

**The consequences of herbivore-induced plant responses for
the arthropod community of the native tobacco *Nicotiana
attenuata***

Dissertation

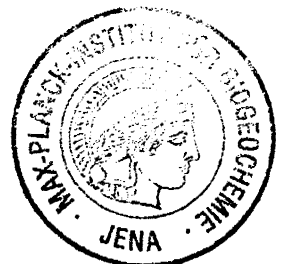
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List of Manuscripts

Manuscript 1

Eco-physiological comparison of direct and indirect defenses in *Nicotiana attenuata*

Rayko Halitschke, André Keßler, Johannes Kahl, Andreas Lorenz and Ian T. Baldwin

(Published: *Oecologia* 2000. **124**: 408-417)

Manuscript 2

***Manduca quinquemaculata*'s optimization of *intra*-plant oviposition to predation, food quality and thermal constraints**

André Kessler and Ian T. Baldwin

(in press: *Ecology* 2002)

Manuscript 3

Defensive function of herbivore-induced plant volatile emissions in nature

André Keßler and Ian T. Baldwin

(Published: *Science* 2001. **291**: 2141-2144)

Manuscript 4

Plant vaccination against herbivore attack in nature

André Kessler and Ian T. Baldwin

(Submitted: 2002)

Manuscript 5

Plant responses to insect herbivory: The emerging molecular analysis

André Kessler and Ian T. Baldwin

(Published: *Annu. Rev. Plant Biol.* 2002. **53**: 299-328)

1. Introduction

Like the Red Queen in Lewis Carroll's (1832-1898) "Through the Looking-Glass and What Alice Found There", both plant-damaging herbivores and their hosts have to keep running frantically just to stay in place. As described by Van Valen (1973) the race reflects the evolutionary forces that prevent plants and their enemies from becoming extinct as each evolves new strategies for defense or attack, respectively. The discovery that herbivore feeding can induce metabolic changes (Green and Ryan 1972, Ryan 1990) and that these changes in turn can influence the population dynamics of a plant's arthropod community (Haukioja and Niemela 1979, Haukioja 1980) provided a starting point for a new research era in chemical ecology. Since this discovery, plant-herbivore interactions have been studied in many different plant and arthropod taxa, and the plant is recognized no longer a passive, but as an active structuring member of the interaction (Karban and Baldwin 1997). As suggested in the Red-Queen-principle, plant-herbivore interactions can be understood as triggering coevolutionary processes whereby herbivores are forced to optimize their foraging efficiency and plants are forced to optimize their defense (Karban and Baldwin 1997).

Plants have evolved many different ways of coping with their enemies (Karban and Baldwin 1997, Paul et al. 2000). They can directly defend themselves against their attackers with physical traits, such as thorns, spines and hairs, or chemical traits, such as toxins, antidigestive and antinutritive compounds (Duffey and Stout 1996). Additionally plants may express traits that facilitate top-down control over the herbivore populations by attracting natural enemies of the herbivores and thus indirectly defend themselves against their attackers (Agrawal 2000, Dicke 2000, Dicke and Grostal 2001).

Many of the direct and indirect chemical defenses are induced by wounding or herbivore damage, suggesting that these defenses have costs. This favors genotypes, which are able to express these defenses only when they are needed, such as in case of herbivore attack (Heil 2001, Heil and Baldwin 2002). These fitness costs can result from either physiological or ecological processes. The physiological costs derive from the allocation of resources from the primary metabolism to the secondary metabolism and are obvious in lower lifetime fitness after the induction of a defense (Baldwin 2001). This fitness loss becomes a benefit for an attacked plant if the trait reduces susceptibility of the plant and/or lowers the fitness of a herbivore (Baldwin 1996). Ecological costs, in turn, result from higher-level interactions that influence plant fitness, such as when (inducible or

constitutive) defensive traits benefit the attacker of the plant. Many specialized insect herbivores are able to sequester plant secondary metabolites and to use them for their own defense against predators and parasitoids (Barbosa et al. 1986, Barbosa et al. 1991). This would make a direct defense useless and render ineffective a top-down control by the third trophic level. A given defense is not necessarily effective against all herbivores attacking a plant species, and ecological costs can arise from specific defenses against different herbivores and require that defense responses be optimized to maximize the plant's reproductive success.

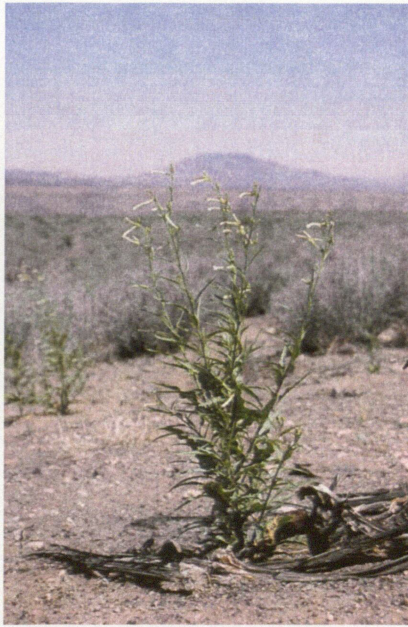


Fig. 1. The wild tobacco plant (*Nicotiana attenuata*) in its natural habitat. Photo: Andre Kessler

In a reductionist approach, we examine how plants metabolically regulate their multiple defense responses against the high diversity of organisms that commonly attack them. With an integrative approach we examine the multitrophic consequences of these defenses. The collection of papers presented here studies the impact of plant defense traits for higher trophic levels and reproductive consequences for the plant under natural conditions. The work focuses on tritrophic consequences of an indirect defense trait,

volatile organic compound (VOC) emission, and the vaccinating effect of the attack by one herbivore species for subsequent attack from another herbivore species.

A number of studies have shown that the feeding activity of herbivores causes plants to emit VOCs (Dicke and van Loon 2000), which are derived from at least three different biochemical pathways: the octadecanoid, the isoprene and the shikimate pathways (Agrawal 2000). These volatiles potentially function as signals that transfer information (infochemicals) from the damaged plant to the foraging predator and parasitoid or host searching herbivore (Turlings and Benrey 1998, Dicke and van Loon 2000, De Moraes et al. 2001, Kessler and Baldwin 2001). VOCs from maize plants, attacked by the larvae of the noctuid *Spodoptera littoralis*, for example, are attractive to the parasitoid braconid wasp *Cotesia marginiventris* (Rose et al. 1996, Rose et al. 1998), which can control the caterpillar population (“top-down” regulation) and subsequently causes a fitness benefit for the plants (Hoballah and Turlings 2001). Spider mite feeding on lima bean induces VOC emission; VOCs are attractive to predatory mites, which can control the spider mite population in greenhouses (Dicke and Sabelis 1988, Dicke et al. 1998). Previously, evidence for the indirect defense function of herbivore-inducible VOC emission came exclusively from laboratory and agricultural systems. But, VOC emission might be able to function as an indirect defense only in simplified agroecosystems, in which, in contrast to natural systems, a single natural enemy species of a herbivore can act as an important biocontrol agent and increase the fitness of a plant (van der Meijden and Klinkhamer 2000). Moreover, both the qualitative and quantitative characteristics of herbivore-induced plumes are known to vary among plant genotypes (Loughrin et al. 1995, Turlings et al. 1998, Gouinguene et al. 2001). The genetic variation commonly found in natural populations may undermine the reliability and specificity of VOCs as a signal for natural enemies, because prior exposure is often needed to associate plant VOCs with the occurrence of a feeding herbivore. By examining indirect defenses under natural conditions, the research presented here not only evaluates the signal function of VOCs but also demonstrates some of the consequences of the combination of direct and indirect defense traits for the arthropod community structure.

In the last two decades, the wild tobacco plant *Nicotiana attenuata* (Fig. 1) has been established as a model system to study the impact of direct and indirect defense traits on plant-herbivore interactions and plant fitness consequences (Baldwin 2001, Baldwin et al. 2001). This solanaceous plant colonizes disturbed desert habitats, such as burns and roadsides, in the southwestern USA (Baldwin and Ohnmeiss 1993). At burns,

N. attenuata dominates the plant community in the first two years after a fire. Its germination is triggered and synchronized by smoke-derived chemical cues and is inhibited by allelopathic substances in the litter of other plant species (Preston and Baldwin 1999). Germinating seedlings experience very low interspecific but high intraspecific competition. Thus, the plant's responses to herbivore attack can be examined in the context of this competition, because the costs of a defense are accentuated when plants compete with other uninduced conspecifics (Baldwin 1996, van Dam and Baldwin 2001). Moreover, the ephemerality of *N. attenuata* forces the plant's herbivore community to reestablish itself with every new population. In the three years of study the most important herbivores were solanaceous specialists: the tomato hornworm (*Manduca quinquemaculata*, Lepidoptera, Sphingidae), the tobacco hornworm (*Manduca sexta*, Lepidoptera, Sphingidae), the true bug *Tupiocoris notatus* (Hemiptera, Miridae), and the flea beetle *Epitrix hirtipennis* (Coleoptera, Chrysomelidae) (Fig. 2).

The plant recognizes herbivore damage by an endogenous jasmonic acid (JA) burst after wounding, which is propagated throughout the plant ahead of the feeding herbivore (Schittko et al. 2000). JA application elicits an increased production of toxins (such as nicotine, flavonoids, phenolic putrescine conjugates and diterpene sugar esters) (Baldwin and Ohnmeiss 1993, Keinänen et al. 2001), antidigestive proteins (proteinase inhibitors) (van Dam et al. 2001) and antinutritive enzymes (polyphenole oxidase) (Karban et al. 2000, Manuscript 2), which may play roles as direct defenses. The expression of such JA-induced defense traits comes with substantial fitness costs to the plant, in part because of the high nitrogen demands of nicotine biosynthesis (van Dam and Baldwin 2001).



