Mechanisms of jasmonate-induced activation of defense responses in *Nicotiana attenuata*

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Manuscript I

Molecular Interactions Between the Specialist Herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and Its Natural Host *Nicotiana attenuata*. III. Fatty Acid-Amino Acid Conjugates in Herbivore Oral Secretions Are Necessary and Sufficient for Herbivore-Specific Plant Responses

Rayko Halitschke, Ursula Schittko, Georg Pohnert, Wilhelm Boland and Ian T. Baldwin

Plant Physiology (2001) **125**: 711-717

This manuscript describes the isolation and characterization of fatty acid-amino acid conjugates (FACs) as elicitors in the oral secretions and regurgitant of *Manduca sexta* and *Manduca quinquemaculata* larvae. These compounds are responsible for the recognition of the feeding herbivore by *Nicotiana attenuata* and elicit several herbivore-specific defense responses including altered transcript accumulation, a jasmonic acid burst, and the release of volatile organic compounds.
I collected, analyzed, and manipulated (by ion-exchange treatment) the caterpillar oral secretions and regurgitant and planned the jasmonic acid accumulation experiment, which I performed together with Ursula Schittko. Furthermore, I designed and performed the experiments to characterize the induced volatile emissions. The characterization of transcript accumulation was planned and realized by U. Schittko and the fatty acid-amino acid conjugates were synthesized by Georg Pohnert in the department of Professor Wilhelm Boland.

Manuscript II

Molecular Interactions Between the Specialist Herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and Its Natural Host *Nicotiana attenuata*. VI. Microarray Analysis Reveals That Most Herbivore-Specific Transcriptional Changes Are Mediated by Fatty Acid-Amino Acid Conjugates

Rayko Halitschke, Klaus Gase, Dequan Hui, Dominik D. Schmidt, and Ian T. Baldwin

Plant Physiology (2003) **131**: 1894-1902

The experiments presented in this manuscript quantitatively evaluate the contribution of the elicitors identified and characterized in Manuscript I to the biological activity of the oral secretions and regurgitant. A cDNA microarray was designed for the expression analysis of herbivore-responsive genes and used to compare the expression patterns in mechanically wounded plants, which received either water or *Manduca* ssp. oral secretions or different solutions of herbivorous elicitors (FACs and glucose oxidase; GOX). A majority of the genes (56%) induced by application of *Manduca* ssp. oral secretions were similarly induced by FAC application. These patterns involved the upregulation of genes involved in oxylipin signaling and defense responses and the downregulation of photosynthetic genes. In contrast, the transcriptional response to a second elicitor, GOX, showed the opposite regulation pattern compared with the response to the larval oral secretions.
I designed the experiments analyzing the transcriptional response to the oral secretions and regurgitants of the two *Manduca* species and FACs and performed the plant treatments, harvest, RNA isolation, and analysis of microarray data. The glucose oxidase experiment was performed by Dominik Schmidt and the cDNA AFLP analysis was performed by Dequan Hui. Klaus Gase designed the microarray and was responsible for the technical development and realization of the microarray analysis.

Manuscript III

Agrobacterium-mediated transformation of _Nicotiana attenuata,_ a model ecological expression system

Tamara Krügel, Michelle Lim, Klaus Gase, Rayko Halitschke, and Ian T. Baldwin

Chemoecology (2002) **12**: 177-183

Manuscript III describes the development of a transformation procedure for *N. attenuata*. The standard transformation protocols for cultivated tobacco (*N. tabacum*) had to be significantly modified for the transformation of *N. attenuata*. The developed method was used to manipulate the expression of genes involved in oxylipin signaling by antisense expression-mediated gene silencing. By silencing the expression of a lipoxygenase (LOX) and allene oxide synthase (AOS) – two enzymes of the octadecanoid pathway – we dramatically reduced the accumulation of wound-induced jasmonic acid (JA) accumulation. Furthermore, we manipulated the release of green leaf volatiles (GLVs) by silencing expression of hydroperoxide lyase. The transgenic plants generated by this procedure represent the tools for the following experiments to elucidate the role of individual oxylipin signals in the defensive response of *N. attenuata*.

Klaus Gase designed and developed the transformation vectors and the DNA extraction procedure described in the manuscript. Tamara Krügel und Michelle Lim developed, optimized, and performed the transformation procedure and generated the first generation of transgenic plants (T_0). I was responsible for the genetic and biochemical characterization of the transformed plants. This involved the confirmation of transformation, segregation analysis, propagation and phenotype characterization. I developed the high-throughput screening procedure for JA and GLV analysis and analyzed these metabolites in the transformed plants.

Manuscript IV

Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata*

Rayko Halitschke and Ian T. Baldwin

Plant Journal (2003) **36**: 794-807

Manuscript IV describes the isolation and characterization of three distinct classes of *LOX* genes in *N. attenuata*. I Identified the isoform (*NaLOX3*) showing expression patterns that strongly correlate with induced JA accumulation for further investigations. I investigated the consequences of reduced JA signaling capacity in transgenic plants (as-*lox*), which were generated by the procedure described in manuscript III. The impaired JA biosynthesis suppressed direct and indirect defense responses and resulted in reduced resistance against herbivory by *M. sexta* larvae.
I was responsible for the experimental planning and realization including bioassays, chemical and statistical analysis. I isolated and characterized the *LOX* genes and characterized the antisense transformed plants.

Manuskript V

Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling cross-talk in *Nicotiana attenuata*

Rayko Halitschke, Jörg Ziegler, Markku Keinänen, and Ian T. Baldwin

In review: Plant Journal

This manuscript describes the isolation and characterization of a hydroperoxide lyase (HPL) gene of *N. attenuata* and the consequences of silencing this HPL and a previously described allene oxide synthase (AOS) by the transformation described in manuscript III. Silencing of HPL (as-*hpl*) and AOS (as-*aos*) resulted in reduced emissions of green leaf volatiles and JA accumulation, respectively. By comparing transgenic plants impaired in two different oxylipin signaling cascades I demonstrate substantial cross-talk between JA and GLV signaling on the level of substrate supply as well as activation of defense responses in *N. attenuata*.
Jörg Ziegler and Markku Keinänen isolated the *HPL* cDNA and performed the initial Northern blot analysis of *HPL* expression. I developed and performed the real-time PCR assays for the analysis of *AOS* and *HPL* transcript accumulation and characterized the as-*hpl* and as-*aos* plants including molecular biological and biochemical analysis and bioassays.

Manuscript VI

Genetically silencing the jasmonate cascade: influences of induced plant defenses on herbivore community composition

André Kessler, Rayko Halitschke, and Ian T. Baldwin

In review: Science

The field experiments presented in manuscript VI demonstrate the value of transgenic plants for the investigation of ecological interactions under natural conditions. The results of the field experiments confirm the resistance phenotype of the transgenic as-*lox*, as-*aos*, and as-*hpl* plants described under laboratory conditions in manuscript IV and V and additionally, demonstrate that JA-induced defense responses influence the herbivore community on wild *N. attenuata* plants and suggest a function for this oxylipin as host plant selection cue for opportunistic herbivores.

All field experiments and the microarray hybridizations were planned and performed by A. Kessler and me. A first draft of the manuscript was written by A. Kessler and optimized by discussions with all co-authors.

1. Introduction

Forced by their sessile nature of growth, which does not allow plants to escape a (potentially) dangerous or unfavorable situation, plants have evolved at least as many protective and defensive traits as there are potential abiotic and biotic stress factors. The defensive mechanisms against herbivory include a bewildering array of mechanical barriers like thorns and hairs (trichomes) and chemical defenses that are toxic to the attacking organism (e.g. alkaloids and glucosinolates) or reduce the nutritional value of the plant material (e.g. protease inhibitors and polyphenol oxidases). These defensive traits can be expressed constitutively or induced in response to an attack (Karban and Baldwin, 1997). The expression of defensive traits mediating resistance, no matter if constitutively expressed or induced, often incurs costs to the plant including direct allocation and autotoxicity costs and indirect ecological costs (Strauss *et al.*, 2002). These costs have been discussed as the driving force for the evolution of induced resistance (Heil and Baldwin, 2002; Karban and Baldwin, 1997). In order to minimize the costs of induced resistance plants have to recognize the attack with a certain degree of specificity and activate the appropriate defense responses. This recognition/activation process involves specific signals produced by the herbivore and transferred during feeding and a complex network of endogenous signaling cascades in the plant.

In the collection of manuscripts presented in this study I analyze the interaction between the wild tobacco plant *Nicotiana attenuata* and two of its most important natural herbivores *Manduca sexta* and *Manduca quinquemaculata*. The study includes the isolation, characterization, and manipulation of chemical cues of the *Manduca* “feeding signature” which are necessary and sufficient to elicit a majority of the herbivore-induced responses in *N. attenuata*. Furthermore, I investigate, by genetic manipulation of individual signaling cascades, the role of specific oxylipin signals in the activation of *N. attenuata*’s defense response.

Elicitors in herbivore oral secretions

The identification of herbivore-specific elicitors has focused on the elicitation of the release of volatile organic compounds (VOCs). The bouquet of VOCs released from herbivore-infested plants differs quantitatively and qualitatively from the emissions of mechanically wounded plants and the specifically released VOCs

function as indirect defense by attracting natural enemies of the herbivore (Dicke and van Loon, 2000). Two classes of elicitors have been identified in the oral secretions and regurgitant (R) of lepidopteran larvae. A β-glucosidase isolated from R of *Pieris brassicae* larvae was shown to elicit the release of parasitoid-attracting VOCs from cabbage (Mattiacci *et al.*, 1995) and lima bean leaves (Hopke *et al.*, 1994). The cleavage of stored glycosidic precursors has been suggested as the release mechanism mediated by this enzymatic elicitor. A second non-enzymatic elicitor has been isolated from R of *Spodoptera exigua* larvae and identified as *N*-(17-hydroxylinolenoyl)-L-glutamine, a fatty acid-amino acid conjugate (FAC). This compound, named volicitin, induces the release of VOCs in corn plants (*Zea mays*) comparable with that induced by larval feeding (Alborn *et al.*, 1997; Turlings *et al.*, 2000). In contrast, volicitin did not elicit the release of VOCs in lima bean leaves indicating the involvement of distinct mechanisms in the elicitation of VOC emissions in different plant species. Nevertheless, several structurally related FACs (Fig. 1) were identified in the R of different lepidopteran species (Alborn *et al.*, 2000; Paré *et al.*, 1998; Pohnert *et al.*, 1999).

In addition to the elicitors of herbivore-induced VOC emissions, glucose oxidase (GOX) in the salivary glands of *Helicoverpa zea* larvae was identified as an enzymatic elicitor inhibiting the wound-induced accumulation of nicotine and inducing herbivore resistance in *Nicotiana tabacum* by the production of H_2O_2 at the wound site (Musser *et al.*, 2002). A novel class of herbivore-specific elicitors has been reported to elicit a novel type of defense response in peas. Bruchins, esterified long chain diols, which were found in pea and cowpea weevils, elicit neoplastic growth in pea leaves and thereby lifts the recently hatched larvae out of the oviposition site and forces it to re-burrow into the pea pod (Doss *et al.*, 2000). While this is not lethal in itself, the neoplasm formation functions as an indirect defense by exposing the young larvae to predators, parasites, and desiccation.

Although the isolation of these elicitors provides evidence for the ability of plants to specifically recognize herbivory, they are limited to the investigation of a single plant trait. A broader characterization of the plant response, including additional defensive and developmental parameters, and the involved signaling cascades are necessary to evaluate the biological activity of individual compounds (or compound classes) in the context of a plant-herbivore interaction.

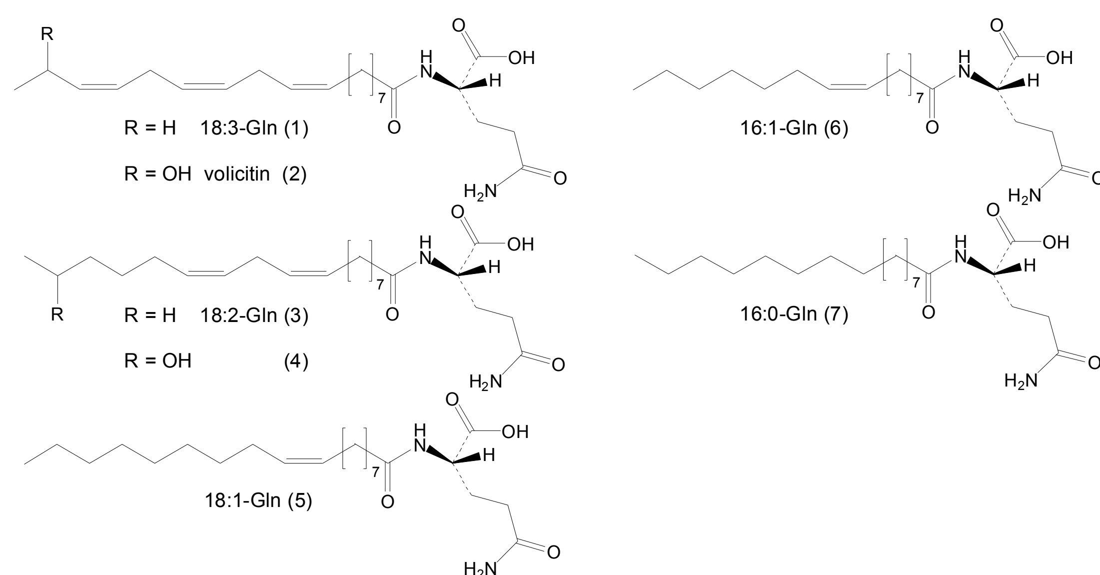

Figure 1. Structures of fatty acid-amino acid conjugates identified in the oral secretions and regurgitant of several lepidopteran larvae: (**1**) *N*-linolenoyl-L-glutamine; (**2**) *N*-(17-hydroxylinolenoyl)-L-glutamine; (**3**) *N*-linoleoyl-L-glutamine; (**4**) *N*-(17-hydroxylinoleoyl)-L-glutamine; (**5**) *N*-oleoyl-L-glutamine; (**6**) *N*-palmitoleoyl-L-glutamine; (**7**) *N*-palmitoyl-L-glutamine

Plant signaling cascades

The activation of wound- and herbivore-induced defense responses involves a complex network of plant signaling cascades (Pieterse *et al.*, 2001) including peptide signals (e.g. systemin) and phytohormones like salicylic acid, ethylene, and lipid derived oxylipins. Jasmonic acid (JA), a linolenic acid-derived oxylipin produced via the octadecanoid pathway, its biosynthetic precursor 12-oxo-phytodienoic acid, and derivatives like the methyl ester (MeJA) or amino acid conjugates, collectively called jasmonates (JAs), represent the best characterized class of signals involved in the defense activation in response to wounding and herbivory (Beale and Ward, 1998; Blee, 2002; Devoto and Turner, 2003; Farmer *et al.*, 2003). The octadecanoid pathway (Vick and Zimmerman, 1984) involves the regio- and stereospecific dioxygenation of linolenic acid by a 13-lipoxygenase (13-LOX); formation of an epoxide by allene oxide synthase (AOS); ring formation by allene oxide cyclase

(AOC); reduction by OPDA reductase (OPR), and side-chain shortening by three consecutive β-oxidation steps (Fig. 2).

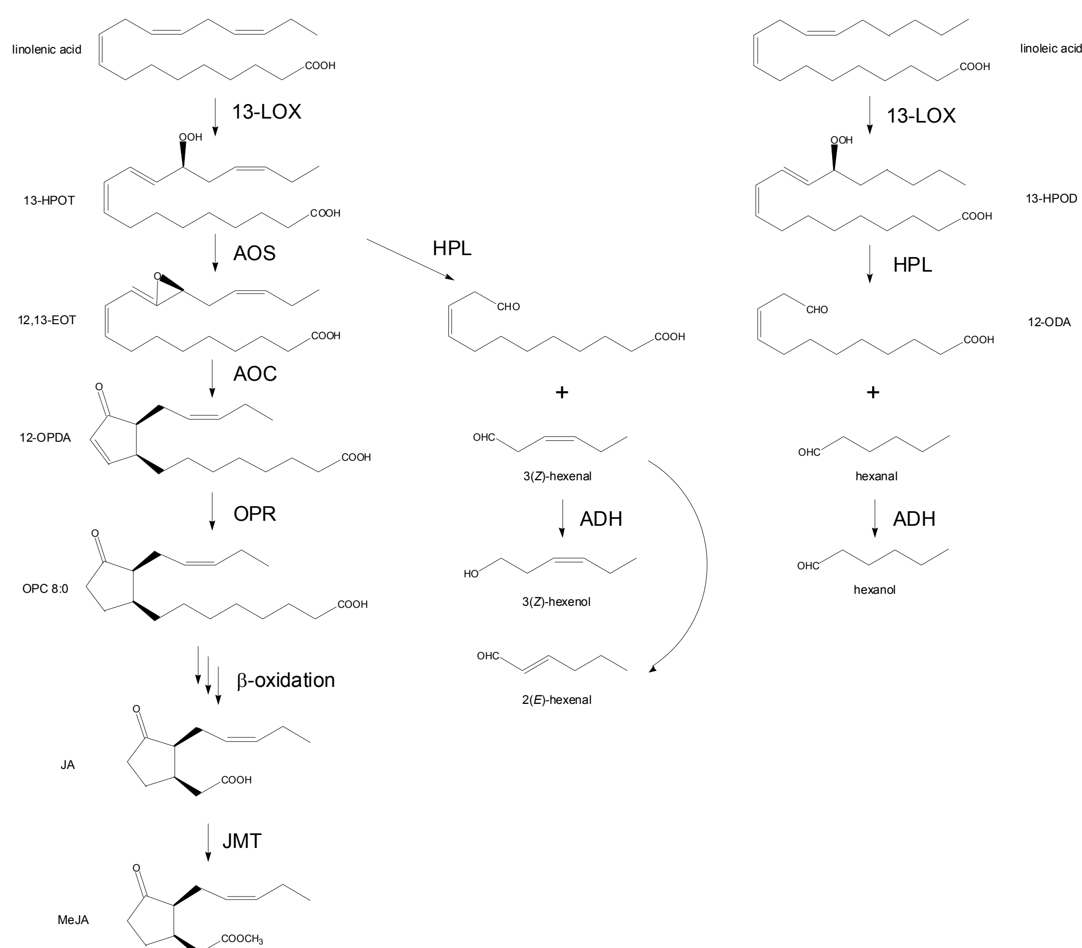

Figure 2. Biosynthesis of jasmonates and green leaf volatiles.

(13-LOX, 13-lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, 12-oxo-phytodienoic acid reductase; JMT, jasmonic acid carboxyl methyltransferase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase, 13-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadeca-dienoic acid; 12,13-EOT, 12,13(*S*)-epoxyoctadecatrienoic acid; 12-OPDA, 9(S)/13(S)-12-oxo-phytodienoic acid; OPC 8:0, 3-oxo-2(2'pentenyl)-cyclopentane-1-octanoic acid; JA, 3(R)/7(S)-jasmonic acid; MeJA, JA methyl ester; 12-ODA, 12-oxo-9(*Z*)-dodecenoic acid)

Enzymes involved in the biosynthesis of jasmonates have been characterized in several plant species, and their genes have been overexpressed or silenced in transgenic plants and mutants (Berger, 2002; Schaller, 2001; Turner *et al.*, 2002). The

genetic manipulation of AOS (Laudert *et al.*, 2000; Park *et al.*, 2002), AOC (Stenzel *et al.*, 2003), or a specific OPR (OPR3; (Sanders *et al.*, 2000; Stintzi and Browse, 2000) has powerfully demonstrated the importance of these enzymes in JA biosynthesis. Additionally, a mutant (*fad3 fad7 fad8* triple mutant) impaired in the biosynthesis of linolenic acid, the fatty acid precursor of JA biosynthesis, has been characterized (McConn and Browse, 1996) and found to be impaired in wound-induced JA accumulation (McConn *et al.*, 1997). A second class of oxylipins, C_6 aldehydes, alcohols, and their esters, is produced in response to wounding and herbivory by the hydroperoxide lyase (HPL) pathway. Due to the importance of GLVs for food quality and flavor the enzymes involved in the biosynthesis of GLVs have been characterized in a variety of plants (Grechkin, 2002; Hatanaka, 1996; Noordermeer *et al.*, 2001). Nevertheless, the release of GLVs was genetically manipulated only in potato plants (Leon *et al.*, 2002; Vancanneyt *et al.*, 2001).

Most of the evidence for the involvement of JAs and GLVs in the plant response to herbivory originates from a) correlative studies comparing oxylipin accumulation (or release) and expression of defensive traits in response to herbivory or wound treatment or b) application of synthetic compounds at more or less realistic concentrations. Despite the high number of available mutants and transgenic plants, their potential for the elucidation of the elicitation mechanisms of herbivore resistance has been appreciated only recently (Kessler and Baldwin, 2002). The *Arabidopsis fad3 fad7 fad8* triple mutant, impaired in wound-induced JA accumulation, has reduced resistance against the fungal gnat *Bradysia impatiens* (McConn *et al.*, 1997) but the defense traits mediating this resistance have not been identified. JA-deficient tomato *def1* mutants show lower resistance against *M. sexta* (Howe *et al.*, 1996) and *S. exigua* (Thaler *et al.*, 2002) larvae and spider mites (Li *et al.*, 2002). The *def1* mutation is thought to result in a biosynthetic defect between the LOX and OPR reactions (Howe *et al.*, 1996), but its function in JA biosynthesis remains to be understood because def1 does not harbor a mutation in the genes coding for either AOS and AOC catalyzing the intermediate biosynthetic steps [Fig. 2; (Li *et al.*, 2002)]. Antisense expression-mediated silencing of a specific lipoxygenase (LOX-H3) abolished the induction of protease inhibitor (PI) in potato plants and enhanced the performance of Colorado potato beetle and beet armyworm larvae feeding on these plants (Royo *et al.*, 1999). However, as no reduction in JA accumulation was observed the mechanism of LOX-H3-mediated herbivore resistance is unclear.

Silencing of a HPL suppressed the production of GLVs in potato leaves and reduced resistance against the green peach aphid (Vancanneyt *et al.*, 2001). The mechanisms of GLV-mediated resistance, which could be a direct effect on aphid fecundity or indirect effects due to induced changes in the plant (Hildebrand *et al.*, 1993), were not characterized.

Because these studies were performed with incompletely characterized mutants or in model systems with poorly understood ecological interaction, they lack either a detailed characterization of the genetic manipulation itself or the phenotypic consequences of the manipulation resulting in the altered resistance. Although the importance of oxylipins in mediating herbivore-resistance has been demonstrated, both the signals and the resistance mechanisms remain to be characterized.

The Nicotiana attenuata model system

The wild tobacco plant *Nicotiana attenuata* Torr. ex Watson (synonymous with *Nicotiana torreyana* Nelson & Macbr.) is an annual plant growing in disturbed desert habitats in the southwestern USA (Fig. 3). *N. attenuata* colonizes and dominates the vegetation of burned areas for the first to years after a fire. This occurrence on nitrogen-rich soils with low interspecific competition is regulated by the synchronization of seed germination by smoke-derived positive cues (Baldwin *et al.*, 1994) and inhibition by allelochemicals in the litter of other plant species (Preston *et al.*, 2002). This synchronized germination behavior results in the occurrence of *N. attenuata* in populations with high intraspecific competition under highly unpredictable environmental conditions. The natural herbivore community, which has to establish with every new population, mainly consists of specialized lepidopteran larvae of *Manduca sexta* and *Manduca quinquemaculata* (Lepidoptera, Sphingidae; Fig. 3), the mirid bug *Tupiocoris notatus* (Hemiptera, Miridae), and the flea beetle *Epitrix hirtipennis* (Coleoptera, Chrysemelidae) with a highly variable (seasonally and spatially) composition (Kessler and Baldwin, 2004).

The majority of leaf area loss is caused by *M. sexta* and *M. quinquemaculata* herbivory to which the *N. attenuata* responds with the induction of a whole array of direct defenses including toxins such as nicotine, flavonoids, phenolics, and diterpene sugar esters (Baldwin, 1999; Keinanen *et al.*, 2001) and protease inhibitors (van Dam *et al.*, 2001) functioning as antidigestive proteins.

Figure 3. The wild tobacco plant *Nicotiana attenuata* in its natural habitat the Great Basin desert in southwest Utah, USA and one of its major herbivores, the tomato hornworm, *Manduca quinquemaculata* (Lepidoptera, Sphingidae).

Additionally, the specific release of herbivore-induced VOCs (Halitschke *et al.*, 2000) functions effectively as an indirect defense (Kessler and Baldwin, 2001). The activation of these defense mechanisms is preceded by a JA burst elicited by larval feeding (Schittko *et al.*, 2000; Ziegler *et al.*, 2001) and the involvement of JA in the defense activation has been demonstrated by the application of synthetic JAs which induce all of the direct and indirect defenses described above (Baldwin, 1999; Halitschke *et al.*, 2000; Keinanen *et al.*, 2001; van Dam *et al.*, 2001). The JA-induced resistance incurs substantial fitness costs but benefits plants under herbivore attack (Baldwin, 1998). In addition to the direct costs of allocating up to 8% of the plant's total nitrogen pool to nicotine production, MeJA treatment reduced the competitive ability for nitrogen uptake representing an ecological cost, in the sibling species *Nicotiana sylvestris* (Baldwin and Hamilton, 2000). Nevertheless, elicitation by treatment with JAs does not completely mimic the response to herbivore feeding and additional signals are involved in the activation of *N. attenuata*'s defense response.

The best described example is the suppression of JA-induced nicotine biosynthesis by an herbivore-induced ethylene burst (Kahl *et al.*, 2000; Winz and Baldwin, 2001) which reduces the large metabolic costs of induced defenses (Voelckel *et al.*, 2001b).

These highly specific responses that allow the plant to fine-tune the activation of appropriate defenses require the specific recognition of the attack by insect-derived cues and the objective of the collection of papers presented here is to elucidate the mechanisms of specificity mediating a plant-herbivore interaction. The following questions were addressed by the identification and manipulation of herbivore elicitors and two distinct plant signaling cascades:

1. How does *N. attenuata* recognize the attack by its specialized herbivores *M. sexta* and *M. quinquemaculata* and what is the specific role of chemical constituents of larval oral secretions in the activation of the plant's defense response?

2. Which endogenous signal cascades are involved in the activation of the defense response in *N. attenuata* and how does the plant regulate the production of these signals?

3. What are the consequences of a reduced signaling capacity for the plant resistance against herbivore attack?

Manuscript I

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Molecular Interactions Between the Specialist Herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and Its Natural Host *Nicotiana attenuata*. III. Fatty Acid-Amino Acid Conjugates in Herbivore Oral Secretions Are Necessary and Sufficient for Herbivore-Specific Plant Responses

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Abstract

Feeding by the tobacco specialist *Manduca sexta* (Lepidoptera, Sphingidae) and application of larval oral secretions and regurgitant (R) to mechanical wounds are known to elicit: (a) a systemic release of mono- and sesquiterpenes, (b) a jasmonate burst, and (c) R-specific changes in transcript accumulation of putatively growth- and defense-related mRNAs in *Nicotiana attenuata* Torr. ex Wats. We identified several fatty acid–amino acid conjugates (FACs) in the R of *M. sexta* and the closely related species *M. quinquemaculata* which, when synthesized and applied to mechanical wounds at concentrations comparable with those found in R, elicited all three R-specific responses. Ion-exchange treatment of R, which removed all detectable FACs and free fatty acids (FAs), also removed all detectable activity. The biological activity of ion-exchanged could be completely restored by the addition of synthetic FACs at R-equivalent concentrations, whereas the addition of FAs did not restore the biological activity of R. We conclude that the biological activity of R is not related to the supply of FAs to the octadecanoid cascade for endogenous jasmonate biosynthesis, but that FACs elicit the herbivore-specific responses by another mechanism and that the insect-produced modification of plant-derived FAs is necessary for the plant's recognition of this specialized herbivore.

Introduction

Feeding by *Manduca sexta* (Lepidoptera, Sphingidae) larvae on *Nicotiana attenuata* Torr. Ex Wats. elicits responses clearly different from those induced by careful mechanical simulation of larval feeding. The wound-induced increase in jasmonic acid (JA) levels is amplified by herbivore feeding and by application of larval oral secretions and regurgitant (R) to mechanical wounds (McCloud and Baldwin, 1997; Schittko et al., 2000), whereas the wound-induced increase in nicotine-accumulation which strongly correlates with wound-induced JA-levels (Baldwin et al., 1994a, 1997) is suppressed (Baldwin, 1988; McCloud and Baldwin, 1997) by an ethylene-burst released by the plant after herbivore attack (Kahl et al., 2000). Furthermore, herbivore feeding and R application to plant wounds are also known to induce the release of several mono- and sesquiterpenes in *N. attenuata* (Halitschke et al., 2000; Kahl et al., 2000), which, in turn, are thought to function as an indirect defense, guiding parasitoids to feeding larvae. Moreover, whereas both the volatile release and nicotine-accumulation can be elicited by the application of jasmonates to plants (Baldwin, 1999; Halitschke et al., 2000; Kahl et al., 2000), only the wound-induced nicotine-response is suppressed by application of inhibitors of endogenous JA-biosynthesis (Baldwin et al., 1997; Halitschke et al., 2000). In short, R results in direct and indirect defense responses in this specialist herbivore-plant system, and these responses appear to involve the octadecanoid cascade.

In addition to these well-described phenotypic responses to herbivory, extensive transcriptional reorganization was recently revealed by mRNA differential display of *N. attenuata* in response to *M. sexta* feeding. In 1/20th of the insect-responsive transcriptome, 27 genes displayed altered expression patterns (Hermsmeier et al., 2001). A subset of seven genes was found to differentially respond to R as compared with mechanical damage. Larval R of *M. sexta* and *M. quinquemaculata* antagonistically (type I genes) or synergistically (type II genes) modified wound-induced transcriptional responses of these seven genes (Schittko et al., 20001). Given that chemical attributes of larval feeding mediate extensive changes in transcript accumulation and phenotypic responses, characterization of active components of R is of great interest.

Two types of elicitors have been identified in lepidopteran R that result in the release of plant volatiles responsible for attracting parasitic wasps. First, an enzymatic

elicitor, β-glucosidase, isolated from *Pieris brassicae* R, was shown to elicit the release of parasitoid-attracting volatile emissions from cabbage leaves. This elicitor is thought to release signal compounds by cleaving stored glycosidic precursors (Hopke et al., 1994; Mattiacci et al., 1995). Second, volicitin, *N*-(17-hydroxylinolenoyl)-L-glutamine, a fatty acid-amino acid conjugate (FAC) identified in the R of *Spodoptera exigua* induces the release of volatiles in corn plants (*Zea mays*) comparable with that induced by larval feeding (Alborn et al., 1997; Turlings et al., 2000). Volicitin and several structurally related FACs have been identified in R of different lepidopteran species (Paré et al., 1998; Pohnert et al., 1999a; Alborn et al., 2000).

Because linolenic acid is a precursor of JA in the octadecanoid cascade, the inducing activity of the FACs may be due to the supply of fatty acid substrates introduced to the plant after hydrolytic cleavage of the FAC amide-bond (Koch et al., 1999). This mechanism is supported by investigations with the lima bean (*Phaseolus lunatus*) in which (a) treatment of leaves with free linolenic acid results in the release of volatiles comparable with that elicited by treatments with *N*-linolenoyl-L-glutamine (18:3-Gln), and (b) treatment of the leaves with inhibitors of the octadecanoid pathway suppresses the volatile response elicited by the application of free FAs (Koch et al., 1999). Unfortunately, in these studies the FAs and FACs were supplied in concentrations far exceeding those found in larval R. Moreover, other mechanisms that do not invoke substrate supply for the octadecanoid pathway can account for the activity of the conjugates. For example, the conjugates may be recognized by specific receptors that subsequently trigger the octadecanoid pathway.

Here we identify and quantify the FAs and FACs in the R of *M. sexta* and *M. quinquemaculata* larvae, synthesize these FACs, and investigate their role in eliciting the volatile release, endogenous JA-accumulation and changes in transcript accumulation of six mRNAs of *N. attenuata* that are known to be specifically altered by R from *M. sexta* and *M. quinquemaculata*. We critically evaluate the biological roles of the identified compounds by removing all FAs and FACs in R by anion-exchange chromatography and add back synthetic FAs and FACs to the ion-exchanged R (exR) at naturally occurring concentrations.

Results and Discussion

Chemical Analysis of R

Oral secretions and regurgitant of *M. sexta* larvae fed on *N. attenuata* foliage were separated by HPLC (gradient C-18: CH_3CN/H_2O; 0.5% [v/v] HAc; 0.7 mL/min: 0% [v/v] CH_3CN, 20 to 25 min 100% [v/v] CH_3CN) and analyzed by atmospheric pressure chemical ionization-mass spectrometry. Analysis of fragmentation patterns (Pohnert et al., 1999a) revealed the presence of a series of FACs as minor constituents in the medium polar region of the chromatogram (Fig. 1A). Adjustment of the separation conditions (HPLC gradient C18: CH_3CN/H_2O; 0.5% [v/v] HAc; 0.7 mL/min: 40% [v/v] CH_3CN, 7 min 68% [v/v] CH_3CN, 18 min 80% [v/v] CH_3CN, 28 min 100% [v/v] CH_3CN) allowed us to separate the FACs (Fig. 1, B and C) and identify seven structurally-related FACs by comparison of their retention times and APCI mass spectra with synthetic references (see "Materials and Methods"). The FACs in the R of *M. sexta* are dominated by glutamic acid conjugates of C16- and C18-fatty acids (Fig. 1B, compounds 3, 5 and 8), which contrasts with the composition of seven other lepidopteran larvae R, which, in turn, are dominated by the glutamine conjugates of these fatty acids (Paré et al., 1998; Pohnert et al., 1999a; Alborn et al., 2000). The glutamine conjugates (Fig. 1B, compounds 1, 4 and 6) are relatively minor components of *M. sexta* R compared with the corresponding glutamic acid conjugates. This unusual composition was also found, in nearly identical relative ratios, in the R of another specialist herbivore of *N. attenuata*, *M. quinquemaculata* (Fig. 1C). Remarkably, no functionalized FACs (e.g. volicitin), often present in the R of lepidopteran larvae (Alborn et al., 1997, 2000; Pohnert et al., 1999a; Turlings et al., 2000), could be detected in the R of *M. sexta* or *M. quinquemaculata*.

The total concentration of FACs in the R of *M. sexta* reared on fresh *N. attenuata* foliage in the laboratory varied from 0.6 to 1.2 mM. Analysis of free fatty acids showed two major FAs, linolenic acid (18:3) at a concentration of 1.3 mM and linoleic acid (18:2) at a concentration of 0.4 mM. Ion-exchange chromatography on R and the synthetic mixture of FACs removed all detectable amounts of FACs (Fig. 1D, detection limit = 30 nM) and FAs (detection limit = 200 nM).

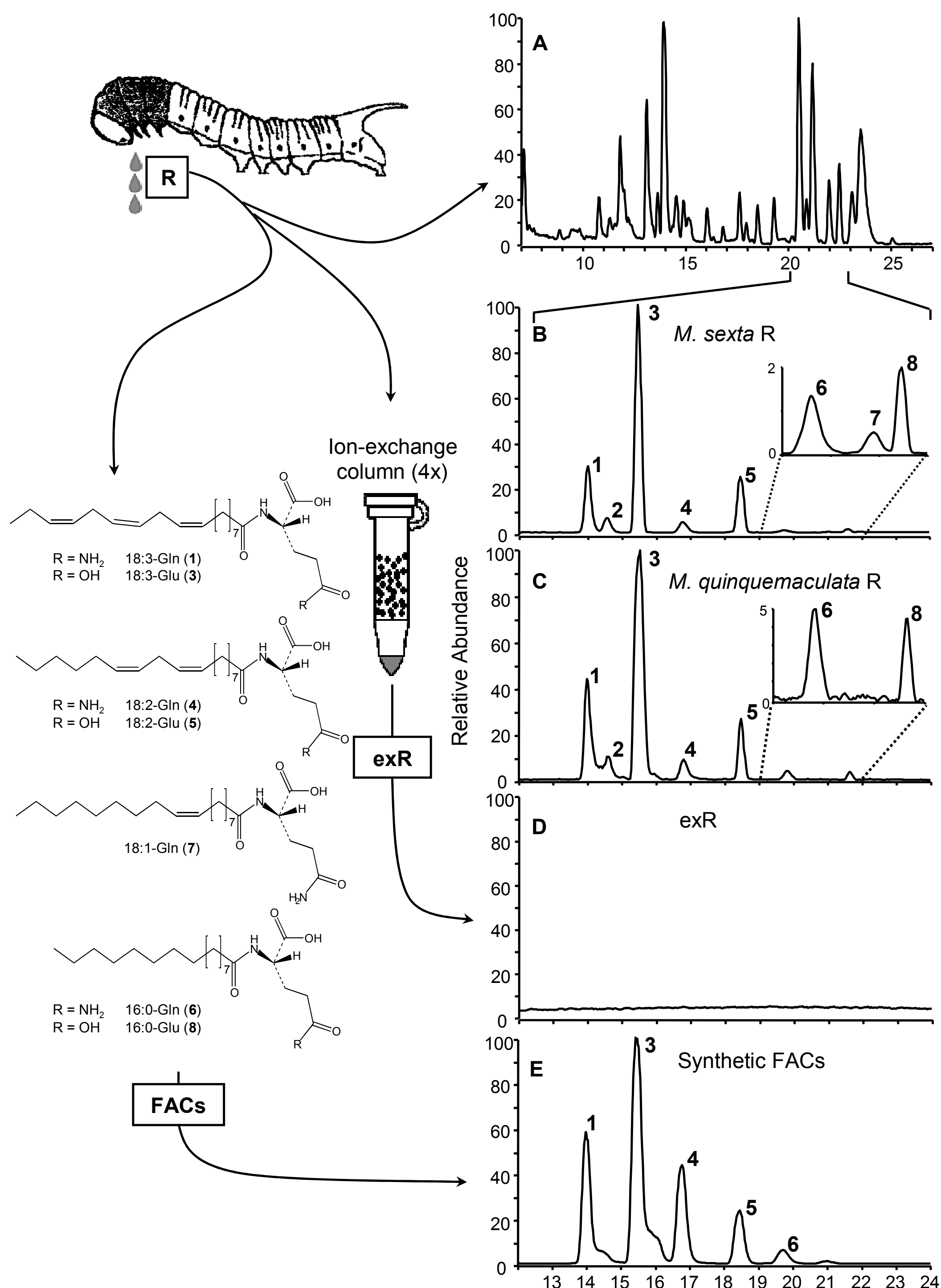

Figure 1. Scheme of experimental setup of the ion-exchange approach and structures of identified FACs (left) and HPLC-MS-Base peak profiles of 10-µL injections of test solutions (right): **A**, oral secretions and regurgitant (R) from *Manduca sexta* larvae. HPLC gradient (C18): CH_3CN/H_2O; 0.5% (v/v) HAc; 0.7 mL/min: 0% (v/v) CH_3CN, 20 to 25 min 100% (v/v) CH_3CN. Separation of the FACs in *M. sexta* (B) and *M. quinquemaculata* (C) R: HPLC gradient (C18): CH_3CN/H_2O; 0.5% (v/v) HAc; 0.7 ml/min: 40% (v/v) CH_3CN, 7 min 68%

(v/v) CH_3CN, 18 min 80% (v/v) CH_3CN, 28 min 100% (v/v) CH_3CN. 1, *N*-linolenoyl-L-glutamine; 2, unidentified; 3, *N*-linolenoyl-L-glutamate; 4, *N*-linoleoyl-L-glutamine; 5, *N*-linoleoyl-L-glutamate; 6, *N*-palmitoyl-L-glutamine; 7, *N*-oleoyl-L-glutamine; and 8, *N*-palmitoyl-L-glutamate. Base peak profiles of ion-exchanged *M. sexta* R (D) mixture of synthetic FACs at concentrations found in *M. sexta* R (E) analyzed with the HPLC gradient as in B and C.

Induction of cis-*α-Bergamotene Release*

Treatment of standard puncture wounds on a single leaf with *M. sexta* R elicited significant increases in whole-plant (WP) emissions of *cis*-α-bergamotene compared with untreated plants or plants that were comparably damaged but had water applied to their puncture wounds (Fig. 2A, ANOVA $F_{9,70} = 14.565$, $P < 0.0001$). Application of *M. quinquemaculata* R induced elevated WP *cis*-α-bergamotene emissions comparable with those elicited by R of the closely related species *M. sexta*. The volatile-inducing activity of R was completely eliminated by ion-exchange chromatography. Application of the exR, which did not contain any detectable amounts of the analyzed FAs or FACs (Fig. 1D), did not induce *cis*-α-bergamotene emissions. However, the addition of synthetic FACs (Fig. 1E) to the ion-exchanged R at their original concentrations completely restored the volatile-inducing activity. Moreover, an aqueous solution of synthetic FACs at concentrations found in R was as active as the larval R. The free fatty acids, when applied as aqueous solution or dissolved in the exR at concentrations found in *M. sexta* R, did not induce volatile emissions, even though the molar concentrations of the applied test solutions were higher than those of the FAC treatment. From these results, we conclude that the FACs found in *M. sexta* R, but not the FAs, are necessary and sufficient for the elicitation of the volatile release *in N. attenuata* plants. However, in excised leaves of lima beans, linolenic acid and its amino acid conjugate 18:3-Gln both induce homoterpene emissions (Koch et al., 1999), which were not detected among the volatiles released by *N. attenuata* (Halitschke et al., 2000). Even though the compounds were applied at higher concentrations than in this study, different mechanisms of volatile-induction may exist in different plant-herbivore systems.

