responses may occur with grazing insects, which are "recognized" by plants when components of their OS, such as FACs, β-glucosidases or proteolytic products of ATPases (inceptins) are introduced into wounds during feeding (Mattiacci et al., 1995; Halitschke et al., 2003; Schmelz et al., 2006). Given the plant's adaptation to fend off its attackers, it is possible that by harboring plant pathogens (or their elicitors) in their midguts, grazing insects elicit SA

signaling to antagonize the more effective JA-mediated defenses, as whiteflies apparently do.

The effects of SA treatment on JA-mediated defenses are commonly interpreted as evidence of the SA/JA antagonism. When SA, its methyl ester (MeSA), or SA mimics are applied to wounded or herbivore-attacked plants, the JA burst, JA-mediated gene expression, levels of JA-elicited defensive metabolites, as well as resistance to some herbivores are suppressed (Doherty et al., 1988; Péna-Cortes et al., 1993; Doares et al., 1995; Baldwin et al., 1997; Fidantsef et al., 1999; Stout et al., 1999; Stotz et al., 2002; Cipollini et al., 2004). Suppressed JA signaling in Arabidopsis plants mutated in *mpk4* can be partially attributed to the plants' high SA levels (Wiermer et al., 2005). In WT *Arabidopsis* SA is thought to antagonize JA signaling during pathogen infection, which is corroborated by the diminishment of this antagonism in pathogen-elicited SA-deficient *NahG* plants that have high levels of *LOX2* transcripts and JA (Spoel et al., 2003). Additionally, *N. tabacum* plants inoculated with tobacco mosaic virus showed attenuated wound-induced JA and nicotine levels (Preston et al., 1999). We recently reported that in *N. attenuata* NPR1 silencing dramatically increases OS-elicited levels of free and total SA and that the resulting SA/JA antagonism inhibited a number of JA-elicited direct and indirect defenses. Ir-npr1 plants, silenced in their *NPR1* expression, were highly vulnerable to herbivores in both the field and the glasshouse (Rayapuram and Baldwin, 2007).

These examples show that the SA/JA antagonism is real, and therefore plants should be able to control the antagonism if they are to adaptively tailor their defense responses. Here we demonstrated that while FACs in OS are not responsible for

eliciting the SA burst, the FAC-elicited ET burst plays an important role in suppressing the OS-elicited SA burst. ET thereby presumably mitigates SA/JA antagonism. OS-elicited WT plants pre-treated with a competitive inhibitor of ethylene receptors, 1-MCP, as well as OS-elicited transgenic 35s-*etr1b* plants, which are impaired in ET perception, accumulate up to 3-fold more SA than OS-elicited WT plants (Fig. 10C). Both 35s-*etr1b* plants and 1-MCP treated WT plants are impaired in their ability to perceive ET and are known to synthesize more ET in response to OS elicitation than WT plants (von Dahl et al., 2007). This suggests that either ET production or ET-perception is responsible for the altered SA burst. Since the increases in total SA mirror the increases in free SA (Suppl. Fig. 1), we infer that the SA burst results from *de novo* biosynthesis. In line with this observation it is unlikely that the ET burst regulates the release of free SA from the larger pool of conjugated SA. Interestingly, besides the increased SA burst and severely impaired JA-mediated defenses of NPR1-silenced *N. attenuata* plants their OS-elicited ET burst does not differ from that of OS-elicited WT plants (Rayapuram and Baldwin, 2007). This suggests that the FAC-elicited ET burst represents a conditional means of modulating SA/JA antagonism in addition to that provided by NPR1 (Scheme 1).