Fig. 2. The most common insect herbivores of *N. attenuata* in southwest Utah. A) Tomato-Hornworm (*Manduca quinquemaculata*), B) Tobacco-Hornworm (*Manduca sexta*), C) leaf bug (*Tupiocoris notatus*), D) flea beetle (*Epitrix hirtipennis*). Photos: Andre Kessler-

Interestingly, the nicotine biosynthesis is reduced in plants attacked by *M. sexta* caterpillars, compared to mechanically damaged plants, although the JA burst is amplified (Kahl et al. 2000). A herbivore-induced ethylene burst attenuates the wound-induced transcriptional increase of the nicotine biosynthetic genes for PMT (putrescine methyl transferase), and thus antagonizes a JA-elicited response (Winz and Baldwin 2001). The

differential activation of various pathways can be understood as the consequence of the specific characteristics of wounding caused by the herbivore and the introduction of specific elicitors into the wounds (Halitschke et al. 2001). Insect-specific alterations of the wound responses are likely to result from crosstalk between the different signaling pathways, which gives plants the potential to optimize their defense (Genoud and Metraux 1999).

The consequences of such herbivore-specific and potentially defensive plant responses for the herbivore community and the reproductive success of the plant are poorly understood. The actual defensive value (measured in a currency of plant fitness) of most widely-recognized plant 'defenses' remains to be established. This situation is particularly acute for traits thought to mediate indirect defenses. In other words, it remains to be proven whether VOCs function as indirect defenses and increase plant fitness in nature and not only under the homogeneous conditions of agricultural and laboratory systems. The *N. attenuata* system has emerged as an ideal study system for these questions. The following collection of papers examines the role played by VOCs in indirect defense and investigates how a plant's chemical responses can structure its herbivore community under natural conditions. Specifically, the following 5 questions were addressed in five manuscripts:

1. Do *N. attenuata* plants emit increased amounts of VOCs after herbivore feeding? To obtain an answer, a detailed study of phenology and ontogeny of VOC emission is performed, culminating in a comparison of physiological cost of direct and indirect defenses.
2. What factors influence host plant selection of ovipositing *M. quinquemaculata* moths? Correlations of the oviposition preferences with predation risk, food quality and thermal constraints are demonstrated. These results lay the groundwork for further studies of "top-down" control of *M. quinquemaculata* populations.
3. Do herbivore-inducible VOCs function as indirect defenses in nature? Whether plants even released VOCs with potential signal function under natural conditions was previously unknown. Moreover, whether naturally occurring predators or parasitoids could decode the kairomonal message remained to be tested.
4. Does previous damage of the plant by one herbivore species influence subsequent attacking herbivores?

5. What are the future perspectives in plant-herbivore interaction research? Modern research approaches and actual publications are reviewed.

Manuscript 1

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**Eco-physiological comparison of direct and indirect defenses in
*Nicotiana attenuata***

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Abstract

After herbivore attack, plants launch a suite of direct and indirect defense responses that must be coordinated if plants are to realize a fitness benefit from these responses. Here we characterize the volatile emissions in the native tobacco plant, *Nicotiana attenuata* Torr. ex Wats., that are elicited by tobacco hornworm (*Manduca sexta* L.) attack and are known to function as attractants for parasitoids. To provide the first eco-physiological comparison of examples of both types of defenses in the same species, we characterize the elicitation and signaling mechanisms, the resources required, and the potential costs and benefits of the volatile release and compare these traits with those of the well-described induced direct defense in this species, nicotine production.

The release of (*E*)- β -ocimene, *cis*- α -bergamotene and linalool is dramatically induced within 24 h by application of methyl jasmonate (MeJA), caterpillar feeding and the treatment of mechanical wounds with larval oral secretions (OS), but not by mechanical damage alone. Plants from different geographic locations produce volatile blends that differ in composition. The most consistently released component from all genotypes, *cis*- α -bergamotene, is positively related to the amount of MeJA and the level of wounding if OS are applied to the wounds. The volatile release is strongly light dependent, dropping to undetectable quantities during dark periods, even when temperatures are elevated to match those of the light period. Inhibitors of wound-induced jasmonate accumulation (salicylates and auxins), which are known to inhibit wound-induced nicotine production, do not inhibit the release of volatiles. By individually inducing different leaf positions with OS and, on other plants, excising them after induction, we demonstrate that the emission is largely a systemic, whole-plant response, which is maximally triggered when the second fully expanded leaf is induced.

We conclude that while both are whole-plant, systemic responses that utilize recently acquired resources for their production and are activated by the jasmonate cascade, the elicitation of the volatile release exhibits greater tissue sensitivity and utilizes additional signaling components than does nicotine production. In contrast to the large investment of fitness-limiting resources required for induced nicotine production or the resources used in benzyl acetone release from flowers for pollinator attraction, the resource requirements for the volatile release are minor. Hence the argument that the

volatile release incurs comparatively large physiological costs can not be supported in this system.

Key Word Index: *Nicotiana attenuata*; nicotine; *cis- α -bergamotene*; indirect defense; jasmonic acid; oral secretion; *Manduca sexta*; diurnal variation.

Introduction

Herbivore attack results in multivariate changes in plant chemistry, morphology and physiology that frequently increase the resistance of plants to further attack (Karban and Baldwin, 1997; Baldwin and Preston, 1999). This induced resistance can be due to direct defenses, such as toxic secondary metabolites which can poison attacking herbivores, or defensive proteins, such as protease inhibitors (PIs) or polyphenoloxidases (PPOs), which decrease nutrient availability and slow the growth of herbivores. In addition, plants use indirect defenses that facilitate "top-down" control of herbivore populations by the herbivore's predators, parasitoids, and pathogens. For example, some plants release volatile organic compounds when attacked and these volatiles are thought to guide parasitoids or other members of the third trophic level to the feeding herbivore (Turlings et al., 1995; Takabayashi and Dicke, 1996; DeMoraes et al., 1998; Paré and Tumlinson, 1999). However, the ecological complexity of chemical defense may make certain combinations of direct and indirect defenses incompatible. For example, plant toxins are frequently sequestered by specialist herbivores for their own defense against their parasitoids and predators (Smiley et al., 1985; Barbosa et al., 1986; Barbosa et al., 1991; Duffey and Stout, 1996; Malcolm and Zalucki, 1996; Turlings and Benrey, 1998) which might make the production of a sequesterable toxin functionally incompatible with the deployment of an indirect defense that attracts parasitoids.

Such incompatibility is strongly suggested by the patterns of expression of direct and indirect defenses in the native tobacco plant, *Nicotiana attenuata*. When this species and its sibling species, *N. sylvestris*, are mechanically damaged or attacked by mammalian herbivores, they respond with dramatic increases in the production and whole-plant accumulation of the highly toxic defense compound, nicotine (Baldwin, 1999). Nicotine is synthesized in the roots and its *de novo* synthesis is regulated by the

endogenous wound signal, jasmonic acid (JA) (Baldwin et al., 1994b; Baldwin et al., 1997b). Leaf wounding dramatically increases endogenous JA levels (5-500 ng g⁻¹) within 90 min after wounding in proportion to the amount of wounding and these endogenous levels of JA are quantitatively correlated with the whole-plant nicotine response 5 d later (Baldwin et al., 1997b; Ohnmeiss et al., 1997). Applications of the auxins, 1-naphtylacetic acid (NAA) and indole-3-acetic acid (IAA) or the salicylates, methylsalicylic acid (MeSA) and acetylsalicylic acid (Asp) directly to leaf wounds inhibit both the rapid wound-induced increase in endogenous JA and the nicotine response 5 d later, but does not inhibit nicotine production induced by the exogenous addition of JA or MeJA (Baldwin, 1989; Baldwin et al., 1990; Baldwin et al., 1996; Baldwin et al., 1997b). In short, nicotine induction is a whole plant trait, which is induced by exogenous jasmonate addition and wound-induced changes in endogenous jasmonate pools. Interestingly, herbivory by the nicotine tolerant herbivore, *Manduca sexta*, or the application of its oral secretions (OS) to leaf wounds results in a dramatic amplification (4-20 fold) of the normal wound-induced JA response but, surprisingly, in a suppression of the subsequent nicotine response (Baldwin, 1988; McCloud and Baldwin, 1997; Schittko et al., 2000).

Recently, Kahl et al. (Kahl et al., 2000) reported the mechanism for the uncoupling of the JA and nicotine responses in *N. attenuata* by *M. sexta* attack. Feeding by *M. sexta* larvae or the application of their OS to leaf wounds results in an ethylene burst. The amount of ethylene produced is sufficient to suppress the induced nicotine response, but has no effect on yet another herbivore-specific response in this species, namely the release of volatile terpenes (Kahl et al., 2000), which in cultivated tobacco and other plant systems, attract parasitoids to actively feeding larvae and possibly functions as an indirect defense (Takabayashi and Dicke, 1996; DeMoraes et al., 1998). The suppression of the induced direct defense may reflect the incompatibility of the defensive use of nicotine against nicotine-tolerant herbivores whose parasitoids are sensitive to nicotine. For example, *Cotesia congregata*, a specialist parasitoid of *M. sexta*, suffers greater mortality when parasitizing larvae feeding on high nicotine leaves than when parasitizing larvae feeding on low nicotine leaves (Thurston and Fox, 1972; Barbosa and Saunders, 1985; Thorpe and Barbosa, 1986).

Insect-pollinated plants frequently attract their pollinators by releasing volatiles from flowers and analogous incompatibilities may arise between the use of a direct defense that is broadly insecticidal to protect flowers in these volatile-mediated

synomonal interactions. Moreover, many of the compounds that are used as floral fragrances are also implicated as indirect defenses when released from attacked foliage (Loughrin et al., 1994; Takabayashi and Dicke, 1996). Evidence for such an incompatibility can be seen in the patterns of release of floral volatiles and nicotine pools in the corollas of *N. attenuata* plants (Euler and Baldwin, 1996). The emission of a single compound, benzyl acetone (BA), from the outer lip of the corollas increases 50-fold in the evening, a pattern characteristic of many moth-pollinated flowers. Nicotine is also found in the headspace of these flowers, but is principally located in the proximal parts of the corolla, below the attachment of the filaments and the nectar reward. The corolla pools of nicotine remain stable throughout the day, but during the period of peak BA emission the pools decrease dramatically (Euler and Baldwin, 1996). The removal of nicotine from the corolla during the scotophase when pollinators are attracted may be the plant's resolution of the conflicting demands of defense and pollinator attraction. Determining whether the function of indirect and direct defenses are incompatible under field conditions is a challenging task that requires extensive field work with natural populations of herbivores and their parasitoids and techniques to uncouple the different induced responses from each other. However, while the ecological explanation of functional incompatibility remains to be rigorously tested, it should be noted that other processes may account for the inverse pattern of expression of induced direct and indirect defenses.