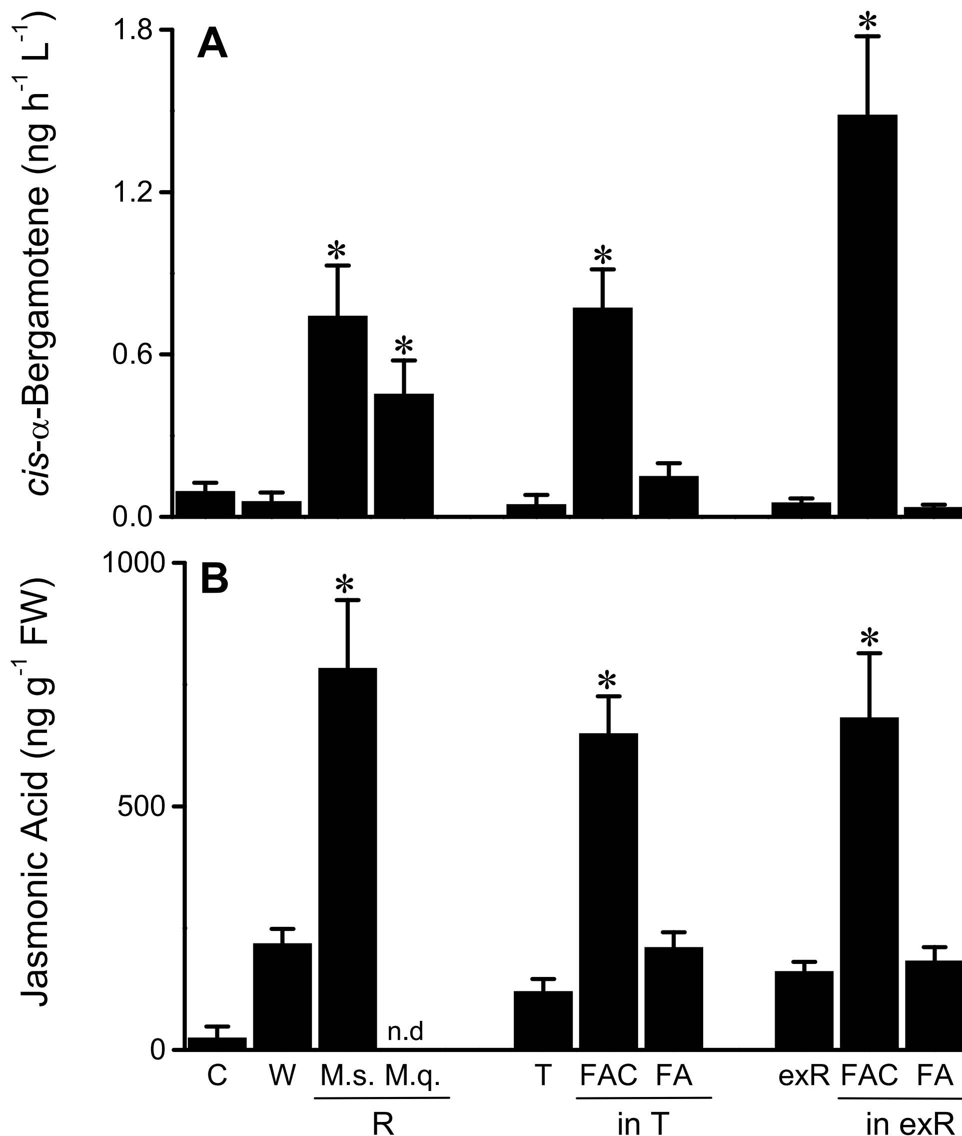

Figure 2. Mean (± SE) WP *cis*-α-bergamotene trapped per hour, per liter air sampled from individual (eight per treatment) *Nicotiana attenuata* plants (A) and mean (± SE) jasmonic acid concentrations (n.d. = not determined) of node two leaves of four replicate plants per treatment (B) 35 min (time of maximum JA induction) after the node two leaf was wounded and treated with 20 µL of the following test solutions: water (W), oral secretions and regurgitant (R) from *Manduca sexta* larvae (M.s.) or *M. quinquemaculata* (M.q.), ion-exchanged R (exR), triton X-100 in water (T), fatty acid–amino acid conjugate mixture (FAC) in concentrations found in R and fatty acid mixture (FA) in concentrations found in R in the triton solution (in T) or in ion-exchanged R (in exR). Control plants (C) remained undamaged. Stars represent significantly ($P < 0.05$) increased emissions as compared with wounded plants treated with water (W) as determined by Fisher's protected least significant difference from ANOVAs.

Induction of Endogenous JA Burst

As previously described (Kahl et al., 2000; Schittko et al., 2000), application of *M. sexta* R to puncture wounds on *N. attenuata* leaves transiently elicits higher JA concentrations than does the addition of water to identical puncture wounds (Fig. 2B, ANOVA $F_{8,26} = 14.551$, $P < 0.0001$). The ion-exchange treatment removed the JA inducing activity of R so that application of exR did not amplify the wound-induced JA accumulation. We tested the mixture of synthetic FACs (Fig. 1E) in a triton-containing aqueous solution and in exR at concentrations comparable to those found in R. Both solutions elicited a dramatic amplification of the wound-induced increase in JA concentrations as observed after application of larval R. No amplification of JA induction was observed after treatments with triton control solution and FA mixtures in exR or triton-containing aqueous solution compared with the wound treatment. These results demonstrate that other induction-mechanisms than a simple supply of FA as substrate for endogenous JA biosynthesis must account for the response activation processes in the *N. attenuata*-*M. sexta* system. The JA response is known to be very sensitive to *M. sexta* R, which retain their activity even when diluted to 1/1,000 with water (Schittko et al., 2000). This sensitivity also argues against a substrate supply mechanism, because the quantity of FAs delivered to a leaf as FACs in this highly diluted, but still active R is not sufficient to supply the quantity of fatty acid substrate required for the observed endogenous JA burst.

Changes in Transcript Accumulation

We also investigated the effect of the identified R-components on transcript accumulation. We applied the same test solutions as described for volatile- and JA-analysis and examined changes in transcript accumulation of a set of genes that specifically respond to R (Schittko et al., 2001). We found that FACs, supplied either in triton-containing aqueous solution or exR, caused specific changes in transcript

accumulation exactly as larval R did, whereas transcript accumulation in response to FA solutions (in triton-containing aqueous solution or exR) did not differ from wound-induced transcript levels (Fig. 3). As described by Schittko et al. (2001), two types of expression patterns were distinguished. Wound-induced transcript accumulation was specifically repressed (type I) by R- or FAC-treatments for threonine deaminase (pDH14.2; Hermsmeier et al., 2001) and an unknown gene encoded by pDH23.5, whereas the wound response of the other four investigated genes was amplified (type II; Fig. 3). Wound-induced transcript accumulation of pathogen-induced oxygenase (pDH41.6; Hermsmeier et al., 2001) was further up-regulated and wound-suppressed transcript accumulation of genes encoded by pDH61.1 (similar to the tomato gene for a light harvesting complex II subunit, *lhb C1;* Schwartz et al., 1991), pDH39.1, and pDH68.1 was further down-regulated (Fig. 3). No changes in transcript accumulation compared with the wound-treatment were observed after application of exR or triton control solution (Fig. 3).

Conclusion

In this study we identified FACs in R of the closely related herbivores *M. sexta* and *M. quinquemaculata* that, when applied to leaves of *N. attenuata*, are sufficient to activate the three investigated herbivore-specific plant responses in the signal transduction hierarchy (JA accumulation, changes in transcript accumulation, and volatile release). The chromatographic inactivation of a complex mixture of elicitors by ion-exchange, and the restoration of activity by re-addition of synthetic FACs, powerfully demonstrates the biological activity of FACs. Future investigations are necessary to examine the contribution of each individual compound to the activity of R and to elucidate the structure-function relationship of the identified FACs.

Because FAs in *Manduca* R were not active elicitors of herbivore-induced responses in *N. attenuata*, the process of their conjugation with amino acids to form FACs in the insect (Paré et al., 1998) suggests that the insect controls the production of its own elicitors. Although the function of FACs in insects is not absolutely clarified, FACs are likely to function as emulsifiers and detergents (Collatz and Mommsen, 1974). Hence, the plant distinguishes the feeding activity of this herbivore

from other agents that cause leaf damage by recognizing compounds essential for the insect's digestive processes.

The FACs could serve as useful tools for the study of plant-herbivore interactions because they allow researchers to uncouple herbivore-specific plant responses from herbivory and the damage it causes.

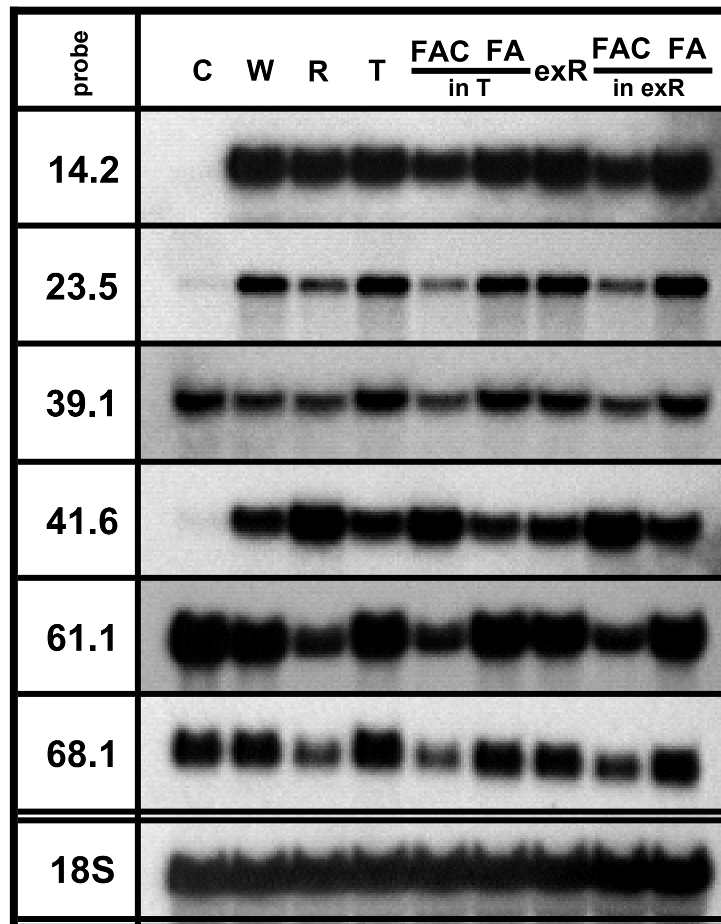

Figure 3. Northern analysis of transcript accumulation in response to different test solutions. The node two leaf of five replicate rosette-stage plants was continuously wounded and supplied with water (W), *Manduca sexta* larval oral secretions and regurgitant (R), ion-exchanged R (exR), Triton X-100 in water (T), a fatty acid-amino acid conjugate mixture (FAC) or a fatty acid mixture (FA) in concentrations found in R, dissolved in either T or exR, for 80 min, creating one row of puncture wounds every 20 min and harvesting 20 min after the final treatment. Untreated node two leaves were harvested as controls (C). Hybridization with an 18S rRNA probe indicates equal loading.

Materials and Methods

Plant Growth and Insect Rearing

Nicotiana attenuata Torr. ex Wats. seeds (collected at the DI ranch, UT, T40S R19W, section 10, 1988) were germinated in smoke-treated soil (Baldwin et al., 1994b). For JA experiments, seedlings were transferred to soil and grown for 3 to 4 weeks. Plants for volatile experiments and Northern analysis were grown as described in Hermsmeier et al. (2001) in no-nitrogen hydroponic solution (Baldwin and Schmelz, 1994). To provide nitrogen, 2 mL of 1mM KNO_3-solution were added to each 1-L chamber, followed by another 1 mL, 10 to 12 days later (the day before the experiment started). All plants were grown under a 32 °C, 16-h/27°C, 8-h day/night regime and were in the rosette-stage of growth at the time of the experiment.

Manduca sexta Linnaeus (Lepidoptera, Sphingidae) larvae were hatched from eggs (Carolina Biological Supply, Burlington, NC) and reared on fresh *N. attenuata* foliage under a 28 ºC, 16-h/8-h day/night regime. Eggs of *Manduca quinquemaculata* Haworth were collected at Pachoon Springs Burn in Utah in 1999 and the larvae were reared in the laboratory as described for *M. sexta*.

Analysis of Oral Secretions and Regurgitant

Atmospheric Pressure Chemical Ionization (APCI)-HPLC-MS analyses of *M. sexta* and *M. quinquemaculata* R and synthetic FAC mixtures were performed as previously described (Pohnert et al., 1999a) using a reversed phase HPLC separation (LiChrospher 100 RP-18, 5 µm, 250 x 4 mm, Merck, Darmstadt, Germany) with acetonitrile, water, and acetic acid as eluent. Detection and identification of the FACs was performed with a Finnigan (San Jose, CA) LCQ ion trap MS (APCI, vaporizer 560°C) by comparison with synthetic standards. Details on the LC-MS procedure, the synthesis and spectroscopic data of FACs 1 and 3 to 7 are published elsewhere (Pohnert et al., 1999a). Synthesis of the newly identified FAC 8, found in *Manduca* R, proceeded from free palmitic acid and unprotected glutamate following a published protocol (Pohnert et al., 1999b).

The following selected spectroscopic data were obtained by MS and NMR analysesof *N*-palmitoyl-L-glutamate (16:0-Glu, 8) [^{1}H]NMR (CD_3OD, 500 MHz) δ: 0.9 (t, J = 7.1, 3H); 1.25-1.35 (m, 26H); 1.62 (t, J = 7.2, 2H); 1.89-1.97 (m, 1H); 2.14-2.22 (m, 1H); 2.25 (t, J = 7.5, 1H); 2.4 (t, J = 7.8, 1H); 4.43 (dd, J = 5, 9.17, 1H);

[^{13}C]NMR (CD_3OD, 125 MHz) δ: 14.54; 23.83; 27.01; 27.94; 30.36; 30.56; 30.57; 30.74; 30.83; 30.83, 30.85; 30.86, 30.87; 30.88; 30.89; 30.9; 31.35; 53.01; 175.08; 176.39; 176.58; MS (70 eV): 385($M^{+\bullet}$, 8), 367(10), 341(8), 256(7), 239(10), 189(89), 171(30), 130(47), 102(100), 84(59), 57(65); and HR-MS: *m/z* calculated for $C_{21}H_{39}NO_5$: 385.2828, observed: 385.2828.

Free fatty acids were extracted from 80 µL of R after addition of 4 µg *cis*-10-nonadecenoic acid as an internal standard with a ternary solvent composition (water–methanol–chloroform), as described by Bligh and Dyer (1959). The extract was derivatized with 600 µL of freshly prepared solution of diazomethane in ether. The solvent was evaporated and the residue dissolved in 20 µL *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Macherey-Nagel, Düren, Germany). One-microliter aliquots were injected and analyzed on a Varian Saturn 2000 GC-MS (Walnut Creek, CA). Methylated FAs were separated on a 30 m x 0.25 mm DB-Wax column (0.25 µm film thickness; J & W Scientific, Folsom, CA). The injector temperature was held at 225ºC and the column oven temperature was programmed as follows: initial column temperature 120ºC held for 3 min, ramped from 120ºC to 170ºC at 10ºC/min, held at 170ºC for 6 min, ramped from 170ºC to 230ºC at 3ºC/min, ramped from 230ºC to 240ºC at 20ºC/min and finally held at 240ºC for 10 min. The carrier gas flow throughout the program was maintained at 1 mL/min.

Test Solutions

Oral secretions and regurgitant were collected with Teflon tubing connected to a vacuum from 4th to 5th instar *M. sexta* and *M. quinquemaculata* larvae reared on *N. attenuata* leaves and stored under argon at –80°C. They were diluted 1:1 (v:v) with water prior to the treatment. To remove FAs and FACs, 400 µL of R were eluted consecutively through four ion-exchange columns containing 400 mg of the basic anion exchange resin Amberlite IRA-400 (Sigma, Steinheim, Germany). The final eluate was called “ion-exchanged oral secretions and regurgitant” (exR). For application of FAs and FACs at concentrations similar to those found in R, aqueous solutions containing 0.005 % (w/w) Triton X-100 (Fluka, Buchs, Switzerland) were prepared and diluted 1:1 (v:v) with water or exR prior to the treatment. The FA solution contained 120 ng µL^{-1} (0.4 mM) of linoleic acid and 350 ng µL^{-1} (1.3 mM) of linolenic acid. A mixture of the four main FACs was prepared at concentrations of 50

ng μL^{-1} (0.12 mM) *N*-linolenoyl-L-glutamine (18:3-Gln, 1), 138 ng μL^{-1} (0.34 mM) *N*-linolenoyl-L-glutamate (18:3-Glu, 3), 41 ng μL^{-1} (0.10 mM) *N*-linoleoyl-L-glutamine (18:2-Gln, 4) and 26 ng μL^{-1} (0.06 mM) *N*-linoleoyl-L-glutamate (18:2-Glu, 5). The FAC mixture differed from the FAC composition of R in that it contained approximately 4 times the amount of FAC 4 and did not contain FAC 8. An aqueous solution containing 0.0025 % (w/w) Triton X-100 was used to control for the potential inducing activity of this detergent.

Volatile and JA Analysis

To determine the JA- and volatile-inducing activity of different test solutions, 20 μL samples were added to the leaf lamina immediately after three rows of puncture wounds were created on each leaf half with a fabric pattern wheel (Dritz, Spartanburg, SC). All treatments were applied to a single leaf node (node two) of each plant with the youngest fully-expanded leaf, the leaf that had just completed the source-sink transition (as defined in Wait et al., 1998) defining node one.

Leaves scheduled for JA-analysis were harvested 35 min after the induction of four replicate plants per treatment. Jasmonate concentrations were determined with $^{13}C_{1,2}$-JA as an internal standard and analyzed by GC-MS as described by Schittko et al. (2000).

Volatile collection commenced 24 h after the treatment and lasted for 8 h. Eight replicate plants per treatment were covered with 1-L open-top WP volatile collection chambers, and volatiles were collected by adsorption on 30 mg of SuperQ at a mean flow rate of 300 mL min^{-1} through the WP-chamber and analyzed by GC-MS as previously described (Halitschke et al., 2000). Because *cis*-α-bergamotene is the most consistently systemically released volatile from different genotypes of *N. attenuata* (Halitschke et al., 2000), we used the WP emission of this sesquiterpene to quantify the induced volatile response. The released amounts were calculated from peak areas using calibration curves with tetraline as an internal standard and normalized to trapping efficiencies by peak areas of a trapped sesquiterpene that was abundant in the surrounding growth room air (Halitschke et al., 2000). Given that induced volatile emissions are known to be influenced by many environmental factors (Loughrin et al., 1994; Takabayashi and Dicke, 1996; Paré and Tumlinson, 1999; Halitschke et al., 2000), our open-flow trapping system has the distinct experimental advantage of allowing the simultaneous analysis of 80 plants. Statistical comparisons

of volatile and JA data were performed with protected contrasts (Fisher's protected least significant difference) from ANOVAs.

Northern Analysis

A fabric pattern wheel (Dritz, Spartanburg, SC) was used to create one row of puncture wounds in parallel to the leaf midrib every 20 min, and 5 μL aliquotes of the respective test solution were applied to the fresh wounds. A total number of five rows were applied to a leaf at node two and the treated leaf of five replicate plants per treatment was harvested 20 min after the last wounding. Total cellular RNA was isolated according to Pawlowski et al. (1994). Agarose gel electrophoresis, Northern blotting, probe labeling and hybridizations were performed as described in Hermsmeier et al. (2001). GenBank accession numbers of the template sequences are AW191811 (pDH14.2), AW191815 (pDH23.5), AW191819 (pDH39.1), AW191821 (pDH41.6), AW191826 (pDH61.1), AW191828 (pDH64.4), and AW191830 (pDH68.1). Hybridization with an 18S rRNA probe (pDH64.4) was used to monitor loading. The wound-induced response of the other six mRNAs of *N. attenuata* is known to be specifically altered by *M. sexta and M. quinquemaculata* R (Schittko et al., 2001).

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Manuscript II

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Molecular Interactions between the Specialist Herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and Its Natural Host *Nicotiana attenuata*. VI. Microarray Analysis Reveals That Most Herbivore-Specific Transcriptional Changes Are Mediated By Fatty Acid-Amino Acid Conjugates

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Abstract

Evidence is accumulating that insect-specific plant responses are mediated by constituents in the oral secretions and regurgitants (R) of herbivores, however the relative importance of the different potentially active constituents remains unclear. Fatty acid-amino acid conjugates (FACs) are found in the R of many insect herbivores and have been shown to be necessary and sufficient to elicit a set of herbivore-specific responses when the native tobacco plant *Nicotiana attenuata* is attacked by the tobacco hornworm, *Manduca sexta*. Attack by this specialist herbivore results in a large transcriptional reorganization in *N. attenuata* and 161genes have been cloned from previous cDNA differential display-polymerase chain reaction and subtractive hybridization with magnetic beads analysis. cDNAs of these genes, in addition to those of 73 new R-responsive genes identified by cDNA amplified fragment-length polymorphism display of R-elicited plants, were spotted on polyepoxide coated glass slides to create microarrays highly enriched in *Manduca*- and R-induced genes. With these microarrays, we compare transcriptional responses in *N. attenuata* treated with R from the two most damaging lepidopteran herbivores of this plant in nature, *M. sexta* and *Manduca quinquemaculata*, which have very similar FAC compositions in their R., and with the two most abundant FACs in *Manduca* R. More than 68% of the genes up- and down-regulated by *M. sexta* R were similarly regulated by *M. quinquemaculata* R. A majority of genes up-regulated (64%) and down-regulated (49%) by *M. sexta* R were similarly regulated by treatment with the two FACs. In contrast, few genes showed similar transcriptional changes after H_2O_2- and R-treatment. These results demonstrate that the two most abundant FACs in *Manduca* R can account for the majority of *Manduca*-induced alterations of the wound response of *N. attenuata*.

Introduction

When herbivores attack plants, they cause wounding, but the response of a plant to herbivore attack can not, in many cases, be mimicked by mechanical wounding (Baldwin, 1988; Baldwin et al., 2001; Kessler and Baldwin, 2002). Several different types of elicitors in the oral secretions and regurgitant (R) of herbivorous insects have been reported to alter the wound response of a plant. For example, a β-glucosidase in the R of *Pieris brassica* larvae elicits the release of volatile organic compounds that function as indirect defenses (Mattiacci et al., 1995). A second enzymatic elicitor found in the salivary glands of *Helicoverpa zea* larvae was identified as glucose oxidase (GOX; Musser et al., 2002). GOX activity was shown to inhibit wound-induced nicotine accumulation and induced resistance in tobacco (*Nicotiana tabacum*) and is thought to function by the production of H_2O_2 at the wound site. *N*-(17-hydroxylinolenoyl)-L-Gln (volicitin), identified in R of *Spodoptera exigua* larvae (Alborn et al., 2000; Alborn et al., 1997; Turlings et al., 2000) was the first fatty acid-amino acid conjugate (FAC) that showed biological activity in inducing volatile emissions in corn (*Zea mays*) plants. Subsequently, several FACs have been identified in R of other herbivorous insect larvae (Halitschke et al., 2001; Pohnert et al., 1999; Turlings et al., 2000).

As demonstrated in other papers in this series, attack by *Manduca sexta* larvae elicits a suite of direct and indirect defense responses in its native host plant *Nicotiana attenuata.* These induced defense responses are accompanied by a large-scale transcriptional reorganization (Hermsmeier et al., 2001; Hui et al., 2003; Schittko et al., 2001; Winz and Baldwin, 2001). Many of the defensive and transcriptional responses elicited by *Manduca* attack can be mimicked by applying *Manduca* R to mechanically produced puncture wounds (Halitschke et al., 2000; Kahl et al., 2000; McCloud and Baldwin, 1997; Schittko et al., 2000, 2001; Winz and Baldwin, 2001) and FACs in *Manduca* R have been shown to be necessary and sufficient to elicit the release of terpenoid volatiles, an endogenous jasmonic acid burst, and changes in transcript accumulation of six herbivore-responsive genes in *N. attenuata* (Halitschke et al., 2001). Because herbivores transfer a bewildering array of potential elicitors to the plant during feeding and plants respond to herbivore attack with a bewildering array of responses, we provide a quantitative analysis of the proportion of

transcriptional changes elicited by R from different *Manduca* species and two different constituents of R, namely: FACs and H_2O_2.

To conduct this analysis we created a microarray enriched in *Manduca*- and R-induced *N. attenuata* genes. Companion papers in this series utilizing cDNA differential display (DDRT)-PCR and subtractive hybridization with magnetic beads (SHMB) techniques to identify differentially expressed genes in *N. attenuata* by comparing mRNA from *M. sexta*-attacked plants with that from developmentally synchronized unattacked control plants (Hermsmeier et al., 2001; Hui et al., 2003) did not discriminate between herbivore-specific and wound-induced transcript accumulations. The expression of a subset of the identified genes was shown to be specifically regulated by constituents of *M. sexta* R (Schittko et al., 2001). In this study we compared the mRNA of plants mechanically wounded and had their wounds treated with either water or with *M. sexta* R with a cDNA-amplified fragment-length polymorphism (cDNA-AFLP; Bachem et al., 1998) analysis to identify additional genes exhibiting R-specific patterns of expression. We spotted PCR fragments of the newly identified differentials together with previously characterized *N. attenuata* expressed sequence tags and genes derived from DDRT-PCR, SHMB, and cDNA-AFLP display of *Manduca* attacked plants (Hermsmeier et al., 2001; Hui et al., 2003) on polyepoxide coated glass slides to create a cDNA microarray containing 241 genes.

The larvae of two *Manduca* species (*M. sexta* and *Manduca quinquemaculata)* have been responsible for the majority of leaf area lost from *N. attenuata* plants to insect herbivores in the past 15 years of field observations in Utah (I.T. Baldwin unpublished data). To determine the relevance of FACs in organizing the transcriptional response of a plant, we first compared the transcriptional response of *N. attenuata* to R from these two species. Although FACs are a minor constituent of R, the two species have very similar FAC compositions (Halitschke et al., 2001) and if the transcriptional responses are not similar, we could infer that FACs play a minor role in determining herbivore-specific responses. After finding that more than 68% of the genes are similarly regulated by the R of the two *Manduca* species, we analyze the contribution of the two most abundant FACs in *Manduca* R, *N*-linolenoyl-L-glutamine and *N*-linolenoyl-L-glutamate at concentrations equivalent to that found in R. We find that these two minor constituents of R account for 56% of the R-specific transcript accumulation. Furthermore, we examine the role of H_2O_2, a product of

GOX activity, in the elicitation of R-elicited transcripts and find only 18% of the genes elicited by GOX treatment responded similarly to treatment with larval R, whereas a majority (42%) showed opposite changes in transcript accumulation. These results underscore the importance of FACs in determining *N. attenuata*'s "recognition" of *Manduca* attack.

Results

cDNA-AFLP Analysis

We amplified the *Eco*RI/*Mse*I cDNA fragments by PCR after subtractive hybridization, and we identified, isolated, and sequenced fragments of 73 genes, of which 22 had similarity to known genes in the databases (Table 1). The microarray analysis of plants elicited by a single treatment of R to wounds (Fig. 1A) confirmed the differential expression of 53% of the 73 genes derived from the cDNA-AFLP analysis, but this may be an underestimation because the cDNA-AFLP analysis was conducted on mRNA from plants elicited multiple times, whereas in the microarray analysis, plants were only elicited by a single treatment. All sequences, with one exception, had not been identified in previous display analyses of *Manduca*-attacked *N. attenuata* plants and confirm the pattern that a substantial proportion of the transcriptome of a plant is altered during attack. Comments on a selection of these genes follow:

DH54 provided a 438 bp fragment of the *N. attenuata* α-dioxygenase (α-DOX), which catalyzes the α-oxidation of fatty acids to hydroperoxy fatty acids and may be involved in signal generation (Hamberg et al., 1999; Sanz et al., 1998). α-DOX had been previously cloned twice by DDRT-PCR (Hermsmeier et al., 2001; Voelckel and Baldwin, 2003) with arbitrary primer R1 and a full length sequence was isolated by a cDNA library screen (Hermsmeier et al., 2001). DH45 had sequence similarity to TSC40-4 from *N. tabacum*, a 60S ribosomal protein L34, which is known to be wound-induced (Gao et al., 1994). DH02, DH24, DH48, and DH57 had similarity to *N. tabacum* chloroplast genome DNA (Shinozaki et al., 1986). DH49 had similarity to a *Solanum tuberosum* mRNA for a serine/glycine hydroxymethyl-transferase (Kopriva and Bauwe, 1995), which catalyzes the interconversion of serine and glycine and is a component of the photorespiratory pathway which recycles

carbon and nitrogen lost from the Calvin cycle by the oxygenation of ribulose 1,5-bisphosphate. DH19 had similarity to a *N. plumbaginifolia* (P.C. LaRosa and A.C. Smigocki, unpublished data) and a *Lycopersicon esculentum* (Giritch et al., 1998) metallothionein-like protein. Some type 2 metallothioneins are thought to function as potent metal chelators but others play roles in different cell death pathways, including senescence and the hypersensitive response after pathogen attack (Butt et al., 1998). DH60 had similarity to *L. esculentum* PTO-responsive protein (rg1) mRNA (R.L. Thilmony and G.B. Martin unpublished data) and again implicates pathogen recognition after herbivore attack. Prior work with DDRT-PCR of *N. attenuata* plants attacked by *M. sexta* larvae provided clone, RC144, which has similarity to a putative tomato *pto* gene (Hui et al., 2003). DH44 had similarity to a *Populus kitamiensis* phenylalanine ammonia-lyase (PAL) gene. PAL represents the keystone enzyme in synthesis of phenolics. Several phenolic compounds are induced in *N. attenuata* by feeding of *M. sexta* larvae (Keinanen et al., 2001; Roda et al., 2003). DH58 had similarity with a *Phleum pratense* mRNA for putative protein translation factor (R. Suck, S. Hagen, O. Cromwell, H. Fiebig unpublished data). DH63 had similarity with a *Petunia hybrida* mRNA for triose phosphate isomerase (Bennissan and Weiss, 1995) which catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate and plays an important role in gluconeogenesis, fatty acid biosynthesis, pentosephosphate pathway, and photosynthetic carbon fixation. In *Petunia* corollas, this gene is induced by gibberellins and its expression is highly correlated with respiration (Bennissan and Weiss, 1995). DH47 had similarity to a *Pisum sativum* mRNA for chloroplast outer membrane pore protein (Pohlmeyer et al., 1997) which may mediate the transport of glutamine and glutamic acid to and from the choloroplast and thereby regulate the export of reduced nitrogen. DH32 had similarity to a *N. sylvestris* ATPase mitochondrial β-subunit, a family that is known to be stress responsive (Lalanne et al., 1998). DH51 had similarity with a *S. tuberosum* mRNA for a low-temperature and salt-responsive protein (E. Nakane, H. Yoshioka, K. Kawakita, and N. Doke unpublished data).

Table 1: Sequence similarities (BLAST query) of *Nicotiana attenuata* cDNAs derived by cDNA-AFLP analysis of mRNA from plants that were wounded and immediately treated with water or treated with *Manduca sexta* oral secretions and regurgitants.

Clone	Accession No.	Sequence similarity	E-value
DH02	CA591820	*N. tabacum* chloroplast genome DNA (Z00044)	4e-68
DH05	CA591823	*L. esculentum* Hsc70 gene (L41253)	6e-24
DH17	CA591835	*L. esculentum* ripening regulated protein (AF204787)	5e-33
DH19	CA591837	*N. plumbaginifolia* metallothionein-like protein (U35225)	5e-59
DH23	CA591841	*N. tabacum* RUBISCO SSU pseudogene (M32420)	2e-10
DH24	CA591842	*N. tabacum* chloroplast genome DNA (Z00044)	4e-77
DH25	CA591843	*N. tabacum* mRNA C-7 (X64399)	1e-133
DH31	CA591849	*L. esculentum* unknown mRNA (AF261140)	3e-29
DH32	CA591850	*N. sylvestris* ATPase beta subunit nsatp2.2.1 (U96498)	3e-91
DH40	CA591858	*N. tabacum* mRNA C-7 (X64399)	2e-93
DH43	CA591861	*H. annuus* NADPH thioredoxin reductase (L36129)	2e-31
DH44	CA591862	*P. kitakamiensis* phenylalanine ammonia-lyase (D43802)	2e-17
DH45	CA591863	*N. tabacum* (TSC40-4) 60S ribosomal protein L34 (L27107)	1e-134
DH47	CA591865	*P. sativum* pore protein (Z73553)	5e-19
DH48	CA591866	*N. tabacum* chloroplast genome DNA (Z00044)	1e-117
DH49	CA591867	*S. tuberosum* serine/glycine hydroxymethyltransferase (Z25863)	1e-107
DH51	CA591869	*S. tuberosum* low temperature and salt responsive protein (AB061265)	3e-15
DH54	CA591872	*N. attenuata* pathogen-inducible α-dioxygenase (AF229926)	0.0
DH57	CA591875	*N. tabacum* chloroplast genome DNA (Z00044)	1e-107
DH58	CA591876	*P. pratense* putative protein translation factor SUI (AJ249397)	1e-35
DH60	CA591878	*L. esculentum* Pto-responsive gene (Prg1) (AF146690)	5e-59
DH63	CA591881	*P. hybrida* triosephosphate isomerase (X83227)	3e-29

Quantitative Analyses

Prior analysis of R found the FAC profiles from the two *Manduca* species to be very similar (Halitschke et al., 2001). To determine the relevance of FACs in R-specific responses, we compared the responses to *M. sexta* R with those to *M.*

quinquemaculata R. We spotted the fragments isolated by cDNA-AFLP together with PCR fragments of previously identified genes (Hermsmeier et al. 2001, Hui et al. 2003) to create a microarray highly enriched in *N. attenuata* genes with *Manduca*-responsive expression.

To examine the suitability of the microarray for this analysis, we compared the expression ratios of a set of genes (threonine deaminase, light-harvesting complex protein, α-DOX, and unknowns pDH68.1 and pDH39.1) that had been extensively characterized with Northern-blot analyses in previous studies utilizing similar experimental treatments (Halitschke et al. 2001, Schittko et al. 2001). All of the previously identified patterns of R- and FAC-elicited transcript regulation of these "control genes", namely the up-regulation (α-DOX) or down-regulation (threonine deaminase, light-harvesting complex protein, pDH68.1 and pDH39.1) that *M. sexta* R-treatment alters in the wound response of *N. attenuata* were confirmed with the microarray, despite experimental differences in timing of harvests and treatment applications between the studies.

The microarray analysis revealed *M. sexta*-specific changes in expression levels of 134 (56%) of the 241 genes spotted on the array (expression ratios can be found in Supplementary Table II, which can be viewed at www.plantphysiol.org). The transcripts of 67 genes showed significant up-regulation, while 78 genes showed significant down-regulation at either of the two times (1 or 10 h) after elicitation (Fig. 1B, left panel). The majority (81%) of the transcripts were regulated at 1 h after elicitation and a minority (19%) at 10 h. Therefore elicitation by *M. sexta* R is a rapidly induced and rapidly waning response. This response was largely mimicked by elicitation by R of *M. quinquemaculata* (Fig. 1A). Forty-two (63%) up-regulated genes and 52 (67%) down-regulated genes showed the same transcriptional regulation with a similar kinetic after treatment with *M. quinquemaculata* R (Fig. 1B, right panel). Additionally, two of the up-regulated genes and three of the down-regulated genes showed the same directional regulation but with a different kinetic (data not shown). In summary, even though FACs compose only a minor fraction of the R of the two *Manduca* species, which could differ in other unmeasured potential elicitors, the similarity of the response of the plant suggested that FACs were of fundamental importance.

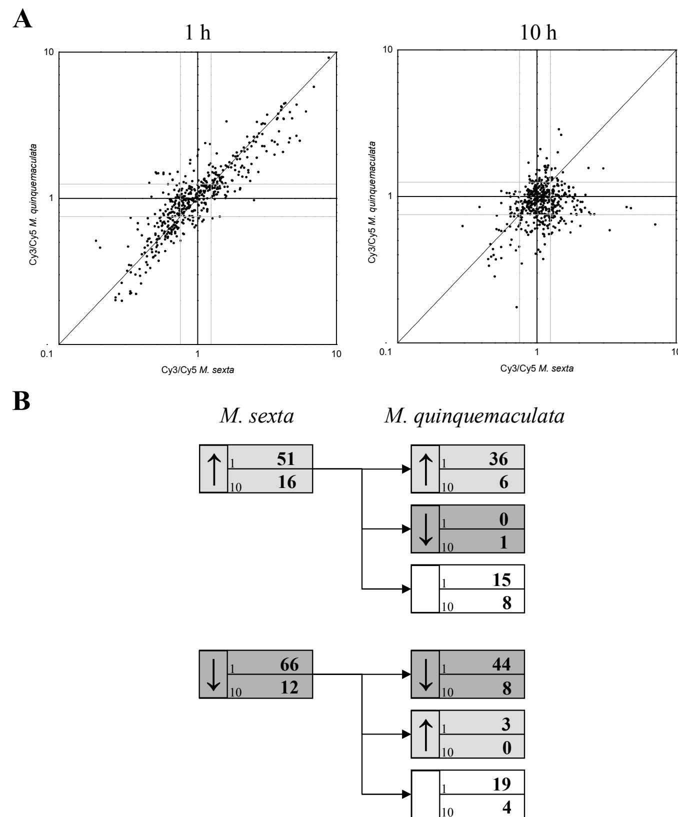

Figure 1: A, Correlation between expression ratios of 250 *N. attenuata* genes 1 h and 10 h after application of *M. sexta* or *M. quinquemaculata* oral secretions and regurgitant (R) to mechanically produced puncture wounds in fully-expanded *N. attenuata* leaves. Each gene is represented by two data points corresponding to the mean expression ratio of 4 replicate spots of the two probes for each gene (see "Materials and Methods"). Each array was hybridized with Cy3- or Cy5-labeled cDNA generated from plants that were wounded and immediately treated with either water (Cy5) or *Manduca* R (Cy3). Expression limits used to define either up- and down regulated expression are depicted as gray lines. B, Number of genes exhibiting significantly altered, up-regulated (↑) or down-regulated (↓), expression at either 1 or 10 h after application of R of *M. sexta* larvae (left panel) and expression patterns (↑, ↓ or not regulated) of these genes in response to application of R of *M. quinquemaculata* (right panel) at the same analysis time. For example, 67 genes were up-regulated by *M. sexta* R treatment of which 42 are also up-regulated, one was down-regulated, and 23 genes were not regulated by *M. quinquemaculata* R treatment at the same analysis time.

To directly determine the elicitor activity of FACs, we applied the two most abundant FACs in the R of both *Manduca* species and compared the response of the plant to that elicited by *M. sexta* R. Since purified FACs are not water-soluble, the

two compounds were dissolved in water containing trace quantities of Triton detergent. To control for potential Triton-mediated effects, the array was hybridized against a cDNA derived from mRNA extracted from plants wounded and treated with only the Triton-containing solution. Because the *M. sexta* R was diluted in water, this array was hybridized against cDNA derived from mRNA extracted from plants wounded and treated with water. Elicitation by the two FACs was remarkably similar to the elicitation by *M. sexta* R. (Fig. 2A). Thirty four (51%) of the up-regulated genes and 33 (42%) of the down-regulated genes after application of *M. sexta* R showed the same transcriptional regulation with a similar kinetic after treatment with the FACs at spit-comparable concentration (Fig. 2B, right panel). Additionally, nine of the up-regulated genes and five of the down-regulated genes showed the same regulation but with a different kinetic (data not shown).

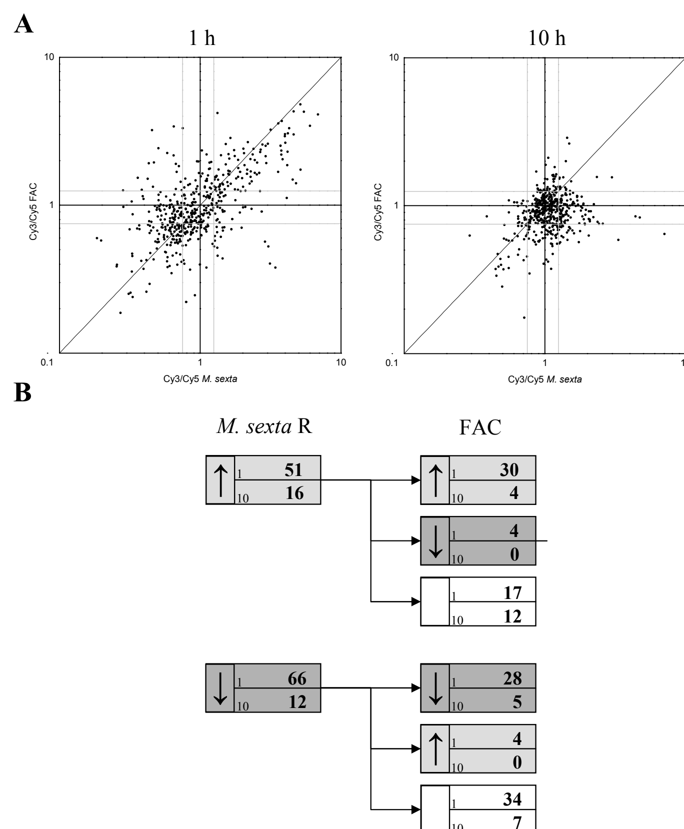

Figure 2: A, Correlation between expression ratios of 250 *N. attenuata* genes 1 h and 10 h after application of *M. sexta* oral secretions and regurgitant (R) or a mixture of the two most abundant fatty acid–amino acid conjugates (FAC) of *Manduca* R dissolved in a triton-containing solution at concentrations equivalent to that of R to mechanically produced puncture wounds in fully expanded *N. attenuata* leaves. Each gene is represented by two data

points corresponding to the mean expression ratio of 4 replicate spots of the two probes for each gene (see "Materials and Methods"). The arrays were hybridized with Cy3- or Cy5-labeled cDNA generated from plants treated with water (Cy5) or *Manduca* R (Cy3) and a Triton control (Cy5) or FAC solution (Cy3), respectively. Expression limits defining up- and down-regulated expression are depicted as gray lines. B, Number of genes exhibiting significantly altered (up-regulated [↑] or down-regulated [↓]) expression either 1 or 10 h after application of R of *M. sexta* larvae (left panel) and expression patterns (↑, ↓ or not regulated) of these genes in response to application of FACs (right panel) at the same analysis time.

In summary, despite the obvious differences between R and Triton matrices, *N*-linolenoyl-L-glutamine and *N*-linolenoyl-L-glutamate at concentrations found in R were sufficient to elicit a majority of the plant's complicated *Manduca*-specific transcriptional response.

GOX-derived H_2O_2 has been implicated in the differential regulation of tobacco defense responses (Musser et al., 2002), and we elicited plants with GOX-derived H_2O_2 and determined the similarity to *M. sexta* R-elicited responses. Note that we identify genes elicited by the GOX treatment and compare these with genes elicited by R treatment. Both microarrays include the appropriate controls (wound + buffer or water controls) and are therefore normalized for the respective wound response for their treatment.

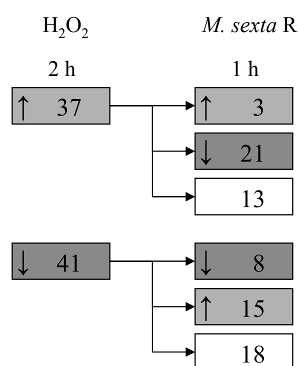

Figure 3: Number of *N. attenuata* genes exhibiting significantly altered expression 2 h after fully expanded *N. attenuata* leaves were infused with a solution of glucose and glucose oxidase to produce H_2O_2 *in situ* (left panel) and expression pattern of these genes 1 h (right panel) after application of R of *M. sexta* larvae.

H_2O_2 treatment up-regulated 37 (15%) and down-regulated 41 (17%) transcripts of the 241 genes spotted on the microarray within 2 h (Fig. 3, left panel). Only 3 (8%) of the up-regulated and 8 (20%) of the down-regulated genes showed comparable changes in transcript accumulation 1 h after treatment with *M. sexta* R (Fig. 3, right panel). We conclude that GOX-derived H_2O_2 plays only a minor role in elicitation of *Manduca*-specific plant responses.

Qualitative Analyses

A complete listing of the mean (+/- SEM) expression ratios of all spotted genes can be found in Supplementary Table II. The observed expression patterns elicited by R- and FAC-treatments reflect a basic shift in plant metabolism in response to herbivore attack. Overall, genes coding for photosynthetic enzymes (small subunit of Rubisco, light-harvesting complex protein) were down-regulated by all treatments including the application of GOX-derived H_2O_2. The exception to this trend was seen with PSII O_2-evolving complex, which was up-regulated at 1 h by *Manduca* R, but down-regulated by FACs and H_2O_2. Two additional subunits of PSII, similar to NtPII10 and a *Spinacia oleracea* PSII polypeptide, were down-regulated by *Manduca* R and FACs but not by H_2O_2. Furthermore, a majority of these photosynthesis-related genes retained their patterns of regulation 10 h after elicitation.

Genes coding for enzymes of the oxylipin signaling cascade (13-lipoxygenase [LOX]; allene oxide synthase [AOS]; hydroperoxide lyase [HPL]) were strongly up-regulated by *Manduca* R- and FAC treatments. Surprisingly, these genes were down-regulated in response to H_2O_2 treatment. Furthermore, a gene coding for a germin homolog, an enzyme involved in production of endogenous H_2O_2, was up-regulated in response to the H_2O_2 treatment but down-regulated by *Manduca* R.