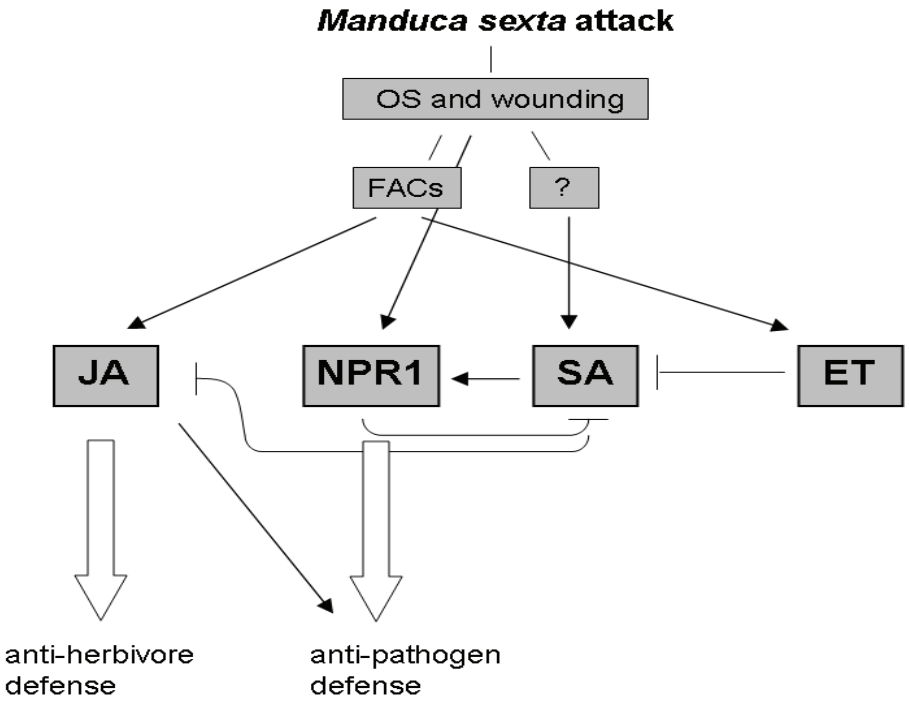
We conclude that FACs indirectly suppress the SA burst by eliciting an ET burst, but the factors in OS which elicit the SA burst remain elusive. Ahmad and Hopkins (1992) demonstrated that *M. sexta* OS contain ß-glucosidase activity and we were able to rule out the hypothesis that ß-glucosidase functioned as the elicitor of the SA burst (Fig. 9). The hypothesis that larval derived ß-glucosidase could potentially hydrolyze sugar conjugated SA to produce a burst of free SA was particularly appealing

in the context of Mattiacci et al. (1995) pioneering work demonstrating that ß-glucosidase in OS of *Pieris brassicae* larvae elicited indirect defenses. However, the application of OS-equivalent activities of ß-glucosidase to leaves did not elicit an SA burst, and ß-glucosidase activity of OS was not correlated with their ability to elicit SA accumulation (Fig. 9). Moreover, the changes in total SA mirrored the changes in free SA (Suppl. Fig.1), making the hydrolysis from conjugates an unlikely explanation for the burst of free SA. The fact that different collections of *M. sexta* OS differ in their ability to elicit an SA burst, suggests that larvae may differently harbor pathogenic factors in their OS. Pathogen-associated molecular patterns (PAMPs) such as flagellin (Felix and Boller, 2003; Zipfel et al., 2006) may be present in larval OS and may be introduced during feeding to elicit the SA burst. Identification of the OS-derived elicitors of the SA burst will help to determine if herbivores actively manipulating SA signaling in plants as a means of using SA/JA antagonism to thwart JA-mediated plant defenses.

The ten fold larger ET burst of *N. attenuata* plants attacked by *M. sexta* larvae as compared to the ethylene emitted by plants attacked by *S. exigua* larvae was accompanied by a 5-fold larger SA burst in *S. exigua*-attacked plants in comparison to plants attacked by *M. sexta* larvae (Fig. 12). These results demonstrate a potential of the SA/ET cross-communication to tune responses to different herbivores. Moreover the data is consistent with the hypothesis that generalist herbivores, such as *S. exigua*, may enhance their fitness by activating the SA pathway concomitantly with the JA pathway to weaken JA-mediated resistance by means of the SA/JA antagonism (Stotz et al., 2002; Cipollini et al., 2004). One issue that should be inserted in the argumentation is

the fact, that Spodoptera exigua larvae fed on artificial diet previously and hence their OS contents could be modified in comparison to larvae which fed on *N. attenuata* leaves. This modification could also account for the differentially regulated SA bursts. But if PAMPs are indeed the elicitors of the SA burst, it would be interesting to understand how generalist herbivores increase PAMP titers in their OS without risking microbial infections. On the other hand this may be the risk that specialist herbivores avoid by the adaptation to their hosts' JA-mediated defenses.