Physiological constraints may limit the simultaneous expression of direct and indirect defenses in plants. For example, both induced responses may make large resource demands and limit a plant's ability to simultaneously launch both defense responses. Additionally, the signal cascades responsible for eliciting the responses may constrain simultaneous elicitation. While a substantial amount of information has been gathered on the spatial (Röse et al., 1996; Paré and Tumlinson, 1998) and temporal scale (Loughrin et al., 1994; Turlings et al., 1998), resource requirements (Paré and Tumlinson, 1997), elicitation (Dicke et al., 1993; Boland et al., 1995; Mattiacci et al., 1995; Alborn et al., 1997) and natural variation (Loughrin et al., 1995; Turlings et al., 1998) in herbivore-induced volatiles emissions, the research has been carried out in different plant species, and in species in which the plants' direct defenses are not well understood. Here, we characterize the volatile release from *N. attenuata* that is induced by *M. sexta* larvae attack to provide a "phytogenic" description of this putative indirect defense that can be compared with the nicotine-based direct defense in the same species. This comparison is

used to evaluate physiological hypotheses for the negative correlation between volatile emissions and nicotine production.

Materials and Methods

Plant growth. We germinated *N. attenuata* Torr. ex Wats. seeds in smoke-treated soil and grew plants in individual 1 l hydroponic chambers as previously described (Baldwin and Schmelz, 1994; Ohnmeiss and Baldwin, 1994) for all plants from which whole-plant emissions were collected. After 10-14 days of growth in 1 l hydroponic chambers, plants received an additional 7 mg N (KNO₃) and were randomly assigned to treatment groups 24 h before starting treatments. Plants were grown in soil for the single leaf volatile emission measurements from greenhouse-grown selfed seeds originating from three different natural populations in the SW USA [genotypes: I, Apex Mine UT (sites in Baldwin 1998); II, Crossing Roads, CA; III, DI ranch, UT: T40S-R19W section9]. All plants were grown in a growth room with the following conditions: 28 °C/16 h L, 28 °C or 25 °C/8 h D, and 800-1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation at plant height from high-pressure sodium lamps.

Treatments. *Manduca sexta* L. larvae were hatched from eggs (Carolina Biological Supply, Burlington, NC, USA) and reared on *N. attenuata* foliage. Two 3rd or 4th instar larvae per plant were allowed to feed freely on plants for 24 h prior to the start of volatile collections. All larvae were removed from plants before volatile collection. Similarly, all leaf treatments were initiated at 9-10 am, 24 h before volatile collection, with the exception of the time series experiment. Methyl jasmonate (MeJA; Bedoukian Research, Danbury, CT, USA), which was close to its thermodynamic equilibrium (90.1% 1R, 2R MeJA and 8.3% 1R, 2S MeJA) for the two naturally occurring epimers, was applied to the adaxial laminar surface in 20 μl of lanolin paste. Control plants received the same amount of pure lanolin. The *M. sexta* OS were collected (McCloud and Baldwin, 1997) from 4th- 5th instar larvae fed *N. attenuata* leaves, stored under Argon at – 20 °C and diluted 1:1 (v:v) with water prior to the treatment. To mimic the wounding resulting from caterpillar feeding, the leaf was mechanically damaged by rolling a fabric pattern wheel over the leaf surface which produced 100-110 puncture wounds per leaf; 20 μl of OS were applied to the fresh puncture wounds. Control plants were similarly

wounded but had 20 μl of distilled water added to their wounds. Different numbers of punctures per leaf were obtained by increasing the number of passes across the length of the leaf lamina with the pattern wheel. Aqueous solutions of salicylates (Asp and MeSA) and auxins [IAA; indole-3-butyric acid (IBA) and NAA] were prepared individually at 9 mg ml^{-1} concentrations. These solutions were diluted 1:1 (v:v) with OS and applied to the leaf as described to deliver 90 μg to the wounded leaf, an amount previously determined to completely inhibit the local wound-induced JA response and the systemic nicotine response in *N. sylvestris* (Baldwin et al., 1996; Baldwin et al., 1997b). The same solutions were diluted with water and applied to puncture wounds on separate plants as controls.

Induction of different leaf positions. To examine the effect of inducing leaves growing at specific nodal positions on whole plant emissions, we induced on separate plants the following 5 phyllotactically consecutive leaf positions: 2 young leaves, which had not yet attained full expansion (Fig. 5; positions 1 and 2), the source/sink transition leaf (position 3) which had just attained full expansion (as defined in Wait et al., 1998), and two older, fully expanded leaves (positions 4 and 5). To determine if the volatile release is a systemic response, the leaf at position 4 was treated with OS and excised 2 h after the treatment. In all other experiments, the treated leaf was always at position 4. Four to six replicate plants were used for each treatment.

Volatile collection. Volatiles were trapped from either single leaves or whole plants. For WP collections, entire hydroponically grown plants were enclosed in individual 1.5 l open-top chambers before leaf treatments. For single-leaf collections, the treated leaf at position 4 of soil-grown plants was enclosed in a 20 ml single leaf volatile collection chamber (Fig. 1A). Volatiles were trapped on 30 mg of a polymeric adsorbent (SuperQ, Alltech, Inc., Deerfield, IL, USA) secured with glass wool in small glass cylinders cut from 1 ml glass syringes. Ambient air from the growth chamber was pulled into each of the individual open-top plant chambers at 300 ml min^{-1} with a vacuum pump (model DAA-V114-GB, Gast Mfg., Benton Harbor, MI, USA) and through the trap for 8 h. The trap sampled air from within each chamber 3 cm above the surface of the plant (Fig. 1A). The pumps were connected to a vacuum manifold, which allowed volatiles to be collected from up to 80 chambers simultaneously. Empty chambers were used to determine the amount of volatiles in the ambient air. The flow-rate through each trap was measured using a mass-flow-meter (0-500 ml min^{-2} ; Aalborg Instruments & Controls, Inc., Orangeburg, NY, USA). Prior to collection, 390 ng of ^{13}C -labelled methylsalicylate

(MeSA) was added as an internal standard to each trap. ^{13}C -MeSA was synthesized by the methylation of salicylic acid (Sigma, St. Louis, M.O., USA; Lot 28F3421) with diazald-N-methyl- ^{13}C (Sigma, St. Louis, MO, USA; Lot 29,598-1).

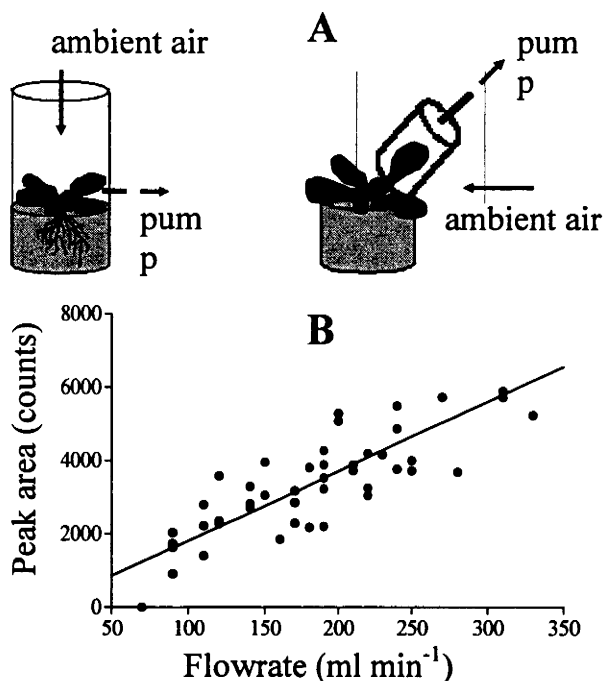


Fig. 1.A) Schematic of whole-plant and single leaf volatile collection chambers. Arrows indicate direction of airflow across plants and through volatile adsorption traps (black bar). **B)** Relationship between flow rate through the trap and peak area of the sesquiterpene in the ambient air that was used to quantify volatile emissions ($R^2 = 0.716$; ANOVA $F_{1,45} = 111.01$, $P < 0.0001$).

Analysis and identification of volatile compounds. Compounds were eluted from the traps with 250 μl of CH_2Cl_2 and 1 μl aliquots were analyzed by capillary GC on a 30 m x 0.25 mm (i.d.) fused silica-column with 0.25 μm thickness (DB-WAX; J & W Scientific, Folsom, CA, USA). The GC was programmed as follows: injector held at 225 $^\circ\text{C}$, initial column temperature at 45 $^\circ\text{C}$ held for 6 min, then ramped at 10 $^\circ\text{C min}^{-1}$ to 130 $^\circ\text{C}$, at 5 $^\circ\text{C min}^{-1}$ to 180 $^\circ\text{C}$ and with 20 $^\circ\text{C min}^{-1}$ to 230 $^\circ\text{C}$, held for 5 min and finally at 20 $^\circ\text{C min}^{-1}$ to 250 $^\circ\text{C}$, held for 5 min. The carrier gas flow was set to 1 ml min^{-1} with an electronic pressure control unit. All compounds were analyzed by a Varian Saturn 2000

ion trap mass-spectrometer operated in the electron impact (EI) ionization mode. Compounds were identified by comparison of GC retention times with those of authentic standards and by comparison of mass spectra with spectra of a Varian terpene database. The absolute amounts were calculated from the peak area of the substance and the internal standard and were corrected by the trapping efficiency, which was calculated from the regression of the peak area of an unidentified sesquiterpene (which occurred in the ambient chamber air as a natural contaminant) against the flow rate (Fig. 1B). The limits of detection were determined to be $1 \text{ pg h}^{-1} \text{ l}^{-1}$.