The same expression pattern observed for genes of the oxylipin signaling cascade, namely up-regulation by *Manduca* R and FACs, but down-regulation by H_2O_2, were found in transcripts coding for WRKY transcription factors, a luminal binding protein, a glutamate synthase, and a ATPase β-subunit. A set of genes involved in herbivore-induced activation of secondary metabolism, 3-hydroxy-3-methylglutaryl-conzyme A reductase, α-DOX, and NADPH thioredoxin reductase, was up-regulated by R and FAC but did not respond to H_2O_2.

Similarly, the majority of genes down-regulated by *Manduca* R and FACs was either down-regulated by (histone H3, major intrinsic protein MIP2, and

metallothionein-like proteins) or not responsive to (GAL83, serine carboxypeptidase) H_2O_2 treatment. In addition to these genes, for which the R-induced regulation could be attributed to FACs in the *Manduca* R, some genes showed R-induced accumulation that could be elicited by neither FACs nor H_2O_2. Threonine deaminase, a C-7 mRNA, membrane channel protein, protein translation factor *sui1*, PTO-responsive gene 1, RNA polymerase II, and triosephosphate isomerase were down-regulated by *Manduca* R but not by FACs and H_2O_2. Genes up-regulated by *Manduca* R, but not by FACs or H_2O_2 code for Mg protopophyrin IX chelatase, thiazole, and glycine/serine hydroxymethyltransferase.

Discussion

By comparing the transcriptome of *N. attenuata* plants elicited by either wounding alone or wounding plus the addition of *M. sexta* R to the wounds, we identified fragments of 73 genes (Table 1). The list of genes identified by this cDNA-AFLP display analysis contained only one previously identified gene from prior analyses: α-dioxygenase (Hermsmeier et al., 2001). The lack of overlap is consistent with earlier predictions of a large herbivore-induced transcriptome (Hermsmeier et al., 2001; Hui et al., 2003; Voelckel and Baldwin, 2003). Moreover, the microarray proved to be an efficient tool for the verification of differential gene expression and when combined with various display procedures, provides a powerful means of analyzing ecological questions in non-model systems without the attendant bioinformatics overload.

We investigated the eliciting mechanism of the transcriptional reorganization in *N. attenuata* by analyzing two characterized types of elicitors identified in larval R, GOX enzyme activity (Musser et al., 2002) and FACs (Alborn et al., 1997; Halitschke et al., 2001; Turlings et al., 2000). A large proportion of the specific alteration in transcript accumulation could be attributed to the activity of FACs in *Manduca* R. Treatment of *N. attenuata* with R of two closely related herbivore species, *M. sexta* and *M. quinquemaculata* which had similar FACs profile (Halitschke et al., 2001), induced similar expression patterns (Fig. 1). Treatment of wounds with only two (of the 8) FACs found in *Manduca* R, elicited more than 55% of the response (Fig. 2). The total influence of FACs in the herbivore-induced transcriptional reorganization is

likely underestimated in our study, as the applied FACs represent only a limited portion of the total FAC bouquet of R (Halitschke et al., 2001). Furthermore, the synthetic FACs were applied in a chemical environment differing (in pH, matrix constituents, etc.) from that found in natural R collected from larvae. The large number of co-regulated genes in response to *Manduca* R and FACs points to the existence of an unknown *trans*-activating factor in *N. attenuata* which responds to FACs and organizes the transcriptional response of the plant. The identification of the putative *Manduca*-recognition element would represent a major milestone in understanding plant-insect interactions.

Changes in transcript accumulation elicited by GOX-produced H_2O_2 do not correlate with R-induced responses (Fig. 3). The majority of genes elicited by H_2O_2 treatment had the opposite patterns of regulation as was elicited by *Manduca* R and FAC treatments, suggesting the activation of different signaling cascades. Therefore, a signaling cascade suggested for activation defense genes in tomato, involving H_2O_2 downstream of octadecanoid and systemin signaling (Orozco-Cardenas et al., 2001) is unlikely to mediate the activation of *Manduca*-specific defense responses in *N. attenuata*.

Materials and Methods

Plant Growth

Nicotiana attenuata Torr. Ex Watts. (7 times inbred line of seeds collected from the DI ranch, Utah) seeds were germinated in smoke-treated soil and plants were grown in individual 1-L hydroponic chambers as previously described (Hermsmeier et al., 2001). After 10-14 days of growth in 1-L hydroponic chambers, plants received additional 7 mg of N as KNO_3 and were randomly assigned to treatment groups 24 h before starting treatments. All plants were grown in a growth room under the following conditions: 28°C/16 h light, 25°C/8 h dark, and 800 to 1000 $\mu mol\ m^{-2}\ s^{-1}$ PAR at plant height from high-pressure sodium lamps.

Treatments

To simulate herbivore damage, the second fully expanded leaf of rosette stage *N. attenuata* plants was treated in all experiments. For plants used in the cDNA-AFLP

analysis, one row of puncture wounds was created on each leaf half with a pattern wheel (Dritz, Spartanburg, S.C., USA) and 5 µL of either deionized water or a 1:10 (v:v) dilution of *M. sexta* oral secretion and regurgitant was applied to the fresh wounds. The treatment was repeated two times in 20 min intervals to create a total of three rows of puncture wounds on each leaf half. The treated leaf of 15 individual plants was harvested 20 min after the final treatment and pooled together for RNA extraction.

For the microarray analysis, the second fully expanded leaf of 10 individual rosette stage *N. attenuata* plants was wounded by creating three rows of puncture wounds on each leaf half as described for the cDNA-AFLP treatment and 20 µL of the different treatment solutions was applied to the fresh wounds. Treatment solutions were the following: deionized water, 1:1 (v:v) dilutions of R collected from 3rd to 4th instar *M. sexta* and *M. quinquemaculata* larvae, or a synthetic mixture of the two most abundant FACs in *Manduca* R, *N*-linolenoyl-L-glutamine and *N*-linolenoyl-L-glutamate (Halitschke et al., 2001). The FACs were dissolved at R-equivalent concentrations in an aqueous solution of 0.005% Triton X-100 (Fluka, Buchs, Switzerland) and diluted 1:1 (v:v) with deionized water prior the treatment. To control for possible Triton effects, we applied a 0.0025% Triton solution to wounds as a control. The treated leaves from 10 replicate plants were harvested at 1 h and 10 h after the treatment and flash frozen in liquid nitrogen.

To generate H_2O_2 *in situ*, GOX and glucose solutions were injected into unwounded leaves (Orozco-Cardenas et al., 2001). Glucose (25 mM) and GOX (from *Aspergillus niger*; 50 units mL^{-1}) were introduced to the leaf in phosphate buffer P (20 mM sodium phosphate, pH 6.5) by pressing a 1-mL syringe onto the leaf surface and twice injecting 200 µL. Control plants received 2 x 200-µL injections of buffer P without enzyme and glucose. The treated leaf of 10 individually treated plants in the rosette stage of growth was harvested and flash frozen in liquid nitrogen. Pooled samples were stored at -80 degree Celsius until RNA extraction.

cDNA-AFLP

RNA was extracted as described in Hermsmeier et al. (2001) and mRNA isolated from 100 µg of total RNA using magnetic beads (Hui et al., 2003). RNA from R-treated plants was used as the tester sample, while RNA from wounded and water treated plants was used as the driver. First strand was synthesized with

SuperScript II RNase H^{-} reverse transcriptase (Invitrogen, Groningen, The Netherlands), the second strand synthesized with DNA Pol I (NEB, Beverly, MA) and dsDNA blunt-ended with T4 DNA polymerase (NEB). The blunt ended DNA was extracted with phenol/chloroform, precipitated and processed following the procedures of Bachem et al. (1998), with the modification that *Eco*RI/*Mse*I restriction enzymes were used. The adaptors were created by annealing the following primer pairs: 5'–CTAACAAGATCTACTCTAGGGCCTCGTAGACTGCGTACC-3' and 3'–CATCTGACGCATGGTTAA-5' for *Eco*R1; 5'–CTAACAAGATCTACTCTAGG GCGACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT-5' for *Mse*I.

Samples were double digested with *Eco*RI/*Mse*I (NEB) and used according to ligation, hybridization, and amplification procedures described in Bachem et al. (1998). The PCR fragments amplified with *Eco*RI/*Mse*I adaptor-specific primers (5'-CTCGTAGACTGCGTACCAATT-3' and 5'-GACGATGAGTCCTGAGTAA-3') were cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced on a ABI Prism 377 XL DNA sequencer with the Big Dye terminator kit (PE-Applied Biosystems, Weiterstadt, Germany), and analyzed with the Lasergene software package (DNASTAR, Madison, WI).

Fabrication of cDNA microarray

The cDNAs cloned in the pCR2.1-TOPO and pUC18 vectors (Hermsmeier et al., 2001; Hui et al., 2003) were PCR amplified using the following primers derived from vector sequences close to the insert: TOP9-22 (5'-CTAGTAACGGCCGCCAGT GTGC-3); TOP10-24 (5'-CGCCAGTGTGATGGATATCTGCAG-3′); SMA1-19 (5'-GAATTCGAGCTCGGTACCC-3′); SMA4-23 (5'-CAGGTCGACTCTAGAGGATC CCC-3′); SMA3-22 (5'-TACGAATTCGAGCTCGGTACCC-3′); SMA2-20 (5'-GTC GACTCTAGAGGATCCCC-3'). For pCR2.1-TOPO, Top10-24 and Top9-22 were used. For pUC18, SMA3-22 and SMA2-20, as well as SMA1-19 and SMA4-23 were used. The *N. attenuata* control gene PCR products to be spotted onto the chip were synthesized as follows [primer sequences and templates were described previously (Hui et al., 2003)]: *pi*, *hpl*, *pmt1*, *aos*, *xet*, *wrky* with primers ASV5-21, ASV6-22, templates pNATPI1, pNATHPL1, pNATPMT1, pNATAOS1, pNATXET1 and pNATTFN1, respectively; 3′ region of *lox* with primers LOX4-22, ASV6-22, template pNATLOX1 and 5′ region of *lox* with primers ASV5-21, LOX3-21, template pNATLOX1. For each cDNA two PCR fragments, with 5'-Aminolink C6

modification (Sigma-ARK, Darmstadt, Germany) on either strand, were synthesized. Even numbered fragments (Supplementary Table I) carry the Aminolink modification at primers TOP9-22, SMA4-23 or ASV6-22, while odd numbered fragments carry the modification at primers TOP10-24, SMA3-22 or ASV5-21. PCR products were purified by a PCR purification kit (QIAquick, Qiagen, Hilden, Germany) following the manufacturer's instructions. Agarose gel electrophoresis was performed to confirm the purity and to determine the concentration of the amplified products. Commercially available epoxy coated slides (Quantifoil Micro Tools GmbH, Jena, Germany) were used. Before spotting, all the cDNA samples were purified through a micron-MultiScreen-PCR (Millipore, Bedford, MA) and concentrated to approximately 0.3 to 0.6 µg μL^{-1} in 1x QMT Spotting Solution I (Quantifoil Micro Tools GmbH). All cDNA samples, including the seven well-characterized *Manduca*-induced genes as controls, were commercially spotted four times by Quantifoil Micro Tools GmbH according to their procedure on the slides using a robot equipped with six printing tips (Biorobotics MicroGrid II Microarrayer: Genemachine, Apogent Discoveries, Hudson, NH). Hence each gene was represented on the microarray by two independent PCR fragments that, in turn, were spotted in quadruplicate. A complete list of identities and positions of spotted PCR products on the microarray can be found in Supplementary Table I www.plantphysiol.org. After processing, sample slides were hybridized with 9-mer random primers 5′-labeled with Cy3 and Cy5, respectively, to examine qualitative and quantitative characteristics of the microarrays.

Microarray Hybridization and Quantification

Pooled leaf samples were ground under liquid nitrogen and total RNA was extracted as described in Winz and Baldwin (2001). To exclude the unspecific wound response, we hybridized cDNA probes derived from plants that received the same mechanical damage but different treatment solutions. The R- and FAC-, and H_2O_2-treated samples served as treatment (Cy3) and the water- and Triton-treated samples, respectively, were labeled and hybridized as controls (Cy5).

Poly$(A)^+$ RNAs were isolated from 400 µg total RNA with Dynabeads Oligo $(dT)_{25}$ (Dynal Biotech, Oslo, Norway) and used for reverse transcription. To synthesize the first strand, 2 µg of Poly$(A)^+$ RNAs were mixed with 4 µg of random hexamer oligonucleotide, 2 µg of oligonucleotide $(dT)_{21}$ in 15.5 µL and incubated at

65°C for 10 min. Subsequently, 0.6 µL of 50x 5-(3-aminoallyl)-2'-dUTP/dNTPs (42.5 µL of each 100mM dATP, dGTP, dCTP; 25.5 µL of 100 mM dTTP, 17 µL of 100 mM 5-(3-aminoallyl)-dUTP, Sigma), 6 µL of 5x first strand buffer (Invitrogen), 3 µL of dithiothreitol (0.1 M), 1.9 µL of SuperScript II RNase H^- reverse transcriptase (Invitrogen); and 3 µL of water were added to a volume of 30 µL and incubated at 42°C for 2 h. cDNA/mRNA hybrids were hydrolyzed with 10 µL of NaOH (1 N) and 10 µL of EDTA (0.5 M) and incubated at 65°C for 15 min, followed by neutralization with 25 µL of 1 M Tri,s pH 7.4.

The cDNA mixtures were cleaned with a Microcon 30 concentrator (YM-30: Millipore) and dried in a vacuum concentrator (Eppendorf, Hamburg, Germany). The pellets of both induced and control sample were resuspended in 9 µL of $NaHCO_3$ buffer (0.5 M, pH 9.0), added to the dried aliquot of monofunctional *N*-hydroxysuccinimid-ester Cy3 dye and to Cy5 dye (Amersham Pharmacia Biotech, Little Chalfont, UK), respectively, for labeling at room temperature in darkness. After 1.5 h, the Cy3 and Cy5 reactions were quenched with 4.5 µL of hydroxylamine (4 M). After purification with QIAquick PCR purification kit (Qiagen), concentration and labeling efficiency of the purified cDNA was checked spectrophotometrically, and samples were dried in a vacuum concentrator (Eppendorf).

The probe solution was prepared by re-suspending the dried pellets in 3 µL of water, mixing them and adding 20 µL of polyadenylic acid (Sigma) and 2.5 µg of yeast tRNA (Sigma). After heating at 95°C for 2 min, 90 µL of Quantifoil Hybridization buffer was added. The probe solution was placed onto the chip prepared according to the Quantifoil protocol. Hybridization was carried out for 16 h in a wet hybridization chamber at 55°C. After hybridization, the slides were immediately washed at room temperature, initially with a solution of 2x SSC and 0.2% (w/v) SDS for 10 min, then with 2x SSC and 0.2x SSC solutions for 10 min each, before being dried in a 3.5-bar nitrogen stream.

An array scanner (428, Affymetrix, Inc., Santa Clara, CA) was used to scan the hybridized microarrays with sequential scanning for Cy5- and then for Cy3-labeled cDNA at a maximum resolution of 10 µm $pixel^{-1}$ with a 16-bit depth. The images were evaluated using the program AIDA Image Analyzer (Raytest Isotopenmessgräte GmbH, Straubenhardt, Germany). Each image was overlaid with a grid to assess the signal strength for both dyes from each spot. The background correction was calculated with the "non spot" mode of the AIDA software package.

To calculate a microarray-specific normalization factor, the measured Cy5 and Cy3 fluorescence intensities were ranked independently and after discarding of the 12.5% maximum and minimum values, the remaining 75% of the values were summed. The array-specific normalization factor was obtained by dividing the calculated sum of Cy3 values by those of the Cy5 values. This procedure excludes the confounding effects of massively down- and up-regulated transcripts (the 12.5% at either end of the distribution) from the normalization procedure. The ratios of normalized fluorescence values for Cy3 and Cy5 of each individual spot (expression ratio = ER) and the mean of the four replicate spots for each cDNA (2 for each gene = ER1, ER2) were calculated. Subsequently, log-transformed expression ratios with a hypothetical mean of “0”, corresponding to an ER of 1, were subjected to an untailed t-Test ($P < 0.05$). A transcript was defined as being differentially regulated if both of the following criteria were fulfilled: (a) Both individual ERs (ER1 and ER2) were equal to or exceeded the arbitrary thresholds for differential expression (0.75 and 1.25) representing 25% down-- and up-regulation, respectively; and (b) both individual ERs were significantly different from 1 as evaluated by t-tests to control for ER-variance and ER-sample size.

The use of statistically rigorous criteria to evaluate the within-array variance allowed us to use lower thresholds with this polyepoxide microarray in comparison with the polylysine microarray used in the companion paper (Hui et al., 2003). In addition, the analysis of within-array variance provides valuable information about the quality of the mRNA used in the hybridization and the effects of microarray age (M. Held, K. Gase, and I.T. Baldwin, unpublished data). Moreover, an ER calculated as a mean of replicate ERs (rather than a single value) allows one to use lower arbitrary thresholds with greater confidence.

To evaluate these criteria we hybridized two arrays with the same two cDNA pools (R. Halitschke and I.T. Baldwin, unpublished data) and found that 210 of 241 genes (84%) had the same regulation identified by the criteria described above. Of the 41 genes that did not show consistent regulation between the two repeat hybridizations, 24 had the same direction in mean ER, but did not meet the statistical requirements for a significant change. A complete list of all signal ratios (+/- SEM) can be found in Supplementary Table II.

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Supplemental Material

The following supplementary material is available at www.plantphysiol.org.

Supplementary Table I: Description of all genes spotted on the cDNA microarray (see appendix)

Supplementary Table II: Mean (±SE) expression ratios of all genes on the cDNA microarray (see appendix)

Manuscript III

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Agrobacterium-mediated transformation of *Nicotiana attenuata,* a model ecological expression system

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Summary

Research into the genetic basis of the ecological sophistication of plants is hampered by the availability of transformable systems with a wealth of well-described ecological interactions. We present an *Agrobacterium*-mediated transformation system for the model ecological expression system, *Nicotiana attenuata*, a native tobacco that occupies the post-fire niche in the Great Basin Desert of North America. We describe a transformation vector and a transformation procedure that differs from the standard cultivated tobacco transformation protocols in its use of selectable markers, explants, media and cultivation conditions. We illustrate its utility in the transformations with genes coding for key enzymes in the oxylipin cascade (lipoxygenase, allene oxide synthase, hydroperoxide lyase) in antisense orientations and present high-throughput screens useful for the detection of altered phenotypes for the oxylipin cascade (green leaf volatiles and jasmonic acid after wounding).

Keywords: *Nicotiana attenuata* - *Agrobacterium* transformation – lipoxygenase - allene oxide synthase - hydroperoxide lyase - green leaf volatiles

Introduction

Transformation is rapidly becoming one of the most important tools for the 'post-genomics' era in plant biology. The ability to silence or over-express individual genes remains the most robust tool for determining gene function and has catalyzed dramatic advances in plant biotechnology (Dixon & Arntzen, 1997; Pereira, 2000). Advances in understanding the traits responsible for the ecological function of plants (in pollinator-, herbivore-, pathogen-interactions, etc.) are similarly dependent on the ability to manipulate the expression of individual genes (Kessler & Baldwin, 2002; Roda & Baldwin, 2002). However, the model plant systems that are currently readily transformable are frequently not the optimal choices for ecological research. Crop plants have been selected for yield maximization, a process that has likely altered ecological responses and *Arabidopsis thaliana* (L.) Heynh. lacks important ecological interactions, which can be analyzed in near relatives (Mitchell-Olds, 2001) but those are currently not readily transformable. Cultivated tobacco, *Nicotiana tabacum* L., was one of the first species to be routinely transformed, but this tetraploid species has never been found outside of human cultivation and has clearly been under strong selection for the particular requirements of smokers. Its diploid relative, *Nicotiana attenuata* Torrey ex Watson, has emerged as a model system for understanding the molecular basis of ecological sophistication in the "agricultural niche" (Baldwin, 2001), a niche characterized by synchronized seed germination into nitrogen-rich soils which, in turn, results in intense intra-specific competition, selection for rapid growth and inducible responses to biotic challenges. As a result of *N. attenuata*'s unusual fire-chasing behavior, which, in turn is determined by its unusual seed germination behavior (Preston & Baldwin, 2000), this species has evolved in exactly this niche.

Unfortunately, *N. attenuata* belongs to the group of native *Nicotiana* species which rapidly 'habituates' after a short exposure to exogenous hormones either before or after *Agrobacterium*-mediated transformation, and grows vigorously for long periods of time as callus without shoot or root differentiation on media without hormone supplements (Bogani et al., 1997). As a consequence of this habituation behavior, the standard procedure for transformation of cultivated tobacco (Gallois & Marinho, 1995; Hall, 1991; Horsch, 1988) does not work for this species. Here we describe a procedure for the efficient transformation and rapid regeneration of this unique model ecological expression system. To illustrate the utility of this

transformation procedure for ecological research, we provide examples of transformations that silenced key enzymes in the oxylipin signal cascade (allene oxide synthase: AOS; lipoxygenase: LOX; and hydroperoxide lyase: HPL), a signal transduction cascade that strongly influences *N. attenuata*'s responses to herbivore attack (Baldwin, 2001). In a high-throughput (HTP) phenotypic screen, we analyzed wound-induced jasmonate (JA) levels and green leaf volatile (GLV) releases of plants transformed with AOS, HPL, and LOX, in an antisense orientation, to identify lines in which the transformation caused the desired phenotypic effect.

Materials and Methods

Materials

Chemicals were from Sigma, oligonucleotides used as primers for PCR and sequencing were synthesized by Sigma-ARK, DNA modifying enzymes were obtained from New England Biolabs, and RNase A was from Machery & Nagel.

Vector Construction

The streptothricin-acetyl-transferase gene (*sat-1*, GeneBank accession number X15995), present on pXGSAT, was PCR amplified with primers SAT3-40 (5'-GGATCTGGATCGTTTCGCATGAAGATTTCGGTGATCCCTG-3') and SAT4-40 (5'-GCGGCGGGTACCGGGCCCGTTAGGCGTCATCCTGTGCTCC-3'). Using primers PBI3-21 (5'-GTTCAATCGGACCAGCGGAGG-3') and SAT2-40 (5'-CAGGGATCACCGAAATCTTCATGCGAAACGATCCAGATCC-3') the nopaline synthase (NOS) promoter sequence of pBI121 (Jefferson et al., 1987) was amplified. In a subsequent polymerase reaction, in which both resulting PCR products served as template for each other, a fusion fragment containing *sat-1* downstream from the NOS promoter was synthesized, digested with *Kpn*I and cloned into pUC19, which had been cut with *Sma*I and *Kpn*I, to produce pUCNAT2. The *Nco*I-*Bst*EII *gusA* gene fragment of pCAMBIA-1301 (GeneBank accession number AF234297) was replaced by the adequately digested PCR fragment obtained with primers PMT6-36 (5'-GCGGCGGGTCACCGGTACCAACACAAATGGCTCTAC-3'), PMT7-31 (5'-GCGGCGCCATGGAGCCCTTAAAGACTTGACG-3') and pBI121-ASPMT (Voelckel et al., 2001) as template. The 9.0 kb partial *Kpn*I-*Bam*HI fragment of the

resulting plasmid pCAMPMT1 served as cloning vector for the 0.9 kb *Kpn*I-*Bcl*I fragment from pUCNAT2. The resulting plant transformation vector pCAMNAT1 contained two selectable plant resistance markers: *hgh* (K01193) *or aph(4)* (V01499) driven by the CaMV 35S promoter and *sat-1* driven by the NOS promoter.

The 8.8 kb partial *Xho*I-*Aat*II-fragment of pCAMNAT1 was ligated with the annealed primers ASV1-23 (5'-GGCCATGGCTGCAGGGTGACCGG-3') and ASV2-31 (5'-TCGACCGGTCACCCTGCAGCCATGGCCACGT-3'). The resulting plasmid pCAMNAT2 was cut with *Xho*I, blunt ended with mung bean Nuclease and partially recut with *Bst*EII. The generated 8.8 kb fragment served as vector for cloning the 2.1 kb *gusA* fragment obtained from pCAMBIA-1301 by *Nco*I digestion, blunt ending with T4 DNA Polymerase and *Bst*EII digestion. The surplus *Bst*EII site near the 3' end of *sat-1* was removed from the resulting plasmid pNATGUS1 by partial *Bst*EII digestion, T4 DNA polymerase treatment and subsequent recircularization. The obtained cloning vector pNATGUS3 (Fig. 1) carried *sat-1* as plant selectable marker and enabled the transcription of transgenes in *N. attenuata*. For the construction of the antisense gene silencing vectors PCR fragments of *N. attenuata hpl* [primers: HPL1-34 (5'-GCGGCGGGTCACCACACTCATGGCGAAAATGATG-3'), HPL2-33 (5'-GCGGCGCCATGGCACAGGTGGACTAAGTCTAAG-3'); template: *hpl* cDNA cloned on plasmid pSKIIHPL, unpublished result], *N. attenuata lox* [primers: LOX1-34 (5'-GCGGCGGGTCACCGGAACAAGAACAAGGAAGATC-3'), LOX2-32 (5'-GCGGCGCCATGGCTACATGTTACTCCAGGGCC-3'); template: *lox* cDNA cloned on plasmid pNaLoxLSA-II, unpublished result] and *N. attenuata aos* [GeneBank accession number AJ295274; primers: AOS1-35 (5'-GCGGCGGGTCAC CGTGTTCTTTCTTATCTTGATCC-3'), AOS2-31 (5'-GCGGCGCCATGGAAGTA GGAAAACCAAGAAC-3'), template: chromosomal *N. attenuata* DNA] were synthesized, digested with *Nco*I and *Bst*EII (*aos* fragment partially) and cloned in pNATGUS3 cut with the same enzymes, yielding pNATHPL1, pNATLOX1 and pNATAOS2, respectively.

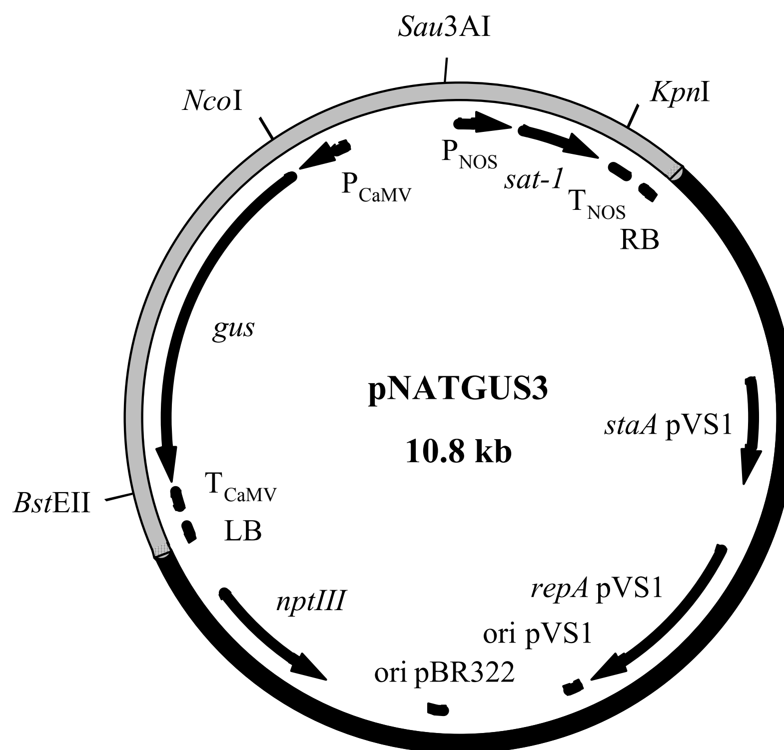


Fig. 1 *N. attenuata* transformation vector pNATGUS3 with *sat-1* as plant selectable marker gene. DNA to be transcribed can be inserted as *Nco*I-*Bst*EII fragment. Functional elements on T-DNA (grey): LB/RB, left/right border of T-DNA; P_{CaMV}/T_{CaMV}, 35S promoter/terminator of cauliflower mosaic virus; P_{NOS}/T_{NOS}, promoter/terminator of nopaline synthase gene; *sat-1*, nourseothricin resistance gene; *gusA*, *E. coli* beta-glucuronidase gene containing intron 1 of *Ricinus communis* catalase gene. Genes outside T-DNA are from pCAMBIA-1301

Plant DNA Extraction

200 mg plant material were flash-frozen in liquid nitrogen, ground to powder and suspended in 750 µl of 100 mM Tris/50 mM EDTA (pH 8.0), containing 250 µg/ml RNase A. Eight µl liquid laundry detergent (Ariel, Procter & Gamble, Schwalbach, Germany) were added. After 60 min incubation at 60ºC and subsequent addition of 80 µl of 5 M NaCl, the suspension was centrifuged for 5 min at 16,000 x g. The supernatant was removed carefully and extracted with phenol/chloroform. The DNA was precipitated with 600 µl isopropanol, pelleted 5 min by centrifugation at 16,000 x g, washed with 200 µl 70% ethanol and dissolved in 50 µl of water.

Plant material and Agrobacterium *suspension cultures*

N. attenuata seeds (derived from a 1988 collection from a native population in Washington County, Utah, USA, and subsequently selfed for 6 generations) were used for all transformations and subsequent analysis with wild type plants. Seeds were sterilized for 7 min in 5 ml aqueous solution of 0.1 g dichloroisocyanuric acid (DCCA: Sigma, St. Louis, MO, USA), supplemented with 50 µl of 0.5% (v/v) Tween-20 (Merck, Darmstadt, Germany). Seeds were washed 3 times with sterile water before incubation for 1 h in 5 ml sterile liquid smoke (House of Herbs, Inc.; Passaic, New Jersey, USA) solution, 50 x diluted in water and supplemented with 50 µl of 0.1 M gibberellic acid, GA_3 (Roth, Karlsruhe, Germany). After this treatment, seeds were washed 3 times with sterile water and 25 seeds were transferred individually to a petri dish containing germination medium [Gamborg's B5 medium: (Sigma) and 0.6% (w/v) phytagel (Sigma)]. The plates were maintained in a growth chamber (Percival, Perry Iowa, USA) at 26ºC/16 h 155 µm/s/m² (measured a LCA3: ADC, Hoddesdon, England) light, 24ºC/8 h dark. *A. tumefaciens* (strain LBA 4404: Life Technologies-Gibco BRL, Eggenstein, Germany) was maintained on solidified yeast-peptone-NaCl (YEP) medium containing 50 mg/l kanamycin at 4ºC in the dark. Two days before transformation, the desired *A. tumefaciens* strain was grown overnight at 28ºC in YEP medium with 25 mg/l kanamycin. The liquid culture was spread onto solid YEP medium containing 50 mg/l kanamycin and grown overnight at 26ºC. The bacteria were washed with 3 ml liquid MS medium containing 0.02 mg/l indole-3-acetic acid (IAA: Duchefa, Netherlands) and 1 mg/l 6-benzylaminopurine (BAP: Duchefa) and the optical density (OD) was measured at 600 nm. An OD of 0.6 proved to be optimal for transformation. Since hygromycin (Duchefa) is labile, all media containing this antibiotic were freshly prepared just before use, and the media was cooled to 56ºC before the addition of hygromycin. Nourseothricin (NTC: Werner BioAgents, Jena, Germany) is significantly more stable, and media containing NTC can be stored for at least two weeks until use.

Transformation procedure

An extensive analysis of callus derived from different plant material revealed that optimal transformation and regeneration results were obtained when hypocotyls of 8-10 day old seedlings, which have a high potential for cell division, were used for transformation. Each seedling was cut below the apical meristem and above the roots.

The excised hypocotyl was subsequently cut into 2-3 (ca. 3 mm) pieces and placed on co-cultivation medium (Table 1). Before each cut, the tip of the scalpel was dipped into the *Agrobacterium* suspension. Clean cuts were essential for good infection. After 3 days of co-cultivation with *Agrobacterium*, explants were transferred to callus induction medium (Table 1) containing the antibiotic timentin (tricarcillin disodium/potassium clavulanate: Duchefa) to inhibit growth of *Agrobacterium*. After 2-3 weeks, when explants had developed callus with green shoot primodia, the callus was sub-cultured onto regeneration medium (Table 1).

Table 1 Steps in *Agrobacterium*-mediated transformation of *Nicotiana attenuata.* Average duration, media composition used, and culture conditions of each stage.

Time required (days)	Type of medium	Medium content	Conditions
3	Co-cultivation	#Basal medium, 0.02 mg/l IAA, 1 mg/l BAP	Dark, 26ºC
14-21	Callus induction	#Basal medium, 0.02 mg/l IAA, 1 mg/ml BAP, §selectable antibiotic, 125 mg/l timentin	16 h day, 30ºC; 8 h dark, 26ºC
14-21	Regeneration	#Basal medium, 0.5 mg/l BAP, selectable antibiotic, 125 mg/l timentin	16 h day, 30ºC; 8 h dark, 30ºC
14-21	Maturation	#Basal medium, selectable antibiotic, 125 mg/l timentin	16 h day, 28ºC; 8 h dark, 26ºC
21	Rooting	1 x Peter's Hydro-Sol 0.292 g/l, 0.6% (w/v) plant agar, *vitamin mixture	16 h day, 26ºC; 8 h dark, 24ºC

*Vitamin mixture:	glycine	2.0 mg/l
	myoinositol	100 mg/l
	nicotinic acid	0.5 mg/l
	pyridoxine HCL	0.5 mg/l
	thiamine	0.1 mg/l
#Basal medium:	MS incl Vitamins	4.41 g/l
	sucrose	3% (w/v)
	phytagel	0.3% (w/v)
§Selectable antibiotic:	hygromycin (20 mg/l) or NTC (50-75 mg/l)	

When light green shoots started to develop from the shoot primodia (typically within 2 weeks after transfer to the regeneration media), the callus with shoots was transferred to the maturation medium (Table 1), and subsequently sub-cultured every 3 weeks until plantlets were formed. These plantlets were separated into single plants and cultured on rooting medium (Table 1), which contained no antibiotics. At this stage, removal of all traces of callus from the base of the plantlet was essential.

Plantlets were sub-cultured on rooting media every 3 weeks until roots appeared, after which plants were carefully removed from the gel and planted into soil (30 l peat moss, 15 l Vermiculite, 125 ml ground limestone, 125 ml bone meal) in Magenta boxes (77 x77 x 77 mm) maintained in growth chambers at 24ºC/16 h light (200-250 µm/s/m²), 24ºC/8 h dark. Once established, plants were transferred to 2 l pots in soil [60 l peat moss, 30 l Vermiculite, 30 l Perlite, 250 ml ground limestone, 250 ml bone meal and 400 ml Osmocote 14:14:14 pellets (N:P:K; 2-3 month release rate)] and grown in the glasshouse at 26-28ºC under 16 h supplemental light from Philips Sun-T Agro 400 or 600W Na lights.

Confirmation of transformation

PCRs with chromosomal DNA of potentially transformed plants as template and primer pairs HYG1-18 (5'-CCGGATCGGACGATTGCG-3'), HYG2-18 (5'-CTGACGGACAATGGCCGC-3') for *hgh*/*aph (4)* and NAT1-18 (5'-CTCTGCTTGC TATGGCGC-3'), NAT2-18 (5'-CGTCATCCTGTGCTCCCG-3') for *sat-1* were used to verify the insertion of the resistance marker and used as an indicator of successfully transformed plants. In addition, leaf disks from T_0 plants were tested for their resistance to the antibiotic under which they were selected. This leaf disc selection procedure provided highly reliable results with a minimum effort and is therefore described in detail. A whole leaf was excised at the petiole from putatively transformed plants and immediately placed into water. The leaf was sterilized for 7 min in DCCA and washed 3 times with sterile water. The leaf was cut into small pieces (ca. 0.5 cm x 0.5 cm) and incubated on MS medium (Murashige & Skoog, 1962) supplemented with 0.1 mg/l IAA, 1 mg/l BAP, selectable antibiotic (250 mg/l NTC or 35 mg/l hygromycin), and 125 mg/l timentin for at least 7 days. Concomitant with each experiment, leaves of wild type plants, which invariably died slowly and developed no callus, were used as a control. The average transformation efficiency, determined from 175 regenerated and evaluated plants was 97%.

Progeny selection

The number of independent loci at which integration occurred can be estimated by the segregation ratio of resistant to sensitive seedlings in the progeny and we used both seed and tissue selection to determine segregation ratios. T_1 seeds from plants transformed with constructs containing *hpt-II* were sterilized and germinated (as above) on Gamborg's B5 medium with 35 mg/l hygromycin. Since roots and shoots appear to have different sensitivities to NTC, we were not able to select seedlings with this antibiotic, but tissue selection worked well for both antibiotics. For tissue selection, half of one cotelydon of 8-10 day old seedlings was removed with a pair of sterile scissors and placed on MS medium supplemented with 0.1 mg/l IAA, 1 mg/l BAP, 250 mg/l NTC or 25 mg/l hygromycin, and 125 mg/l timentin for callus induction at 26ºC under 16 h 125 µm/s/m² light. If vigorous callus growth was observed within 7 days, the seedling from which the cotyledon was excised, was planted into soil and gradually adapted to the high light levels of the glasshouse. This procedure identified and rescued homo- and heterozygous plants and subsequent crossing and selection was used to identify homozygous plants.

AOS, HPL, and LOX HPT phenotypic screens

Plants silenced in 13-LOX, (because it supplies substrates, 13-hydroperoxides of linoleic and linolenic acid, for AOS and HPL: Fig. 3B) and those silenced in AOS and HPL, were identified by their reduced ability to produce JA and release the GLVs, hexanal and (*Z*)-3-hexenal, after wounding. T_1 plants from independently transformed as-LOX, as-AOS, and as-HPL lines were screened. Wound-induced levels of JA and GLVs of five plants per line for a total of 515 plants from 103 independent lines (one wild type, 35 as-LOX, 30 as-AOS, and 37 as-HPL) were analyzed.

Wound-induced JA analysis

The second fully-developed leaf of rosette-stage plants was wounded with a pattern wheel producing 3 rows of puncture wounds on each leaf half. The wounded leaves of wild-type *N. attenuata* plants were harvested at the different time points (5; 15; 35; 45; 60; 90; 120; 180; and 300 min) after wounding and immediately frozen in liquid nitrogen. The 0 min time point indicates harvests of leaves from unwounded

control plants. The leaves of as-AOS and as-LOX transformed plants were harvested 35 min after the treatment. Samples were analyzed by GC-MS after addition of 172 ng of $^{13}C_{1,2}$-JA (Baldwin et al., 1997) as an internal standard and sample preparation as described by Schittko et al. (2000) with the following modifications of the extraction procedure. Leaf samples were homogenized in the extraction buffer with the FastPrep extraction system FP120 (Savant Instruments, Holbrook, NY, USA). Tissue was homogenized by reciprocating shaking at 6.0 m/sec for 90 sec in extraction tubes containing 900 mg of lysing matrix (BIO 101, Vista, CA, USA).

Volatile analysis

Volatiles released into the headspace of a leaf during the first minute after wounding were collected and analyzed with a portable gas analyzer. The zNose™ (EST, Newbury Park, CA, USA) is a portable gas chromatograph equipped with an internal sample pump, a pre-concentration trap, a 1 m stainless column (DB-5 stationary phase) and a surface acoustic wave (SAW) detector. The short column length allows rapid chromatographic separation of analytes and is therefore highly suitable for HTP analysis of large sample numbers. The unique sensitivity of the SAW detector obviates requirements for long preconcentration periods and the time-consuming sample preparation of conventional trapping-desorption methods and allows for very short (2 min) sampling intervals.

The treated leaf was placed for 30 sec in an open volatile collection chamber (125 ml) immediately after wounding (see JA analysis) and volatiles were collected by adsorptive trapping on the internal trap of the zNose™ for 30 sec. Sample flow was set to 30 ml/min. Column temperature was maintained at 45°C for 1 sec and raised to 175°C at 5°C/sec. The SAW detector temperature was set to 45°C. The identity of hexanal and (*Z*)-3-hexenal was verified by co-chromatography with synthetic standards.

Results and Discussion

The principle difficulty of the transformation and regeneration of native *Nicotiana* species is their taxonomically-determined proclivity to rapidly ‘habituate’ and grow vigorously for long periods of time without shoot or root differentiation

after a short exposure to exogenous hormones (Bogani et al., 1997). If the published procedures for *Nicotiana tabacum* callus induction and regeneration (Murashige & Skoog, 1962; Zambryski et al., 1983; Guerineau et al., 1990) are used for *N. attenuata*, no regeneration from callus was observed after more than 4 years of vigorous callus growth. The aggressive addition of auxin-antagonists and transport inhibitors (p-chlorophenoxyisobutyric acid and triiodobenzoic acid) and the complete removal of exogenous auxins did not result in regeneration (data not shown). Two considerations are essential for *N. attenuata* regeneration: the type and length of exposure to auxins and the choice of explant. A minimal exposure (0.02 mg/l, Fig. 2; Table 1) to short-lived auxins (IAA and not 1-naphtylacetic acid) was sufficient to stimulate callus growth and resulted in the highest regeneration rates. This short exposure period produced friable callus of low density that rapidly initiated shoot primordia. High temperatures (30ºC) as well as subculturing to auxin–free regeneration media as soon as shoot primordia were observed, were also required at this stage (Table 1). Since the timing of auxin exposure is critical, it was essential to select an explant tissue that produced consistent regeneration. Only explants from the hypocotyls of germinating seeds (and not leaves, cotyledons or roots) provided reliable material (data not shown), and this reliable response could be due to the particular hormonal balance of this tissue.