In this study we found, that SA is induced by herbivory, depending on the herbvivores saliva and we show that ET suppresses SA accumulation during herbivore attack, which minimizes SA/JA antagonism and as a result allows for the unhampered activation of JA-mediated defense responses (Scheme 1). How and whether the ET burst interacts with NPR1 signaling in modulating SA/JA antagonism and the analysis of the herbivore-derived elicitor of the SA burst are questions that deserve more attention in the future.



Scheme 1. *Model of OS-elicited signaling crosstalk in* M. sexta-*attacked* N.attenuata *leaves.* When *M. sexta* larvae attack and feed on plants, fatty acid-amino acid conjugates (FACs) from the larval oral secretions (OS) are introduced into wounds during feeding. Treating wounds with the two most abundant FACs in *M. sexta* OS is sufficient to elicit both ET and JA bursts, but not the SA burst, that are elicited when wounds are treated with OS. The elicitors of the SA burst in OS remain unknown. Increased ET emissions suppresses the SA burst, which, in turn, is known to inhibit the JA burst and JA-dependent defenses. We propose that the FAC-elicited ET burst represents a conditional means of modulating SA/JA antagonism and thereby adds flexibility to the regulation of the SA/JA antagonism provided by NPR1 in *N. attenuata* (Rayapuram and Baldwin, 2007).

Supplemental Figures:

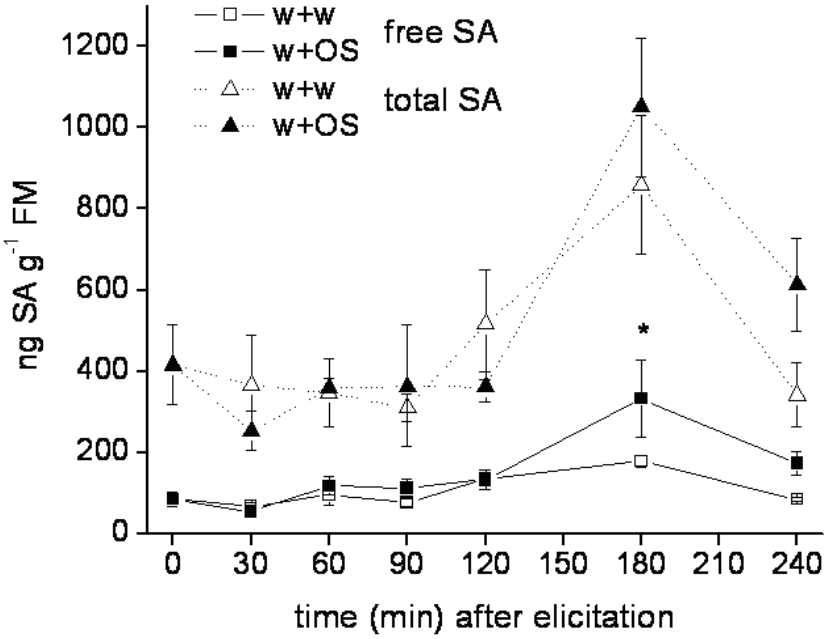


Figure S1. *Conjugated SA increases concomitantly with free SA*
Mean ± SE (n= 5) free (square symbols) and total (triangles) SA per fresh mass (FM) of wild type *N. attenuata* plants growing in a native population in SW Utah. Fully mature leaves were wounded with a fabric pattern wheel and the wounds were either treated with water (w + w, dashed line) or M. *sexta* oral secretions (w + OS, solid line). Control plants (0 min) were left unwounded. Asterisks indicate significant differences among treatments (ANOVA, $F_{12,51} = 5.327$, $P < 0.05$).

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Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der aufgeführten Hilfsmittel und Literatur-Quellen angefertigt habe.

Jena, den 03.10.2007