Results

Genotypes differ in volatile emissions. Treatment of a single leaf with $150 \text{ } \mu\text{g}$ MeJA induced the release of several terpenoids from all three genotypes (Table 1). Single leaf emission of (*E*)- β -ocimene, *cis*- α -bergamotene and an unidentified sesquiterpene from MeJA-induced plants were significantly (pairwise t-test, all *P*'s < 0.05; Table 1) higher than those from lanolin treated controls. Interestingly the genotypes differed dramatically in their emission patterns.

Table 1. Mean (\pm SE) $\text{ng h}^{-1} \text{ l}^{-1}$ volatiles trapped from individual leaves of plants of 3 different genotypes of *Nicotiana attenuata* (4 replicates), which had been treated with $20 \text{ } \mu\text{l}$ of lanolin paste (control) or with $20 \text{ } \mu\text{l}$ of lanolin paste containing $150 \text{ } \mu\text{g}$ of MeJA 24 h before the volatile collection started.

Geno- type	Volatiles trapped ($\text{ng h}^{-1} \text{ l}^{-1}$)							
	<i>(E)</i> - β -Ocimene		Linalool		<i>cis</i> - α -Bergamotene		Sesquiterpene	
	Lanolin	MeJA	Lanolin	MeJA	Lanolin	MeJA	Lanolin	MeJA
I	0.10 \pm 0.01	8.01\pm1.93	0.04 \pm 0.02	0.15\pm0.10	0.03 \pm 0.01	9.01\pm2.80	- ^a	0.15\pm0.09
II	0.57 \pm 0.33	5.88\pm1.58	0.05 \pm 0.05	0.07\pm0.04	- ^a	3.90\pm0.96	- ^a	0.36\pm0.21
III	0.01 \pm 0.01	0.13\pm0.03	- ^a	- ^a	- ^a	23.21\pm12.94	- ^a	2.52\pm1.24

^a below detection limit ($< 0.001 \text{ ng h}^{-1} \text{ l}^{-1}$)

The (*E*)- β -ocimene emissions from genotype I and II were dramatically higher (up to 80x) than that from genotype III, while genotype III emitted much more (up to 10x) *cis*- α -

bergamotene and the unidentified sesquiterpene (Table 1). MeJA treatment significantly increased linalool emissions from genotype I, but not from genotype II and linalool was not detected in the headspace of genotype III.

OS and caterpillar feeding, but not mechanical damage elicits volatile release. Caterpillar feeding and OS application induced significant increases in whole plant emission of (*E*)- β -ocimene ($F_{3,12} = 8.9$, $P = 0.002$) and *cis*- α -bergamotene ($F_{3,12} = 4.06$, $P = 0.033$) (Table 2). No significant difference was found in the emissions between either control and wound+water-treated plants [(*E*)- β -ocimene, Fisher's PLSD, $P = 0.36$; *cis*- α -bergamotene, Fisher's PLSD, $P = 0.92$], or between the OS- and caterpillar-damaged plants [(*E*)- β -ocimene, Fisher's PLSD, $P = 0.61$; *cis*- α -bergamotene, Fisher's PLSD, $P = 0.73$; Table 2]. The release of linalool was significantly induced by OS-treatment ($F_{3,12} = 12.9$, $P = 0.0005$).

Table 2. Mean (\pm SE) $\text{ng h}^{-1} \text{l}^{-1}$ volatiles trapped from individual (4 per treatment) *Nicotiana attenuata* plants grown in individual whole plant chambers 24 h after a single leaf was either damaged mechanically and the puncture wounds treated with water or oral secretions from *Manduca sexta* larvae (OS) or were damaged by the feeding of a *M. sexta* larvae (caterpillar). Control plants remained undamaged.

Treatment	Volatiles trapped ($\text{ng h}^{-1} \text{l}^{-1}$)		
	(<i>E</i>)- β -Ocimene	Linalool	<i>cis</i> - α -Bergamotene
Control	0.21 \pm 0.08	0.01 \pm 0.01	0.06 \pm 0.04
Wound + water	0.13 \pm 0.03	- ^a	0.03 \pm 0.01
Caterpillar	0.49 \pm 0.08	0.03 \pm 0.02	0.89 \pm 0.43
Wound + OS	0.44 \pm 0.03	0.23 \pm 0.06	0.78 \pm 0.16

^a below detection limit ($< 0.001 \text{ ng h}^{-1} \text{ l}^{-1}$)

The volatile release is strongly light dependent, even under constant temperature. The increase in the emission of the different terpenoids occurred at different rates. The

increase in (*E*)- β -ocimene and *cis*- α -bergamotene emission started within the first 8 h after application of 150 μ g MeJA to a single leaf. The first detectable increase in linalool release occurred only 24 h after treatment (Fig. 2). The emissions of all three compounds showed a strong light dependence [(*E*)- β -ocimene, $F_{1,18} = 8.4$, $P = 0.009$; linalool, $F_{1,18} = 4.1$, $P = 0.057$; *cis*- α -bergamotene, $F_{1,18} = 8.1$, $P = 0.011$] and were almost not detectable in the headspace of plants during dark periods. Increasing the growth chamber temperature during the dark-period to match that of the light period during the 3rd night after treatment did not influence this striking diurnal pattern. Additionally, in a separate experiment in which MeJA was applied to leaves at the beginning of the dark period, the volatile release did not start until the next light period (data not shown).

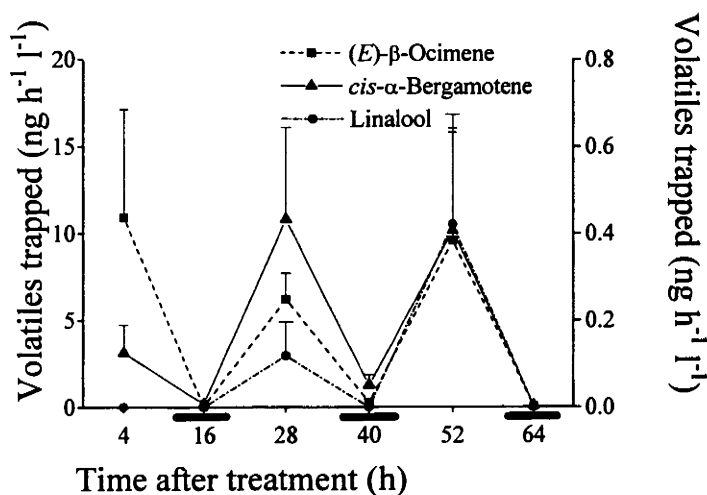


Fig. 2. Mean (+ SE) *cis*- α -bergamotene, (*E*)- β -ocimene (left) and linalool (right) trapped from individual leaves of 4 *Nicotiana attenuata* plants over 3 consecutive light-dark (16:8 h) cycles after treating a single leaf on each plant with 150 μ g of MeJA at time = 0 h. Black bars signify dark periods, which were adjusted to match the temperature of light periods (28 °C) during the 3rd cycle. (For clarity, only positive error bars are shown.)

Emissions increase with increasing MeJA and OS treatments. The whole plant emission of *cis*- α -bergamotene 24 h after treatment with 0-150 μ g MeJA increased in proportion with the amount of MeJA applied (Fig. 3A; $F_{8,36} = 11.2$, $P < 0.0001$). The application of 5 μ g MeJA was the smallest amount tested which significantly (*t*-test; $P =$

0.01) increased emission above that measured in lanolin-treated control plants. The whole plant response to OS application exhibited a similar dependence on the number of leaf punctures into which OS was applied (Fig. 3B; $F_{3,28} = 6.5$, $P = 0.002$), with the emission rates of *cis*- α -bergamotene increasing 10-fold over a 5-fold increase in the number of punctures.

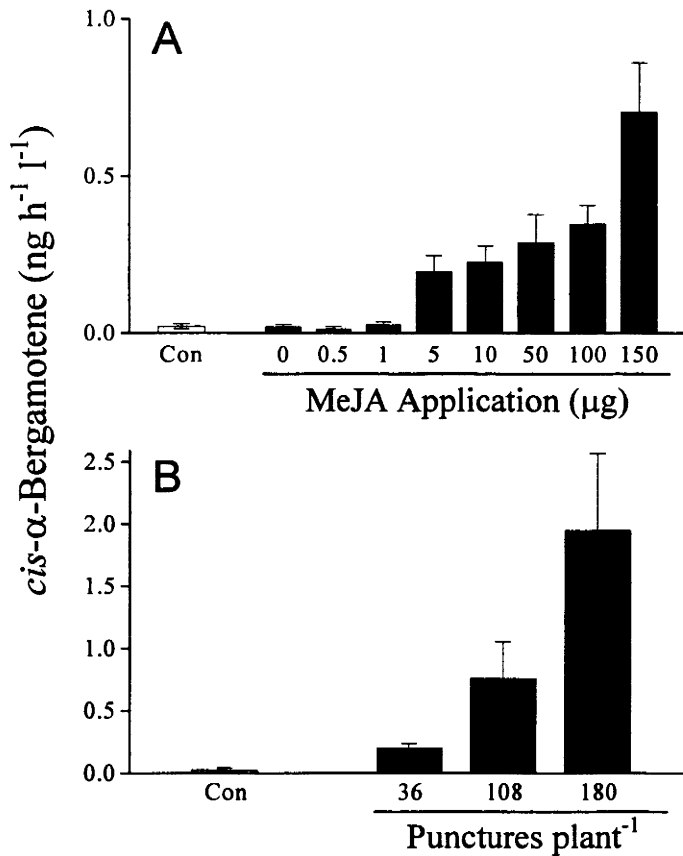


Fig. 3. A) Mean (+ SE) *cis*- α -bergamotene trapped from individual (5 per treatment) *Nicotiana attenuata* plants grown in whole-plant chambers (Fig. 1A) 24 h after 10 µl of lanolin paste containing 0-150 µg of MeJA had been applied to a single leaf of each plant. Plants labeled “con” received neither lanolin nor MeJA. **B)** Mean (+ SE) *cis*- α -bergamotene trapped from individual plants (8 per damage level) grown in whole-plant chambers after a single leaf had received 36-180 punctures and immediately treated with 20 µl of oral secretions from *Manduca sexta* larvae (OS) 24 h prior to the measurement.