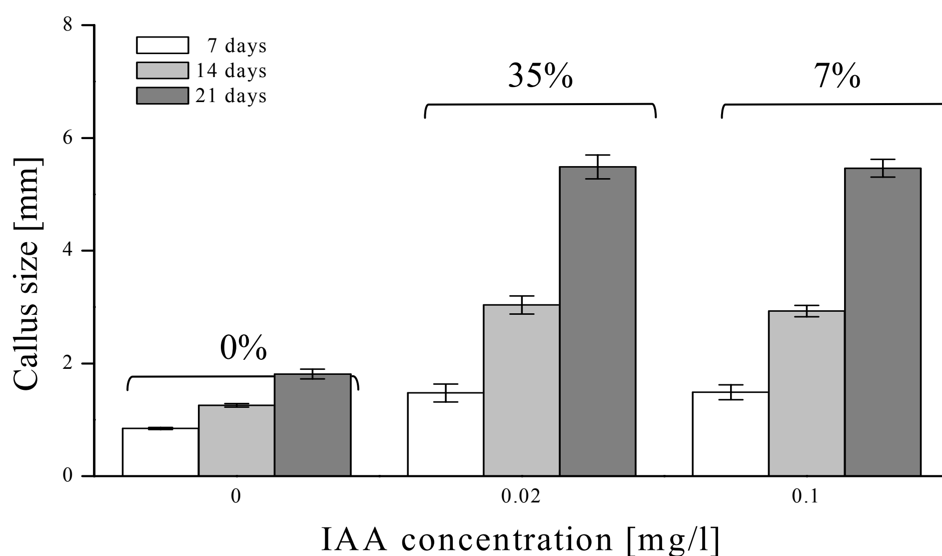
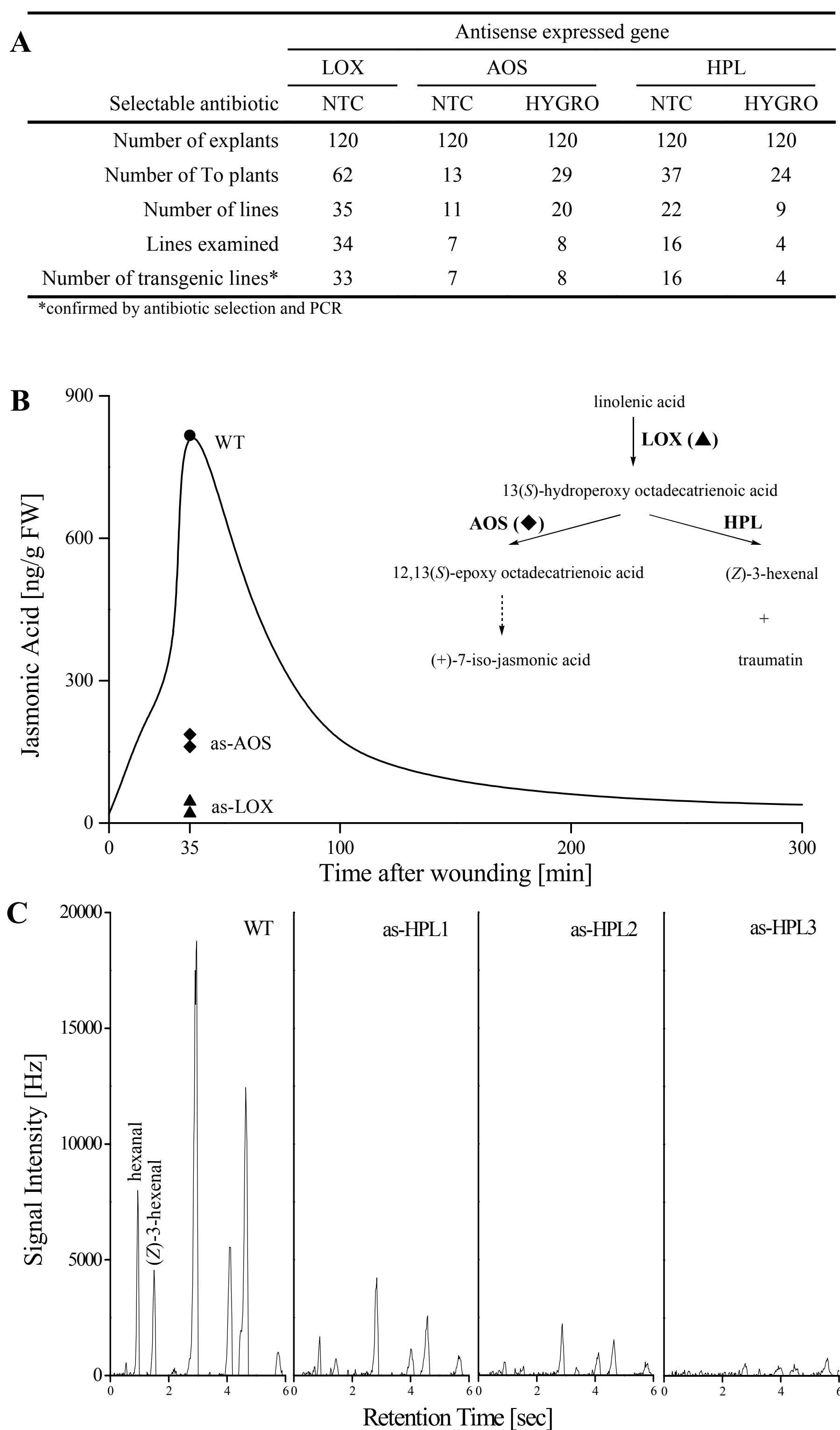


Fig. 2 Size of callus (mean ± SE of 3 independent experiments) regenerated from hypocotyls of seedlings transformed with pCambia1301. Bars indicate callus size at 7, 14, and 21 days

after transfer to MS media containing 20 mg/l hygromycin (see Materials and Methods). Numbers on the brackets indicate regeneration rates of the respective experiments, which were determined at day 28 after transformation

Other considerations proved to be important for attaining a high frequency of transformation and regeneration. Using the lowest concentration of selectable antibiotic dramatically increased regeneration rates. The values recommended in Table 1 represent an optimization of maximized regeneration rate and a minimization of the production of non-transformed escapes or chimerical plants. Given hygromycin's lability, the maintenance of consistent selection proved more difficult with this antibiotic than with NTC, but with experience, both antibiotics produced acceptable results (Fig. 3A). Hygromycin selection, on the other hand, has the advantage of being useful in seedling screens that facilitate the analysis of segregation ratios in progeny. Avoidance of the apical meristem in preparing explants proved to be essential in reducing the production of escapes, for this tissue was invariably resistant to *Agrobacterium* transformation. We worked primarily with the LBA4404 strain of *Agrobacterium,* which also works well with other *Nicotiana* species. The choice of antibiotic to control *Agrobacterium* infestation after transformation dramatically influenced regeneration. We found that timentin, at the concentrations listed in Table 1, allowed for regeneration, while others (cefotaxime, gentamicin and vancomycin) dramatically inhibited regeneration. Because of the delicate nature of hypocotyls, we obtained superior results when *Agrobacterium* was introduced on the scalpel during cutting, rather than dipping the explant in the *Agrobacterium* culture. We selected a single regenerating plant from each hypocotyl to guarantee that each plant represented an independent transformation event.

Our analysis of 5 plants from each of 102 independently transformed lines with as-LOX, as-AOS, and as-HPL containing constructs underscored the importance of appropriate HPT screens for ecological research. Only 25 (71%) of the 35 as-LOX lines and 2 (5.4%) of the 37 as-AOS lines exhibited more than an 80% reduction in wound-induced JA accumulation observed in wild-type plants and only 12 (40%) of the 30 as-HPL lines exhibited more than a 50% reduction in wound-induced GLVs release (Fig. 3B, C).



A

	Antisense expressed gene				
	LOX	AOS		HPL	
Selectable antibiotic	NTC	NTC	HYGRO	NTC	HYGRO
Number of explants	120	120	120	120	120
Number of To plants	62	13	29	37	24
Number of lines	35	11	20	22	9
Lines examined	34	7	8	16	4
Number of transgenic lines*	33	7	8	16	4

*confirmed by antibiotic selection and PCR

Fig. 3 A) Number of T_0 plants regenerating from 120 explants transformed with *N attenuata* LOX, AOS and HPL constructs in an antisense (as) orientation with either NTC or hygromycin as the selectable antibiotic, and the number of lines examined and found to be transformed (by antibiotic selection and PCR). **B)** Scheme of the biochemical reactions

catalyzed by LOX, AOS, and HPL enzymes in the plant oxylipin cascade leading to jasmonic acid and green leaf volatiles. Kinetic of the wound-induced JA accumulation (ng/g fresh mass) in wild-type *Nicotiana attenuata* plants (solid line) and wound-induced JA levels in the treated leaves of the wild type (circle, ●), as-AOS (diamonds, ◆), and as-LOX (triangles, ▲) transformed lines Leaves were harvest 35 min after a standardized mechanical wounding procedure. **C)** Profile of headspace volatiles released by wild type (WT) *Nicotiana attenuata* plants, and three plants representing independently as-HPL transformed lines Volatiles were sampled and analyzed with the zNose^{TM} during the first minute after leaves had been mechanically damaged by a standardized wounding procedure

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This phenotypic variation presumably results from positional effects, resulting from the insertion of transgenes into random positions in the genome, which, in turn, differ in transcriptional activity. This phenotypic variation is enormously useful for ecological analyses, for it provides the best controls for the transformation process and allows for quantitative analysis of a trait. Positional variation can produce plants that have been through the entire transformation-regeneration process and contain the transgene, but have wild-type phenotypes, and as such represent the best controls for a phenotypic analysis. Since transposable elements can be activated during callus induction (Grandbastien, 1998), control plants used in a phenotypic analysis should have been through the entire transformation-regeneration process. Moreover, since transformation is essentially a mutagenic process, which inserts transgenes at random places in the genome and thereby potentially disrupts other genes in the process, it is important that the phenotypic analysis is conducted with multiple independently transformed lines. If a number of different lines exhibit the same phenotype, it is highly unlikely that the phenotype results from the disruption of an endogenous gene by the insertion of the transgene.

If little positional variation is found and all lines show strong phenotypic effects, ‘empty-vector’ transformants - plants transformed by the same procedure and contain the selectable marker but lack the gene of interest, its promotor and terminator - are the next best controls to use in the phenotypic analysis. Only when the phenotypic analysis includes these controls, one can be certain that the phenotype does not stem from the expression of the selectable marker, disruption caused by the insertion of the plasmids or mutations induced during the transformation-regeneration process.

While we have developed a transformation system for this ecological expression system, it is clear that the system will require additional refinements. In particular, vectors will be required to increase the frequency of 'knock-out' phenotypes and we are currently exploring the utility of constructs containing inverted repeats of the gene of interest to this end, as has been pioneered by Waterhouse and associates (Wesley et al., 2001). Second, constitute expression strongly limits the choice of genes to those that can be manipulated without deleterious consequences for plant growth or development. The development of an inducible expression system, which could be activated by ecologically-relevant external stimuli would greatly increase the types of genes that could be manipulated. Third, since *Agrobacterium*-mediated transformation can produce multiple-copy insertions and rearrangements of the T-DNA, including deletions from the ends, direct repeats, inverted repeats, and concatamerization, albeit at lower frequencies than other transformation techniques (Barcelo et al., 2001), it will be valuable to rapidly analyze the insertion processes in particular transformed lines by rescuing the inserted T-DNA from plant genomic DNA. These three refinements are currently being developed for this transformation system.

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Manuscript IV

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Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata*

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Summary

Inhibition of jasmonate (JA) signaling has been shown to decrease herbivore resistance, but the responsible mechanisms are largely unknown because insect resistance is poorly understood in most model plant systems. We characterize three members of the lipoxygenase (LOX) gene family in the native tobacco plant *Nicotiana attenuata* and manipulate, by antisense expression, a specific, wound- and herbivory-induced isoform (LOX3) involved in JA biosynthesis. In three independent lines, anti-sense expression reduced wound-induced JA accumulation but not the release of green leaf volatiles (GLVs). The impaired JA signaling reduced two herbivore-induced direct defenses, nicotine and trypsin protease inhibitors (TPI), as well as the potent indirect defense, the release of volatile terpenes that attract generalist predators to feeding herbivores. All of these defenses could be fully restored by methyl-JA (MeJA) treatment, with the exception of the increase in TPI activity, which was partially restored, suggesting the involvement of additional signals. The impaired ability to produce chemical defenses resulted in lower resistance to *Manduca sexta* attack, which could also be restored by MeJA treatment. Expression analysis using a cDNA microarray specifically designed to analyze *M. sexta*-induced gene expression in *N. attenuata,* revealed a pivotal role for LOX3-produced oxylipins in up-regulating defense genes (protease inhibitor, PI; xyloglucan endotransglucosylase/hydrolase, XTH; threonine deaminase, TD; hydroperoxide lyase, HPL), suppressing both downregulated growth genes (RUBISCO and photosystem II, PSII) and upregulated oxylipin genes (α−dioxygenase, α-DOX). By genetically manipulating signaling in a plant with a well-characterized ecology, we demonstrate that the complex phenotypic changes that mediate herbivore resistance are controlled by a specific part of the oxylipin cascade.

Keywords: jasmonates, herbivore resistance, lipoxygenase, nicotine, trypsin protease inhibitors, quantitative PCR

Introduction

Plant resistance to herbivory is mediated by a diverse set of defense traits that can be constitutively expressed or are elicited by herbivore attack. Many inducible defense responses are activated by the octadecanoid signaling cascade (Reymond and Farmer, 1998; Blee, 2002). The plant hormone jasmonic acid (JA) and its precursor 12-oxo-phytodienoic acid (OPDA), collectively called jasmonates, are induced by mechanical wounding and herbivory. In native tobacco species *Nicotiana sylvestris* (McCloud and Baldwin, 1997) and *Nicotiana attenuata* (Schittko *et al.*, 2000) and in corn (Schmelz *et al.*, 2003b), wound-induced JA accumulation is dramatically amplified by attack from host-specific herbivores via the introduction of constituents in herbivore oral secretions and regurgitant (R) into wounds (Halitschke *et al.*, 2001; Schmelz *et al.*, 2003a).

The JA biosynthetic pathway involves: regio- and stereospecific dioxygenation of linolenic acid by a lipoxygenase (LOX); formation of an epoxide by allene oxide synthase (AOS); ring formation by allene oxide cyclase (AOC); reduction by OPDA reductase (OPR), and side-chain shortening by three consecutive β-oxidation steps. Enzymes involved in the biosynthesis of jasmonates have been characterized in several plant species, and their genes have been overexpressed or silenced in transgenic plants (Schaller, 2001; Berger, 2002; Turner *et al.*, 2002). Manipulation of different enzymes in the octadecanoid cascade, such as AOS (Laudert *et al.*, 2000; Park *et al.*, 2002), AOC (Stenzel *et al.*, 2003), or a specific OPR (OPR3; Sanders *et al.*, 2000; Stinzi and Browse, 2000) by antisense or overexpression of endogenous genes; as well as with insertional mutants, has demonstrated the importance of these enzymes in JA biosynthesis. Additionally, a mutant (*fad3 fad7 fad8* triple mutant) impaired in the biosynthesis of linolenic acid, the fatty acid precursor of JA biosynthesis, has been characterized (McConn and Browse, 1996) and found to be impaired in wound-induced JA accumulation (McConn *et al.*, 1997).

Plant LOXs occur in a gene family and can be grouped into two main subfamilies (class 1 and 2) according to their primary sequence similarities (Shibata *et al.*, 1994; Feussner and Wasternack, 2002). According to this classification, the LOXs catalyzing dioxygenation of fatty acids at the 13-C position (13-LOX) are members of the type 2 class, which possess a chloroplast transit peptide. Biosynthesis of JA requires a 13-LOX to produce the initial intermediate of the cascade, 13(S)-

hydroperoxy linolenic acid. A specific lipoxygenase (AtLOX2), which is involved in JA biosynthesis, has been identified in *Arabidopsis thaliana*. Antisense expression of *AtLOX2* reduced wound-induced JA accumulation (Bell *et al.*, 1995). In tomato plants, expression of a specific chloroplast-targeted 13-*LOX* (*TomLOXD*) is rapidly and transiently induced by wounding (Heitz *et al.*, 1997), whereas other *LOX* genes are expressed only in seeds or fruits (*TomLOXA* and *TomLOXB*; Ferrie *et al.*, 1994) and are not induced by wounding (*TomLOXC*; Heitz *et al.*, 1997). Three distinct classes of LOXs have been defined in potato (Royo *et al.*, 1996): LOX1 is expressed in roots and tubers and is involved in tuber development (Royo *et al.*, 1996; Kolomiets *et al.*, 2001); LOX2 isozymes are expressed in leaves; and LOX3 is expressed in leaves and roots (Royo *et al.*, 1996). Both LOX2 and LOX3 possess putative chloroplast transit peptides, but show distinct expression patterns in response to wounding (Royo *et al.*, 1996). Potato *LOX*2 (*LOX-H1*) transcript accumulation increases steadily for 24 h after wounding, whereas transcripts of *LOX*3 (*LOX-H3*) are induced much faster and transiently with highest accumulations 30 min after wounding (Royo *et al.*, 1996). Co-suppression of *LOX-H1* reduced the release of green leaf volatiles (GLVs), C6 alcohols and aldehydes that are products of the hydroperoxide lyase (HPL) reaction, but did not influence basal and wound-induced JA levels (Leon *et al.*, 2002). Surprisingly, antisense expression of *LOX-H3* influenced neither the release of GLVs (Leon *et al.*, 2002) nor the basal and wound-induced JA accumulation (Royo *et al.*, 1999).

Three systems of transgenic plants impaired in JA biosynthesis have been used to study the octadecanoid signaling cascade in eliciting resistance to herbivory. These studies, while providing tantalizing evidence for the importance of octadenaoids in mediating plant resistance, lack either a detailed characterization of the genetic manipulations or the phenotypic consequences of the manipulation. In the first system, the resistance of *Arabidopsis* against the fungal gnat *Bradysia impatiens* has been studied in two mutants, *opr3* and *fad3 fad7 fad8* triple mutant, both of which lack wound-induced JA accumulation. However, while the *fad3 fad7 fad8* triple mutant has impaired resistance to the fungal gnat, the *opr3* mutant exhibits resistance comparable to wild type (WT) plants (McConn *et al.*, 1997; Stintzi *et al.*, 2001). Therefore, oxylipins derived from linolenic acid other than JA are likely to mediate resistance against the fungal gnat. The defense traits elicited by the unknown oxylipin have not been identified. In the second system, tomato *def1* mutants, deficient in

wound-induced JA accumulation, show lower resistance to feeding *Manduca sexta* (Howe *et al.*, 1996) and *Spodoptera exigua* (Thaler *et al.*, 2002a) larvae and spider mites (Li *et al.*, 2002). However, the *def1* mutation remains to be understood in terms of its function in JA biosynthesis. The *def1* mutation is thought to result in a biosynthetic defect between the LOX and OPR reactions (Howe *et al.*, 1996), but is not a mutation in the genes coding for either AOS and AOC catalyzing the intermediate biosynthetic steps (Li *et al.*, 2002). In a third system, the induction of protease inhibitor (PI) activity was abolished in antisense *LOX-H3* potato plants, enhancing the performance of Colorado potato beetle and beet armyworm larvae feeding on these plants. However, as no reduction in JA accumulation was observed (Royo *et al.*, 1999) the mechanism of LOX-H3-mediated herbivore resistance is uncertain. In short, although the importance of oxylipins in mediating herbivore-resistance has been demonstrated, both the signals and the resistance mechanisms remain to be characterized.

In this study, we isolated and characterized three classes of the lipoxygenase gene families in the wild tobacco plant *N. attenuata*, and analyzed their tissue-specific and wound-induced expression patterns. Antisense expression-mediated silencing of one specific isoform reduced accumulation of JA in response to wounding and application of *M. sexta* R in three independent antisense (as-*lox*) lines, but the silencing did not reduce the release of GLVs. The reduction in JA signaling capacity decreased *N. attenuata*'s resistance to herbivory by *Manduca sexta* larvae. The deficiency in resistance could be rescued by methyl-JA (MeJA) treatment. We analyzed the expression of several direct and indirect defense traits in response to *M. sexta* feeding and demonstrate reductions in induced nicotine accumulation, PI activity, and release of R-induced terpenoid volatiles, known to function as defenses against *Manduca* larvae attack (Voelckel *et al.*, 2001; Glawe *et al.*, 2003, Kessler and Baldwin, 2001). With a cDNA microarray which was specifically designed to detect *M. sexta*-induced changes in transcript accumulation in *N. attenuata*, we analyzed the expression of 240 genes in *M. sexta*-attacked as-*lox* plants in comparison to attacked WT plants. We demonstrate the reduced expression of genes involved in defense activation and altered expression of growth-related genes.

Results

Isolation and characterization of LOX *cDNAs*

A partial 1.98-kb genomic DNA fragment of *N. attenuata* lipoxygenase was used to screen cDNA libraries prepared from *N. attenuata* root and shoot tissue induced by feeding of *M. sexta* larvae (Hermsmeier *et al.*, 2001). Three of the 26 initial positive shoot cDNA clones with expected fragment sizes were sequenced. The isolated cDNAs, 2832, 2740, and 2740 bp, designated *NaLOX1a*, *NaLOX1b*, and *NaLOX1c*, respectively differed only in the polyadenylation site and two nucleotide substitutions in *NaLOX1b,* which might have resulted from the fact that the cDNA library was prepared from plants of six different *N. attenuata* populations (Winz and Baldwin, 2001). Sequence comparisons showed 81 to 82% nucleotide identity with potato lipoxygenase genes *PotLOX1* and *PotLOX2* (Kolomiets *et al.*, 1996) and tomato lipoxygenase *TomLOXA* (Ferrie *et al.*, 1994).

A second lipoxygenase gene was identified by the random sequencing of a flower cDNA library. The cDNA sequence of the 2836-bp clone, designated as *NaLOX2*, showed 84 and 83% identity with potato *LOX2* (clone H1; Royo *et al.*, 1996) and tomato lipoxygenase *TomLOXC* (Heitz *et al.*, 1997), respectively.

A third class of *LOX* genes was isolated by screening the *N. attenuata* shoot and root cDNA libraries with a probe derived from tomato lipoxygenase gene *TomLOXD* (Heitz *et al.*, 1997). Initial sequencing of four leaf and three root cDNA clones revealed high sequence similarity; only the longest root and leaf cDNA clones were sequenced completely. The two sequences were identical and showed only a 40-bp truncation in the 5' end of the clone obtained from the root cDNA library. The 3042-bp leaf cDNA clone, designated as *NaLOX3*, showed 87 and 83% nucleotide identity with potato *LOX3* (clone H3; Royo *et al.*, 1996) and tomato lipoxygenase *TomLOXD* (Heitz *et al.*, 1997), respectively.

Individual LOXs show distinct expression patterns

Specific probes for detecting individual *LOX* transcripts were designed and cross-hybridized against plasmids containing the different cDNAs (Figure 1a). Northern analysis of different tissues from *N. attenuata* plants grown in hydroponic culture (flowers, stem, leaves and roots) revealed low to undetectable constitutive expression of all three *LOX* genes. Only weak constitutive expression of *NaLOX2* was

detected in leaf tissue of untreated plants (data not shown). To investigate the potential involvement of the three enzymes in biosynthesis of wound- and R-induced JA, we analyzed the expression levels of *NaLOX1*, *NaLOX2*, and *NaLOX3* in *N. attenuata* leaves at different times after wounding and treatment of the wounds with either water or R.

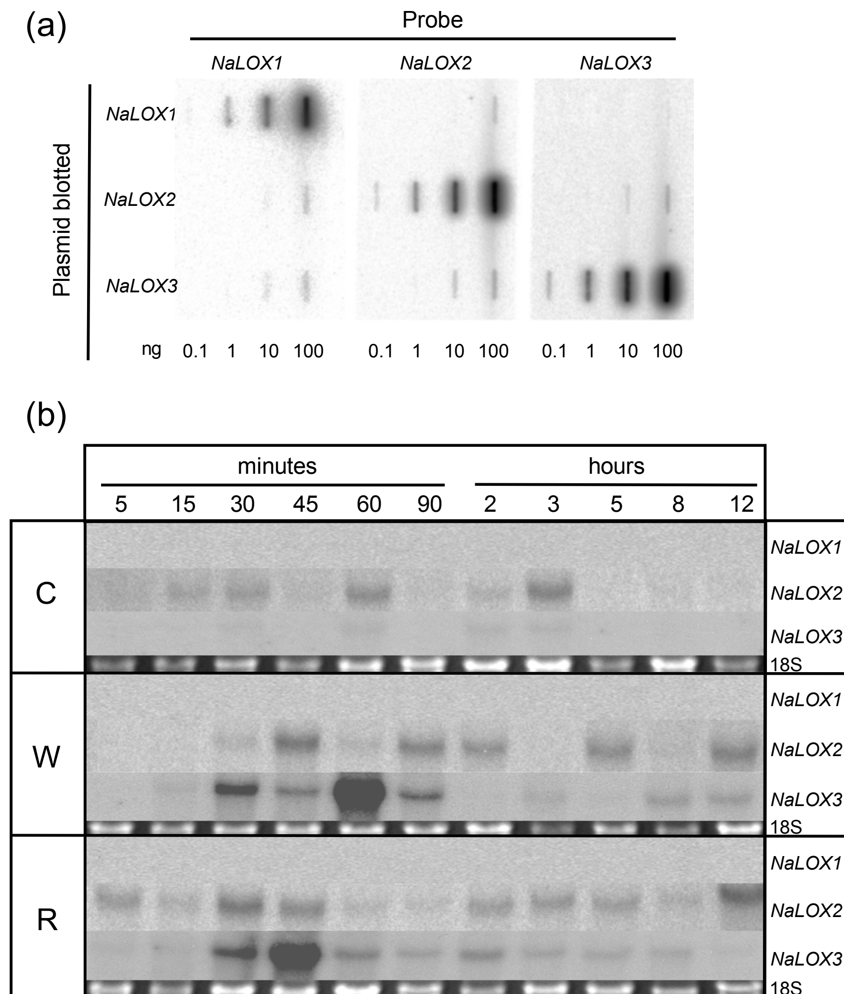

Figure 1. *NaLOX3* is rapidly and transiently wound- and herbivore-induced.
(a) Plasmids (0.1-100 ng) containing full-length cDNAs of *NaLOX1*, *NaLOX2*, and *NaLOX3* were slot-blotted and hybridized with specific probes for the individual *LOX*s.
(b) Total leaf RNA (10 µg) of individual *N. attenuata* plants harvested at the indicated times after treating single leaves by wounding and application of water (W) or *M. sexta* oral secretion and regurgitant (R) was hybridized with specific probes for *NaLOX1*, *NaLOX2*, and *NaLOX3*. Control plants (C) remained untreated. Ethidium bromide stained 18S rRNA is shown as loading control.

NaLOX1 transcripts were not detectable in leaves of unwounded control plants and were not induced by water and R treatment of mechanical wounds (Figure 1b). Northern analysis of root RNA from soil-grown plants showed clear transcript accumulation of *NaLOX1* in root tissue (data not shown). Transcript accumulation of *NaLOX2*, which was detectable in control and treated plants, did not show a clear expression pattern after water or R treatment of plant wounds (Figure 1b). In marked contrast, *NaLOX3* showed a rapid and transient increase in transcript accumulation, starting at 15 min and peaking between 45 and 60 min after induction by water or R treatment (Figure 1b). Transcript levels declined to basal level 2-3 h after the treatment (Figure 1b). These expression patterns suggest that NaLOX3 is involved in induced JA biosynthesis in *N. attenuata*, which follows a similar kinetic in response to wounding (Kahl *et al.*, 2000; Schittko *et al.*, 2000).

Antisense expression of NaLOX3 *specifically reduces elicited JA accumulation*

Transgenic plants expressing *NaLOX3* in an antisense orientation (as-*lox*) were produced by *Agrobacterium*-mediated transformation (Krügel *et al.*, 2002), and transgene incorporation was verified by Southern analysis (Figure S1). Three independently transformed lines (A363, A211, and A300) showing the largest reduction in JA accumulation in a high throughput phenotype screen (Krügel *et al.*, 2002), were further characterized. The basal JA levels of the as-*lox* lines as measured in unwounded leaves of plants from each line did not differ significantly from WT levels (Figure 2a; ANOVA, $F_{3,13}$ = 0.445, P = 0.7249). The wound-induced JA concentrations in the as-*lox* lines A363, A211, and A300, which were measured 35 minutes after mechanical wounding and water treatment were significantly reduced by 45, 54, and 65%, respectively, compared to the wound-induced WT level (Figure 2a; ANOVA, $F_{3,16}$ = 10.703, P = 0.0004, Fisher's protected least significant difference (PLSD) ≤ 0.0051). A similar reduction of 32% (A363), 22% (A211), and 50% (A300) in comparison to WT JA levels was observed in plants induced by wounding and by application of larval R (Figure 2a; ANOVA, $F_{3,14}$ = 13.062, P = 0.0002, Fisher's PLSD ≤ 0.0492), which amplified wound-induced levels in WT plants by 38%.

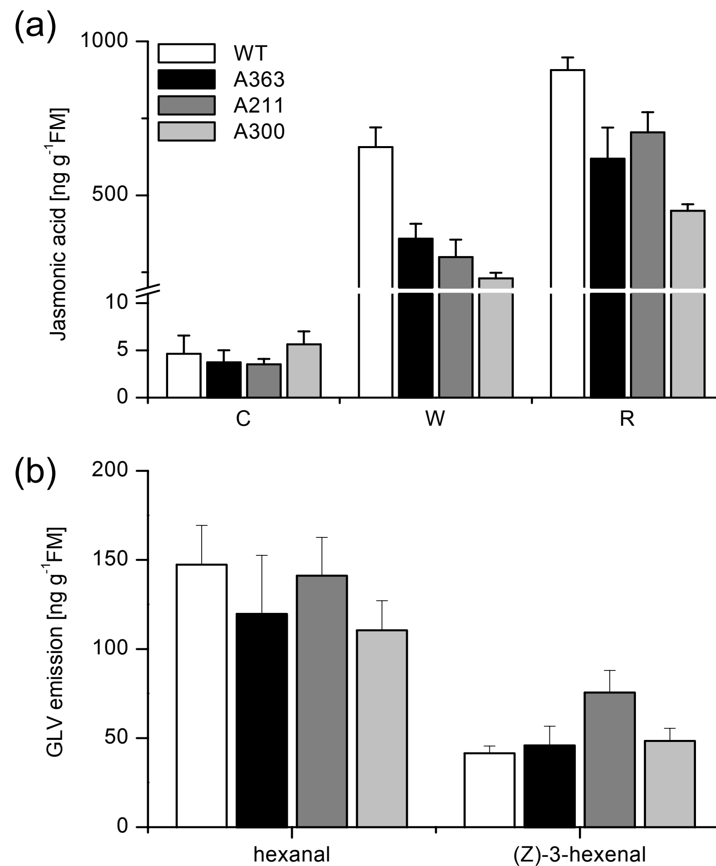

Figure 2. Silencing of *NaLOX3* specifically suppresses JA accumulation.

(a) Jasmonic acid was analyzed in single leaves of 5 replicate wild type (WT) *N. attenuata* plants and three independently transformed antisense-*lox* lines (A363, A211, A300) after wounding with a pattern wheel and immediately suppling water (W) or *M. sexta* oral secretion and regurgitant (R). Control plants (C) remained untreated. The treated leaves were harvested 35 min after treatment (time of maximum JA accumulation) for the analysis of endogenous JA levels (mean + SE).

(b) Mean (+ SE) hexanal and (*Z*)-3-hexenal release from five replicate plants in the first minute after mechanical wounding of a single leaf of the same genotypes described in (a). Unwounded plants had no detectable emissions (data not shown).

Real-time PCR analysis of *NaLOX3* expression demonstrated a reduction of wound- and R-induced *NaLOX3* transcript accumulation by 73-83% in A363 plants and 83-92% in A300 plants, respectively (Figure 3). The greater silencing efficiency of *NaLOX3* transcripts in the as-*lox* line A300 is accompanied by a larger accumulation of as-*lox* transcripts, which are not detectable in WT plants (Figure 3).

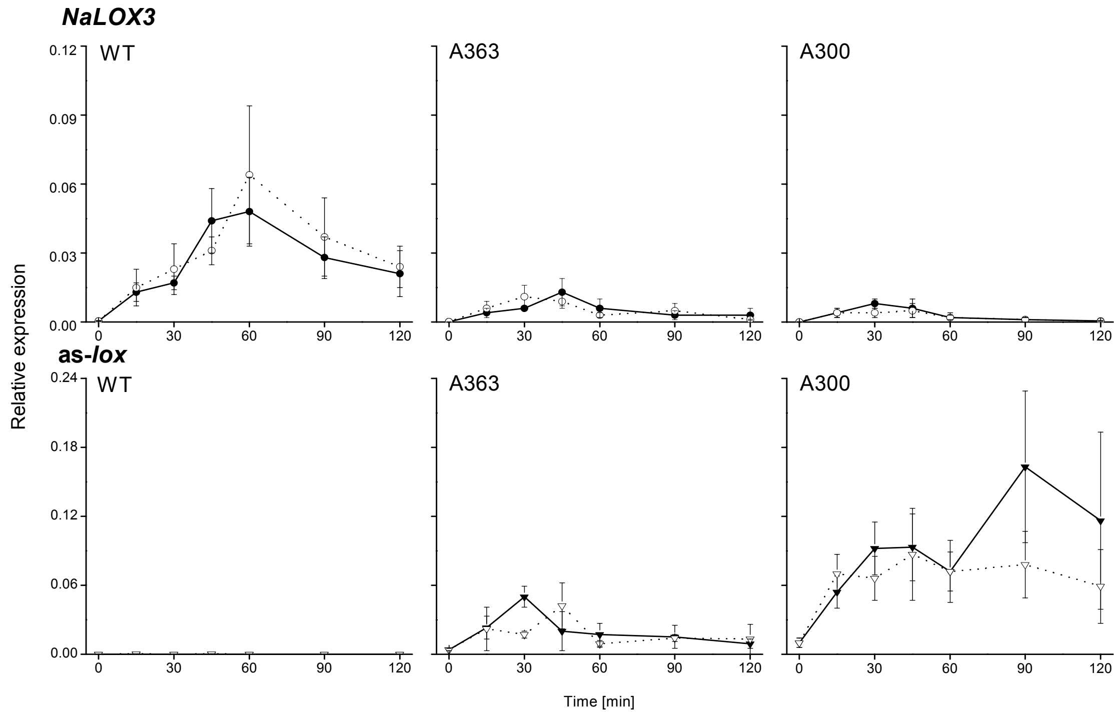

Figure 3. Antisense expression suppresses wound- and herbivore-induced *NaLOX3* transcript accumulation.

Accumulation of endogenous *NaLOX3* (circle) and as-*lox* (triangle) transcripts was analyzed by real-time PCR. cDNAs were prepared from 20 ng total leaf RNA of four replicate plants harvested at the indicated times for each of WT and as-*lox* lines (A363 and A300). At time 0, plants were elicited by wounding single leaves and applying water (open symbol, dotted line) or *M. sexta* oral secretion and regurgitant (closed symbols, solid line). Expression is shown as mean value (±SE) of four independent experiments in arbitrary units resulting from a calibration with a 10x dilution series of cDNAs prepared from RNA samples containing *NaLOX3* and as-*lox* transcripts.

As 13-LOX catalyzes the formation of fatty acid hydroperoxides and thereby supplies the substrate for an additional downstream and potentially competitive reaction, namely the formation of GLVs, we analyzed the emissions of hexanal and (*Z*)-3-hexenal, which are released exclusively after wounding and not from undamaged control plants (data not shown). Emissions of hexanal (Figure 2b; ANOVA, $F_{3,16}$ = 0.557, P = 0.6511) and (*Z*)-3-hexenal (Figure 2b; ANOVA, $F_{3,16}$ = 2.670, P = 0.0826) from wounded as-*lox* lines did not differ from levels released by

WT plants. These findings demonstrate that NaLOX3 supplies the substrates for induced JA biosynthesis but not for the release of GLVs.

Antisense expression of NaLOX3 *reduces resistance to* M. sexta *attack*

Manduca sexta larvae feeding on as-*lox* lines A363 and 300 gained more mass than did caterpillars feeding on WT plants by 72% and 63%, respectively (Figure 4a; ANOVA, $F_{3,20}$ = 3.306, P = 0.0412, Fisher's PLSD ≤ 0.0290). Larvae feeding on plants of line A211 did not differ significantly in their mass from larvae feeding on WT plants (Figure 4a; Fisher's PLSD = 0.6350). Treatment with MeJA recovered the resistance of as-*lox* plants and diminished the increase in mass gain of larvae feeding on as-*lox* plants (Figure 4a; ANOVA, $F_{3,19}$ = 2.505, P = 0.0900). Larvae feeding on plants of line A300 consumed significantly more leaf tissue than did larvae feeding on WT plants (Figure 4b; ANOVA, $F_{3,21}$ = 4.261, P = 0.0169, Fisher's PLSD ≤ 0.0185), whereas no significant difference in leaf area consumption was observed on plants of as-*lox* lines A363 and A211 in comparison to WT plants (Figure 4b; Fisher's PLSDs ≥ 0.2613). Leaf area consumption on MeJA-treated plants of all three as-*lox* lines did not show significant differences in comparison to MeJA-treated WT plants (Figure 4b; ANOVA, $F_{3,23}$ = 0.880, P = 0.4664). The concentrations of two secondary metabolites known to function as direct defenses were analyzed in plants damaged by continuous larval feeding in order to understand differences in larval mass gain.

Antisense expression of NaLOX3 *reduces elicitation of direct defenses*

The *Manduca*-induced accumulation of nicotine was significantly reduced in plants of all three as-*lox* lines by 33-42% (Figure 4c; ANOVA, $F_{3,22}$ = 7.125, P = 0.0016, Fisher's PLSD ≤ 0.0025) compared to WT plants. Treatment with MeJA restored nicotine induction, and MeJA-treated WT and as-*lox* plants accumulated similar levels of nicotine (Figure 4c; ANOVA, $F_{3,22}$ = 1.536, P = 0.2333). In contrast, the reduction in *Manduca*-induced trypsin PI (TPI) activity was significant only in as-*lox* line A211 (Figure 4d; ANOVA, $F_{3,22}$ = 3.511, P = 0.0331, Fisher's PLSD = 0.0165) and not in as-*lox* lines A363 and A300 (Figure 4d; Fisher's PLSD ≥ 0.0772). However, treatment with MeJA elicited significantly higher TPI activity in WT plants than in as-*lox* plants A363 and A300 (Figure 4d; ANOVA, $F_{3,21}$ = 4.935, P = 0.0095, Fisher's PLSD ≤ 0.0153). These results demonstrate the involvement of endogenous

JA biosynthesis in the elicitation of both direct defense responses but with distinct elicitation signals or mechanisms.

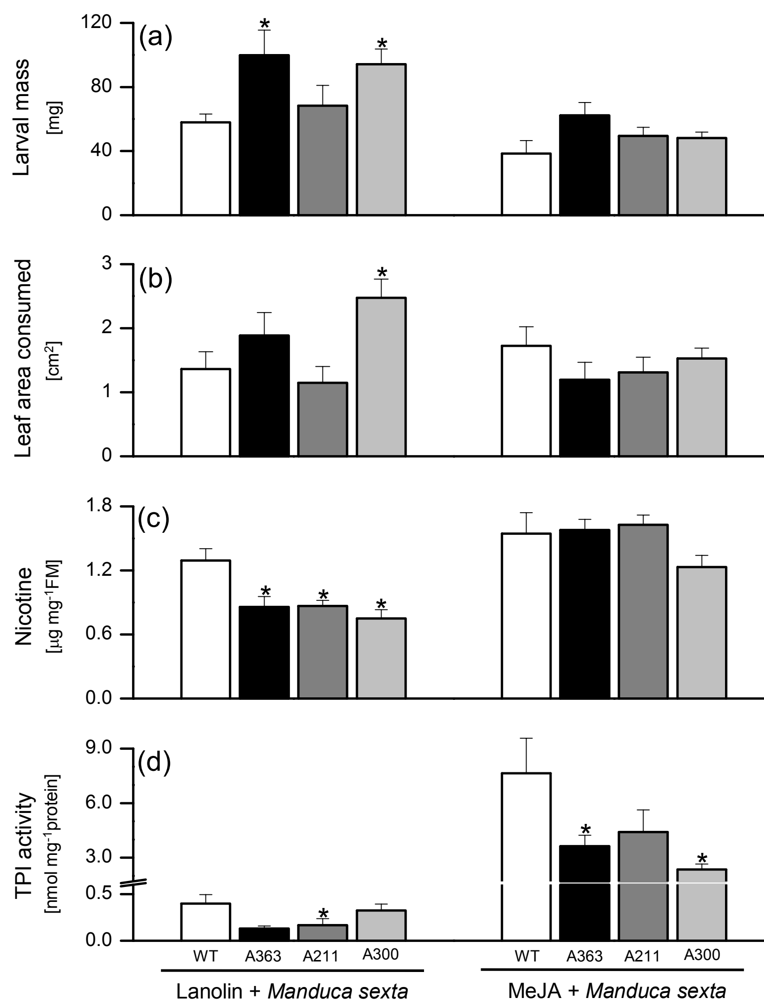

Figure 4. Silencing of *NaLOX3* reduces herbivore resistance and direct defense activation. The second and third fully expanded leaf of 8 replicate plants of wild type (WT) *N. attenuata* plants and three independently transformed antisense-*lox* lines (A363, A211, and A300) were treated with lanolin paste (Lanolin) or 224 µg MeJA in 20 µl of lanolin paste (MeJA), and a freshly eclosed *M. sexta* larva was placed on the second leaf. Larvae were allowed to feed for 7 days after which larval mass was recorded and plants were harvested to analyze defense metabolites and leaf area consumed.

(a) Mean (+ SE) mass of individual *M. sexta* larvae was recorded after 7 days of feeding on separate plants.

(b) Mean (+ SE) square centimeter leaf area consumed was calculated from image analysis of digitized pictures of leaves damaged by *M. sexta* feeding for 7 days on the replicate plants from each genotype.

(c) Mean (+ SE) nicotine concentration was analyzed in the first fully developed leaf, which remained unattacked, of the replicate plants from each genotype.
(d) Mean (+ SE) TPI activity was analyzed in the damaged third fully developed leaf of the replicate plants from each genotype. Statistical comparisons (ANOVA) were performed within treatment groups, and stars represent significant differences in the as-*lox* lines compared to WT plants (Fisher's PLSDs $P < 0.05$).

Antisense expression of NaLOX3 *inhibits elicitation of indirect defenses*

To investigate the involvement of the octadecanoid signaling cascade in elicitation of the volatile release, we analyzed the wound- and R-induced *cis*-α-bergamotene emission in WT and the as-*lox* plants with the lowest R-induced JA accumulation (A300 and A363). Wounding alone did not induce the release of *cis*-α-bergamotene in WT and as-*lox* plants (Table 1; *t*-test, $P > 0.1384$) in comparison to unwounded control plants, whereas the addition of R to the wounded leaf elicited three- to eightfold increases in the emissions of *cis*-α-bergamotene in WT plants (Table 1; *t*-test, $P \leq 0.0033$) but not in as-*lox* plants (Table 1; *t*-test, $P \geq 0.4068$).

Table 1. Inhibition of terpenoid volatile emissions in as-*lox* plants.
Mean (± SE) *cis*-α-bergamotene release from individual wild type (WT) and as-*lox* (A363, A300) *N. attenuata* plants 24 h after treating the second fully expanded leaf by wounding with a pattern wheel and immediately supplying water (W; n = 6) or *M. sexta* oral secretion and regurgitant (R; n = 6) or applying 224 µg MeJA in 20 µl lanolin (MeJA; n = 4). Control plants (C; n = 4) remained untreated. Significant differences compared to the volatile release of untreated control plants (unpaired *t*-tests; $P < 0.005$) are shown in bold numbers.

	cis-α-Bergamotene release (ng h^{-1} per plant)			
Line	C	W	R	MeJA
WT	1.98±0.92	2.22±0.44	**15.53±2.43**	**33.55±3.70**
A363	1.84±0.46	0.77±0.21	2.92±0.86	**13.13±6.10**
WT	5.60±1.03	5.23±0.52	**14.68±1.95**	**42.49±13.80**
A300	5.00±1.14	5.50±1.23	4.22±1.50	**55.48±5.23**

MeJA elicitation induced 7- to 17-fold increases in emissions of *cis*-α-bergamotene in all WT and as-*lox* plants (Table 1; *t*-test, $P \leq 0.0038$), demonstrating that terpenoid biosynthesis in all plants was unimpaired and that the inability of as-*lox* plants to release volatiles after R-elicitation results from a defect in JA signaling.

Antisense expression of NaLOX3 *alters JA-dependent gene expression*

We compared transcript accumulation in response to herbivore feeding in as-*lox* plants (A300 and A363) and WT plants to identify JA-dependent transcriptional responses by cDNA microarray analysis. A complete list of the spotted genes can be found in Table S1. Presented are mean values of the two expression ratios (ERs) calculated from two PCR fragments (ER1 and ER2), spotted in quadruplicate on the array for each analyzed gene (Figure 5).

To evaluate the criteria applied to detect significant changes in transcript accumulation, we analyzed the reproducibility of results obtained from repeated hybridizations of A300- against WT-derived cDNAs (Figure 5). Two independent hybridization experiments identified identical expression patterns for 201 genes (84%). Moreover, for an additional 11% (26) of the genes, expression patterns were significant (meeting both criteria) in one of the experiments while failing for only one criterion in the second hybridization. The remaining 5% (13) of the genes showed a significant response in one experiment but not in the other.

The *Manduca*-induced transcript accumulation was significantly increased for 30 genes and significantly decreased for 24 genes in both as-*lox* lines. Additionally, 31 genes showed significantly increased (23 genes) or decreased (8 genes) transcript accumulation in one of the as-*lox* lines but a non-significant trend in the second analyzed line. We discuss genes with known or putative function that showed altered expression in response to *M. sexta* feeding in the as-*lox* lines (Figure 5). ERs of all genes including genes without significant similarities to known genes in the databases are available in Table S2.