Inhibitors of wound-induced JA accumulation do not suppress the OS-induced volatile release. All tested salicylate and auxin inhibitors of wound-induced JA accumulation were ineffective in suppressing the OS-induced whole plant emission of *cis*- α -bergamotene (Fig. 4, pairwise t-tests, all P's > 0.786). All OS treatments induced the release of *cis*- α -bergamotene compared to the respective water treatments (all P's < 0.05) and the inhibitors when applied in water to puncture wounds without OS did not increase emission rates above those found in water+wound control plants (Fig. 4; P's > 0.519).

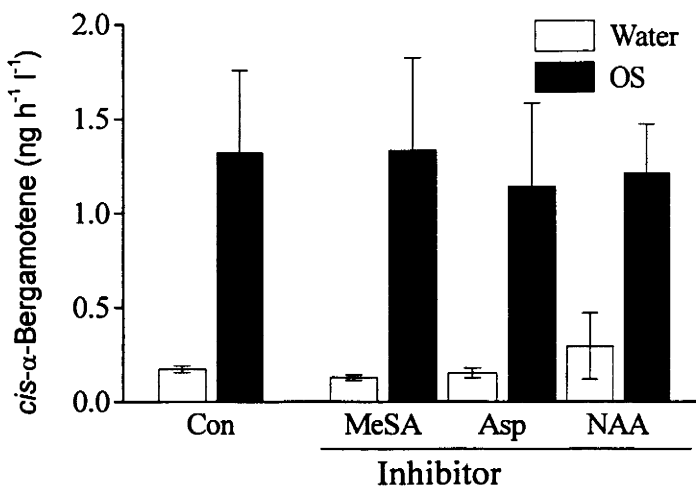


Fig. 4. Mean (+ SE) *cis*- α -bergamotene trapped from individual (4 per treatment) *Nicotiana attenuata* plants grown in whole-plant chambers 24 h after a single leaf was treated. Immediately after mechanical wounding plants received 20 μ l water (open bar) or oral secretions from *Manduca sexta* larvae (OS) (solid bar) or a solution of 90 μ g MSA, NAA or Asp in 20 μ l water (open bars) or 20 μ l OS (solid bars).

Systemic volatile emission is highly dependent on the developmental stage of the treated leaf. The whole plant *cis*- α -bergamotene emission rate 24 h after OS treatment of an individual leaf during different stages of development differed significantly (Fig. 5; ANOVA for leaf stage, $F_{6,21} = 7.6$, $P = 0.0002$). Treatment of a single leaf (position 4) growing at a node two positions older than the source-sink transition leaf resulted in a dramatic 50-fold increase in *cis*- α -bergamotene emission rate. In marked contrast, the treatment of leaves which were just undergoing source-sink transition and younger leaves

(positions 1-3), as well as older leaves (position 5) resulted in only 7-17-fold increases in emission rates compared to water+wound controls (Fig. 5). This effect of leaf age or position is highly significant (Fisher's PLSD, $P < 0.004$). Moreover, this experiment demonstrated that the emission of *cis*- α -bergamotene is a systemic response which is activated by a signal which exits the treated leaf within 2 h. When the OS-treated leaf at position 4 was excised 2 h after treatment, the whole plant release was not significantly different (Fig. 5; t-test, $P = 0.74$) from that of similarly-treated plants without the excision of the OS-treated leaf at position 4 (Fig. 5).

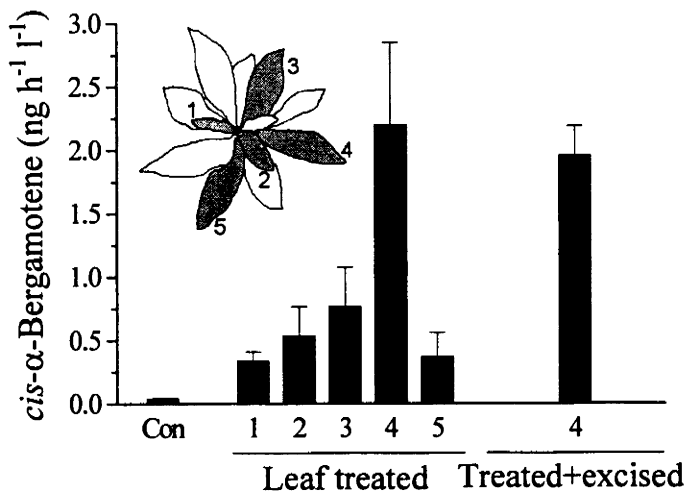


Fig. 5. Mean (+ SE) *cis*- α -bergamotene trapped from individual (4 per treatment) *N. attenuata* plants grown in whole-plant chambers 24 h after a single leaf, growing at nodes 1-5, was treated with 20 μ l of OS. Leaves growing at node 3 had just attained full expansion and completed the sink→source transition. For plants with leaf 4 treated, the treated leaf remained on the plant or was excised 2 h after the treatment.

Discussion

The analysis of herbivory-induced volatile emissions in *N. attenuata* allows us to compare the signaling and resource-requirements of the volatile release with those of nicotine induction and assess physiological explanations for the inverse patterns of expression of these two induced responses. The analysis revealed numerous parallels with

other plant species in which the volatile emission is better characterized physiologically (Paré and Tumlinson, 1999) and provided new insights into the control of this whole plant response. The most abundant volatiles induced, (*E*)- β -ocimene, linalool and *cis*- α -bergamotene, are all terpenoids that have been reported in the headspace of herbivore-elicited tobacco, cotton, corn and lima bean plants and are thought to attract parasitoids when these plants are attacked by herbivores (Dicke, 1994; Takabayashi and Dicke, 1996; Paré and Tumlinson, 1999).

A comparison of the signals that elicit the whole plant volatile and nicotine responses discounts the hypothesis that the simple constraint of using the same signal cascade to activate both responses can account for their inverse patterns of expression. While both responses can be quantitatively elicited by exogenous jasmonate additions in proportion to the amount applied (Fig. 3), the role of endogenous jasmonates in eliciting the two responses clearly differ. A significant increase in *cis*- α -bergamotene emissions was observed 24 h after applying 5 μ g of MeJA in lanolin paste (Fig. 3A) and similarly, whole plant nicotine pools are known to double 5 days after addition of 11 μ g of MeJA to the roots of hydroponically-grown plants (Baldwin, 1996). Both responses increase with increasing amounts of applied MeJA, which is readily de-esterified to JA in the plant (Zhang and Baldwin, 1997). When *Nicotiana* plants are quantitatively wounded with a fabric pattern wheel, endogenous JA pools increase in proportion to the number of puncture wounds 90 min after wounding (the time of peak JA accumulation) which results in proportional increases in whole plant nicotine biosynthesis and accumulation (Baldwin et al., 1997b; Ohnmeiss et al., 1997). Moreover, if these wound-induced increases in JA are inhibited by applying salicylates or auxins to the wounded tissues, the nicotine response is inhibited (Baldwin et al., 1996; Baldwin et al., 1997b). These results establish that the endogenous JA response to wounding is both necessary and sufficient for the elicitation of the wound-induced nicotine response.

In contrast, wounding does not elicit a volatile release unless OS from *M. sexta* larvae are applied to the wounds (Table 2). Once OS are applied to the wounds, the whole plant emission is proportional to the number of wounds into which OS has been applied (Fig. 3B). OS from *M. sexta* larvae elicit a dramatic endogenous JA burst in the treated tissues (McCloud and Baldwin, 1997; Kahl et al., 2000) which spreads rapidly (within 40 sec) through the attacked leaf (Schittko et al., 2000). The JA burst is so fast that even the considerable appetite of fifth instar *M. sexta* larvae is unable to inhibit its spread by

consuming the tissues propagating this OS induced signal (Schittko et al., 2000). Interestingly, the inhibitors (MeSA, Asp, IAA, NAA and IBA) which are so effective in suppressing the wound-induced JA and nicotine responses, are ineffective in inhibiting either the OS-induced volatile release (Fig. 4) or the ethylene burst (Kahl et al., 2000). These results demonstrate that while exogenous additions of jasmonates can elicit the volatile release, the elicitors of this response activate signal cascades which are different from those activated by wounding and are likely upstream of the jasmonate cascade. Since it is clear that the release of volatiles is elicited by additional signal components, the negative correlation between the two induced responses is unlikely to be due to simple constraints arising from the use of a common signal cascade.

This difference in signaling of the two responses highlights the fact that this plant can distinguish among different agents which cause wounding, and provides a cautionary tale for ecologists interested in simulating herbivore-induced responses with exogenous additions of jasmonates. Although MeJA application clearly activates both responses, it does not simulate the responses elicited by the attack of *M. sexta* larvae. Recently, an analysis of the performance of herbivorous larvae and their parasitoids in a field study with tomato plants treated with jasmonates (Thaler, 1999) suggested that both direct and (unmeasured) indirect defenses functioned in concert to reduce the performance of herbivorous larvae. Our results demonstrate that the treatments of *N. attenuata* plants with jasmonates elicits both the volatile release and nicotine induction, responses which are not simultaneously expressed when *M. sexta* larvae attack these plants. While the factors in *M. sexta* OS which are responsible for eliciting both the JA burst and the volatile release are still under investigation, significant progress has been made in other plant-herbivore systems. From the OS of beet armyworms (*Spodoptera exigua* H.) the elicitor responsible for the volatile release in corn (*Zea mays*), *N*-(17-hydroxylinolenoyl)-L-glutamine (“volicitin”), has been isolated and identified (Alborn et al., 1997). In lima bean (*Phaseolus lunatus*), Boland and co-workers (Koch et al., 1999) have shown that the blend of the volatiles is determined by the activation of two components of the jasmonate cascade, JA and its biosynthetic precursor, 12-oxo-phytodienoic acid. Continued advances in this area will help clarify the signals that elicit direct and indirect responses and will provide the tools to enable ecologists to selectively activate defense responses.

Another dramatic difference between the two induced responses in *Nicotiana attenuata* is the remarkable tissue sensitivity in the elicitation of the volatile release. Quantitative wounding of leaves growing at different developmental stages on *N.*

sylvestris plants revealed only minor differences in their ability to elicit whole plant nicotine responses and most of the observed differences were likely due to the inability of young, assimilate-importing leaves (sink leaves) to transmit the wound signal to the nicotine-producing roots (Ohnmeiss et al., 1997). Once root nicotine biosynthesis has been activated, the newly synthesized nicotine is transported preferentially to leaves and plant parts of high fitness value, tissues that directly or indirectly contribute to seed production (Ohnmeiss and Baldwin, 2000). In contrast, the whole plant volatile release is highly dependent on the leaf position that has been induced. Elicitation of a leaf that has recently gone through the source-sink transition (nodal position 4) results in the largest whole plant volatile release (Fig. 5). While the reason for the particular sensitivity of this leaf position is not known, we can exclude two hypotheses. First, since it was possible to excise this leaf 2 h after elicitation without influencing the quantity of released volatiles, it is clear that this leaf position contributes little to the whole plant response (Fig. 5). Second, since both older and younger leaves have comparable leaf sizes, leaf area cannot account for the particular sensitivity of this position (Fig. 5).

We hypothesize that the unique sensitivity of this leaf position relates to its ability to export the signal to the plant parts that release the volatiles. The leaf excision experiment (Fig. 5) demonstrates that the signal activating the volatile release exits the leaf within 2 h, a rate comparable to the exodus of the nicotine-inducing signal from a wounded *Nicotiana* leaf (Baldwin et al., 1994b). The excision experiment also demonstrates that the volatile release is largely a systemic response, as has been elegantly demonstrated in cotton with ^{13}C -labeling experiments (Röse et al., 1996; Paré and Tumlinson, 1998) and in cultivated tobacco, with excision experiments similar to those used here (DeMoraes et al., 1998). The small volatile response that occurs after the elicitation of leaves that are undergoing the source-sink transition or are younger and are still importing photoassimilates (Fig. 5) may simply reflect the difficulty of exporting a wound signal against the direction of primary photoassimilate transport into these immature leaves. This “signal export” hypothesis can not explain why the elicitation of fully mature, exporting leaves at nodal position 5 produces such small whole plant volatile responses (Fig. 5). These leaves may export their photoassimilates to below-ground sinks (roots), while those at position 4 may be exporting to younger sink leaves. This hypothesis is consistent with the pattern observed in herbivore-induced cotton in which young leaves are responsible for a majority of the systemic volatile release (Röse et al., 1996; Paré and Tumlinson, 1998).

The resource demands required for the simultaneous expression of both responses represent additional potential physiological constraints. Assessing the resource demands of a response is an onerous task that requires the quantification of the used resources in a fitness-limiting currency. Such an analysis has been performed for the nicotine (Baldwin, 1997; Baldwin and Hamilton, 2000) but not for the volatile response. However, a comparison of the quantities of volatiles released after herbivore attack with those released from flowers during pollinator attraction allows one to draw inferences about their relative costs and additionally draw comparisons with the resource-demands of nicotine induction.

Induced plants released less than 10 μg per day of the three most abundant induced terpenoids and it is instructive to compare this quantity with the amount of benzyl acetone (BA) released from *N. attenuata* flowers to attract pollinators. Plants grown under the same conditions as those used in this study produce on average 20 open flowers, which release on average 50 μg BA per night (Euler and Baldwin, 1996; Baldwin et al., 1997a). In summary, plants release at least 5-fold more volatiles during pollinator attraction than they do after herbivore feeding. If one assumes that BA is synthesized from the deamination of phenylalanine, as other phenylpropanoid-derived volatiles are (Schnitzler et al., 1992) and that the N from phenylalanine is lost for other uses, then one can compare the amount of N used for pollinator attraction on a whole plant basis per night with the amount of N used in the whole plant nicotine response. Such calculations reveal that the BA-release, which is 5 times larger than the herbivore-induced volatile emission on a whole plant basis, utilizes 0.1 % of the amount incorporated in nicotine after wounding (Baldwin et al., 1998). Clearly the resource demands of the induced nicotine response dwarf those of the volatile release. Moreover, the volatiles released consist only of C, H, and O and it is very unlikely that the carbon demands of *N. attenuata* volatile release represent significant physiological constraints, since this desert annual sustains carbon assimilation rates of 20 $\mu\text{Mol m}^{-2} \text{s}^{-1}$ under field conditions (I. T. Baldwin unpublished results). This comparative analysis challenges the assumption that the volatile release incurs large physiological costs (Paré and Tumlinson, 1999). Relative costs, rather than absolute costs, are the more appropriate measures for the description of the fitness consequences of inducible responses.

The volatile release exhibited a strong diurnal pattern (Fig. 2), which coincides with the light period (and presumably photosynthetic activity) but is independent of the

temperature differences between the light and dark cycles. Moreover, if plants are induced at the beginning of the scotophase, no release occurs until the following light period. Similar results have been reported from studies with cotton (Loughrin et al., 1994). The timing of the release of different compounds is dependent on the molecule and plant species. Whereas the fatty-acid derived green leaf volatiles (mainly C6-aldehydes, -alcohols and -acetates) are released immediately after the feeding started, the induction of terpenoid-biosynthesis starts with a delay of 2-24 h (Turlings et al., 1998). Moreover, with ^{13}C pulse-chase experiments with cotton, the release of terpenoids has been shown to arise largely from recently assimilated carbon (Paré and Tumlinson, 1998). In contrast, the nicotine response is not strongly light-dependent and unlike the volatile release, nicotine production makes large demands on a plant's nitrogen (N) budget, requiring at least 6 % of whole plant N (Baldwin et al., 1998) an investment which can not be recouped by metabolism (Baldwin et al., 1994a; Baldwin and Ohnmeiss, 1994). In spite of the large N requirements, the nicotine response is clearly given a high priority since it is sustained even under N deprivation (Lynds and Baldwin, 1998) and is produced with resources limiting for growth and seed production (Baldwin, 1998; Baldwin et al., 1998). Under normal growing conditions plants use recently acquired and assimilated NO_3 in nicotine biosynthesis and when plants are given the choice, they preferentially use NH_4 over NO_3 (Lynds and Baldwin, 1998), suggesting that nicotine biosynthesis utilizes the least energetically costly available N-source. In contrast, the volatile release is not strongly buffered from variations in carbon availability and does not draw deeply on stored reserves of carbon, since its release is completely terminated in the night.

In summary, our analysis (Table 3) provides little support for the hypothesis that physiological constraints, arising from either the signaling or the resource-demands of the responses, can account for the suppression of nicotine accumulation and amplification of the volatile release after attack from the specialist herbivore, *M. sexta*. If the coordinated changes between direct and indirect defenses represent an optimization of defense responses against an adapted herbivore then it is possible that the observed variations in the composition of the volatile blend relate to the defensive function of the volatile release. We found that the emission pattern varied dramatically among the three geographically isolated genotypes of *N. attenuata* (Table 1) as has been shown for corn (Turlings et al., 1998) and cotton (Loughrin et al., 1995). For example, genotype III collected in Utah produces 80 times less (*E*)- β -ocimene and no detectable linalool in

comparison to the other two genotypes measured (Table 1). The value of volatile signals as indirect defense lies in its information content to the third trophic level. However, a plant has little control over the response of its ecological community to an increase in emission. Under some circumstances, a release of volatiles may increase a plant's apparancy and attract its herbivores (Takabayashi and Dicke, 1996). Since the plant no longer controls the signal once it has been released into the environment, the functional consequences for the plant are likely to be more unpredictable than they are for direct defenses. For example, herbivore-induced volatile emissions from both crabapple and grape appear to serve as host location cues for the Japanese beetle and contribute to the formation of large feeding aggregations of this pest species (Loughrin et al., 1996). Since parasitoids frequently must learn to associate the particular characteristics of a volatile signal from a plant with an actively feeding herbivore (Kester and Barbosa, 1991; Vet and Dicke, 1992), the variation among genotypes in the volatile release, or even within the same genotype over plant development, may help reduce the potentially negative kairomonal consequences of emitting these signals. However, alternatives to the adaptive explanation, such as drift or neutral mutations may account for the inter-population variation.

Clearly, the functional analysis of the interplay between direct and indirect defenses in a plant will require extensive field-based experimentation, which is currently in progress for the *Nicotiana attenuata* system. The fitness consequences of the volatile emissions and those of direct defenses will be highly contingent on the ecological context and, will be understood only in the habitats in which the plant has evolved.

Table 3. Eco-physiological comparison of direct (nicotine) and indirect (volatile release) defenses in *N. attenuata*.

<i>Trait</i>	<i>Nicotine</i>	<i>Volatile emission</i>
Elicitation by		
- Wounding	Yes	No
- Applied jasmonates	Yes	Yes
- <i>M. sexta</i> oral secretions	Suppresses induction	Yes
Inhibition by		
- Salicylates	Yes	No
- Auxins	Yes	No
Light dependence	No	Yes - no release in the dark.
Spatial scale of response	Systemic - preferential allocation of recently synthesized nicotine to young leaves and reproductive parts.	Systemic - excision of triggered leaf doesn't reduce whole plant emission.
Temporal pattern of response	Max. accumulation after 5 days. Rate of <i>de novo</i> synthesis increased within 21 h of wounding.	Monoterpene release (ocimene) within 8 h; sesquiterpene release within 24 h of elicitation.
Tissue sensitivity	Low - wounding of most tissues elicits response.	High - maximal elicitation occurs when an exporting leaf is induced.
Resources used	Preferential use of recently acquired NO ₃ over stored N reserves.	Likely current photosynthetic assimilation.
Costs		
- Physiological	High - 6% of whole-plant N is in nicotine after induction.	Low - quantity of emitted compounds is less than 1/5 of floral emissions.
- Ecological	Fitness disadvantage of induced plants in herbivore-free environments.	Kairomonal attraction of herbivores.
Ecological benefit	High - but potentially counter-productive against nicotine-tolerant herbivores.	Potentially high – but completely dependent on predator and parasitoid community.