As expected, the *NaLOX3* probe on the microarray, which was designed from the region of *NaLOX3* that was transformed into the as-*lox* plants, detected higher *NaLOX3* transcript accumulation in the as-*lox* lines. This high ER was because of the constitutive expression of the transgene (see also Figure 3) under the control of the 35S promoter of cauliflower mosaic virus (CaMV). AOS transcripts, which are induced by *M. sexta* feeding in *N. attenuata* WT plants (Ziegler *et al.*, 2001), showed

mixed responses. One allele (Ziegler *et al.*, 2001) did not show as-*lox*-dependent regulation (data not shown), while the second (Hui *et al.*, 2003) exhibited a slight upregulation (Figure 5). *Manduca*-induced accumulation of HPL transcripts was strongly reduced in both as-*lox* lines, whereas transcript accumulation of α-dioxygenase (α-DOX) was strongly amplified in as-*lox* plants in response to *M. sexta* feeding (Figure 5), demonstrating differential JA-mediated modulation within the oxylipin biosynthetic pathways.

Many defense-related genes that include threonine deaminase (TD), xyloglucan endotransglucosylase/hydrolase (XTH), and TPI are strongly induced by *M. sexta* feeding (Glawe *et al.*, 2003; Halitschke *et al.*, 2003; Hui *et al.*, 2003). As expected, transcripts of these genes were strongly suppressed in as-*lox* plants (Figure 5), demonstrating the importance of a LOX3-derived oxylipin for their elicitation after herbivore attack. While not strictly associated with defense, two additional genes, a major intrinsic protein (MIP2) and a RNA-binding glycine-rich protein (RGP-1a), which have been found to be up-regulated by *M. sexta* feeding (Hui *et al.*, 2003), were both downregulated in as-*lox* plants, suggesting a similar dependence on a LOX3-mediated oxylipin for their elicitation.

In addition to the strong upregulation of genes thought to be involved in herbivore resistance, attack from *M. sexta* larvae and elicitation by MeJA also results in a dramatic downregulation of photosynthesis- and growth-related genes in *N. attenuata* (Hermsmeier *et al.*, 2001; Halitschke *et al.*, 2003; Hui *et al.*, 2003). The *Manduca*-induced downregulation of two photosynthetic genes (light-harvesting complex protein LHCII and photosystem II (PSII) oxygen evolving complex polypeptide) was suppressed (ER > 1; Figure 5) in as-*lox* plants, suggesting LOX3-dependent downregulation in WT plants. Surprisingly, additional three genes involved in photosynthesis (RUBISCO small subunit (SSU), and two peptides of PSII), which are also downregulated by herbivore attack in WT plants (Hermsmeier *et al.*, 2001; Halitschke *et al.*, 2003; Hui *et al.*, 2003), showed amplified downregulation in *M. sexta*-attacked as-*lox* plants (ER < 1; Figure 5), suggesting that for these genes, a LOX3-derived oxylipin attenuates an even larger downregulation in WT plants. A similar response was found for three genes that are thought to be involved in the metabolic re-configuration elicited by herbivore attack, and have been found to be upregulated after *M. sexta* attack: sulfite reductase, α-amylase, and ferridoxin-

dependent (FD) glutamate synthase. These genes were all upregulated in as-*lox* plants and hence a LOX3-derived oxylipin likely suppresses upregulation in WT plants.

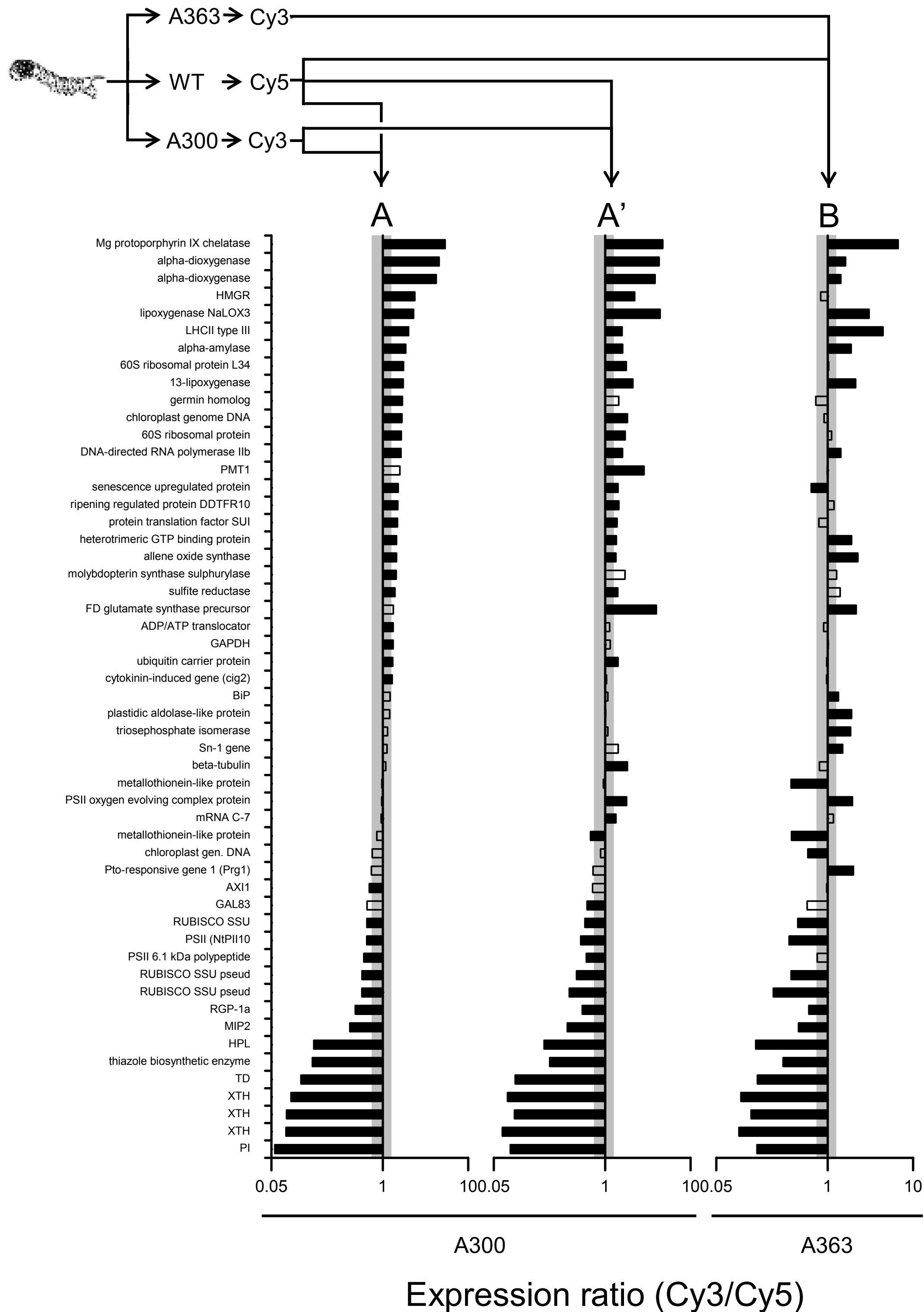

Figure 5. Silencing of *NaLOX3* suppresses herbivore-induced transcriptional reorganization.

A cDNA microarray designed to analyze changes in gene expression (up- and down-regulation) elicited by *M. sexta* attack was used to identify genes with altered expression in antisense-*lox* lines. Fluorescencently labeled cDNA derived from *M. sexta* attacked leaves of as-*lox* plants A300 and A363 (Cy3) were hybridized against cDNA derived from similarly attacked WT *N. attenuata* plants (Cy5, see experimental scheme and Experimental procedures for ER criteria). Mean ER of genes that fulfilled both criteria for significant regulation for both ER1 and ER2 individually are depicted as closed bars. Open bars represent mean ERs of genes for which the significance criteria were not fulfilled in both experiments. The arbitrary thresholds for up- or downregulation (1.25 and 0.75, respectively), one of the criteria for significant expression, are shown as gray shaded areas. The analysis of as-*lox* line A300 cDNA was repeated in an independent hybridization experiment to determine experimental variation between microarray experiments.

Discussion

We identified three different lipoxygenases in *N. attenuata,* which we designated as NaLOX1, NaLOX2, and NaLOX3 to correspond to the potato LOX classification (Royo *et al.*, 1996). The sequences and expression patterns of the identified enzymes strongly resemble previously described LOXs in other solanaceous plants. NaLOX1, which is expressed only in root tissue and most likely possesses 9-lipoxygenase activity, is unlikely to be involved in wound-induced production of JA. While both NaLOX2 and NaLOX3 putatively possess 13-lipoxygenase activity, the inducibility and expression kinetic of *NaLOX3* (Figures 1 and 3) suggested that it is involved in JA biosynthesis. A function in JA biosynthesis and signal transduction has been suggested for the potato (*LOX-H3*; Royo *et al.*, 1996) and tomato (*TomLOXD*; Heitz *et al.*, 1997) homologs of *NaLOX3*. Antisense expression of *NaLOX3* reduced the wound- and R-induced transcript accumulation of *NaLOX3* by up to 92% (Figure 3) and JA levels in the three as-*lox* lines by as much as 65% (Figure 2a). These results clearly demonstrate that JA biosynthesis in *N. attenuata* is mediated by NaLOX3. In contrast, antisense expression of potato homolog (*LOX-H3*) in potato did not result in reduced accumulation of wound-induced JA levels (Royo *et al.*, 1999).

While antisense expression of *NaLOX3* reduced the wound- and herbivore-elicited increases in JA, no effects on the wound-induced release of GLVs (Figure 2b)

were found. This demonstrates that NaLOX3 specifically supplies fatty acid hydroperoxide substrates to the octadecanoid pathway but not to the HPL reaction. In potato, LOX-H1, a homolog of NaLOX2 characterized in this study, supplies the substrate for the HPL reaction required for the release of GLVs, but does not supply the hydroperoxide to the octadecanoid pathway (Leon *et al.*, 2002). While the wound-induced release of GLVs was unaffected by antisense expression of *NaLOX3,* the normal wound-induced levels of HPL transcripts are clearly reduced in as-*lox* plants (Figure 5). The apparent disconnect between transcript accumulation and enzyme activity may reflect the high constitutive HPL activity detected in *N. attenuata* leaves (Ziegler *et al.*, 2001).

Antisense expression of *NaLOX3* decreased the plant resistance and increased the performance *M. sexta* larvae (Figure 4a), which in turn, was correlated with reduced expression of two direct defenses: nicotine accumulation and TPI activity. In previous research, inhibition of both of these direct defenses individually increased the performance of *M. sexta* larvae (Voelckel *et al.*, 2001; Glawe *et al.*, 2003). Here, we find that reductions in nicotine accumulation correlate more strongly with caterpillar performance than do the reductions in TPI activity (Figure 4c,d). Our laboratory study measured only the effects of direct defenses and excluded indirect defenses that possibly enhance the defensive function of particular direct defenses. Under natural conditions, the defensive function of TPIs that slow the growth of herbivores and keep them for longer in smaller life stages that are more vulnerable to predators, may be much greater when coordinately expressed with indirect defenses, such as the release of volatiles that attract predators (Kessler and Baldwin, 2001; Glawe *et al.*, 2003).

A LOX3-derived oxylipin was demonstrated to elicit numerous transcriptional and secondary metabolite responses in *N. attenuata*. The induction of resistance responses is reduced in as-*lox* plants despite the larger amount of damage caused by *M. sexta* feeding, which positively correlates with JA accumulation and defense activation in WT plants (Baldwin *et al.*, 1997; Halitschke *et al.*, 2000). The observation that deficiencies in both induced nicotine accumulation (Figure 4c) and *cis*-α-bergamotene emissions (Table 1) in as-*lox* plants could be fully restored by MeJA treatment is consistent with an important role for JA in eliciting these defensive responses (Halitschke *et al.*, 2000; Winz and Baldwin, 2001). In WT plants, *M. sexta* attack and MeJA treatment elicit dramatic increases in defense-related transcripts and

decreases in transcripts of the light-harvesting complex protein and oxygen-evolving complex polypeptide of PSII (Hermsmeier *et al.*, 2001; Hui *et al.*, 2003). In *M. sexta*-attacked as-*lox* plants, the defense-related transcripts, PI, TD, XTH, and HPL were decreased, while the transcripts of the two photosynthetic genes were increased, demonstrating that an oxylipin produced via the LOX3-mediated octadecanoid pathway regulates these genes.

However, the involvement of signals in addition to LOX3-derived oxylipins is implicated in the elicitation of other herbivore-induced responses in *N. attenuata*. For example, TPI activity was not fully recovered by MeJA treatment (Figure 4d), suggesting the involvement of an additional herbivore-induced signal in TPI activation. Possible signals could be ethylene, which is induced by *M. sexta* feeding (Kahl *et al.*, 2000) and has been shown to synergize PI elicitation in tomato (O'Donnell *et al.*, 1996), or intermediates in the octadecanoid cascade upstream of JA, such as OPDA, which has been shown to elicit the production of numerous secondary metabolites (Blee, 2002).

In addition to the strong evidence for a LOX3-dependent oxylipin in up-regulating several defense responses and downregulating some photosynthetic genes, our results also demonstrate an antagonistic effect of the LOX3-dependent signal on the activity of other signaling pathways. For example, an additional branch of the complex oxylipin signaling network in plants is mediated by α-oxygenation of fatty acids (Hamberg *et al.*, 1999). α-DOX transcripts are known to increase in response to wounding and to be further amplified when R is added to mechanical wounds in *N. attenuata* (Schittko *et al.*, 2001). Here, we found the *Manduca*-induced α-DOX transcript levels to be amplified in as-*lox* plants in comparison to WT plants (Figure 5), suggesting that a LOX3-mediated oxylipin normally suppresses the amplification process. Pathogen attack is known to elicit α-DOX (de Leon *et al.*, 2002), and signal cross-talk between pathogen- and herbivore-induced responses (Reymond and Farmer, 1998) may be involved in the α-DOX elicitation. In addition to suppressing amplification of α-DOX transcripts, LOX3-derived oxylipins suppress the down-regulation of a set of photosynthetic genes in response to *M. sexta* feeding and MeJA treatment in WT plants (Hermsmeier *et al.*, 2001; Hui *et al.*, 2003). In as-*lox* plants, the transcript accumulation of RUBISCO SSU and two enzymes of PSII were further reduced in response to *M. sexta* feeding (Figure 5). In contrast to the photosynthetic

genes that showed strong JA-dependent downregulation, the downregulation of these genes is negatively modulated by the oxylipins of the LOX3-dependent octadecanoid cascade, again suggesting cross-talk between JA-dependent and JA-independent signaling cascades.

The traits responsible for herbivore resistance have long been studied by ecologists and are known to involve complex adaptations that include direct and indirect defenses, as well as tolerance responses (Karban and Baldwin, 1997). The majority of molecular work on plant-herbivore resistance has been conducted in model plant systems in which a detailed understanding of the phenotypic traits responsible for herbivore resistance is lacking or only recently being examined (Thaler *et al.*, 2002). In this study, we generated transgenic plants impaired in a JA-biosynthetic enzyme with reduced wound- and R-induced JA accumulation and reduced resistance against herbivore attack. Because these transformations were conducted in a species in which the traits responsible for herbivore resistance have been thoroughly characterized (Baldwin, 2001), the transformants provided valuable insights into the complex signaling processes involved in activating induced herbivore resistance. In particular, the laboratory-based experiments presented here have established causal associations among a signal cascade, particular direct defenses, and herbivore resistance. We anticipate that by examining herbivore resistance of these plants in the complex habitats of their natural environment, we will eventually understand how direct and indirect defenses function together to provide herbivore resistance.

Experimental procedures

Plant material

Wild-type *N. attenuata* plants were grown from seeds of a field collection from a native population (DI Ranch, Santa Clara, UT, USA) after subsequent selfing for 11 generations. Seeds were germinated as described in Krügel *et al.* (2002). After 10 days seedlings were transferred into potting soil and grown for 2-3 weeks under 16 h light (28ºC)/8 h dark (24ºC). Two- to three-week-old rosette stage plants were used in all experiments.

Isolation of lipoxygenase genes

A genomic DNA fragment (L7a2) of *N. attenuata* LOX gene was synthesized by PCR with primers derived from highly conserved regions of tomato *TomLOXA* and *TomLOXB* (Ferrie *et al.*, 1994) and potato *LOX1* (Royo *et al.*, 1996) genes, cloned into the pCR®2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), and sequenced. An exon-specific fragment of L7a2 was PCR-amplified and served as a probe for a cDNA library screen. A second probe was derived from tomato *TomLOXD* (Heitz *et al.*, 1997) by PCR.

Two hundred thousand plaque-forming units (pfu) of cDNA libraries (λZAP II; Stratagene, La Jolla, CA, USA) prepared from root (Winz and Baldwin, 2001) and shoot (Hermsmeier *et al.*, 2001) mRNA of *N. attenuata* plants exposed to *M. sexta* feeding for 24 h were plated, blotted and screened according to the manufacturer's instruction. The PCR-derived probes described above were labeled with ^{32}P using a random prime labeling kit (RediPrime II; Amersham-Pharmacia, Little Chalfont, UK) and purified on G50 columns (Amersham-Pharmacia). Blots were washed four times with 2x SSC, 0.1% SDS at 65°C for 30 min after hybridization at 65°C and analyzed by autoradiography. Initial positive clones were analyzed for full length by PCR, and selected plaque-pure clones were excised *in vivo* according to the λZAP II protocol (Stratagene), and sequenced on an ABI310 sequencer using the Big Dye terminator kit (Applied Biosystems, Darmstadt, Germany). Additionally, 96 flower cDNA containing plasmids were sequenced with plasmid-specific primers after *in vivo* excision from a cDNA library prepared by directional cloning of poly(T)-primed flower cDNA into the *Eco*RI/*Xho*I site of the Uni-ZAP XR vector (λZAP II; Stratagene). Two cDNA clones with similarities to known *LOX* sequences were sequenced completely.

Generation and characterization of transgenic plants

The *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure and the transformation vector pNATLOX1 are described by Krügel *et al.* (2002). Transformation was confirmed by PCR and resistance screening of potentially transformed plants and progeny of verified transformants was screened for the desired phenotype, namely reduced wound-induced JA accumulation in a high throughput screen (Krügel *et al.*, 2002). Progeny of homozygous plants were selected by nourseothricin (NTC) resistance screening and used for further experiments.

Nucleic acid analysis

Extraction of total RNA and Northern blot analysis was performed as described by Winz and Baldwin (2001). Probes for *NaLOX1*, *NaLOX2*, and *NaLOX3* were synthesized by PCR with gene specific primers and labeled with ^{32}P using a random prime labeling kit (RediPrime II; Amersham-Pharmacia). Blots were washed after overnight hybridization at 42°C three times with 2x SSPE and one time with 2x SSPE/2% SDS at 42°C for 30 min and analyzed on a phosphoimager (model FLA-3000; Fuji Photo Film Co. Ltd., Tokyo, Japan). The specificity of the designed probes was analyzed by slot-blotting (model PR 648; Hoefer Scientific Instruments, San Francisco, CA, USA) a dilution series (0.1, 1, 10, and 100 ng) of the plasmids containing the full-length cDNA of *NaLOX1a*, *NaLOX2*, and *NaLOX3* respectively, onto a nylon membrane (GeneScreen plus; NEN, Boston, MA, USA) according to the manufacturer's protocols, and hybridization with the radioactively labeled probes. Additionally, *NaLOX3* and as-*lox* transcript accumulation was analyzed by real time PCR (ABI PRISM™ 7000; Applied Biosystems, Darmstadt, Germany). Total RNA was extracted as described above and cDNA was prepared from 20 ng RNA with MultiScribe™ reverse transcriptase (Applied Biosystems), and amplified using the qPCR™ core reagent kit (Eurogentec, Seraing, Belgium) and gene specific primers and probes (Figure S2).

Analysis of M. sexta *performance*

Freshly eclosed *M. sexta* larvae (North Carolina State University, Raleigh, NC, USA) were placed on the second fully developed leaf (position +2) of eight replicate plants from each analyzed line. An additional eight plants of each line received 1 μmol MeJA to the second (+2) and third (+3) fully developed leaves prior to the larva being placed on the former. Larvae were allowed to feed on the plant for 7 days after which larval mass was recorded. Leaf area consumed was calculated from digital pictures of the damaged leaf.

Analysis of direct defense traits

Samples of the harvested +1 leaves were analyzed by HPLC as described in (Keinanen *et al.*, 2001) with the following modification of the extraction procedure: approximately 100 mg frozen tissue was homogenized in one mL extraction buffer utilizing the FastPrep® extraction system (Savant Instruments, Holbrook, NY, USA).

Samples were homogenized in FastPrep® tubes containing 900 mg lysing matrix (BIO 101, Vista, CA, USA) by reciprocating shaking at 6 m sec^{-1} for 45 seconds.

Trypsin protease inhibitor activity was analyzed by radial diffusion activity assay in the harvested +3 leaves as described by van Dam *et al.* (2001).

Analysis of JA accumulation and GLV release

Five replicate plants of each genotype were treated by wounding the second fully expanded leaf with a pattern wheel and immediately applying 20 µL water or 20 µL *M. sexta* oral secretion and regurgitant, diluted 1:1 (v/v) with water to the puncture wounds. Control plants received no treatment. Leaves were harvested 35 min after elicitation, and endogenous JA levels were analyzed by gas chromatography-mass spectrometry (GC-MS) with a ^{13}C-labeled internal standard as described by Krügel *et al.* (2002).

GLV emissions were analyzed from individual leaves of five replicate plants per genotype before and in the first minute after wounding with a pattern wheel with a portable gas analyzer (zNose™; EST, Newbury Park, CA, USA; Krügel *et al.,* 2002). Released amounts of hexanal and hexenal were calculated by calibration curves and normalized to the fresh mass of the analyzed leaf. The zNose™ instrument was calibrated with gas-phase samples using a calibration system (model 3100; EST). Absolute amounts (0.2 to 200 ng) of hexanal and (*E*)-2-hexenal in 0.2 µL methanol were vaporized and analyzed. Linearity of response was demonstrated by regression analysis (correlation coefficient of 0.9957 and 0.9914 for hexanal and (*E*)-2-hexenal, respectively). The chromatographic separation of (*Z*)-3-hexenal and (*E*)-2-hexenal did not provide sufficient resolution to definitely identify the released isomer. Therefore, wound-induced volatiles were analyzed by proton-transfer reaction mass spectrometry (PTR-MS; Ionicon Analytik, Innsbruck, Austria) to identify the released hexenal isomer. Excised leaves were wounded as described above and placed into a 100-mL glass chamber. The chamber was flushed with a stream of purified air (200 mL min^{-1}), of which a portion (15 mL min^{-1}) was introduced continuously into the PTR-MS. Fragmentation pattern of the headspace sample of freshly wounded leaves from WT plants was dominated by the mass ion 81 and showed low abundance of mass ions 57 and 99. This fragmentation pattern is identical to the fragmentation pattern of (*Z*)-3-hexenal and does not match the fragmentation pattern of (*E*)-2-hexenal, which, in turn, is dominated by the mass ion 55 (Fall *et al.*, 1999). This fragmentation was

confirmed by the analysis of synthetic standards (Bedoukian, Danbury, CT, USA) and clearly identified the plant-released hexenal as the (*Z*)-3-isomer.

Analysis of terpenoid volatile release

We analyzed volatile emission of WT and as-*lox* transformed plants from the A300 and A363 lines. Plants (four to six replicate plants per treatment) were treated by wounding the second fully expanded leaf with a pattern wheel and immediately supplying 20 µL water (W; n = 6) or 20 µL *M. sexta* oral secretion and regurgitant, diluted 1:1 (v:v) with water (R; n = 6) or applying 1 µmol MeJA in 20 µL lanolin (MeJA; n = 4) to undamaged leaves. Control plants (C; n = 4) remained untreated. Plants were enclosed in open-top volatile collection chambers 24 h after elicitation, and volatiles were collected for 8 h and analyzed by GC-MS as described by Halitschke *et al.* (2000).

cDNA microarray analysis

Total RNA was extracted from pooled +2 leaves of the replicate plants of each line used in the *M. sexta* performance experiment described above. Isolation of mRNA and cDNA synthesis was performed as described by Halitschke *et al.* (2003). cDNA derived from WT plants was labeled with Cy5 and cDNA of as-*lox* lines was labeled with Cy3 fluorescent dye (Halitschke *et al.*, 2003). The microarray, described extensively in Halitschke *et al.* (2003), contains PCR fragments of 240 *N. attenuata* genes identified by differential display analysis of *M. sexta*-induced gene expression (Hermsmeier *et al.*, 2001; Hui *et al.*, 2003, Halitschke, 2003 #9). The criteria for detection of up- and downregulated expression are as follows. The quadruplicate spotting of each PCR fragment allowed a statistical analysis of the ERs. Therefore, log-transformed ER were analyzed for significant differences from a hypothesized mean of ‘0’, corresponding to an untransformed ER of ‘1’, by one sample *t*-test. A gene was considered to be up- or downregulated if the following two criteria were fulfilled for the ER of both spotted PCR fragments (ER1 and ER2): (i) both individual ER were significantly different from ‘1’ (one sample *t*-test, $P \leq 0.05$) and (ii) both individual ERs were equal to or exceeded an arbitrary threshold of 0.75 or 1.25, representing 25% down- or upregulation, respectively.

Availability of materials

Upon request, all novel material described in this publication will be made available in a timely manner for non-commercial research purposes. Sequence data were submitted to GenBank under accession numbers AY254345 (*NaLOX1a*), AY254346 (*NaLOX1b*), AY254347 (*NaLOX1c*), AY254348 (*NaLOX2*), and AY254349 (*NaLOX3*).

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Supplementary Material

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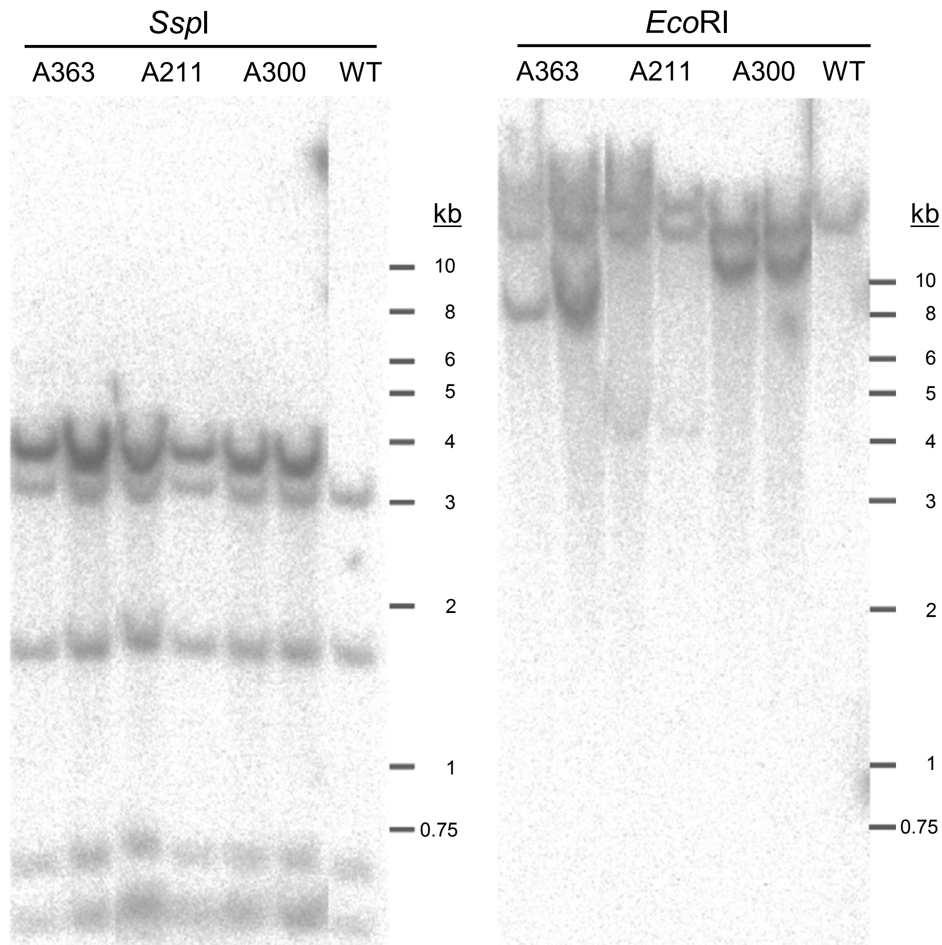

Figure S1. Transgene insertion in plants of three independently transformed antisense-*lox* lines.

Genomic DNA (10 µg) from two individual plants of each as-*lox* line (A363, A211, A300) and WT *Nicotiana attenuata* plants was digested with *Ssp*I or *Eco*RI, respectively, and blotted onto nylon membranes (Winz and Baldwin, 2001). The blots were hybridized with a PCR fragment of the region of *NaLOX3* used for the antisense construct. The digestion with *Ssp*I, having a recognition site inside the *NaLOX3* coding region, and *Eco*RI (without a recognition site in *NaLOX3*), indicate a single copy of the transgene in as-*lox* lines A211 and A300 and potentially two insertions in the genome of A363, in addition to the endogenous *NaLOX3* gene, which was also detected in the WT.

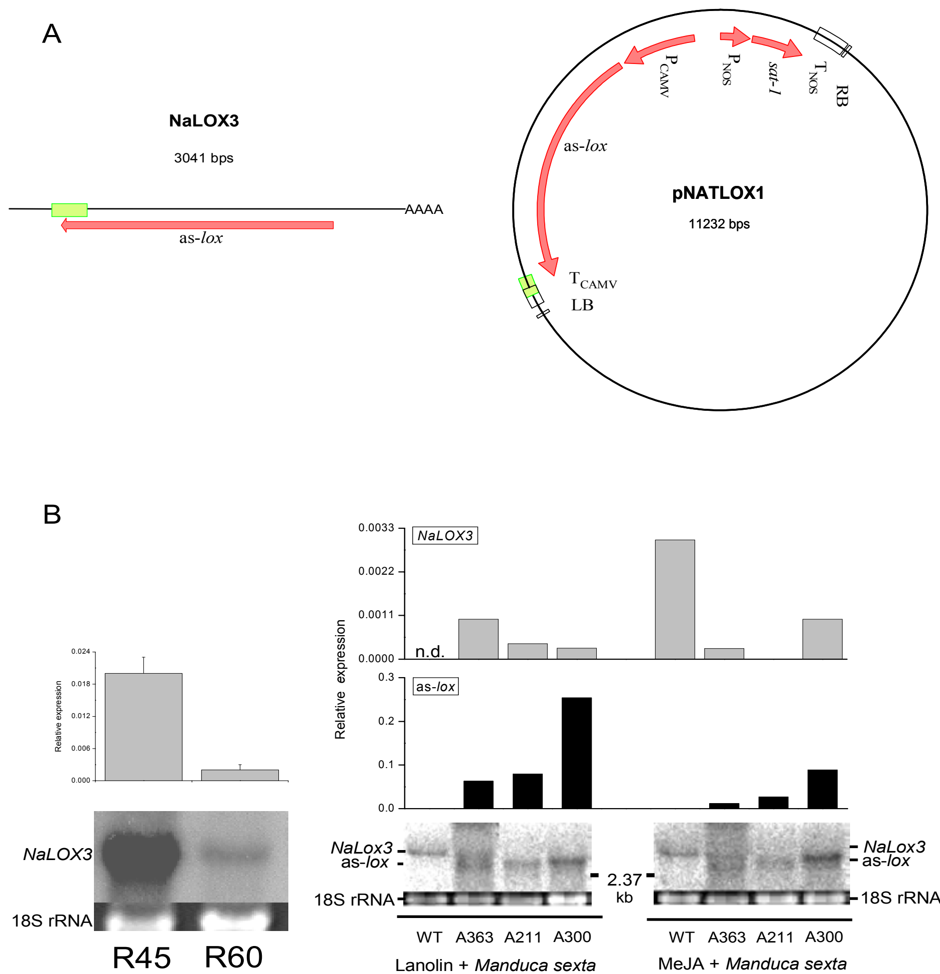

Figure S2. Real time PCR assay for analysis of endogenous *NaLOX3* and antisense-*lox* (as-*lox*) transgene expression.

Real time PCR analysis was performed on a ABI PRISM™ 7000 Sequence detection System (Applied Biosystems, Darmstadt, Germany). A) Specific amplicons (green box), a PCR primer pair and a double fluorescent dye-labeled probe, were designed for the detection of endogenous *NaLOX3* and as-*lox* transgene transcripts . The *NaLOX3* assay amplified the 5' region of *NaLOX3* and spans the transition to the region of *NaLOX3* used for the construction of the transformation vector, pNATLOX1. The as-*lox* assay amplifies the 3' region of the expressed transgene with a gene specific primer annealing to the antisense-*lox* sequence and a second transformation vector-specific primer and the probe annealing to the transcribed cauliflower mosaic virus 35S terminator (T_{CAMV}) sequence. The specificity of the PCR reaction was verified by gel analysis of the reaction products and the quality of the assay was evaluated by analyzing previously characterized RNA samples, analyzed by Northern analysis, and comparing the results of the two independent methods. Expression levels of *NaLOX3* in WT plants 45 (R45) and 60 (R60) minutes after wounding and application of *Manduca sexta* oral secretions and regurgitant were analyzed by real-time PCR and compared

to Northern blot analysis (left panel, see Figure 1b lanes 45 and 60 min). Expression of *NaLOX3* and as-*lox* was analyzed in plants treated with lanolin or methyl jasmonate and attacked by a single *M. sexta* larva (right panel, see Figure 4). n.d. represents a sample not determined due to lack of RNA.

Table S1 Description of all genes spotted on the cDNA microarray (see appendix)

Table S2 Mean (±SE) expression ratios of all genes on the cDNA microarray (see appendix)

Manuscript V

In review: Plant Journal

Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling cross-talk in *Nicotiana attenuata*

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Summary

Recent analysis of the biosynthetic pathways for jasmonic acid (JA) and green leaf volatiles (GLV) suggests that the initial substrate - fatty acid hydroperoxide (HP) – is individually supplied to the downstream processes by separate lipoxygenases. This finding is supported by differential localization of the HP-metabolizing enzymes HP lyase (HPL) and allene oxide synthase (AOS) in the chloroplast membrane. Here we characterize an *HPL* gene from *Nicotiana attenuata* and manipulate the expression of *NaHPL* and the recently characterized *NaAOS* by antisense transformation. Antisense-*AOS* (as-*aos*) and -*HPL* (as-*hpl*) plants had reduced levels of transcript accumulation and induced JA accumulation or GLV emissions, respectively. Induced JA accumulation and the release of (*Z*)-3-hexenal were negatively correlated in the transformants, suggesting substrate "cross-talk" between the two enzymatic cascades. The antisense plants also differed in their elicitation of direct and indirect defenses and transcriptional responses as measured with a microarray, suggesting differential involvement of JA and GLV signaling. Functional JA and GLV signaling cascades were required for the full elicitation of trypsin protease inhibitors (a direct defense), whereas the release of terpenoid volatile organic compounds (an indirect defense) is activated by JA independently of GLV signaling. *Manduca sexta* larvae attacking wild type *N. attenuata* plants grew significantly faster than larvae on as-*hpl* plants without apparent amplification of defense responses in the as-*hpl* plants. Addition of synthetic GLVs to as-*hpl* plants restored caterpillar performance, which was correlated with increased consumption, suggesting that GLVs function as feeding stimulants for this specialist herbivore.

Introduction

Plants encounter diverse biotic and abiotic stimuli to which they respond with a fine-tuned array of induced defenses. The specific activation of these induced responses is regulated by a network of signaling cascades, including peptide signaling (e.g. systemin) and phytohormones such as salicylic acid, ethylene, and lipid-derived oxylipin signals. Oxylipin signals derived from the allene oxide synthase (AOS) pathway, including oxo-phytodienoic acid (OPDA), jasmonic acid (JA), and its methyl ester (MeJA), collectively named jasmonates (JAs), play a major role in the activation of wound- and herbivore-induced responses. A second class of oxylipins, C_6 aldehydes, alcohols, and their esters, is produced in response to wounding and herbivory by the hydroperoxide lyase (HPL) pathway. The biosynthesis of JAs and GLVs has been characterized and most of the involved enzymes have been isolated from several plant species (Blee, 1998; Feussner and Wasternack, 2002). Mechanical wounding induces a rapid increase in *AOS* and *HPL* transcript accumulation in *Arabidopsis* (Bate *et al.*, 1998; Laudert and Weiler, 1998; Matsui *et al.*, 1999), citrus (Gomi *et al.*, 2003), tomato (Howe *et al.*, 2000; Sivasankar *et al.*, 2000) and wild tobacco (Ziegler *et al.*, 2001) but the timing of the induced transcript accumulation is not sufficiently rapid to explain the responses in downstream products, suggesting additional control mechanisms such as substrate availability or compartmentation. Both pathways utilize fatty acid hydroperoxides (HP) as a common substrate, but recent analysis of lipoxygenase (LOX) function suggests specific substrate supply to the AOS and HPL pathways by distinct LOX isoforms. The production of GLVs in potato is dependent on LOX-H1 but independent of LOX-H3 (Leon *et al.*, 2002). In contrast, JA biosynthesis is not reduced by LOX-H1 depletion (Leon *et al.*, 2002). A similar pathway-specific substrate supply has been demonstrated in *Nicotiana attenuata*, in which the silencing of NaLOX3 (the homolog of potato LOX-H3) specifically suppressed wound- and herbivore-induced JA accumulation but not the release of GLVs (Halitschke and Baldwin, 2003). These findings are consistent with the differential localization of AOS and HPL in the chloroplasts. In tomato, HPL is targeted to the outer and AOS to the inner envelope of the chloroplast membrane (Froehlich *et al.*, 2001).

In contrast to the well-characterized biosynthetic mechanisms of both AOS and HPL cascades, JA has received a majority of the effort to elucidate its role in the

regulation of numerous developmental and stress-related processes (Devoto and Turner, 2003). HPL-derived oxylipins are involved in several distinct defense responses. C_6 aldehydes and alcohols exhibit fungicidal and antibacterial activity. (*E*)-2-hexenal is a fungicidal constituent in *Ginko biloba* leaves (Major *et al.*, 1960) and inhibits growth of *Aspergillus flavus* in cultivars (Zeringue *et al.*, 1996). (*E*)-2-hexenal and (*Z*)-3-hexenol, which are released from lima bean leaves in response to inoculation with pathogenic bacteria, inhibit *Pseudomonas syringae* growth (Croft *et al.*, 1993).

In addition to these antimicrobial activities, GLVs are involved in herbivore defense. The herbivore-induced release of (*Z*)-3-hexen-1-ol functions as an indirect defense by attracting predators of *Manduca sexta* larvae in attacked *N. attenuata* plants, thereby effectively reducing herbivore survival (Kessler and Baldwin, 2001). HPL-derived compounds released from *Nicotiana tabacum* plants repel ovipositing *Heliothis virescence* moths (DeMoraes *et al.*, 2001) and are negatively correlated with aphid population growth (Hildebrand *et al.*, 1993). Moreover, GLVs may function as between-plant signals eliciting resistance against aphids in tomato (Hildebrand *et al.*, 1993) and activating wound- and herbivore-induced defense responses. Exposure to synthetic GLVs elicits the accumulation of phytoalexins in wounded cotton bolls (Zeringue, 1992), anthocyanin production in *Arabidopsis* seedlings (Bate and Rothstein, 1998), and the release of terpenoid volatile organic compounds (VOCs) in tomato (Farag and Paré, 2002). The transcript level of genes involved in phenyl propanoid- and oxylipin biosynthesis is increased by GLV treatment in *Arabidopsis* (Bate and Rothstein, 1998), lima bean (Arimura *et al.*, 2001), and citrus (Gomi *et al.*, 2003). Additionally, the accumulation of the tomato systemin precursor was induced by (*E*)-2-hexenal exposure but did not result in the activation of systemin-induced defense responses (Sivasankar *et al.*, 2000).

While a number of JA-deficient or –insensitive mutants have been created and used to study the role of octadecanoid signaling in the activation of induced plant defenses (reviewed in (Halitschke and Baldwin, 2003), GLV release has been genetically manipulated only in potato plants (Leon *et al.*, 2002; Vancanneyt *et al.*, 2001). GLV production was silenced by co-suppression of potato LOX-H1, which specifically supplies substrate to the HPL cascade (Leon *et al.*, 2002) and by antisense expression of potato HPL (Vancanneyt *et al.*, 2001). The latter manipulation reduced

resistance against the green peach aphid, but the mechanism of GLV-mediated resistance (direct or indirect) was not characterized (Vancanneyt *et al.*, 2001).

In this study, we characterize the expression of a HPL gene from *N. attenuata* and silence the activity of NaHPL and the previously described NaAOS (Ziegler *et al.*, 2001) by antisense expression. We demonstrate the 13-HP-metabolizing activity of NaHPL and NaAOS by reduced emissions of the GLVs, hexanal and (Z)-3-hexenal, and the reduced wound- and herbivore-induced JA accumulation in antisense-*HPL* (as-*hpl*) and antisense-*AOS* (as-*aos*) plants, respectively. A negative correlation between the products of the two oxylipin pathways revealed possible biosynthetic cross-talk at the level of substrate supply. Analysis of several defense traits in the transformed *N. attenuata* plants revealed different roles for the two signaling cascades. The elicitation of an indirect defense (VOC emissions) requires only JA signaling, whereas the induction of trypsin protease inhibitor (TPI) activity requires both GLV and JA cascades. *Manduca sexta* larvae attacking wild type (WT) *N. attenuata* plants grew significantly faster than larvae on as-*hpl* plants without apparent amplification of defense responses in the as-*hpl* plants. Addition of synthetic GLVs to as-*hpl* plants restored caterpillar performance, which was correlated with increased consumption. We analyzed herbivore-induced changes in transcript accumulation in as-*hpl* and as-*aos* plants in comparison to similarly attacked WT plants with cDNA microarrays. Extensive overlap of GLV- and JA-regulated transcript accumulation suggests cross-talk between signals derived from these two oxylipin pathways.

Results

NaHPL *and* NaAOS *transcript accumulation*

Northern blot analysis revealed a constitutive accumulation of *NaHPL* transcripts in roots and all green tissues, including leaves, stems, and flowers of *N. attenuata* plants (Fig. 1A). The expression was higher in young stem leaves, stem tissue, and sepals compared to a weak expression in rosette leaves and roots. No transcripts were detected in petals (Fig. 1A). Mechanical wounding increased the accumulation of *NaHPL* transcripts in *N. attenuata* leaves (Fig. 1B). Transcripts started to increase 75 min after wounding attaining maximum levels between 2 and 8h

and waning to basal levels at 16h. Attack by *M. sexta* larvae induced an increase in *NaHPL* expression after 2h, reached maximum levels between 9-10h and remained elevated throughout the period of active larval feeding (Fig. 1C).

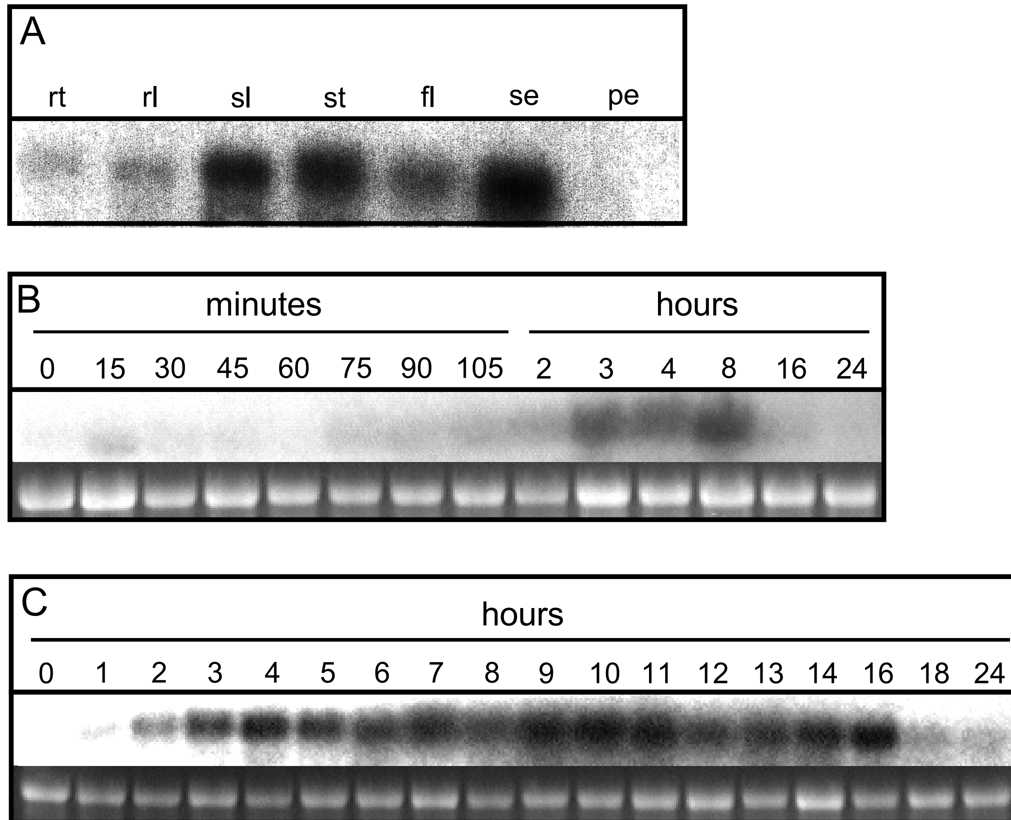

Figure 1. Tissue specific and herbivore-induced *NaHPL* transcript accumulation.
Total RNA isolated from **A)** different tissues (rt, roots; rl, rosette leaves, sl, stem leaves; st, stem; fl, whole flowers; se, sepals; pe, petals) of *Nicotiana attenuata* WT plants or individual leaves harvested at the indicated times after **B)** mechanical wounding, **C)** feeding by 3 first instar *Manduca sexta* larvae was hybridized with a *NaHPL* probe. Etidium bromide-stained 28S rRNA is shown as loading control.

We generated transgenic plants expressing a 1.1kb fragment of *NaHPL* (as-*hpl*) or a 1.2kb fragment of *NaAOS* (as-*aos*) in antisense direction by an *Agrobacterium tumefaciens*-mediated transformation procedure (Krügel *et al.*, 2002). We screened 30 to 35 independently transformed lines of each genotype for reduced levels of wound-induced JA accumulation or GLV release identified homozygous

plants of selected lines by segregation analysis (data not shown). The transgene insertion was confirmed by Southern blot analysis (Supplemental Fig. S1). We designed a quantitative reverse transcription PCR (qRT-PCR) assay for *NaHPL* and *NaAOS* to analyze the expression of these genes in response to wounding in WT and as-*aos* and as-*hpl* plants (Fig. 2). In agreement with the Northern blot analysis (Fig. 1A), a rapid initial peak in *NaHPL* expression was detected 15 min after wounding (ANOVA, $F_{6,20} = 9.686$, $P < 0.0001$; Fisher's PLSD, $P = 0.0184$) and transcript accumulation gradually increased 45 min after wounding (Fig. 2A; Fisher's PLSD, Ps ≤ 0.0017). Treatment of wounds with *M. sexta* R elicited only the initial 15 min peak (ANOVA, $F_{6,20} = 2.799$, $P = 0.0383$; Fisher's PLSD, $P = 0.0304$) but suppressed the later increase in *NaHPL* transcript accumulation (Fig. 2A; Fisher's PLSD, Ps ≥ 0.298). Basal (ANOVA, $F_{2,8} = 8.718$, $P = 0.0098$), wound- (ANOVA, $F_{2,9} = 11.986$, $P = 0.0029$), and R-induced (ANOVA, $F_{2,9} = 7.465$, $P = 0.0123$) levels of *NaHPL* transcript accumulation in as-*hpl* lines A247 and A337 were reduced to 10 to 15 % compared to similarly treated WT plants (Fig. 2A; Fisher's PLSD, Ps ≤ 0.0205).

Quantitative RT-PCR analysis of *NaAOS* expression confirmed the results by Ziegler et al. (2001). Transcript accumulation steadily increased for 2 h in WT plants after mechanical wounding (ANOVA, $F_{6,21} = 13.835$, $P < 0.0001$; Fisher's PLSD, Ps ≤ 0.0078) or treatment of wounds with *M. sexta* R (ANOVA, $F_{6,21} = 10.974$, $P < 0.0001$; Fisher's PLSD, Ps ≤ 0.0178). Constitutive *NaAOS* transcript levels were reduced to 53 and 17 % of the WT level in as-*aos* lines A340 and A160, respectively (Fig. 2B; ANOVA, $F_{2,9} = 8.159$, $P = 0.0095$; Fisher's PLSD, Ps ≤ 0.0390). Wound- (ANOVA, $F_{2,8} = 10.396$, $P = 0.0060$), and R-induced (ANOVA, $F_{2,8} = 14.078$, $P = 0.0024$) levels of *NaAOS* transcript accumulation were reduced to 19 % of WT levels in as-*aos* line A340 and 40 to 45 % in line A160 (Fig. 2B; Fisher's PLSD, Ps ≤ 0.0241).

Additionally, we monitored transgene expression by RT-PCR (data not shown) and Northern blot analysis (Supplemental Fig. S2). As-*hpl* and as-*aos* transcripts were detected only in plants of the as-*hpl* and as-*aos* lines, respectively, and not in WT plants.

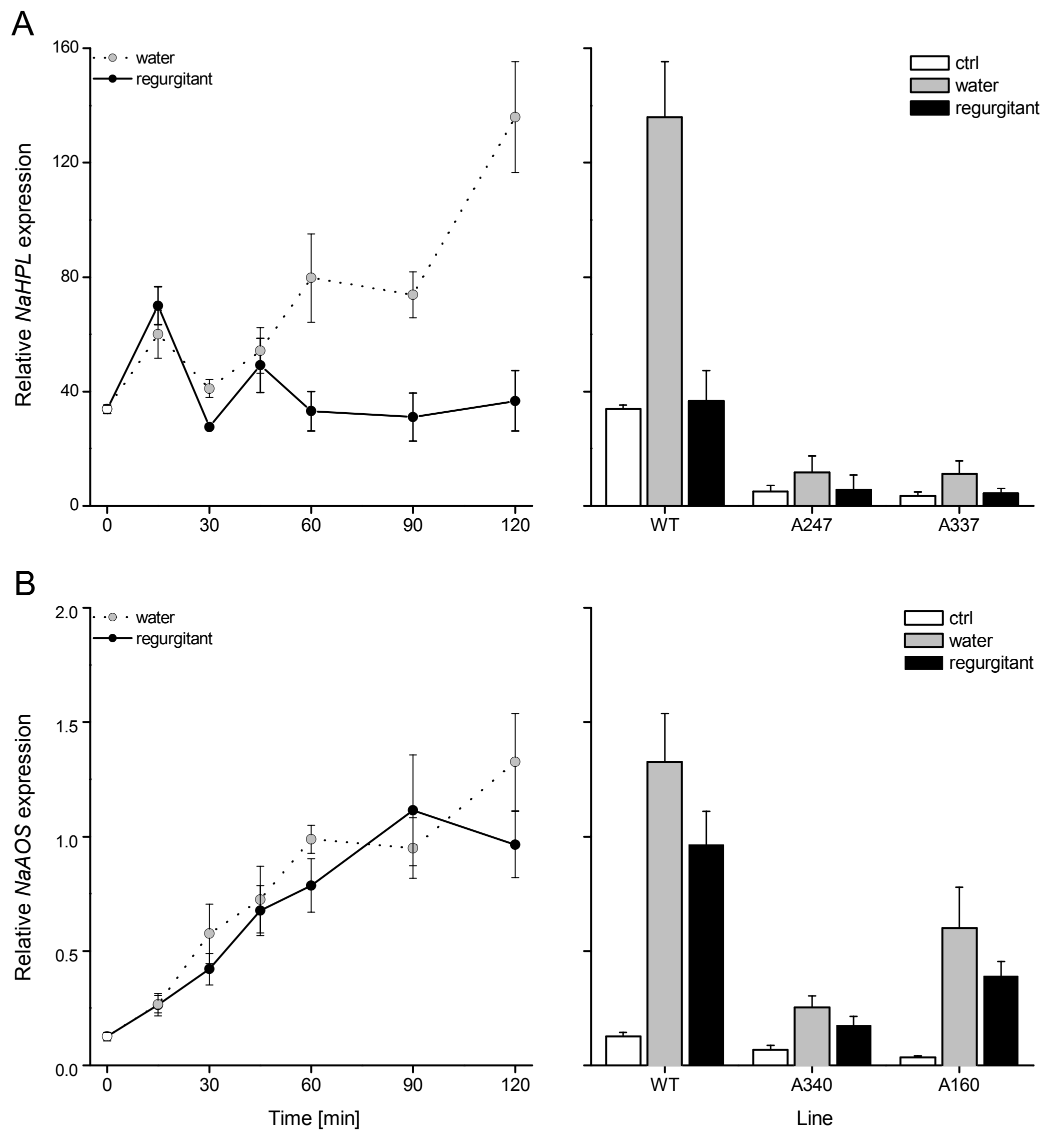

Figure 2. Transcript accumulation in as-*aos* and as–*hpl* plants.

Relative expression levels (mean ± SE of for replicate experiments) of A) *NaHPL* and B) *NaAOS* in wild type (WT), as-*hpl* (A247 and A337), and as-*aos* (A340 and A160) plants, respectively. Real time quantitative PCR was performed on RNA samples of WT leaves (left panel) harvested at the indicated time points after wounding and application of water (grey circles, dashed line) or application of *Manduca sexta* oral secretions and regurgitant (black circles, solid line). Leaves of antisense lines were harvested 120 min after wounding and treatment with water (grey bars) or wounding and regurgitant treatment (black bars). Control plants (open circles and bars) remained untreated. The relative expression was calculated by linear regression from a calibration using a cDNA standard dilution series.

JA accumulation and GLV emissions

JA levels were analyzed before and 35 min after elicitation by wounding and application of water or *M. sexta* oral secretions and regurgitant (R). Basal JA levels in untreated leaves of as-*aos* and as-*hpl* plants did not differ significantly from levels in WT plants (Fig. 3A; ANOVA, $F_{4,13} = 0.932$, $P = 0.4753$). In contrast, wound- and R-induced JA levels were altered by antisense expression of *AOS* and *HPL* (Fig. 3A). Wound-induced JA levels (ANOVA, $F_{4,20} = 36.722$, $P < 0.0001$) were decreased by 58 and 56% in as-*aos* lines A340 and A160, respectively (Fisher's PLSD, *P*s < 0.0001) and increased by 28% in as-*hpl* line A247 ($P = 0.0357$) in comparison to wound-induced JA levels of WT plants. A similar pattern was observed for R-induced JA levels (ANOVA, $F_{4,20} = 45.262$, $P < 0.0001$).

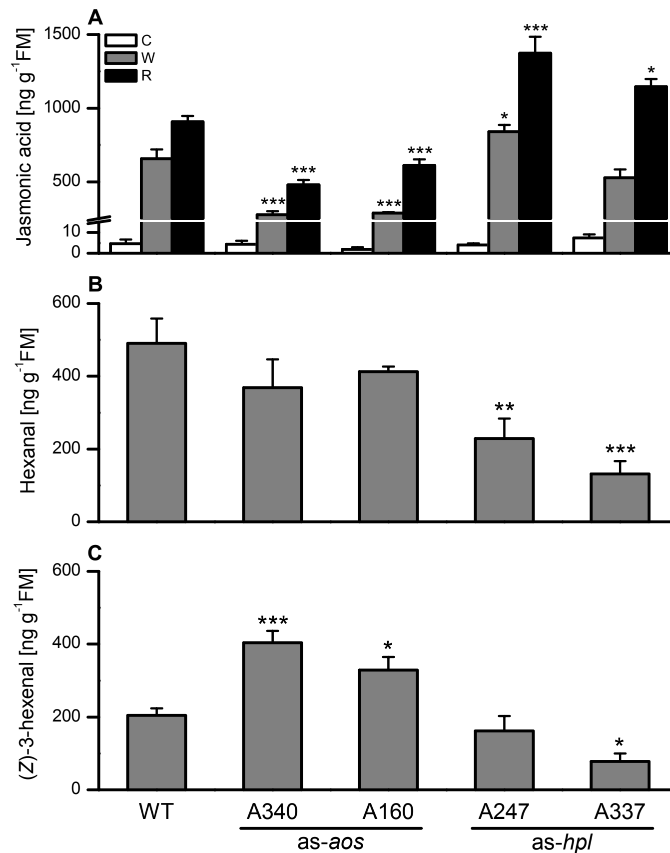

Figure 3. JA accumulation and GLV release.

A) Mean (+ SE; n = 5) jasmonic acid accumulation of wild type (WT), as-*aos* (A340, A160), and as-*hpl* (A247, A337) *Nicotiana attenuata* plants. Single leaves of individual plants were wounded with a pattern wheel and immediately supplied with water (W) or *Manduca sexta* oral secretion and regurgitant (R). Control plants (C) remained untreated. JA levels were

analyzed in the treated leaves 35 min after elicitation. Mean (+ SE; n = 5) emissions of **B**) hexanal and **C**) (*Z*)-3-hexenal released from individual plants of the same genotypes described in A) in the first minute after mechanically wounding a single leaf.

Antisense expression reduced R-induced JA accumulation by 47 and 33% in as-*aos* lines A340 and A160, respectively (Fisher's PLSD, $Ps \leq 0.0003$) and increased JA levels by 51 and 26% in as-*hpl* lines A247 and A337, respectively ($Ps \leq 0.0190$).

The release of the GLVs, hexanal and (*Z*)-3-hexenal, from a single leaf was analyzed before and in the first minute after mechanical wounding. No emission of the analyzed compounds was detected in unwounded plants (data not shown). Mechanical wounding elicited a rapid and transient release of hexanal (Fig. 3B) and (*Z*)-3-hexenal (Fig. 3C) in WT plants. The wound-induced release of hexanal (ANOVA, $F_{4,21} = 6.078$, $P = 0.0021$) was reduced by 53 and 73% in as-*hpl* lines A247 and A337, respectively (Fisher's PLSD, $Ps \leq 0.0049$) but not significantly changed in as-*aos* lines A340 and A160 ($Ps \geq 0.1399$). In contrast, the wound-induced release of (*Z*)-3-hexenal (ANOVA, $F_{4,21} = 17.685$, $P < 0.0001$) was significantly increased by 97 and 61% in as-*aos* lines A340 and A160 compared to WT plants (Fisher's PLSD, $Ps \leq 0.0128$) and reduced by 62% in as-*hpl* line A337 ($P = 0.0112$).

Herbivore resistance

The mass of *Manduca sexta* larvae feeding on WT, as-*aos*, and as-*hpl* plants was recorded for the first 5 d after hatching. Caterpillars feeding on as-*aos* plants A340 and A160 did not develop significantly different from larvae on WT plants (Fig. 4A; Repeated Measurement ANOVA, $F_{6,86} = 2.937$, $P = 0.0118$; Fisher's PLSD, $Ps \geq 0.1118$). In contrast, larvae feeding on plants of as-*hpl* lines A247 and A337 gained significantly less mass compared to larvae feeding on WT plants (Fig. 4A; Fisher's PLSD, $Ps \leq 0.0049$). Treatment of as-*hpl* plants with synthetic GLVs recovered the performance of larvae feeding on both as-*hpl* lines (Fisher's PLSD, $Ps \geq 0.1024$). Elicitation of *N. attenuata* plants by MeJA treatment induced resistance against *M. sexta* larvae to the same extend in WT, as-*aos*, and as-*hpl* plants (Fig. 4A). The reduced mass gain did not differ significantly between larvae feeding on MeJA-treated WT, as-*aos*, and as-*hpl* plants (Repeated Measurement ANOVA, $F_{4,67} = 1.347$, $P = 0.2619$).

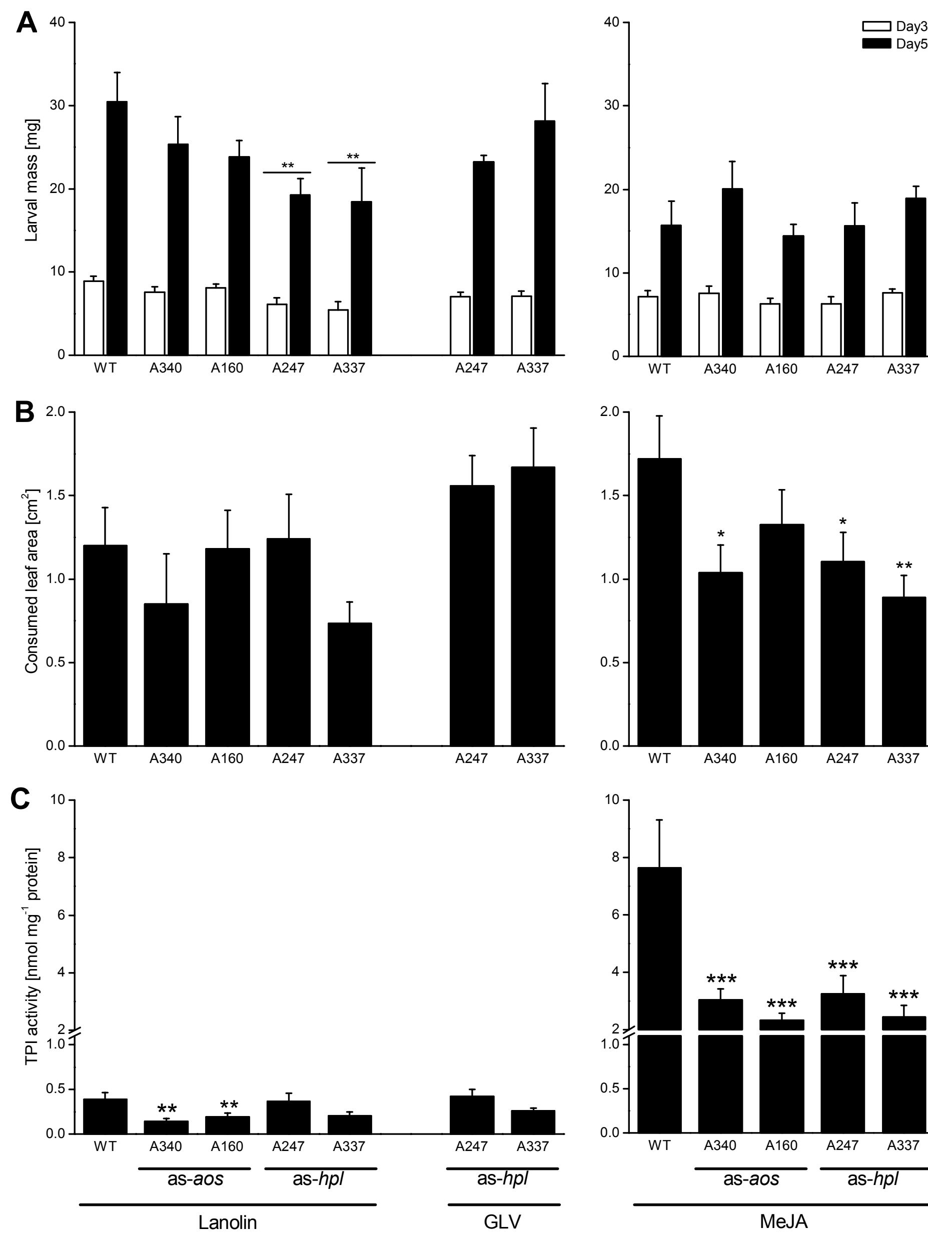

Figure 4. Herbivore performance and activation of direct defense responses.

Freshly hatched *Manduca sexta* larvae were allowed to feed on the second fully developed leaf of wild type (WT), as-*aos* (A340, A160), and as-*hpl* (A247, A337) *Nicotiana attenuata* plants (n = 8) after application of lanolin, synthetic green leaf volatiles (GLV) or methyl jasmonate (MeJA) to the second and third fully developed leaf.

A) Mean (+ SE) mass of *M. sexta* larvae after 3 and 5 days of feeding.

B) Mean (+ SE) leaf area consumed by *M. sexta* larvae after 7 days of continuous feeding.

C) Mean (+ SE) trypsin protease inhibitor (TPI) activity after 7 days of caterpillar feeding.

The leaf area consumed by *M. sexta* larvae after 7 d of continuous feeding on as-*aos*, and as-*hpl* plants was statistically not different compared to the leaf area removed from WT plants (Fig. 4B; ANOVA, $F_{6,45}$ = 2.157, P = 0.0651, Fisher's PLSD, Ps $\geq$ 0.1599). Application of GLVs caused a small increase in leaf consumption by larvae feeding on as-*hpl* plants, which was not statistically significant (Fig. 4B). In contrast, the amount of leaf area consumed by *M. sexta* feeding was significantly reduced in MeJA-treated plants of as-*aos* line A340 and as-*hpl* lines A337 and A247 compared to MeJA treated WT plants (Fig. 4B; Repeated Measurement ANOVA, $F_{4,35}$ = 2.824, P = 0.0394; Fisher's PLSD, Ps $\leq$ 0.0299).

Direct defense activation

Trypsin protease inhibitor (TPI) activity and nicotine accumulation was analyzed after 7 d of continuous feeding by a *M. sexta* larva. No significant difference in herbivore- (ANOVA, $F_{6,45}$ = 0.829, P = 0.5534) and MeJA-induced (ANOVA, $F_{4,33}$ = 0.152, P = 0.9609) nicotine concentration was observed among lines (data not shown). In contrast, TPI activity in herbivore-induced plants was suppressed in as-*aos* lines A340 and A160 (Fig. 4C, ANOVA, $F_{6,44}$ = 3.679, P = 0.0048; Fisher's PLSD, Ps $\leq$ 0.0059). MeJA-induced TPI activity was strongly suppressed in both as-*aos* and as-*hpl* lines (Fig. 4C, $F_{4,35}$ = 8.85, P < 0.0001; Fisher's PLSD, Ps $\leq$ 0.0004).

Indirect defense activation

We analyzed *cis*-α-bergamotene emissions in WT, as-*aos*, and as-*hpl* plants 24 h after elicitation by mechanical wounding and application of water or R or treatment with MeJA. Genotype and treatment had significant effects on release rates (two-way ANOVA; genotype: $F_{4,137}$ = 5.815, P = 0.0002; treatment: $F_{3,137}$ = 42.251, P < 0.0001). Comparisons of *cis*-α-bergamotene emissions from treated and untreated plants in the individual genotypes (Table 1) revealed 4- to 5-fold increased emission in R-treated WT and as-*hpl* (A247 and A337) plants (ANOVA, Fisher's PLSD, Ps $\leq$ 0.0068) but not in as-*aos* (A340 and A160) plants (Ps $\geq$ 0.1874). Wounding and water application did not elicit volatile emissions (Fisher's PLSD, P $\geq$ 0.2424) whereas MeJA treatment elicited increased volatile emissions in all analyzed genotypes (Ps $\leq$ 0.0337), demonstrating that MeJA recovered the WT phenotype in as-*aos* plants.

Table 1. Mean (± SE) *cis*-α-bergamotene release from individual wild type (WT), as-*aos* (A340, A160), and as-*hpl* (A247, A337) *Nicotiana attenuata* plants. Volatiles were analyzed 24 h after wounding a single leaf of each plant with a pattern wheel and treating the wounds with water (W, n = 6) or *Manduca sexta* oral secretion and regurgitant (R, n = 6) or applying 1 μmol methyl jasmonate (MeJA) in 20 μl lanolin (MeJA, n = 4). Control plants (C, n = 4) remained untreated. Bold numbers indicate significantly increased emissions compared to the volatile release of untreated control plants within individual lines (P < 0.05).

		cis-α-Bergamotene release (ng h^{-1})			
Genotype	Line	C	W	R	MeJA
WT	WT	3.81±0.84	7.18±2.77	**15.70±2.04**	**47.52±12.00**
as-*aos*	A340	5.02±1.06	3.25±0.80	4.26±1.04	**42.60±16.25**
	A160	3.66±0.41	3.18±0.56	6.56±1.67	**31.36±6.69**
as-*hpl*	A247	1.67±0.96	1.43±0.91	**8.30±2.80**	**26.92±4.76**
	A337	7.54±1.98	9.45±1.28	**39.41±11.25**	**20.61±5.63**

Herbivore-induced transcript accumulation

We analyzed NaAOS- and NaHPL-dependent transcriptional responses in two independent as-*aos* and as-*hpl* lines by comparing RNA from *M. sexta*-attacked WT and antisense-transformed plants on a cDNA microarray. A list of expression ratios of all genes is available in supplemental Table S1. The expression of 70 and 81 of the 240 genes represented on the array showed altered expression in at least one of the analyzed independent as-*aos* or as-*hpl* lines, respectively. Sixty percent of these genes showed the same expression pattern in both analyzed as-*aos* (20 up- and 21 down-regulated genes) and as-*hpl* (21 up- and 26 down-regulated genes) lines. Genes with known or putative functions that had the same expression pattern in each of two independent lines of as-*aos* or as-*hpl* genotypes are shown in Table 2. In agreement with their JA-induced expression, several defense related genes coding for a proteinase inhibitor (PI), xyloglucan endo-transglucosylase/hydrolase (XTH), threonine deaminase (TD), and a metallothionein-like protein had reduced transcript accumulation in as-*aos* plants compared to WT plants, whereas the majority of photosynthesis-related genes, with the exception of the RUBISCO small subunit

(SSU) and a photosystem II (PSII) protein showed amplified transcript accumulation (Table 2). Interestingly, an almost identical pattern of gene regulation was observed in the as-*hpl* plants.

Table 2. Expression ratios (ER = mean of ER1 and ER2) of genes differentially regulated in plants of as-*aos* line A340 or as-*hpl* line A337. ERs fulfilling all criteria for designation of differential regulation (see "Experimental procedures") in two independently analyzed lines are shown in bold numbers. mRNA from leaves of *Manduca sexta* damaged WT (Cy5) and as-*aos* or as-*hpl* (Cy3) plants was fluorescently labeled and comparatively analyzed on a cDNA microarray. Putative gene functions derived from database comparisons of ESTs are label with the corresponding source organism: *H.b., Hevea brasiliensis*; *L.e., Lycopersicon esculentum*; *N.a., Nicotiana attenuata*; *N.p., N. plumbaginifolia*; *N.s., N. sylvestris*; *N.t., N. tabacum*; *N.to. N. tomentosiformis*; *S.t., Solanum tuberosum*.

Label	Acc. nr.	(putative) function	ER (Cy3/Cy5)	
			as-*aos*	as-*hpl*
414/5	AW191829	*N.s.* Rubisco small subunit	**0.22**	**0.52**
612/3	CA591795	*N.t.* Rubisco small subunit, pseudogene	**0.11**	**0.39**
608/9	CA591793	*N.t.* photosystem II protein	**0.25**	**0.36**
406/7	AW191826	*L.e.* light harvesting complex II protein	**4.63**	**8.11**
548/9	CA591767	*H.b.* plastidic aldolase-like protein	1.88	**3.27**
552/3	CA591769	*N.t.* photosystem II O_2 evolving complex polypeptide	**2.55**	**2.95**
420/1	AW191831	*N.t.* Mg protoporphyrin IX chelatase	**6.07**	**5.27**
432/3	CA591712	*S.t.* α-amylase	**2.30**	**1.91**
438/9	CA591715	*S.t.* lipoxygenase H3	**2.03**	**2.02**
440/1	CA591716	*L.e.* allene oxide synthase	**5.14**	**2.90**
674/5	AJ295274	allene oxide synthase NaAOS	**8.55**	0.85
668/9	AJ414400	hydroperoxide lyase NaHPL	**0.40**	**0.25**
342/3	AW191811	threonine deaminase	**0.19**	**0.34**
676/7	X82685	xyloglucan endotransglucosylase/hydrolase	**0.13**	**0.14**
666/7	AF105340	proteinase inhibitor	**0.37**	**0.12**
660/1	CA591816	*S.t.* major intrinsic protein 2	**0.58**	**0.66**
664/5	CA591818	*N.p.* metallothionein-like protein	**0.41**	**0.57**
382/3	AW191821	α-dioxygenase	0.55	1.17
454/5	CA591723	*N.to.* heterotrimeric GTP binding protein	**2.20**	1.30

The increase in *NaAOS* signal in as-*aos* plants is due to the nature of the probe spotted on the microarray. The spotted PCR fragment includes the region of *NaAOS* used for the antisense construct and therefore does not distinguish between *NaAOS* and as-*aos* transcripts. Northern blot analysis clearly showed the suppression in *NaAOS* transcript accumulation in as-*aos* plants (Fig. S2). The suppression of *NaHPL* transcript accumulation in as-*hpl* plants is detectable with the microarray (Table 2) and was confirmed by Northern blot analysis (Fig. S2). Transcript accumulation of NaHPL was also reduced in *M. sexta*-damaged as-*aos* plants. Further genes involved in oxylipin signaling including a second *AOS* and a *LOX* showed amplified transcript accumulation in both as-*aos* and as-*hpl* plants.

Discussion

Regulation of JA and GLV production

The rapid and highly transient release of GLVs after wounding is not correlated with a corresponding increase in *NaHPL* transcript accumulation suggesting that GLV release is not transcriptionally regulated. Most likely, the release is limited by the supply of fatty acid HP substrate that is readily metabolized by the high constitutive HPL activity in *N. attenuata* (Ziegler *et al.*, 2001) and potato (Vancanneyt *et al.*, 2001) leaves. Antisense expression-mediated silencing of HPL activity reduced the release of wound-induced GLVs and amplified the wound- and herbivore-induced JA burst (Fig. 3). Conversely, silencing of AOS activity resulted in reduced JA accumulation accompanied by an increased release of HPL-derived GLVs (Fig. 3). This negative correlation demonstrates that the cascade-specific HP supply by distinct LOX isoforms (Halitschke and Baldwin, 2003; Leon *et al.*, 2002) does not restrain substrate allocation between downstream oxylipin cascades and thereby could serve as regulatory mechanism to amplify (or suppress) individual components of the complex oxylipin signature. The suppression by larval R of wound-induced *NaHPL* transcript accumulation (Fig. 2) could be the regulatory mechanism responsible for the R-induced amplification of JA accumulation in *N. attenuata*, which could not be explained by expression of JA biosynthetic enzymes analyzed so far (Halitschke and Baldwin, 2003; Ziegler *et al.*, 2001).

Signaling function of JAs and GLVs

Wound-induced GLV emissions play an important role in the attraction of predators of *M. sexta* larvae to attacked *N. attenuata* plants (Kessler and Baldwin, 2001) but are not involved in the specific elicitation of herbivore-induced terpenoid emissions, which in turn are dependent on oxylipin signals produced by the octadecanoid pathway in *N. attenuata* (Table 1; Halitschke and Baldwin, 2003) and tomato (Thaler *et al.*, 2002). In contrast, the induction of TPI accumulation involves both, HPL- and AOS-derived oxylipins (Fig. 4C). Sivasankar *et al.* (2000) proposed the independent activation of systemin signaling by GLVs in parallel with the wound-induced JA cascade to mount the complete defense response in tomato and this synergistic interaction could explain the reduced TPI accumulation in both, JA- and GLV-deficient plants. Alternatively, additional signals that are involved in the elicitation of proteinase inhibitors (e.g. ethylene; O'Donnell *et al.*, 1996) might depend on a functional HPL cascade.

A significant proportion of the large herbivore-responsive transcriptome of *N. attenuata* (Halitschke *et al.*, 2003; Hermsmeier *et al.*, 2001; Hui *et al.*, 2003) is regulated by a NaLOX3-derived signal (Halitschke and Baldwin, 2003). Consistent with a JA-dependent regulation in WT plants, a set of herbivore-induced defense related genes and several photosynthetic genes which are down-regulated in response to herbivory show a highly similar expression pattern in as-*aos* plants compared with the expression in previously described as-*lox* plants (Table 2; Halitschke and Baldwin, 2003). A prominent difference between as-*lox* and as-*aos* plants is the herbivore-induced expression of α-dioxygenase (α-DOX). The accumulation of α-DOX transcripts is induced by wounding, herbivory, and MeJA treatment (Hermsmeier *et al.*, 2001) but silencing of NaLOX3 resulted in an increase in herbivore-induced α-DOX transcripts (Halitschke and Baldwin, 2003). However, in the JA-deficient as-*aos* plants characterized here, α-DOX expression is reduced which suggest a JA-dependent induction mechanism. In the pathogen response of cultivated tobacco, a negative correlation between α-DOX and 9-LOX products has been recently demonstrated (Hamberg *et al.*, 2003), but the function of α-DOX in plant resistance against herbivores remains to be established.

While silencing of the HPL branch of the oxylipin cascade increases the substrate pool available for the biosynthesis of JAs (Fig. 3), the pattern of herbivore-induced transcript accumulation in as-*hpl* <u>and</u> as-*aos* plants show are strikingly

similarity (Table 2), including the reduced transcript accumulation of a *PI* gene which is consistent with the reduced TPI activity in both as-*hpl* and as-*aos* lines (Fig. 4C). This co-regulation of gene expression by HPL- and AOS-derived oxylipins again indicates a critical role for both cascades for the activation of *N. attenuata*'s defense responses. Silencing of a potato HPL did not reduce the expression of genes coding for PI and signaling pathway proteins (Vancanneyt *et al.*, 2001) whereas the specific silencing of the LOX isoform that supplies the substrate for the HPL cascade (LOX-H1) significantly reduced PI expression (Leon *et al.*, 2002). The GLV-induced expression of further defense- and signaling-related genes in *Arabidopsis* (Bate and Rothstein, 1998), tomato (Sivasankar *et al.*, 2000), lima bean (Arimura *et al.*, 2001), and citrus (Gomi *et al.*, 2003) further supports an important role for HPL-derived oxylipins as signals in plant defense activation.

Herbivore Resistance

Although the silencing of AOS activity resulted in a reduction of wound- and R-induced JA accumulation (Fig. 3A) comparable with the reduction in lipoxygenase *NaLOX3*-deficient (as-*lox*) plants (Halitschke and Baldwin, 2003), *M. sexta* larvae feeding on as-*aos* plants did not grow differently from larvae feeding on WT plants. The main difference between as-*aos* and as-*lox* plants is that reduced nicotine accumulations were only observed in the latter. Reduced nicotine accumulations are strongly negatively correlated with the performance of feeding *M. sexta* larvae (Halitschke and Baldwin, 2003; Steppuhn *et al.*, 2004). Nevertheless, a defensive function of TPI has been elegantly shown by directly manipulation the TPI expression in *N. attenuata*. Restoring TPI activity in a mutant defective in TPI expression as well as silencing TPI expression in TPI-expressing lines dramatically affected the development of *M. sexta* larvae (Zavala *et al.*, 2004). In our experiment, larvae feeding on WT plants with elevated TPI activity (Fig. 4C) might be able to compensate for the anti-digestive function of TPI by increasing their consumption of leaf material (Fig. 4B). The reduced performance of *M. sexta* larvae feeding on as-*hpl* plants cannot be easily explained by the pattern of induced defenses (Fig. 4A and C). The performance of aphids feeding on tomato leaves was negatively affected by HPL-derived GLVs (Hildebrand *et al.*, 1993; Vancanneyt *et al.*, 2001), but the mechanism of this decrease in performance has not been characterized. The increased leaf consumption by *M. sexta* larvae feeding on as-*hpl* plants which had been

supplemented with synthetic GLVs (Fig. 4B) suggests a direct role for GLVs as feeding-stimulants for *M. sexta* larvae. Alternatively, increased JA production due to higher substrate availability (Fig. 3) could negatively influence larval performance by inducing an uncharacterized plant defense response or by affecting detoxification responses in the larvae (Li *et al.*, 2002).

The possible shift in the allocation of LOX-derived substrates to the HPL and AOS pathway and the substantial overlap of GLV- and JA-regulated plant defense responses demonstrates a cross-talk between the two cascades that clearly exceeds the competition for a common substrate. The transgenic plants generated and characterized in this study will allow the dissection of the complex interplay of multiple oxylipin signaling cascades that are involved in the elicitation of *N. attenuata*'s defense responses.

Experimental procedure

Plant and insect material

Wild type plants were generated from an inbred line (11 generations) of a seed collection from a native population (DI Ranch, Santa Clara, UT) of *N. attenuata* (synonymous with *N. torreyana* Nelson and Macbr.) and all plants were grown under the conditions described by Halitschke and Baldwin (2003). The as-*aos* and as-*hpl* genotypes were generated by an *Agrobacterium tumefaciens*-mediated transformation procedure (Krügel *et al.*, 2002) and single-copy transgene incorporation was confirmed by segregation analysis for the selective marker (Krügel *et al.*, 2002) and Southern blot analysis (Supplemental Fig. S1). *Manduca sexta* eggs were purchased from North Carolina State University (Raleigh, NC).

Isolation of NaHPL *cDNA*

A cDNA clone isolated from a library prepared from leaves of *N. attenuata* plants exposed to *M. sexta* feeding for 24 h by screening the library for AOS cDNAs (Ziegler *et al.*, 2001) showed now amplification after PCR with AOS gene-specific primers. PCR with degenerated primers HPL-for 5'-CCATTAGACTAYAAYTGGT TYC-3' and HPL-rev 5'-TTTTCACAGATGTGAGTGWNCC-3' amplified a fragment of the expected size of 1.3 kb. The cDNA clone with a size of 1675 bp was

sequenced completely and showed highest sequence similarity to a tomato HPL (Howe *et al.*, 2000).

Nucleic acid analysis

DNA isolation and Southern blot analysis was performed as described by Winz and Baldwin (2001) and RNA extraction and Northern blot analysis was performed as described by Ziegler *et al.* (2001). Blots were hybridized with a radioactively labeled 1.3 kb PCR fragment of *NaHPL* synthesized with the primers described above.

Quantitative real-time PCR assays were developed to analyze transcript accumulation of *NaHPL*, *NaAOS*, and the transgenes as-*aos* and as-*hpl*. Specific amplicons detecting only the endogenous transcript and not the region selected for the antisense construct were designed. Total RNA was isolated from 4 replicate plants of each line for each treatment and time point and cDNA was synthesized from 20 ng of total RNA as described by Halitschke and Baldwin (2003). Transcript accumulation of *NaHPL* and *NaAOS* was analyzed with the following primer and fluorescence dye-labeled probe combinations: HPL_F1 (5'-CACTTAGACTTAGTCCACCTGTGC-3'), HPL_R1 (5'-AACACAAACTTTTCAGGATCATCA-3'), and HPL_P1 (5'-FAM-CCTTTGGATCTCTCATTACCAATGGCTGA-TAMRA-3') and AOS_F2 (5'-GACGGCAAGAGTTTTCCCAC-3'), AOS_R2 (5'-TAACCGCCGGTGAGTTCAG T-3'), and AOS_P2 (5'-FAM-CTTCACCGGAACTTTCATGCCGTCG-TAMRA-3'), respectively. Real-time PCR was performed on a SDS7700 (Applied Biosystems, Darmstadt, Germany) using the qPCR^{TM} reagent kit (Eurogentec, Seraing, Belgium) as described by Halitschke and Baldwin (2003).

Analysis of JA accumulation GLV emissions

JA accumulation was analyzed in five replicate plants of each genotype after elicitation by wounding the second fully expanded leaf with a pattern wheel and supplying 20 µl water or 20 µl *M. sexta* oral secretion and regurgitant (R), diluted 1:1 (v:v) with deionized water, to the fresh wounds. Treated leaves were harvested after 35 min and analyzed as described by Krügel *et al.* (2002). Hexanal and (*Z*)-3-hexenal emissions were sampled for 1 min from individual plants (5 replicates per genotype) before and immediately after elicitation by mechanical wounding (Halitschke and Baldwin, 2003).

Analysis of M. sexta *performance, nicotine accumulation and TPI activity*

The second (+2) and third (+3) fully developed leaf of 8 replicate plants from each genotype were treated 20 µl lanolin containing 1 µmol of MeJA or a mixture of (*E*)-2-hexenal, (*Z*)-3-hexenol, (*E*)-2-hexenol, (*Z*)-3-hexenyl acetate, and (*Z*)-3-hexenyl butyrate (1 µmol each). Control plants received 20 µl pure lanoline paste. A single freshly eclosed *M. sexta* larva was placed on the +2 leaf. Larvae were allowed to feed for 7 days and larval mass was recorded on day 3, 5. The first fully developed (+1), +2, and +3 leaf of each plant was harvested for further analysis. Consumed leaf area was calculated from digitized images of the +2 leaf. Trypsin protease inhibitor (TPI) activity in the +3 leaf and nicotine accumulation in leaf +1 was analyzed as described by Halitschke and Baldwin (2003).

Analysis of cis-*α-bergamotene emissions*

The second fully-expanded leaf of four to six individual plants was treated by wounding with a pattern wheel and immediately supplying 20 µl water (W, n = 6) or 20 µl *M. sexta* oral secretion and regurgitant, diluted 1:1 (v:v) with water (R, n = 6) or applying 1 µmol MeJA in 20 µl lanolin (MeJA, n = 4) to undamaged leaves. Control plants (C, n = 4) remained untreated. Volatiles were collected 24 h after elicitation by adsorptive trapping for 8 h and analyzed by gas chromatography-mass spectrometry as described by Halitschke *et al.* (2000).

cDNA microarray analysis

We analyzed the herbivore-induced transcript accumulation of WT, as-*aos*, and as-*hpl* plants by cDNA microarray analysis. Eight replicate plants of each genotype were exposed to herbivory by a first instar *M. sexta* larva placed on the +2 leaf for 7 days after which the damaged leaves of the replicate plants were harvested and pooled. The extraction of total RNA, isolation of mRNA and cDNA synthesis was performed as described by Halitschke *et al.* (2003). cDNA from WT plants was labeled with Cy5 and cDNA from antisense lines was labeled with Cy3 fluorescent dye and the pooled cDNAs were hybridized on a microarray representing 240 herbivore-responsive *N. attenuata* genes and analyzed as described by Halitschke *et al.* (2003). Two PCR fragment of each gene were spotted in quadruplicate on an epoxy coated slide and several main criteria for the identification of differentially regulated genes were established and evaluated by repeated hybridization experiments

(Halitschke and Baldwin, 2003). The mean value of expression ratios (ERs) for both PCR fragments (ER1 and ER2) have to exceed an arbitrary threshold ($ER > 1.3$ or $ER < 0.76$) and significantly different from 1 (one-sample t-test; $P < 0.05$). If both criteria were fulfilled for both PCR fragments of each gene the expression was considered as differentially regulated.

Statistical analysis

Data were analyzed by ANOVAs using the StatView software (SAS, Cary, NC). Relative expression and TPI activity data were log-transformed. Fisher's partial least significant differences (PLSD) were calculated to compare the antisense-transformed plants with similarly treated WT plants.