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Manuscript 2

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***Manduca quinquemaculata's* optimization of *intra-plant* oviposition to
predation, food quality and thermal constraints**

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Running title: *Intra-plant oviposition pattern*

Abstract

To examine the hypothesis that *intra*-plant oviposition preferences of *Manduca quinquemaculata* on *Nicotiana attenuata* optimize predation risk and nutritional needs of developing larvae and eggs, we measured oviposition behavior of adults and larval mortality, movement, performance and body temperatures at different leaf positions in a natural population. Nearly 70% of the eggs were oviposited on young central stem leaves of elongated plants. *Intra*-plant movement was very rare in the 1st and common in the 2nd to 4th larval instars. The oviposition preference for, and larval movement towards, younger leaves was correlated with a 40% lower predation risk and a 6.3-fold greater weight gain, suggesting higher nutritive value despite 2.1-fold higher nicotine concentrations and 4.6-fold higher polyphenol oxidase activities. The predatory bug, *Geocoris pallens*, which consumed eggs and larvae with instar- and leaf position-specific preferences, was responsible for the vast majority of *M. quinquemaculata* mortality and may shape the moth's oviposition preference.

Keywords: Lepidoptera, Sphingidae, oviposition, top-down control, *Nicotiana attenuata*, food quality, predation

Introduction

The selection of appropriate oviposition sites by adult females is crucial for the larval success of numerous Lepidoptera species with early instars of low vagility. Optimal host selection means not only selecting the right host plant in the right habitat but also selecting the right place on the plant. Larval performance is known to be dependent on various microclimatic factors (Casey 1976), nutrition availability (Feeny 1976, Slansky and Feeny 1977, Schroeder 1981), and metabolic, physical and enzymatic defenses of the plant (Appel and Martin 1992, Duffey and Stout 1996), which can function as toxins, antinutritive and antidigestive compounds (Duffey and Stout 1996). How these factors influence larval performance and thereby potentially shape the evolution of *intra*-plant preferences for oviposition sites by the mothers is largely unknown and is the subject of this study.

These factors have both direct and indirect effects on larval performance. Physical defenses incur handling costs while some chemical defenses incur metabolic costs for their detoxification [metabolic load hypothesis, (Whittaker and Feeny 1971, Appel and Martin 1992)] and both can reduce larval growth resulting in increased predation risk through longer exposure to natural enemies (Bjorkman et al. 1997, Williams 1999). The fitness consequences of certain direct defenses that slow larval growth may be more pronounced if attacked plants attract natural enemies of the herbivores by emitting volatile organic compounds (VOCs, Kessler and Baldwin 2001). Since these factors that influence larval performance are known to vary within plants, they may provide the selective pressure that shapes and optimizes the oviposition behavior of the adults.

The moths *Manduca sexta* L. and *M. quinquemaculata* Haworth (Lepidoptera: Sphingidae) have similar morphology (Sannino et al. 1993), behavior (Peterson et al. 1993) and ecology (Bossart and Gage 1990, Kester and Barbosa 1994). Both are significant pests on solanaceous plants in North America and have been the subject of recent interest in plant-herbivore interactions (Raguso and Willis 1997, van Dam et al. 2000, Schittko et al. 2000, Hermsmeier et al. 2001). One of their host plants in the Great Basin Desert of SW Utah is the annual tobacco *Nicotiana attenuata* Torr. ex Wats., whose defense responses to these herbivores have been extensively studied (Baldwin 1999, Kahl et al. 2000, Schittko et al. 2000, Hermsmeier et al. 2001, Voelckel et al. 2001, Winz and Baldwin 2001). *N. attenuata* occurs in recently disturbed areas, washes and, predominantly, on newly burned sites (Baldwin and Ohnmeiss 1993). It dominates the plant community in the first year after a fire, decreasing in numbers during the following two growing seasons until seed dormancy is again enforced until the next fire (Preston and Baldwin 1999).

The plant “recognizes” damage by an endogenous jasmonate (JA) burst (Schittko et al. 2000). JA application results in the induction of secondary metabolites such as nicotine, phenolics, flavonoids, phenolic putrescine conjugates and diterpene sugar esters (Keinanen et al. 2001), digestibility reducers such as proteinase inhibitors (PIs; van Dam et al. 2001b) and antinutritive enzymes such as polyphenol oxidase (PPO; Karban et al. 2000). Intriguingly, while nicotine and PIs are known to reduce larval growth, the nicotine response is attenuated by an ethylene burst that occurs in response to *Manduca* feeding or application of their regurgitant to mechanical wounds (Kahl et al. 2000, Winz and Baldwin 2001). In contrast *N. attenuata* plants emit a suite of VOCs (Halitschke et al.

2000) which are elicited by fatty acid-amino acid conjugates in the regurgitant of the feeding caterpillars (Halitschke et al. 2001). This emission attracts a generalist predator, *Geocoris pallens* (Heteroptera: Geocoridae), to damaged plants, where they consume *Manduca* eggs and larvae in the early instars (Kessler and Baldwin 2001). Nicotine is likely toxic to such predators as it is to endoparasitic wasps (Barbosa et al. 1991) and hence the attenuation of the nicotine response may represent an optimization of the plant's direct and indirect defenses (Kahl et al. 2000). Moreover, adult *M. quinquemaculata* moths show a strong oviposition preference for undamaged plants, and use specific components of the plant's VOC signal for their oviposition decision, thereby reducing intraspecific competition, predation pressure or the effect of induced phytochemicals on their offspring (Kessler and Baldwin 2001). The proximate mechanisms of these oviposition choices are likely mediated by *Manduca*'s responses to olfactorial and contact chemosensory information (Yamamoto et al. 1969).

Here we examine oviposition behavior, hornworm mortality and movement, body temperature, larval performance and predation pressure on a *M. quinquemaculata* population ovipositing and feeding on native *N. attenuata* host plants to understand the consequences of this insect's *intra*-plant oviposition choices. These consequences may provide the ultimate explanations for this species' oviposition behavior.

Methods

The study was performed in a natural *M. quinquemaculata* population foraging and ovipositing on *N. attenuata* from May to July 1999. We used a *N. attenuata* population of more than 1×10^6 plants growing on a 2588.5 ha burn located NW of St. George, Utah, which had burned several times, after lightning strikes and human influence in June and July 1998 (Pahcoon Spring Burn R310; T41S, R18W). *M. quinquemaculata* use *Datura wrightii*, *Proboscidea* spp and *N. attenuata* as natural host plants in the study area. The crepuscularly-active adults usually oviposit only one egg per *N. attenuata* plant. Larvae must consume up to 5 flowering *N. attenuata* plants to reach pupation.

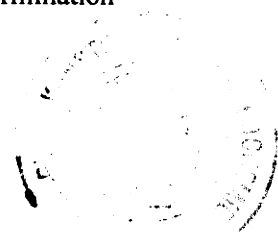
Manduca spp. census. To determine larval mortality, oviposition rates and *intra*-plant preferences, we conducted visual censuses of *N. attenuata* for eggs and larvae of *Manduca* spp. On June 9-10, during the peak period of oviposition, 1476 rosette and 1288 elongating plants were censused for eggs. All plants in 14 haphazardly selected 4-m² plots

were examined, hence the census reflects the relative proportion of different plant developmental stages at that time. Plant developmental stage, egg number and leaf position, on which the eggs were found, were recorded. The oviposition preferences for rosette versus elongating plants was analyzed by using a two-way contingency test (Pearson chi-square test; SYSTAT 7.0 for Windows) of the following four categories of plants: elongated with/without eggs, rosette with/without eggs. For each of the elongating plants that received eggs ($n=508$) the position of the first hornworm egg was registered so that for each of the plots the *intra*-plant distribution of eggs was monitored on a plant level. Using the data from the 14 plots, the *intra*-plant distribution of eggs was analyzed by ANOVA.

Hornworm mortality and movement. On another haphazardly selected set of flowering plants ($n=76$), all eggs and larvae ($n=89$) were observed for 12 d (June 10 – 22). Every 3rd d during this time, the *intra*-plant position, the disappearance and appearance of eggs and larvae and larval movement were used to determine mortality and oviposition. The movements of a subset of caterpillars (35 individuals) were observed over their development. The probability of moving in different instars was analyzed with a two-way contingency test (Pearson Chi^2) and the distances moved were compared with an ANOVA.

An egg was considered dead if it: a) disappeared without any sign of a hatched larvae; or b) was found emptied with only a small hole, characteristic of attack by predatory bugs. A 1st to 4th instar hornworm was considered dead if it: a) disappeared without any sign of moving to another adjacent plant; or b) was found dead on the plant, as was commonly found when larvae were attacked by predatory bugs which pierce and drain early instar larvae. Fifth instar hornworms are able to disperse long distances over the ground, making their disappearances from plants difficult to categorize and hence were excluded from the mortality tabulation. The differences in mortality of different developmental stages were analyzed with the nonparametric Kruskal-Wallis-H-test, while leaf position specific mortality rates were analyzed with an ANOVA, since the data met the requirements for a parametric analysis. We grouped the analysis of the leaf position specific mortality rates into three categories to reflect the observed oviposition pattern [rosette, middle stem leaves s2-s4 (receiving more than half of the eggs), and upper stem leaves, s5-flowers].

Differentiation between *M. sexta* and *M. quinquemaculata* during early instars is difficult and only by rearing larvae through to adults, is a definitive determination



possible (Mechaber and Hildebrand 2000). Two points of evidence strongly suggest that our observations were mainly, if not exclusively, of *M. quinquemaculata*. First, we reared 117 haphazardly collected larvae from the field to the 5th instar and all proved to be *M. quinquemaculata*. Secondly, all 5th instar larvae observed in the field during the census were *M. quinquemaculata*.