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Supplementary Material

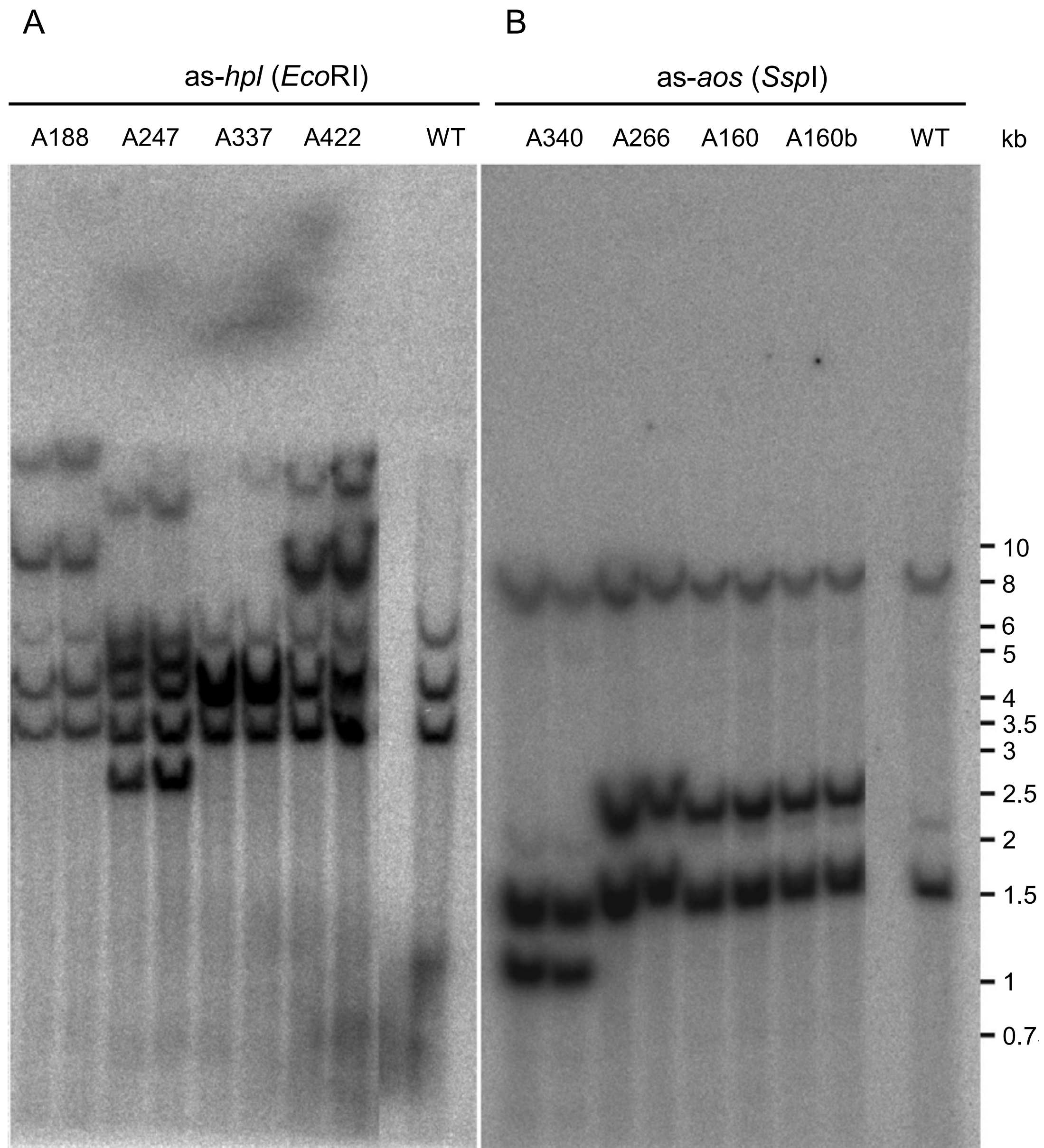

Figure S1. Southern blot analysis of antisense-transformed lines.

A) Ten µg of genomic DNA isolated from two individual plants of 4 independent as-*hpl* lines and WT *Nicotiana attenuata* plants was digested with *Eco*RI and hybridized with a PCR fragment of the region of *NaHPL* used for the as-*hpl* transformation vector. B) Ten µg of genomic DNA isolated from two individual plants of 3 independent as-*aos* lines (A160 and A160b represent progeny of two T1 plants generated from a single parent plant and not independent lines) and WT *N. attenuata* plants was digested with *Ssp*RI and hybridized with a PCR fragment of the region of *NaAOS* used for the as-*aos* transformation vector.

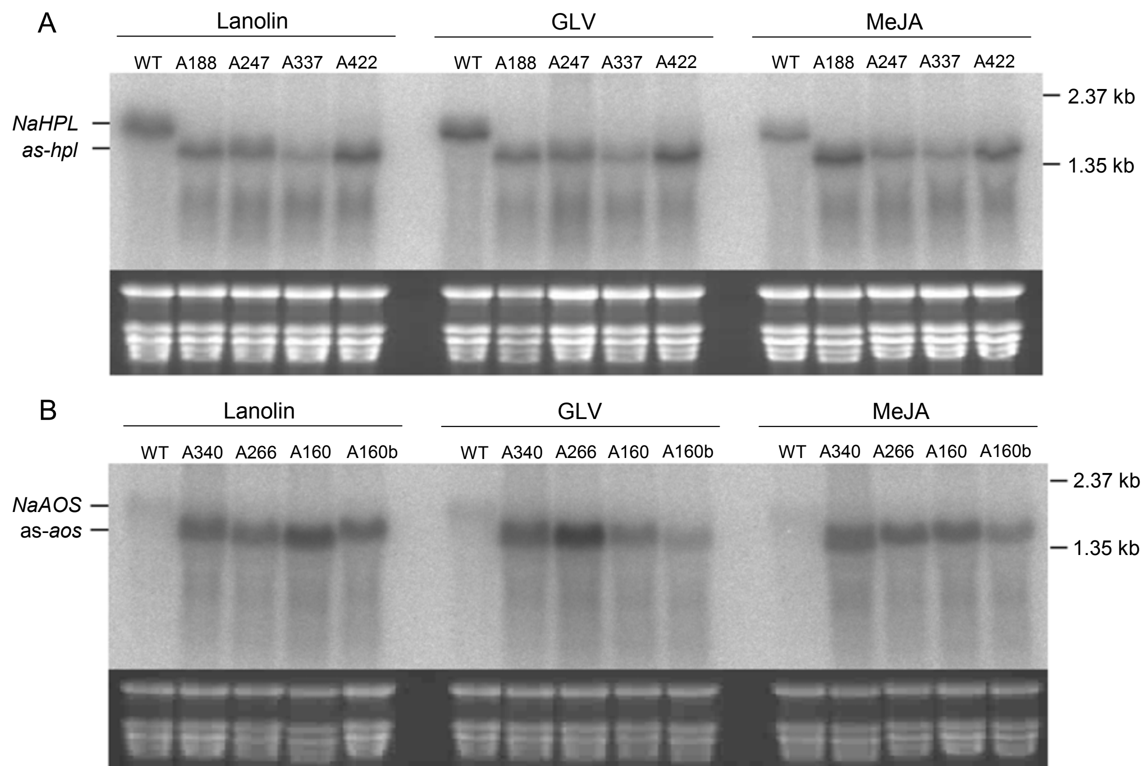

Figure S2. Transcript accumulation in antisense-transformed lines.

The second and third fully-expanded leaf of 8 replicate plants of the lines described in supplemental Fig. 2 were treated with lanolin paste (Lanolin), or MeJA, or a mixture of GLV in 20 µl of lanolin paste (see Experimental Procedures), and a freshly eclosed *Manduca sexta* larva was placed on the second leaf. Larvae were allowed to feed for seven days after which attacked leaves of the replicate plants were harvested and pooled for RNA extraction. Total RNA (10 µg) of plants from **A)** four independent as-*hpl* lines and B) three independent as-*aos* lines was probed with a *NaHPL* and *NaAOS* PCR fragment respectively. The PCR fragments were synthesized from the regions of *NaHPL* and *NaAOS* that were used for the antisense construct and therefore simultaneously detect endogenous (*NaHPL* and *NaAOS*) and transgene (as-*hpl* and as-*aos*) transcripts. Ethidium bromide stained 18S rRNA is shown as loading control.

Table S1. Description, expression ratios (Mean ± SE) and one-sample *t*-test results for all genes spotted on the cDNA microarray (see appendix)

Manuscript VI

In review: Science

Genetically silencing the jasmonate cascade: influences of induced plant defenses on herbivore community composition

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Ecology is complex and difficult to study. We transformed the native tobacco, *Nicotiana attenuata*, to silence its lipoxygenase, hydroperoxide lyase and allene oxide synthase genes to inhibit oxylipin signaling, which is known to mediate the plant's direct and indirect defenses. When planted into native habitats, lipoxygenase-deficient plants were more vulnerable to *N. attenuata*'s adapted herbivores but also attracted novel herbivore species, which fed and reproduced successfully. While highlighting the value of genetically silenced plants in studying ecological interactions in nature, these results demonstrate that jasmonate signaling determines host selection for opportunistic herbivores and that induced defenses influence herbivore community composition.

Scientists agree that a deep understanding of the complex ecological interactions that occur in nature is essential for sustainable agriculture. The plant traits that are important for resistance to herbivore attack in nature are complex and operate on many spatial scales, for they involve both direct defenses (toxins, digestibility reducers etc.)(*1*), that themselves protect plants as well as indirect defenses, that work with components of the plants' community (natural enemies, diseases, etc.)(*2,3*). Moreover, plant defenses can be constitutively expressed or produced in response to an attacking pathogen or herbivore, when they are needed (*4*). Genetic transformation provides a valuable tool to manipulate traits that mediate these complex interactions and allows an integrative analysis of single traits (*5*). However, the evaluation of transformants is usually performed with known challenges, not the vast number of unknowns that occur in nature. We used transformed lines of the wild tobacco species *N. attenuata*, which express *LOX3*, *HPL* and *AOS* in an antisense orientation (as-*lox*, as-*hpl*, as-*aos* respectively)(*6*), to study herbivore-induced plant responses in nature. All three enzymes are key regulators in the oxylipin pathways and are known to play a major role in the plants' wound recognition and signaling (*7*), but their defensive function outside the laboratory is unknown.

Phenotypic plastic responses such as herbivore-induced plant defenses are thought to be an adaptation to unpredictable environments (*8*). In native populations of *N. attenuata*, herbivory is an unpredictable selective factor. The plant grows in large ephemeral populations after fires in desert habitats in southwestern USA and germinates from long-lived seed banks in response to factors in wood smoke (*9*). This fire-chasing behavior forces *N. attenuata*'s herbivore community to reestablish itself

with every new plant population, and the plant produces a wide array of direct and indirect chemical defenses in response to attack by a diverse and unpredictable herbivore community (*10*). Many of the responses are specifically elicited by signals introduced to the wound during feeding (*11*). The defensive compounds derive from various biosynthetic pathways and may function as toxins (e.g. nicotine) or inhibit digestion (proteinase inhibitors). Although some of the compounds are known to increase the fitness of plants in natural populations under herbivore attack (*3,12*) they may incur fitness costs when elicited in the absence of herbivores (*12,13*). Moreover, the herbivore-induced emission of volatile organic compounds (VOCs) functions as a defense by both repelling ovipositing *Manduca* moths (direct defense) and attracting a generalist predator, *Geocoris pallens*; this behavior facilitates top-down control of the herbivores (indirect defense)(*3*).

Most herbivore-induced responses require oxylipin signaling (*7,14*). Plants deficient in the expression or recognition of products derived from the octadecanoid pathway are unable to elicit defense compounds and are more susceptible to herbivore attack in laboratory studies (*15-17*). LOX catalyzes the oxygenation of linolenic acid to the 9- and 13-hydroperoxides. 13-hydroperoxy linolenic acid (13-HPOT) is further elaborated by enzymatic dehydration, cyclization, reduction, and β-oxidation to form jasmonic acid (JA). LOX3 deficient *N. attenuata* plants respond with an attenuated JA burst to herbivore attack and produce less nicotine, *cis*-α-bergamotene, and proteinase inhibitors than do wildtype (WT) plants (*16*). Similarly, antisense LOX-H3 transformed potato plants have reduced herbivore-induced expression of the pin2 gene (*15*). The LOX-derived 13-HPOT is a substrate for several other enzymes in the oxylipin pathways that are thought to be involved in plant-insect interactions, such as HPL and AOS. AOS produces the unstable allene oxide, which, in turn, is cyclized by allene oxide cyclase to form 12-oxo-phytodienoic acid (OPDA). The subsequent reduction and ß-oxidation of OPDA produces JA. HPL cleaves the 13-hydroperoxide into C_6-aldehydes and C_{12}-oxoacids. C_6-aldehydes and the derived alcohols (green leaf volatiles or GLVs) are antimicrobial and can function as direct defenses against some herbivores (*18*). Moreover, some C_6-compounds may function as indirect defenses (*3*) or play a role in eliciting defense gene expression (*19*) and signaling within or between plants (*20*).

The function of oxylipins is not restricted to wound signaling and defense induction but includes the regulation of tuber growth (*15*), trichome (*17*) and flower

development (*21-23*) and UVB protection. Thus it was not at all clear how LOX-, AOS- and HPL-deficient plants would behave in the natural environment with its complexity of stressors, although the antisense lines were extensively characterized in the laboratory (*16,24*). Testing the performance of these genetically transformed lines in the rough and tumble of their natural environment is essential for understanding the function of oxylipins. In addition to an extensive laboratory profiling of the genetically transformed plant lines (*16,24*), we characterized their growth, induced production of VOCs, and evaluated their susceptibility to a specialized herbivore under field conditions.

We transplanted young rosette plants into the field plots and allowed them to establish for at least one week before experimenting (*6*). The growth rates (ANOVA, $F_{3,118}$=1.837, P=0.144) and morphology of the three transformed plant lines did not differ from those of WT plants under the non-competitive conditions of the experiment (*25*). The plants, however, did differ in their production of VOCs, a demonstrated proxy of oxylipin signaling.

The release of herbivore-induced terpenoid compounds, in particular the sesquiterpene *cis*-α-bergamotene, is a proxy for octadecanoid signaling, which in turn is responsible for other herbivore-induced changes in *N. attenuata* plants (*10,16,26*). We used an open-flow trapping design to collect VOCs individually from WT plants and from LOX-, HPL-, and AOS-deficient plants in their natural environment (*6*). Each plant had one leaf attacked by one third-instar *M. sexta* larva for 24h or remained undamaged. Undamaged plants of all 4 genotypes released similarly low levels of *cis-a*-bergamotene (ANOVA, $F_{3,11}$=0.876, P=0.483). WT plants and HPL-deficient plants released large amounts of *cis-a*-bergamotene and a suite of other terpenoid compounds after *Manduca* damage (*6*). In contrast, *cis-a*-bergamotene emission from herbivore-damaged as-*lox* and *as-aos* (ANOVA, $F_{3,10}$=44.56, P<0.0001; Bonferroni *post hoc* P<0.05) plants remained low (Fig. 1A). These results confirm earlier laboratory findings (*16,24*) and demonstrate the silencing of octadecanoid signaling of plants grown in a natural environment.

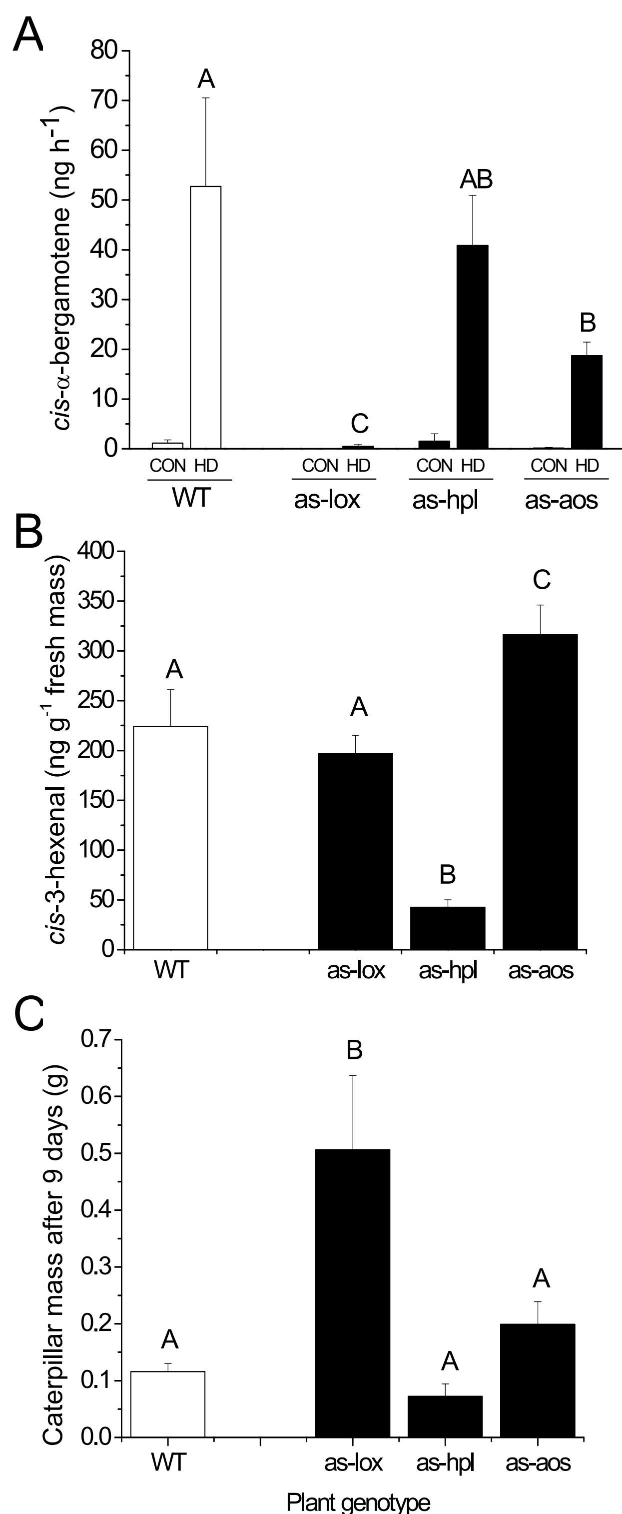

Fig. 1. Volatile organic compound emission and herbivore susceptibility of *Nicotiana attenuata* wild type (WT) plants and plants transformed to silence LOX (as-*lox*), HPL (as-*hpl*) and AOS (as-*aos*) activity. (A) Mean (+ SEM) *cis*-α-bergamotene emission (ng h^{-1}) from undamaged (CON) and *Manduca sexta* hornworm-damaged (HD) plants of the four genotypes. (B) Mean (+ SEM) emission of *cis*-3-hexenal (ng g^{-1} fresh mass) measured with a

portable GC (z-Nose™) in the headspace of excised leaves that had been mechanically wounded. (C) Mean (+ SEM) caterpillar mass (g) after 9 days of development on WT plants compared to the genetically transformed lines. Different letters designate significantly different means as informed by a Bonferroni *post hoc* (P<0.05) test of an ANOVA.

In addition to the open-flow VOC trapping design, we used a portable gas chromatograph (z-Nose™)(*27*) to characterize the wound-induced emissions of the green leaf VOC, *cis*-3-hexenal, from WT and transformed plants (*6*). As-*hpl* plants released significantly less *cis*-3-hexenal than did WT, as-*aos* and as-*lox* lines immediately after damage to leaf tissues (ANOVA, $F_{3,19}$=33.07, P<0.0001; Bonferroni *post hoc* P<0.05; Fig. 1B), which demonstrates that the production of green leaf VOCs, such as *cis*-3-hexenal, requires the activity of HPL but not LOX3. Interestingly, AOS-deficient plants had significantly higher *cis*-3-hexenal emission levels after the mechanical damage than did all other plant lines (Bonferroni *post hoc* P<0.05; Fig. 1B), which suggests a potential channeling of AOS substrates into the HPL cascade (*24*).

The altered production of secondary metabolites, such as terpenoids, in as-*lox* plants was correlated with reduced resistance to attack by the specialist herbivore *Manduca sexta*. Freshly hatched *M. sexta* caterpillars, which fed on rosette-stage, field-grown WT plants and similar-sized plants of the three different transformed lines (*6*), had the fastest weight gain on LOX-deficient plants (ANOVA, $F_{3,23}$=5.83, P=0.0041). After 9 days of development on as-*lox* plants, they were 4.4-fold heavier than caterpillars feeding on WT plants and were 7-fold and 2.5-fold heavier than those on HPL- and AOS-deficient plants (Bonferroni *post hoc* P<0.05), respectively (Fig. 1C). The increased caterpillar performance on as-*lox* plants confirms earlier laboratory results (*16,24*) and characterizes as-*lox* plants as hosts of increased susceptibility. Interestingly, in laboratory experiments, herbivore resistance can be restored by treating as-*lox* plants with methyl jasmonate (*24*).

The initial field trials demonstrated that the three transformed plant lines (as-*lox*, as-*aos*, as-*hpl*) have similar characteristics in the field as in the laboratory and differ in their responsiveness to damage by one particular herbivore. However, the herbivore community of *N. attenuata* is diverse and includes piercing-sucking, leaf-mining, and leaf-chewing herbivores. Moreover, the actual composition of the

herbivore community can be extremely variable and is influenced by the random occurrence of fire-chasing *N. attenuata* plants (*9*) as well as by plant-mediated interactions with different herbivore species and members of the third trophic level (*3,10*). Therefore, the exposure of genetically manipulated plants to their natural herbivore community provides a realistic evaluation of the role played by oxylipin-mediated herbivore-induced responses in structuring the herbivore community.

We transplanted 80 young rosette-stage plants in groups of four into a field plot along a linear transect with at least 1m between the groups (*6*). Each group comprised one WT plant and one plant of each of the following transformed lines: as-*lox*, as-*hpl*- and as-*aos*. An extensive prior analysis of plants transformed with empty vector constructs revealed no differences in any measured herbivore-resistance trait or growth and reproduction when compared to WT plants (*13*). Within the groups in our field trial, the positions were randomly assigned with 40cm between the plants (*6*). During the 2002-3 field seasons, we observed damage from herbivores of all three feeding guilds (leaf miners, leaf chewers, piercing-sucking) but found very few plants that were attacked by the most damaging herbivores of previous field seasons: the leaf-chewing sphingid hornworms *Manduca quinquemaculata* and *M. sexta,* and the piercing-sucking mirid bug *Tupiocoris notatus* (*3,10*). Thus the observed damage remained relatively low but allowed a clear evaluation of the distribution of herbivory on the antisense lines.

If plants within a population show induced defense responses to herbivory and herbivores can freely choose among plants, herbivory would be predicted to be equally distributed. Plants lacking the ability to respond to herbivore attack are more susceptible and should be preferred over responsive plants (*4*). The results of our field trials support these predictions. We found dramatically more herbivore damage on as-*lox* plants than on WT, as-*hpl,* or as-*aos* plants (ANOVA, $F_{3,120}$=24.19, P<0.0001; Bonferroni *post hoc* P<0.05; Fig. 2A; Fig. S1A), which corresponds to the increased performance of *M. sexta* caterpillars on as-*lox* plants in the laboratory (*16*) and in the field trials reported here. Similarly, herbivores attacked a significantly greater proportion of as-*lox* plants compared to all other lines in the experimental population (Fig. 2A). While herbivory was equally distributed among WT, as-*aos and* as-*hpl* (Pearson chi-square test χ^2<2.518, P>0.05), it was significantly higher on plants with attenuated induced responses (as-*lox*; Pearson chi-square test χ^2>49.207, P<0.0001),

which suggests that a plant's ability to elicit defenses influences the distribution of herbivory within a plant population.

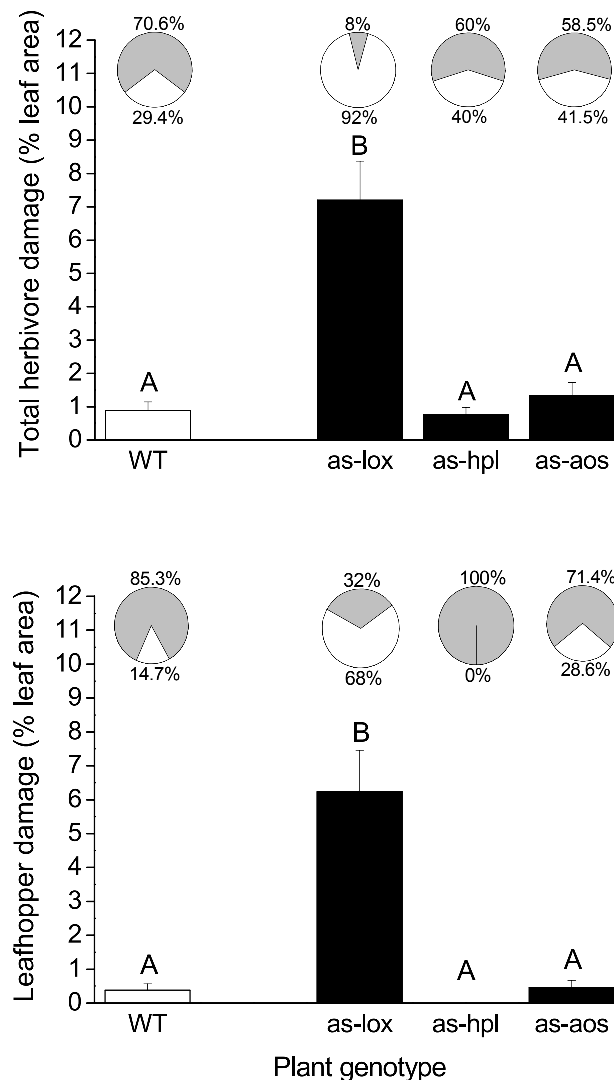

Fig. 2. Herbivore damage on field-grown *Nicotiana attenuata* wild type (WT) plants and plants transformed to silence LOX (as-*lox*), HPL (as-*hpl*) and AOS (as-*aos*) activity. (A) Mean (+ SEM) percentage of leaf area damaged by herbivores (bar graph) and proportion of plants in the population that were attacked by herbivores (white in the pie chart) or remained undamaged (grey in the pie chart). (B) Mean (+ SEM) percentage of leaf area damaged by *Empoasca* spp. leafhoppers (bar graph) and proportion of plants in the population that were attacked by leafhoppers (white in the pie chart) or remained undamaged (grey in the pie chart). Different letters designate significantly different means as informed by a Bonferroni *post hoc* ($p<0.05$) test of an ANOVA.

Moreover, a more detailed analysis of the herbivore community revealed that the herbivore-induced plant responses can alter the host breadth of generalist herbivores. In comparison to previous study years, we found two novel herbivore species on the experimental plants: the leafhopper *Empoasca* spp.*(28)*(Fig. 3) and the western cucumber beetle *Diabrotica undecimpunctata tenella* Le Conte (Fig. S1C). In fact, most of the observed damage on LOX-deficient plants resulted from just one of these, *Empoasca* spp., which is an unusual herbivore on *N. attenuata* plants.

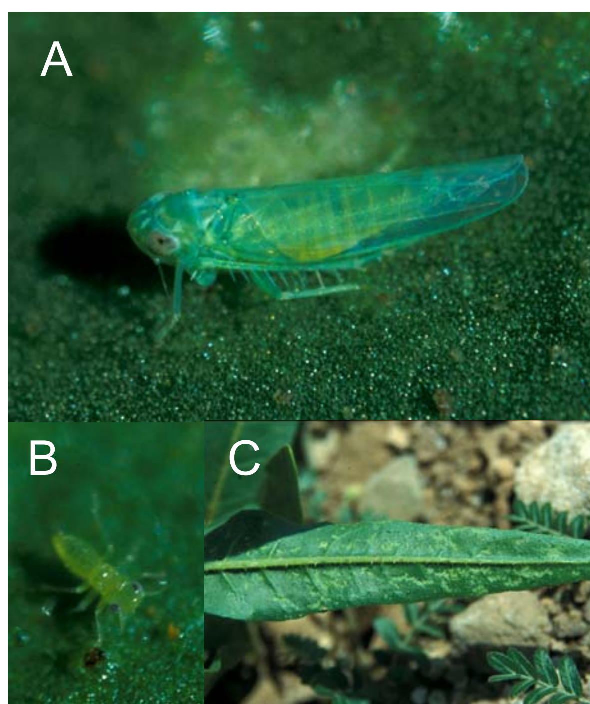

Fig. 3. *Empoasca* spp. leafhoppers on as-*lox Nicotiana attenuata* plants. (A) Adult *Empoasca* spp. (B) *Empoasca* spp. nymphs were exclusively found on as-lox plants. (C) Visual damage by *Empoasca* spp. on as-*lox N. attenuata* plants.

In 12 quantitative formal surveys of 600 plants conducted over four consecutive years, we found a total of only 4 *Empoasca* spp. specimens on *N. attenuata* plants growing in native populations (Table S1). In none of these surveys, as well as in informal observations of more than 40,000 plants over the past decade of field work has there been evidence of *Empoasca* feeding damage. *Empoasca* spp. is a highly mobile, opportunistic herbivore that attacks *Datura wrightii*, *Solanum americanum,* and *Mirabilis multiflora*, which were abundant in the study area. In our

field trials with different *N. attenuata* lines, the as-*lox* plants received significantly more *Empoasca* damage than did the other transformed lines (as-*aos*, as-*hpl*) and the WT (ANOVA, $F_{3,121}$=30.25, P<0.0001, Bonferroni *post hoc* P<0.05; Fig. 2B). More than 68% of the as-*lox* plants in the experimental population were damaged by *Empoasca,* while the proportion of damaged plants in the other lines did not exceed 29% (Fig. 2B). More importantly, *Empoasca* females oviposited on as-*lox* plants, and these plants were susceptible to attack from the emerging leafhopper offspring (Fig. 3B). We found *Empoasca* nymphs on 81% of the attacked as-*lox* plants but none on WT, HPL-, and AOS-deficient lines. Moreover, when given the choice (*6*), adult leafhoppers clearly prefer as-*lox* plants over WT plants (Paired Student's t-test, t=-4.919, P=0.0012; Fig. 4). Interestingly, we found no *Empoasca* damage on as-*hpl* plants, which suggests, as demonstrated in laboratory experiments with *M. sexta* (*24*), that wound-induced GLVs may function as feeding stimulants.

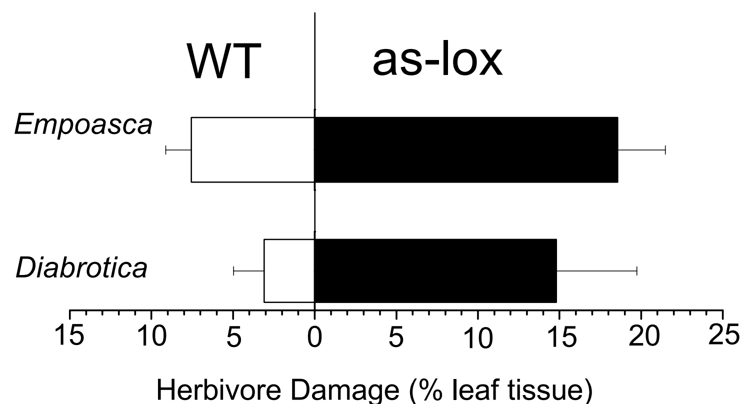

Fig. 4. Mean (+ SEM) leaf tissue damage on wildtype (WT) and as-*lox* plants after 3 days of attack by *Empoasca* spp. leafhoppers or *Diabrotica undecimpunctata* leaf beetles respectively. Ten *Empoasca* and two *D. undecimpunctata*, respectively, were allowed to choose between a WT and an as-*lox* plant that had been potted together and covered with insect mesh. Both experiments were replicated 10-times.

The reduced resistance of LOX-deficient plants was correlated with an altered octadecanoid-dependent gene expression in response to *Empoasca* attack. With a cDNA microarray analysis, we compared transcript accumulation in response to *Empoasca* feeding in WT and as-*lox* plants, to identify octadecanoid-dependent

transcriptional responses (Fig. S2; Table S2). Of 240 *N. attenuata* genes spotted on the microarray, 54 genes showed significantly increased LOX-dependent transcript accumulation after *Empoasca* damage and 50 showed decreased accumulation (*6*). A series of defense-related genes such as trypsin proteinase inhibitor (TPI) and threonine deaminase had increased expression levels, while others, such as xyloglucan endotransglucosylase/ hydrolase or GAL83 and many of the photosynthesis-related genes (e.g. small subunit of ribulose bisphosphate carboxylase), were down-regulated in response to *Empoasca* attack and showed little if any regulation in as-*lox* plants (Table S2). These results suggest that a complex LOX-dependent regulation of primary and secondary metabolism in *N. attenuata* mediates resistance to the piercing-sucking *Empoasca* leafhoppers. Moreover, LOX-dependent octadecanoids may play a direct role in host-plant selection by enabling herbivores to differentiate between responsive and non-responsive plants. For example, the corn earworm, *Helicoverpa zea*, uses induced jasmonate and salicylate to activate four of its cytochrome P450 genes that are associated with detoxification either before or concomitantly with the biosynthesis of allelochemicals (*29*). However, our field results demonstrate that octadecanoid signaling plays a role in plant defense against both piercing-sucking and leaf-chewing insects, suggesting common response mechanisms against attack from members of these two feeding guilds.

In addition to *M. sexta*, which was affected by LOX-mediated plant resistance traits, we found a novel leaf-chewing herbivore on *N. attenuata*, the leaf beetle *Diabrotica undecimpunctata tenella* Le Conte (Fig. S1C). It often feeds on *Datura wrightii* and *Cucurbita foetidissima* flowers in the study area and was observed on *N. attenuata* plants exclusively in this study and only on as-*lox* plants. To test whether or not this clear preference in the field is caused by the decreased expression of LOX, we allowed the beetles to choose between WT and as-*lox* plants. The choice experiment revealed a clear preference for as-*lox* plants compared to WT plants (Paired Student's t-test, t=-4.050, P=0.003; Fig. 4).

Our results demonstrate that the octadecanoid-mediated inducibility of plants is crucial for the oviposition decision and the opportunistic host selection behavior of *Empoasca* spp. and *D. undecimpunctata* and thereby defines host breadth. Host selection seems determined not only by the plant's chemical phenotype and external mortality factors (predation pressure, abiotic stress)(*30*) but also by the plant's ability to elicit responses to herbivory. The additional finding that induced responses to

herbivory influence the distribution of herbivory within a plant community, points to the usefulness of genetically silenced plants in ecological research, which has been an unfortunate casualty of the polarized debate surrounding the use of transformed plants in agriculture.

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Supplementary Material

Material and Methods

All experiments were conducted at our field station at the Lytle Preserve, Beaver Dam Wash in Santa Clara, 84765, Utah (37°08'45"N, 114°01'11"W) in two consecutive years 2002, 2003.

Plant genotypes and plant growth

The wild-type (WT) *N. attenuata* plants were grown from seeds that were field collected from a native population (DI Ranch, Santa Clara, UT, USA) and subsequently inbred for 11 generations. The resulting WT line was transformed by *Agrobacterium tumefaciens* (strain LBA 4404) to express *N. attenuata* lipoxygenase NaLOX3 (as-*lox*), allene oxide synthase (as-*aos*) and hydroperoxide lyase (as-*hpl*) in antisense direction. Vector construction and transformation procedure are described by Krügel et al.(*1*). The characterization of the genetically transformed plants revealed different levels of phenotype expression. We chose the plant lines, which expressed the strongest phenotypes in previous laboratory studies, A300-1 (as-*lox*), A337-2 (as-*hpl*), A340-1 (as-*aos*) (*2,3*). Interestingly, the as-*aos* line with the strongest phenotype (A340-1) while having similar reductions in elicited JA, *cis*-α-bergamotene and TPIs, they were not significantly impaired in elicited nicotine accumulations or *M. sexta* resistance in laboratory feeding trials *(2,3)*. We interpret this difference between the observed resistance phenotypes of as-*lox* and as-*aos* plants as reflecting either the greater importance of substrate flow or the requirement of LOX-derived elicitors other than JA. However, *N. attenuata* LOX3 is a member of a three-gene family, and extensive metabolic profiling (JA and GLVs) revealed no

evidence for silencing of LOX2 or LOX1 in the asLOX3 lines (*2*). Additional transcriptional analysis is ongoing.

The seeds were germinated on growth media [Gamborg's B5 medium with minimal organics (Sigma) and 0.6% (w/v) phytagel (Sigma)], using a standard procedure (*1*) and the seedlings remained on the medium for 5 days. In the young seedling stage, plants were transplanted into peat soil on seed trays and watered with distilled water. After 10 days of acclimation to soil, the seedlings were transferred to root trainers, where they remained an additional 5 days, before being transplanted into 4L pots in which the plants remained until the end of the experiment. By growing plants in pots allowed us to control the water and fertilizer supply. Plants were fertilized in 7-day cycles with 20mL fertilizer (Miracle-Gro™ all purpose plant food, Scotts Miracle-GroProducts, Inc. Marysville, OH, USA)

VOC trapping using an open-flow design

We transplanted potted young rosette plants in groups of four into a field plot on 11th June 2002, each group with a size-matched WT plant and a LOX-, HPL- and AOS-deficient plant, respectively. The pots were completely dug into the ground and covered with ambient soil. Within the groups the positions were randomly assigned with 40cm between the plants. The groups were planted along a linear transect with at least 1m between groups. After one week of growth, four plants of each genotype received one 3rd instar caterpillar on the source-sink transition leaf. Another four plants of each genotype remained undamaged. After 24h of damage, the source-sink transition leaves of all 32 plants in the experiment were each enclosed in a 400-mL polystyrene chamber fitted with holes at both ends. With a portable 12V DC vacuum pump (Gast Mfg., Benton Harbor, MI), we pulled air through the chamber at 450-500mL min^{-1} (measured by a mass flow meter; Aalborg Instruments, Orangeburg,

NY) and subsequently through a charcoal air-sampling trap (ORBO-32™; SUPELCO, State College, PA) for 6h. The VOCs were eluted from the traps using dichloromethane and further analyzed by gas chromatography-mass spectrometry (GC-MS). The analysis was performed at the field site with a Shimadzu quadrupole GC-MS (Model 5000) programmed as described elsewhere (*4*). Compounds were identified by comparing retention times and mass spectra with those of authentic standards. The sampled amounts were calculated using tetraline as an internal standard that had been applied to the traps before elution. Here we present only data of *cis*-α-bergamotene emission because we had shown in earlier studies that the emission of this sesquiterterpene is correlated with the production of a series of other secondary metabolites, which require both wounding and the application of an herbivore-specific elicitor and intact oxylipin signaling to be activated (*2,5,6*). The amounts of induced *cis*-α-bergamotene released from the four different lines in this experiment (Fig. 1) were compared using a Bonferroni *post-hoc* test of an ANOVA of the log-transformed data.

Green leaf VOC trapping using the z-Nose™

To analyze the GLVs released immediately after a mechanical damage to leaves, we developed a field method for a portable GC (z-Nose™) that allows for a rapid and online measurement of low molecular weight compounds in a leaf's head-space (*7*). We used 8 undamaged plants of each of the four genotypes, which were grown in the same experimental plot under the same conditions as the plants in the VOC trapping experiment described above. We excised the source-sink transition leaf from 10 plants of each line and damaged each with 10 lines of puncture holes using a pattern wheel. The damaged leaf was weighed and placed in a 200-mL glass chamber

into which the headspace VOCs accumulated for 1min. The head-space sample was immediately drawn from the chamber and analyzed in the z-Nose™, which was programmed as follows: pump time, 30s; data rate, 20 ms/point; analysis time 30s; sensor and column start temperature 45°C; inlet temperature 180°C; column temperature ramp, 175°C at 10°C per second. The z-Nose™ was controlled by a laptop computer and operated with a deep-cycling 12V car battery connected to a solar panel. C*is*-3-hexenal was identified by its mass spectra determined by a PTR-MS (*2*). We used *trans*-2-hexenal as the calibration standard for quantification and expressed the measured amounts relative to the leaf mass (*2*). We log-transformed the data and analyzed them with ANOVA across all plant lines.

Manduca *performance on genetically transformed plant lines in nature*

Potted plants of the three different genetically transformed lines and the WT line were transplanted into an irrigated field plot along 4 transects on 12th June 2002. The pots were completely dug into the ground and covered with ambient soil so that the plants were naturally positioned and could initiate root growth out of the pot. The minimal distance between the plants was 40cm and the different plant lines were randomly assigned to the different positions along the 4 transect lines. We allowed plants to grow and to establish for one week after transplanting before experiments were started. To evaluate the herbivore susceptibility of plants under field condition, one freshly hatched *Manduca sexta* caterpillars was placed on each of the rosette-stage WT plants (n=10) and on similar-sized plants of the three different transformed lines (each n=10) and allowed to feed freely. The caterpillars were weighed on site after 9 days of development. We computed the log-transformed caterpillar masses in an ANOVA and compared the results from the different lines using a Bonferroni *post-hoc* test.

Field exposure of the transformed lines

We transplanted young potted rosette-stage plants in groups of four into a field plot on 9th June 2002, each group with size-matched WT, as-*lox*-, as-*hpl*- and as-*aos*-plants,. Within the groups, the positions were randomly assigned with 40cm between plants. The groups were planted along a linear transect with at least 1m of each other. As in the earlier experiments, the pots were completely dug into the ground. Two days after transplanting, we monitored performance, the occurrence of herbivores and their damage for 14 days until the plants started flowering. As plants began to elongate and produce flowers, plants were examined daily and all flowers were removed before opening and anthesis to meet the performance standards determined in the Code of Federal Regulations (7CFR340.3(c)) for the Introduction of Organisms Altered or Produced Through Genetic Engineering. Consequently, direct fitness measures were unattainable in this experiment. The herbivore damage levels on the different plant genotypes were compared with a Bonferroni *post-hoc* test of an ANOVA.

Choice experiments

Rosette-stage WT plants and an as-*lox* plant were planted together in one 3.8L pot on 10th July 2003 and grown together for one week. The pots were covered with insect mesh and 10 *Empoasca* individuals were placed in the enclosure and allowed to freely choose and feed on the plants. After two days we estimated the damage of each of the plant pairs and compared genotypes with a paired Student's *t*-test. In a similar experiment, we allowed two field-collected *Diabrotica undecimpunctata tenella* Le Conte beetles to choose between an as-*lox* and a WT plant and recorded the damage after two days.

Nucleic acid analysis

Potted as-*lox* and WT plants were transplanted into an irrigated field plot along 4 transect lines on 4th June, 2002. The pots were completely dug into the ground and covered with ambient soil as described above and were randomly assigned to different positions along 2 linear transects. After 24 days we randomly selected two *Empoasca*-attacked (after 4 days of attack) and two un-attacked plants of each of the two genotypes, WT and as-*lox*, from the entire experimental population and harvested the second and third stem leaves. A total of four leaves from two plants were pooled for each treatment: WT *Empoasca*-damaged, WT undamaged, as-*lox* *Empoasca*-damaged, as-*lox* undamaged. In general the *Empoasca* damage observed on WT plants was dramatically less than that on as-*lox* plants (Fig. 2B), which should be considered when interpreting the results of the expression analysis. We conducted three complementary microarray hybridizations, which allowed a critical and conservative evaluation of the transcriptional patterns (see below and Table S2 and Fig. S2).

The harvested leaves were immediately transferred into a 50mL Falcon™-tube filled with 10mL TRI-Reagent™ (SIGMA) and homogenized in the field using a battery operated Polytron™ homogenizer (KINEMATICA). After one hour, RNA was extracted at the field station following the TRI-Reagent™ protocol. The RNA was stored at -20°C in 75% ethanol (SIGMA) and later transported to our laboratory in Germany, were it was further processed for microarray hybridization.

The isolation of mRNA and cDNA synthesis was performed as described by Halitschke et al. (*8*). We conducted 3 complementary microarray hybridizations. In the first, the cDNA derived from *Empoasca*-damaged WT plants was labeled with Cy3 fluorescent dye and hybridized against cDNA, which originated from undamaged WT plants and was labeled with Cy5 fluorescent dye. On the second microarray, we

hybridized cDNA from *Empoasca*-damaged as-*lox* plants (Cy3) against cDNA derived from undamaged as-*lox* plants (Cy5). On the third microarray we hybridized cDNA from *Empoasca*-damaged as-*lox* plants (Cy3) against cDNA from *Empoasca*-damaged WT plants (Cy5). The microarray, described by Halitschke et al. (*8*), contains PCR fragments of 240 *N. attenuata* genes identified by differential display analysis of *M. sexta*-induced gene expression. The criteria for detection of up- or down-regulation are also described by Halitschke et al. (*8*).

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Table S1. *Empoasca spp*. observations on *N. attenuata* at various field sites in Southwest Utah in three consecutive years. We observed no *Empoasca* damage or *Empoasca* nymphs in the censused field populations.

Date	Population	Number of plants censused	Number of *Empoasca* individuals
29.May 1999	Pahcoon spring	140	0
15.June 1999	Pahcoon spring	120	0
02.July 1999	HI91(98)	30	1
15.June 2000	Apex mine (99)	66	1
16.July 2000	Apex mine (99)	19	0
10.May 2001	Montaqua wash	17	0
10.May 2001	Pahcoon flat	38	0
12.June 2001	Beaver Dam Wash	18	2
16.June 2001	HI91 (00)	60	0
10.June 2003	Apex mine	36	0
20.June 2003	Montaqua	30	0
07.July 2003	Apex mine	36	0

Table S2. (see appendix) Mean expression ratio (ER) of genes after *Empoasca* damage in (A) wildtype *N. attenuata* plants,(B) in plants transformed to silence lipoxygenase and (C) in as-*lox* plants compared to WT plants (see Fig. S2 for hybridization scheme). Presented are the mean values of ERs calculated from two PCR fragments, which were each spotted in quadruplicates on the array. The arbitrary thresholds for up- (red) and down- (green) regulation are 1.25 and 0.75 respectively. The complementarity of the hybridizations allows an accurate and conservative identification of genes whose expression depends on LOX-associated signals and were either increased (↑) or decreased (↓) in response to *Empoasca* attack. Genes for which no clear change in the expression could be observed are labeled with "-".

We identified three major categories of LOX-dependently up-regulated genes. In the first, increased transcript accumulation after *Empoasca* attack occurred only in the WT plants (e.g. #352, #400, #600), suggesting the complete loss of inducibility of the gene expression in as-*lox* plants. In this category we find many of the genes that had been isolated in a differential display analysis (DDRT-PCR) of transcriptional changes in WT *N.attenuata* plants after damage by *M.sexta* attack(*9*). In the second category are genes, which are up-regulated in both the WT and as-*lox* plants, but have a lower relative expression level in *Empoasc*a attacked as-*lox* plants compared to attacked WT plants, suggesting the involvement of additional signals, other than those derived from LOX and/or a reduced constitutive expression of the gene in as-*lox* plants. This category includes defense-related genes such as *N.attenuata* threonine deaminase and *N.attenuata* proteinase inhibitor (e.g. #342, #666). The genes of the third category are up-regulated in WT plants but down-regulated in as-*lox* plants (e.g. #614), suggesting that LOX expression amplifies the expression of these genes and an additional herbivory-induced LOX-independent signal causes a reduced expression.

Similarly, the analysis identified three major categories of LOX-dependently down-regulated genes. The first category was composed of genes that had decreased expression levels only in WT plants in response to *Empoasca* damage (e.g. #348, #410, #604, 644), which suggests a complete loss of elicited reductions in gene expression in as-*lox* plants.

Genes with this expression pattern are defense-related genes such as germin homolog (#348) and *Solanum tuberosum* GAL83 (#410). In the second category, we find genes, which have decreases transcript accumulation in both the WT and as-*lox* plants in response to *Empoasca* attack, but have increased or decreased constitutive transcript levels in as-*lox* plants (e.g. #414 #584 #608 #726), suggesting an alteration of the constitutive expression levels in as-*lox* plants. Most of the photosynthesis-related genes fall into this category. The third category comprises genes, which are down-regulated in WT plants, but up-regulated in as-lox plants in response to *Empoasca* attack (e.g. #346, #446, #470), which suggests LOX-derived products mediate a suppression of gene expression which is removed in as-*lox* plants. Genes, involved in cell wall modification (XTH; #446, #470) are in this category.

Our analysis is likely to underestimate the number of genes that have LOX-dependent regulation because *Emoposca* leafhoppers damaged as-*lox* plants significantly more than they did WT plants (Fig. 2B). We estimate that 23.3% (56 of 240) of the genes identified as being not regulated in a LOX dependent manner (e.g. #382, #418, #564), may be incorrectly classified (Type II error) due to the small amount of damage received by WT plants.

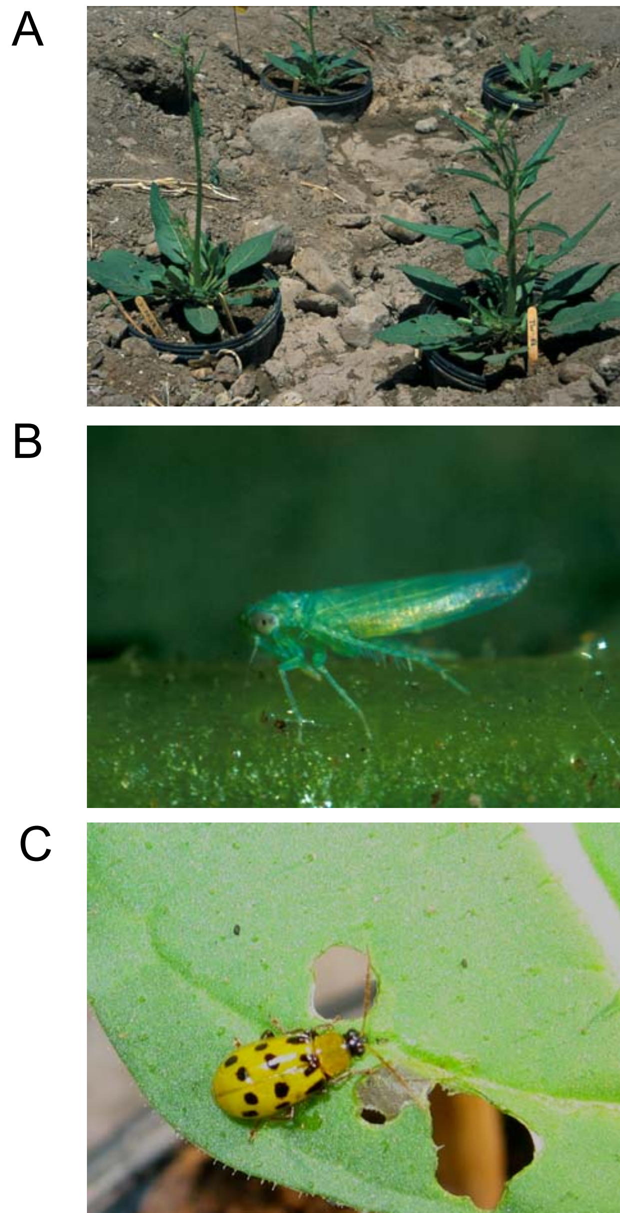

Figure S1. Herbivores on as-*lox* *Nicotiana attenuata* plants. (A) Differential leaf area loss of WT (right) and as-*lox* (left) *N. attenuata* plants after *Manduca sexta* attack in nature. (B) Adult *Empoasca* spp. (C) *Diabrotica undecimpunctata tenella* Le Conte leaf beetle.

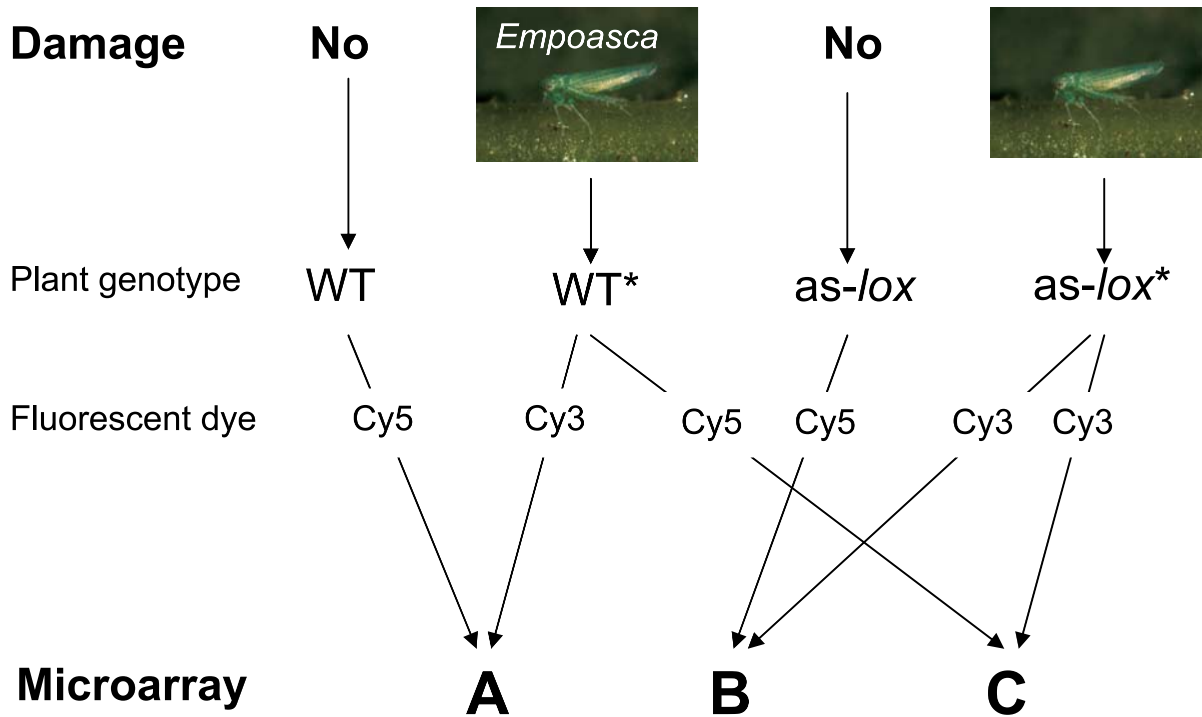

Figure S2. Microarray hybridization scheme. Fluorescently labeled cDNA from *Empoasca*-damaged WT (Cy3)(WT*) plants was hybridized against cDNA derived from undamaged WT plants (Cy5)(WT)—hybridization **A;** cDNA from *Empoasca*-damaged as-*lox* plants (Cy3) (as-*lox**) was hybridized against cDNA from undamaged as-*lox* plants (Cy5)(as-*lox*)—hybridization **B;** and cDNA from *Empoasca*-damaged as-*lox* plants (Cy3) (as-*lox**) was hybridized against cDNA from *Empoasca*-damaged WT plants (Cy5)(WT*)—hybridization **C**.

3. Discussion

In a comprehensive analysis of literature on plant resistance Strauss et al. (2002) found evidence for substantial fitness cost in the majority (ca. 80%) of the surveyed studies. The existence of these direct and ecological costs with magnitudes of up to 400% supports a selective advantage of induced resistance under highly variable or unpredictable conditions. The fire-chasing germination behavior of *N. attenuata* results in an ephemeral occurrence under highly unpredictable environmental conditions that include the composition of the herbivore community (Kessler and Baldwin, 2004) and a concomitant high intraspecific competition and *N. attenuata* expresses several induced defenses. These characteristics and the extensive characterization of *N. attenuata*'s ecology (Baldwin, 2001) motivated the development of a set of sophisticated molecular tools to analyze the mechanism of induced resistance in this model plant. The defensive function of herbivore-induced plant traits including the production of the toxic alkaloid nicotine (Steppuhn *et al.*, 2004; Voelckel *et al.*, 2001a) and antidigestive protease inhibitor (PI) activity (Zavala *et al.*, 2004) have been elegantly demonstrated by loss-of-function studies directly targeting the defense genes. The differential activation of these traits in response to mechanical wounding and herbivory requires specific recognition mechanisms and signaling cascades in the plant, and the identification and manipulation of these regulatory mechanisms was addressed in the collection of manuscripts presented here.

Nicotiana attenuata *specifically recognizes* Manduca sexta *herbivory*

In previous studies the treatment of mechanically wounded *N. attenuata* plants with R of *M. sexta* larvae revealed specific changes in gene expression (Hermsmeier *et al.*, 2001; Schittko *et al.*, 2001), JA accumulation and ethylene release (Kahl *et al.*, 2000; Schittko *et al.*, 2001), nicotine biosynthesis (Kahl *et al.*, 2000; Winz and Baldwin, 2001), PI activity (van Dam *et al.*, 2001), and VOC release (Halitschke *et al.*, 2000). Fatty acid-amino acid conjugates (FACs) in R of *M. sexta* larvae clearly allow *N. attenuata* plants to recognize the received damage as herbivore attack and are necessary and sufficient to elicit the herbivore-specific alterations of the plant's response to wounding (Manuscript I and II). FACs in the larval R are responsible for the R-specific amplification the wound-induced JA accumulation and the subsequent release of VOCs (Manuscript I). Furthermore, similar expression patterns of

herbivore-responsive genes are elicited by treatments with *Manduca* R and FAC solutions (Manuscript II) and the up-regulation of defense-related genes (secondary metabolism) and down-regulation of growth- and photosynthesis-related genes (primary metabolism) suggest a substantial metabolic shift in the plant in response to herbivory. Application of only two of the at least 8 FACs in *Manduca* R (Manuscript I) was sufficient to elicit the majority of the R-induced transcriptional response (Manuscript II) whereas glucose oxidase, which has been identified as an elicitor in the salivary glands of *Helicoverpa zea* larvae (Musser *et al.*, 2002), is unlikely to play a major role in the elicitation of *Manduca*-induced responses in *N. attenuata* (Manuscript II). A recent study confirmed the pivotal role of FACs as elicitors of the R-specific changes in gene expression and JA accumulation shown in manuscript I and II and demonstrates the FAC-dependent induction of trypsin protease inhibitor (TPI) activity (Roda *et al.*, 2004). Extending the investigations of the elicitor activity of FACs on additional signaling cascades (e.g. ethylene) and resistance traits (e.g. nicotine accumulation) will further pinpoint the overall contribution of FACs and potential other elicitors in *Manduca* R to the induced defense response in *N. attenuata*.

Nicotiana attenuata plants do not respond to the application of free fatty acids (Manuscript I) and amino acids (Roda *et al.*, 2004), emphasizing the requirement of a modification of ingested fatty acids in the caterpillar digestive system (Paré *et al.*, 1998). Insect-derived elicitors that induce resistance in the attacked host plant represent an insect trait which is unfavorable in the light of the ‘co-evolutionary arms race’ between plants and herbivores; indicating an important function of the FACs for the herbivore. FACs posses strong emulsifying properties and Collatz and Mommsen (1974) suggested a digestive function for these compounds which they found in several diverse arthropod species (Collatz and Mommsen, 1974). The release of oral secretions can be considered a defensive behavior of lepidopteran larvae and chemical constituents in the secretions could serve as repellents or toxins against predatory or parasitoid enemies of the herbivore. A function of FACs in R of *M. sexta* larvae in the defense against one of its major natural enemies, the predatory bug *Geocoris pallens* (Fig. 4.)(Kessler and Baldwin, 2001) is currently elucidated.

Figure 4. *Geocoris pallens*, a predatory bug representing an important natural predator of *Manduca* larvae (Photograph: André Kessler)

The biosynthetic origin of FACs is still controversially discussed. Spiteller *et al.* (2000) isolated and characterized bacteria from guts of several lepidopteran larvae which were able to synthesize FACs (Spiteller *et al.*, 2000). In contrast, a membrane-associated enzyme catalyzing the synthesis of *N*-linolenoyl-L-glutamine has recently been isolated from tissue of *M. sexta* larvae (Lait *et al.*, 2003). Whereas Lait *et al.* (2003) did not find evidence for a bacterial origin of FAC production we clearly found FAC-producing activity in fresh *M. sexta* R and crude bacterial preparations (Dieter Spiteller and Rayko Halitschke, unpublished data). Further characterizations of the FAC-producing enzymes and the isolation of the corresponding genes are necessary to understand the evolution of the production of FACs and their functional significance for the herbivore.

Regulation of oxylipin signaling in N. attenuata

A wound-induced increase in JA accumulation and GLV emissions have been found in a large number of plant species and seem to be ubiquitous among plants (Sembdner and Parthier, 1993). Additionally, an amplification of the wound-induced JA accumulation in response to herbivore feeding (JA burst) has been observed in the wild tobacco plants *Nicotiana sylvestris* (Baldwin *et al.*, 1997) and *N. attenuata*

(Schittko *et al.*, 2000; Ziegler *et al.*, 2001). This herbivore-specific response can be attributed to the elicitor function of FACs in R of *Manduca* larvae which are necessary and sufficient to elicit the JA burst in *N. attenuata* (Manuscript I). Similar to the response in *N. attenuata* characterized in Manuscript I, the elicitation of a JA burst and the subsequent release of VOCs has recently been demonstrated in corn (*Zea maize*) plants treated with volicitin (Schmelz *et al.*, 2003); but volicitin was not active in eliciting the VOC release from lima bean plants (Koch *et al.*, 1999). In contrast, the non-hydroxylated FAC *N*-linolenoyl-L-glutamine and free linolenic acid induced the VOC release, suggesting a substrate supply for the octadecanoid pathway as the elicitation mechanism of VOC release in lima bean plants (Koch *et al.*, 1999). In *N. attenuata* the elicitor activity of FACs is not due to a simple substrate supply for JA biosynthesis because the application of free fatty acid did not result in amplified JA biosynthesis or amplified JA-induced responses (Manuscript I).

To study the regulation of oxylipin-mediated responses in *N. attenuata* more thoroughly, *N. attenuata* genes involved in oxylipin signaling were isolated and characterized and transgenic plants impaired in different oxylipin cascades were generated (Manuscript III). Antisense expression-mediated silencing of enzymes involved in JA biosynthesis, namely the specific LOX isoform NaLOX3 (as-*lox*; Manuscript IV) and the recently characterized (Ziegler *et al.*, 2001) NaAOS (as-*aos*; Manuscript V), reduced the wound- and R-induced JA accumulation in several independently transformed lines. Similarly, the wound-induced release of GLVs was suppressed by silencing the activity of NaHPL (as-*hpl*; Manuscript V). The manipulation of different steps of the two cascades revealed insight in the substrate channeling and competition between the AOS- and HPL-cascade (Fig. 5).

While antisense expression of NaLOX3 reduced the wound- and herbivore-elicited increases in JA, no effects on the wound-induced release of GLVs were found (Manuscript IV). This demonstrates that NaLOX3 specifically supplies fatty acid hydroperoxide substrates to the octadecanoid pathway but not to the HPL reaction. In potato, LOX-H1, a homolog of NaLOX2 characterized in Manuscript IV, supplies the substrate for the HPL reaction required for the release of GLVs, but does not supply the hydroperoxide for the octadecanoid pathway (Leon *et al.*, 2002). Antisense expression-mediated silencing of HPL activity reduced the release of wound-induced GLVs and amplified the wound- and herbivore-induced JA burst (Manuscript V). Conversely, silencing of AOS activity resulted in reduced JA accumulation

accompanied by an increased release of HPL-derived GLVs (Manuscript V). This negative correlation demonstrates that the cascade-specific supply of fatty acid hydroperoxide by distinct LOX isoforms (Manuscript IV; (Leon *et al.*, 2002) does not restrain substrate allocation between downstream oxylipin cascades and thereby could serve as regulatory mechanism to amplify (or suppress) individual components of the complex oxylipin signature (Fig. 5). The suppression of wound-induced *NaHPL* transcript accumulation by larval R (Manuscript V) could be the regulatory mechanism responsible for the R-induced JA burst in *N. attenuata*, which could not be explained by the expression pattern of the JA biosynthetic enzymes LOX (Manuscript IV) and AOS (Manuscript V; (Ziegler *et al.*, 2001) analyzed so far.

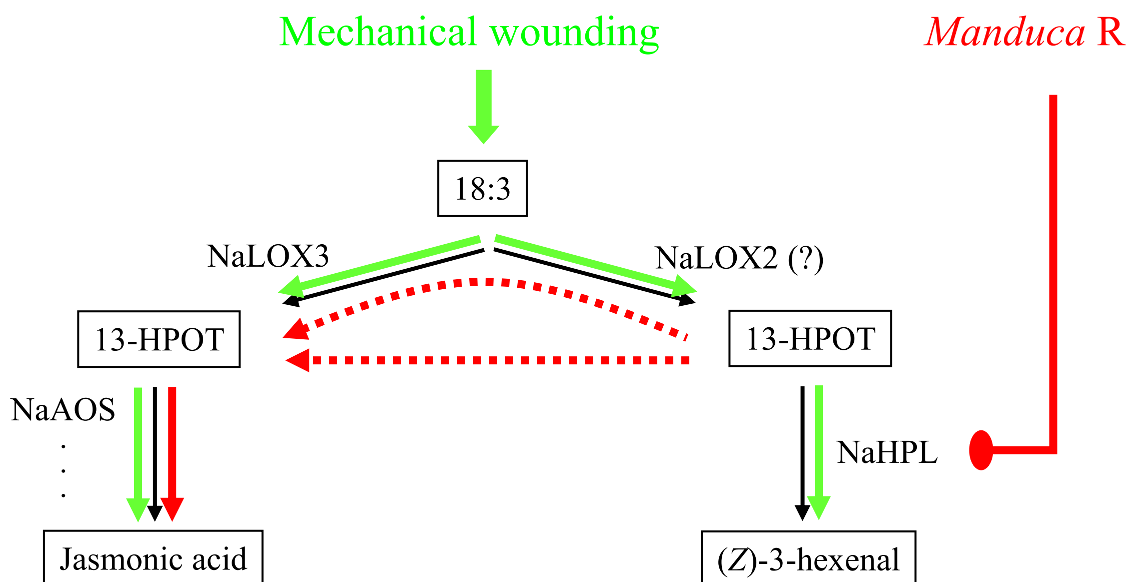

Figure 5. Proposed mechanism for the *Manduca*-induced jasmonic acid burst in *Nicotiana attenuata*. The schematic biosynthetic pathway of jasmonic acid and the green leaf volatile (GLV) (*Z*)-3-hexenal involving the sequential activity of a lipoxygenase NaLOX3/NaLOX2(?), allene oxide synthase NaAOS, and hydroperoxide lyase NaHPL is shown in black. The expression of NaLOX3, NaAOS, and NaHPL is induced by mechanical wounding (green). The application of *Manduca* sexta oral secretions and regurgitant (R) does not affect the expression of JA biosynthetic enzymes and the initial wound-induced GLV production but inhibits the wound-induced increase in NaHPL transcript accumulation. Thereby, additional fatty acid (18:3) or hydroperoxide (13-HPOT) substrate could be channeled from the HPL cascade into the JA branch of the oxylipin biosynthesis (red).

Defense signaling and herbivore resistance N. attenuata

The involvement of JA in the activation of *N. attenuata*'s defense response has been shown by elicitation experiments with synthetic MeJA and JA. The transgenic lines characterized in Manuscript III-V represent ideal tools to analyze jasmonate- and GLV-mediated responses without applying synthetic compounds which only partially mimics herbivory. Here, the consequences of altered JA- or GLV-signaling capacity were determined by analyzing herbivore-induced transcript accumulation, direct (nicotine and TPI activity) and indirect (VOC emissions) defense traits and herbivore performance on antisense-transformed plants (Manuscript IV-VI).

Deficiency in JA biosynthesis decreased plant resistance and increased the performance of *M. sexta* larvae feeding on as-*lox* plants compared with larvae feeding on WT *N. attenuata* plants (Manuscript IV and VI). This reduction in resistance was correlated with reduced expression of two direct defenses: nicotine accumulation and TPI activity (Manuscript IV). The deficiency in induced nicotine accumulation in as-*lox* plants, which showed the strongest correlation with the changes in larval performance in the experiments presented here, could be fully restored by MeJA treatment and is consistent with an important role for JA in eliciting this defensive response (Baldwin *et al.*, 1997; Winz and Baldwin, 2001). However, the involvement of signals in addition to LOX3-derived oxylipins is implicated in the elicitation of the herbivore-induced response in *N. attenuata*. For example, TPI activity was not fully recovered by MeJA treatment (Manuscript IV), suggesting the involvement of an additional herbivore-induced signal in TPI activation. Possible signals could be ethylene, which is induced by *M. sexta* feeding (Kahl *et al.*, 2000) and has been shown to synergize PI elicitation in tomato (O'Donnell *et al.*, 1996), or intermediates in the octadecanoid cascade upstream of JA, such as OPDA, which has been shown to elicit the production of numerous secondary metabolites (Blee, 2002).

Larvae feeding on as-*aos* plants with a similar reduction of wound- and R-induced JA accumulation did not develop differently from larvae feeding on WT plants with an intact JA signaling cascade (Manuscript V and VI). The main difference between as-*aos* and as-*lox* plants is that the induced nicotine production was suppressed only in the latter. The difference in the observed resistance phenotypes of plants impaired in either of the first two consecutive steps of JA biosynthesis (Fig. 2) could reflect the highly substrate-limited biosynthesis of JA or compensation of reduced NaAOS activity by a second AOS enzyme which has been

recently identified (Hui *et al.*, 2003). Alternatively, oxylipins other than JA, which were not analyzed, could be involved in the elicitation of induced resistance in *N. attenuata*. GLV-deficient as-*hpl* plants were more resistant against herbivory by *M. sexta* (Manuscript V and VI), but the reduced larval performance could not be explained by patterns of induced defenses (nicotine accumulation and TPI activity). The recovery of larval development and increased leaf consumption in response to supplementation of as-*hpl* plants with synthetic GLVs (Manuscript V) indicates a direct function of GLVs as feeding stimulants. In contrast, the application of synthetic GLVs had a direct negative effect on aphid population development on tomato plants (Hildebrand *et al.*, 1993) and HPL depletion of potato plants (Vancanneyt *et al.*, 2001) increased aphid performance (Vancanneyt *et al.*, 2001). The latter study did not discriminate between secondary indirect effects resulting from altered plant signaling and direct effects of HPL-derived products but aphids clearly respond to GLVs as has been demonstrated by electroantennogram analyses (Park and Hardie, 1998; Visser and Yan, 1995). Further analysis is required to elucidate the plant resistance traits mediated by the HPL cascade and mechanisms of direct responses of feeding herbivores to GLVs which have been elegantly shown to effect oviposition behavior of herbivores and predator attraction (DeMoraes *et al.*, 2001; Kessler and Baldwin, 2001).

In WT plants, *M. sexta* attack and wounding with application of *Manduca* R elicit increased expression of defense-related genes and reduced accumulation of transcript of genes involved in photosynthesis and primary metabolism (Manuscript II; (Hermsmeier *et al.*, 2001; Hui *et al.*, 2003; Schittko *et al.*, 2001). In *M. sexta*-attacked as-*lox* plants, the defense-related transcripts, including genes coding for TPI, threonine deaminase (TD), xyloglucan endotransglucosylase/hydrolase (XTH), and HPL were decreased, while the transcripts of two photosynthetic genes coding for a light-harvesting complex protein (LHCII) and oxygen-evolving complex polypeptide of photosystem II (PSII-O_2) were increased compared with the expression in WT plants, demonstrating that an oxylipin produced via the LOX3-mediated octadecanoid pathway regulates these genes (Manuscript IV). The total number of genes differentially regulated in as-*aos* plants was attenuated compared with as-*lox* plants but similar patterns, namely the reduced activation of defense genes (TPI, XTH, TD) and suppressed down-regulation of photosynthesis-related genes (LHCII, PSII-O_2), were observed in both JA-deficient genotypes (Manuscript IV and V). Despite the

increased substrate pool available for JA biosynthesis in as-*hpl* plants (Fig. 5), the pattern of transcript accumulation in JA-deficient as-*aos* and GLV-deficient as-*hpl* plants show a striking similarity (Manuscript V). GLVs induce gene expression in *Arabidopsis* (Bate and Rothstein, 1998), tomato (Sivasankar *et al.*, 2000), lima bean (Arimura *et al.*, 2001) and citrus (Gomi *et al.*, 2003) and silencing of a LOX isoform (LOX-H1, the potato homolog of NaLOX2) that specifically supplies the substrate for GLV biosynthesis (Fig. 5) reduces PI expression. These studies, together with the results presented here indicate a pivotal role for HPL-derived oxylipin signals in the activation of plant defense responses. Sivasankar *et al*. (2000) proposed a GLV-dependent activation of systemin signaling in parallel with the wound-induced JA signaling cascade for the elicitation of the complete tomato wound response and this signaling model is confirmed by the reduced TPI activity in both, JA- and GLV-deficient *N. attenuata* plants (Manuscript IV and V). In contrast to the JA- and GLV-dependent induction of TPI activity, the elicitation of the herbivore-induced release of terpenoid VOCs (Halitschke *et al.*, 2000) is independent of the HPL cascade but only dependent on the JA-producing octadecanoid pathway in *N. attenuata* (Manuscript IV-VI).

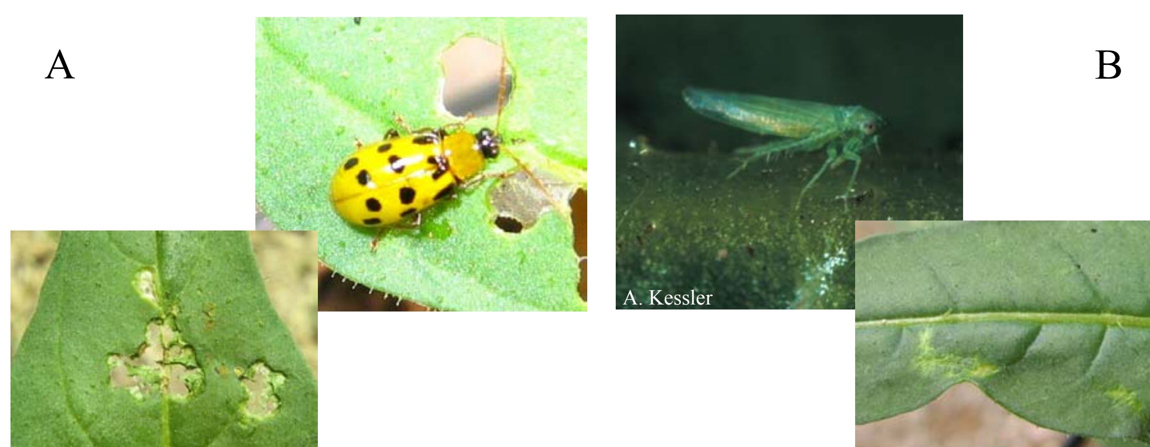

Figure 6. Two novel herbivore species that exclusively attack jasmonate-deficient (as-*lox*) but not wild type *Nicotiana attenuata* plants. **A)** The western cucumber beetle *Diabrotica undecimpunctata tenella* Le Conte) usually feeds on *Datura wrightii* and *Cucurbita foetidissima* flowers and causes extensive mechanical damage by its leaf-chewing feeding behavior. **B)** The leafhopper *Empoasca* spec. usually feeds on *D. wrightii*, *Solanum americanum*, and *Mirabilis multiflora* and the typical damage caused by its piercing/sucking feeding behavior.

Field release experiments confirmed the phenotypic consequences of altered oxylipin signaling characterized in the laboratory and additionally revealed further implications for the JA signaling cascade in the resistance against a broad range of herbivores (Manuscript VI). The analysis of the transformants under natural conditions demonstrated a crucial role for the octadecanoid-mediated inducibility of plants in the host selection behavior of two opportunistic herbivores, the leafhopper *Empoasca* spec. and the chrysomelid beetle *Diabrotica undecimpunctata*, which are abundant in the study area (Fig. 6) but were not observed on WT *N. attenuata* plants. These results clearly demonstrated that the plant's ability to elicit induced responses represents a factor for host selection decisions of herbivores in addition to the plant's chemical phenotype and external mortality factors (Bernays and Graham, 1988).

4. Conclusion

The traits responsible for herbivore resistance have long been studied by ecologists and are known to involve complex adaptations that include direct and indirect defenses, as well as tolerance responses (Karban and Baldwin, 1997). The majority of molecular work on these plant-herbivore interactions has been conducted in model plant systems in which a detailed understanding of the phenotypic traits responsible for herbivore resistance is lacking or only recently being examined (Li *et al.*, 2004; Thaler *et al.*, 2002). *Nicotiana attenuata* represents a model species in which the traits responsible for herbivore resistance have been thoroughly characterized (Baldwin, 2001) and the development and application of sophisticated molecular tools is providing valuable insights into the complex defense processes involved in herbivore resistance. In this study, the elicitors in the oral secretions of *Manduca sexta* and *M. quinquemaculata*, two closely related specialist herbivores of *N. attenuata*, were isolated and characterized. The isolated compounds elicit the herbivore-specific activation of defense responses in *N. attenuata* which is mediated by amplification of wound-induced JA biosynthesis. Transgenic plants impaired in the biosynthesis of JAs and GLVs, two oxylipin signals which play important roles in wound and herbivore responses in several plant species (Blee, 2002) were generated and the consequences of altered inducibility in the transformants were analyzed. In particular, the laboratory-based experiments presented here have established causal

associations among reduced oxylipin signal production, subsequently suppressed activation of particular direct (nicotine accumulation and TPI activity) and indirect (VOC release) defense traits, and the resulting reduction in herbivore resistance against *M. sexta*. Additional field release experiments demonstrated the influence of the plant's inducibility on the herbivore community composition. The identification of additional natural herbivores on JA-deficient plants highlights the usefulness of genetically silenced plants for ecological studies addressing functional mechanisms of induced responses. The characterization of the unknown 'unknowns' that impact plant performance in nature substantially extends the knowledge about factors (biotic and abiotic) that contribute to the selective pressure shaping the evolution of induced plant responses.

5. Zusammenfassung

Als sessile Lebewesen sind Pflanzen nur sehr beschränkt in der Lage (potentiellen) Gefahren aktiv auszuweichen. Im Laufe der Evolution entwickelten Pflanzen daher eine Vielzahl von Abwehrreaktionen, mit denen sie auf die auf sie einwirkenden biotischen und abiotischen Umweltfaktoren reagieren können. Der Befall durch Herbivoren stellt einen wesentlichen biotischen Stressfaktor dar dem Pflanzen mit diversen mechanischen (z.B. Dornen) und chemischen (toxische oder verdauungshemmende Substanzen und Enzyme) Abwehrmechanismen begegnen. Die Abwehr kann sowohl konstitutiv in der Pflanze exprimiert sein oder erst nachdem die Pflanze von einem Herbivoren befallen wurde aktiviert (induziert) werden. Diese induzierten Abwehrmechanismen können Fitnesskosten reduzieren, die durch die Allokation von Ressourcen in die Abwehr entstehen. Diese Kostenersparnis setzt die spezifische Erkennung des Frassfeindes und die Aktivierung der geeigneten Abwehr durch das komplexe Netzwerk von Signalkaskaden in der Pflanze voraus.

Die wilde Tabakpflanze *Nicotiana attenuata* kann zwischen einer rein mechanischen Verwundung und Frassschädigung durch Raupen des Tabak- (*Manduca sexta*) und Tomatenschwärmers (*M. quinquemaculata*), differenzieren. Der Transfer von Raupenregurgitat während des Frassvorgangs ermöglicht diese spezifische Erkennung und induziert eine Reihe herbivoren-spezifischer direkter

(Nikotin, Proteinase Inhibitoren) und indirekter (Duftstoffemission) Abwehrreaktionen in der Pflanze.

Die Zielsetzung der vorgelegten Arbeit war die Identifizierung und Charakterisierung der chemischen Signalstoffe im Raupenregurgitat und der endogenen Signalkaskaden der Pflanze, die die herbivoren-spezifische Abwehrreaktionen in *N. attenuata* induzieren. Zu diesem Zweck wurden die oralen Sekrete der Larven der zwei Schwärmerarten chemisch analysiert, die induzierenden Substanzen (Elicitoren) identifiziert (Manuskript I) und ihre biologische Aktivität charakterisiert (Manuskript I und II). Durch antisense Transformation wurde die Expression von Genen der durch Raupenfrass oder Behandlung mit Elicitoren in der Pflanze aktivierten Signalkaskaden manipuliert (Manuscript III) und die Auswirkungen der verminderten Induzierbarkeit auf die Resistenz von *N. attenuata* in Labor- (Manuskript IV und V) und Freilandversuchen (Manuskript VI) untersucht.

Fettsäure-Aminosäure-Konjugate sind die notwendigen Signale für die Aktivierung der herbivoren-spezifische Abwehr in *Nicotiana attenuata*. Mehrere Fettsäure-Aminosäure-Konjugate konnten aus dem Regurgitat der beiden Herbivoren *M. sexta* und *M. quinquemaculata* isoliert und auf ihre biologische Aktivität untersucht werden. Die von glutaminsäurehaltigen Verbindungen dominierte Zusammensetzung des Regurgitats von *Manduca* Larven unterscheidet sich wesentlich von der anderer lepidopterer Herbivoren. Die Behandlung mechanisch verwundeter Pflanzen mit Raupenregurgitat oder den isolierten Konjugaten induzierte ein vergleichbares Muster an Abwehrreaktionen in *N. attenuata* und die chromatographische Entfernung der Elicitoren inaktivierte die induzierende Wirkung des Raupenregurgitats. Die identifizierten Elicitoren sind für die Induktion herbivoren-spezifischer Geneexpressionsmuster und Duftstoffemissionen verantwortlich. Diese Induktion wird durch die Verstärkung der verwundungsinduzierten Biosynthese von Jasmonsäure (Octadecanoid-Kaskade) reguliert.

Spezifische Lipoxygenase (LOX) Gene sind in die Biosynthese von Jasmonsäure und C_6-Alkoholen und –Aldehyden involviert. Mehrere LOX Gene konnten aus *N. attenuata* isoliert werden (NaLOX1, NaLOX2, NaLOX3). Die Expressionsanalyse zeigte die wurzelspezifische Expression von NaLOX1 und die Transkriptakkumulation von NaLOX2 und NaLOX3 im Blattgewebe. Die verwundungs-

induzierte Expression von NaLOX3 zeigte eine ähnliche Kinetik wie die Jasmonsäurekonzentration im verwundenten Gewebe und wurde daher als Kanditat für die genetische Manipulation der Octadecanoid-Kaskade ausgewählt. Die Expression von NaLOX3 in Antisens-Orientierung (as-*lox*) reduzierte spezifisch die Jasmonsäurebiosynthese hatte aber keinen Einfluss auf die Emission der C_6-Verbindung (Z)-3-Hexenal, die aus dem gleichen Substrat (13-Hydroperoxy-linolensäure) durch die Hydroperoxid Lyase (HPL) Reaktion synthetisiert wird.

Octadecanoid- und HPL-Signalkaskade, spielen eine wichtige Rolle in der Regulation der induzierten Resistenz in *N. attenuata*. Die durch Verwundung induzierte Expression der Hydroperoxid Lyase (HPL) wird durch Elicitoren im Raupenregurgitat unterdrückt. Ein auf der Verschiebung des Flusses von Hydroperoxidsubstrat zwischen HPL- und Octadcanoid-Kaskade basierender Mechanismus für die amplifizierte Jasmonsäurebiosynthese wird diskutiert. Durch genetische Manipulation der Expression von NaLOX3 (as-*lox*), HPL (as-*hpl*), und der Allen Oxid Synthase (as-*aos*) konnten sowohl jasmonsäure- als auch C_6-abhängige Abwehrreaktionen in *N. attenuata* gezeigt und die Resistenz gegen *M. sexta* reduziert (as-*lox*) bzw. erhöht (as-*hpl*) werden.

Proteinase Inhibitoren (PI) und ein grosser Teil der herbivoren-induzierten Genexpression werden durch die parallele Funktion von Jasmonsäure und C_6-Verbindungen aktiviert. Die durch Behandlung mit Methyljasmonat induzierte PI Aktivität war sowohl in Pflanzen mit verminderter Jasmonsäurebiosynthese (as-*lox* und as-*aos*) als auch in Pflanzen mit reduzierter Produktion von C_6-Alkoholen und –Aldehyden (as-*hpl*) geringer als in vergleichbar behandelten Wildtyp Pflanzen. Weiterhin zeigte sich eine erhebliche Überschneidung der durch die Manipulation der beiden einzelnen Signalkaskaden verursachten Veränderungen in der durch Raupenfrass induzierten Genexpression.

Die Induktion von Dufstoffemissionen als indirekte Abwehr ist abhängig von einer intakten Jasmonsäurekaskade aber nicht von C_6-Verbindungen. Die durch Raupenfrass induzierte Abgabe des Sesquiterpenes *cis*-α-bergamotene wurde durch die reduzierte Jasmonsäurebiosynthesekapazität in as-*aos* und as-*lox* Pflanzen unter-

drückt, war aber unverändert in as-*hpl* Pflanzen. Die jasmonsäureabhängige Induktion wurde weiterhin bestätigt, da der Defekt in der Biosynthese durch die Behandlung mit synthetischem Methyljasmonat umgangen werden konnte.

C_6-Verbindungen stimulieren das Frassverhalten von *Manduca sexta*. Die Reduktion der von *M. sexta* konsumierten Blattfläche in as-*hpl* Pflanzen konnte durch die Behandlung mit synthetischen C_6-Verbindungen aufgehoben werden, ohne dass Unterschiede in der chemischen Abwehr der Pflanzen beobachtet werden konnten.

Die jasmonsäureabhängig induzierte Abwehr beeinflusst die Zusammensetzung der Herbivorengemeinschaft auf *N. attenuata* Pflanzen. In Freilandversuchen konnte gezeigt werden, dass Pflanzen mit verringerter Jasmonsäurebiosynthese (as-*lox*) stärker durch Herbivoren geschädigt werden Die in den Laborversuchen beobachete verringerte Resistenz gegen Herbivorie durch *M. sexta* konnte unter natürlichen Bedingungen bestätigt werden. Zusätzlich wurden as-*lox* Pflanzen von zwei oportunistischen Herbivoren (*Diabrotica undecimpunctata* und *Empoasca* spec.), die nicht auf Wildtyp *N. attenuata* Pflanzen beobachtet wurden, attackiert.

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7.1. Supplementary Data

The following microarray results can be found as electronic copy on the supplemental CD-ROM.

Manuscript II

- Supplementary Table I
- Supplementary Table II

Manuscript IV

- Supplementary Table S1
- Supplementary Table S2

Manuscript V

- Supplementary Table S1

Manuscript VI

- Supplementary Table S2

7.2. Publication List

Halitschke R, Kessler A, Kahl J, Lorenz A, Baldwin IT (2000) Ecophysiological comparison of direct and indirect defenses in *Nicotiana attenuata*. Oecologia **124:** 408-417

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Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine is an effective resistance trait not co-opted by herbivores in nature. PLoS Biology **in review**

Oral and Poster Presentations

Feb. 25 - March 2, 2001 GRC on Plant Herbivore Interactions, Ventura, USA

Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient to elicit herbivore-specific plant responses.

Oct. 3-5, 2002 Kurth-Mothes-Doktoranden-Workshop Sekundärstoffwechsel, Jena

Manipulation of defense signaling in *Nicotiana attenuata*.

Nov12-13, 2002 Ecology and Biodiversity Research Symposium, Krakow, Poland

Dissecting the oxylipin-regulated ecological interactions of *Nicotiana attenuata*.

Aug. 3-8, 2003 ESA Meeting, Savannah, USA

Ecological consequences of altered jasmonate signaling capacity in *Nicotiana attenuata*.

7.3. Lebenslauf

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17.09.1999	Friedrich-Schiller-Universität Jena Abschluss als Diplomchemiker (Umweltchemie)
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Dissertation

ab 01/2000	Friedrich-Schiller-Universität Jena Thema: "Mechanisms of jasmonate-induced activation of defense responses in *Nicotiana attenuata*"

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7.5. Selbständigkeitserklärung

Entsprechend der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Personen, die an der Durchführung und Auswertung des Materials und bei der Herstellung der Manuskripte beteiligt waren sind am Beginn der Arbeit („Manuscript Overview“) und jedes Manuskriptes angegeben.

Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen.

Die vorgelegte Arbeit wurde weder an der Friedrich-Schiller-Universität Jena, noch an einer anderen Hochschule als Dissertation eingereicht.

Rayko Halitschke