Body temperature. Since thermoregulation in desert environments is thought to be an important determinant of *M. sexta*'s foraging behavior (Casey 1976), we measured body temperature of 37 haphazardly selected *M. quinquemaculata* 5th instar larvae crawling on the soil or foraging at different heights on *N. attenuata* plants. The correlation between body temperature and distance from the ground was determined with a regression analysis. In addition, we measured the body temperatures of 21 caterpillars feeding on plants before and 1 min after being placed on the soil surface; the data were analyzed with a paired *t*-test. A preliminary experiment revealed no change in body temperature when larvae were returned to the plant, rather than being placed on the ground. We chose 5th instar larvae for the body temperature measurements because they are large enough to introduce a digital electronic thermometer (TEM-ComTM) into the rectum. The thermometer gives stable temperature readings within 60s with 0.1°C accuracy. Height above ground was recorded before the hornworm's rectal temperature was measured. Body temperatures were measured between 10 a.m. and 2 p.m. on July 5, during which soil temperature (69°C) and air temperature (39°C) were characteristically different and remained unchanged as determined hourly.

Hornworm performance. To compare the performance of young hornworms on old vs. younger leaves, we used *M. quinquemaculata* caterpillars hatched from naturally oviposited eggs collected on *N. attenuata* in the field. The freshly hatched larvae were weighed and placed on excised rosette (3 leaf positions older than the first stem leaf) and younger stem (3rd stem leaf) leaves in individual 750-ml-polystyrene boxes, which were placed inside a trailer. The leaves were collected from 12 flowering plants at the Pahcoon field site and used within 2 h for the bioassay. Petioles were placed into 1.5ml vials filled with water to maintain leaf turgor. Young and old leaves (12 replicates per leaf age) received one neonatant hornworm and were replaced by freshly collected leaves every 2nd d. Hornworm mass was measured 4, 6 and 8 d after hatching.

Analysis of nicotine and PPO. We germinated seeds from a *N. attenuata* inbred line (DI 92) in smoke-treated soil and grew the plants in 1-L hydroponic chambers, as previously described (Baldwin and Schmelz 1994). The inbred line originated from a seed

collection in 1992 in Utah, which was sequentially inbred for 9 generations of selfing in a glass house. After 14 d of growth in 1-L hydroponic chambers, the plants received an additional 7 mg N (KNO₃). Two days later, leaf laminas were harvested (without midveins:150-300 mg), by position, flash-frozen in liquid N₂ and ground frozen to a fine powder in 2 ml microcentrifuge tubes. For nicotine analysis, ground material was extracted with 1.5 ml of 40% aqueous MeOH containing 0.5% acetic acid for 2 h and centrifuged (12 min, 13000 rpm). The supernatant was analyzed by HPLC as described in Keinanen et al. (2001). For PPO and protein analyses, the frozen and ground samples were extracted on ice, for 1 h in 0.1 M NaPO₄ buffer (pH 7.2; 10µl/mg fresh mass) containing 0.15 % SDS. The extract was centrifuged (2 min, 12000 rpm) and the PPO activity was determined in the supernatant by measuring the conversion of L-DOPA to dopachrome at 490 nm and 30°C in 96-well microtiter plates. For the analysis, 205 µl of 4.5 mg/ml L-DOPA in 0.1 M NaPO₄ buffer (pH 7.2), previously aerated for 2 min and 56 units of catalase in 20 µl water were added to 15 µl of the plant extract to start the assay [modified by Sherman et al. (1991) and Constabel and Ryan (1998)]. Absorbance was recorded every 11 seconds and the first 4-6 values were used to calculate the reaction rate. Protein content was determined in 1:6 NaPO₄ buffer diluted aliquot (to reduce the SDS concentration) of the plant extract using the Bradford assay (van Dam et al. 2001b).

Hornworm predation. In addition to the census of larval movement and mortality, we performed a field experiment to determine the effects of developmental stage and oviposition site on the within-plant patterns of larval mortality. Naturally oviposited eggs were collected in the field and attached to 3 different leaf positions on twenty 30-40 cm tall and undamaged flowering plants. The selected leaf positions spanned 3 regions of the plant (Fig. 1): the basal rosette (2 positions older than s2), mid-plant (s3) and the upper (s8). Three eggs per plant (1 egg/position) were attached individually to the lower side of the selected leaves using α -cellulose glue, which elicits neither VOC responses nor influences predation (Kessler and Baldwin 2001). Eggs were considered depredated if they were empty with small injection holes, the characteristic feeding damage of the most important predator, *G. pallens*. Egg mortality was checked 24 h after the experimental oviposition and mortality at different leaf positions was compared with a Wilcoxon-Sign-Rank test (StatView for Windows, SAS Institute Inc.), using a Bonferoni-correction of significance levels for 2 comparisons. In addition we used the Friedman test as a

nonparametric analog of a repeated measures analysis of variance (StatView for Windows, SAS Institute Inc.).

Results

Oviposition site selection. *M. quinquemaculata* moth's nocturnal oviposition patterns clearly demonstrated a preference for elongating plants as compared with rosette plants. While the proportions of rosette and elongated plants in the population was nearly similar (rosette plants 53.4%, elongated plants 46.6%), 92.41% of the plants that received eggs were in the elongating stage (Pearson chi-square test; $Chi^2=433.9$, $P<0.001$). In total 559 eggs were found on elongated plants, while 34 were oviposited on rosette-stage plants. Furthermore, a detailed examination of 508 elongating plants on the fourteen 4m²-plots revealed that younger leaves in the middle part of the plant were the preferred site for oviposition (ANOVA, $F_{9, 130}=17.739$, $P<0.0001$; Fig. 1). The majority of eggs (69.4% of 559) were found on the 4 stem leaf positions (s1 to s4; Fig. 1), almost exclusively attached to the lower leaf side. Neither the rosette leaves, with their greater leaf area, nor the smaller leaves of the upper part of the plant, received as many eggs.

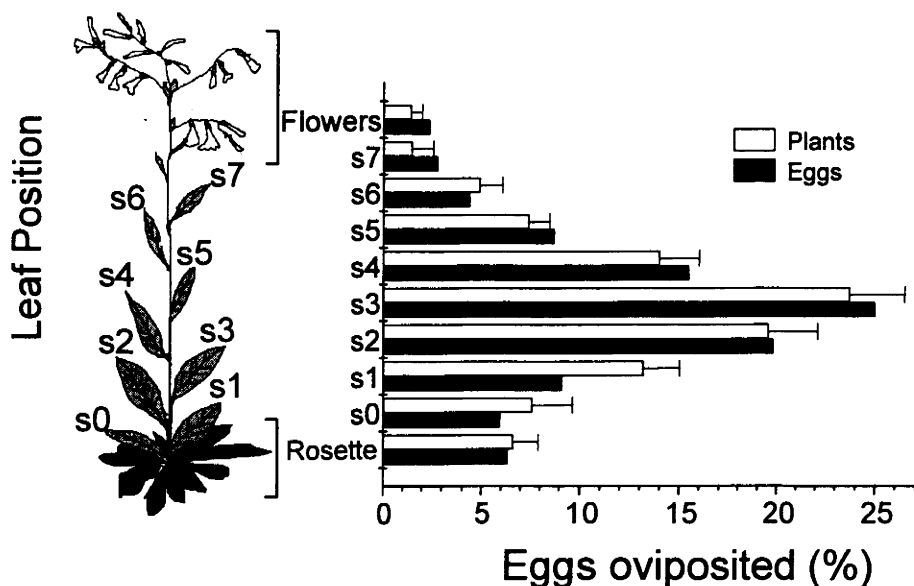


Fig. 1. Percentage distribution of hornworm eggs on different leaf positions of *Nicotiana attenuata* in a natural population in SW Utah. The black bars depict the percentage of a total of 559 eggs distributed on 508 elongating plants that were oviposited on each of the leaf positions s0-s7, rosette and flowers. The white bars depict the average percentage (+ SEM) of plants that

received the first hornworm egg on one of the leaf positions. "Rosette" represents the sum of all 7-11 basal rosette leaves, while "flowers" represents all parts of the inflorescence.

Hornworm movement. Freshly hatched larvae do not leave the leaf on which their egg was laid until the 2nd instar. None of the 35 larvae in the 1st instar were observed leaving their natal leaf. The probability that a hornworm moves to another leaf within the plant increases with age (Pearson $Chi^2=186.01$, $P<0.0001$; Fig. 2A). Occasionally, inter-plant movement was observed when 3rd and 4th instars were feeding on plants with leaves that overlapped those of neighboring plants (3 of 112). Only larvae in the 5th instar crawl on the ground to move from plant to plant. The distance that hornworms are able to move is strongly dependent on their age ($F_{2, 32}=11.245$, $P=0.0002$; Fig. 2B). Fifth instar caterpillars are able to crawl surprisingly long distances across the hot desert soil. The longest distance moved between plants in this study was 151 m on a day when the temperature of the soil surface was 68°C. On the plant, hornworms usually orient parallel to the sun's radiation on the lower side of the leaves.

Body temperature. Larval body temperature was negatively correlated with their distance from the ground (Fig. 3). Hornworms higher on the plant had the lowest body temperature while larvae lower on the plant or which were crawling over the soil surface had the highest body temperature (including caterpillars crawling on soil: $R^2= 0.614$, $P<0.0001$; excluding caterpillars crawling on soil: $R^2= 0.524$, $P= 0.001$). The maximal measured body temperature was 40.9°C, which was measured in a 5th instar caterpillar crawling on soil, independent from the other measurements and therefore not included in the regression. The temperature of hornworms, which were experimentally moved from the plant to the soil increased on average 1.16°C (paired t-test: $t= 2.919$, $P= 0.0085$) within 1 min.

Hornworm performance. The first three instars of *M. quinquemaculata* hornworms developed significantly faster on young excised leaves than on old excised leaves (repeated measures ANOVA. $F_{1, 27}= 14.882$, $P=0.0039$; Fig. 4A). For example, after 8 d, the weight gain of larvae fed young stem leaves was 6.5-fold higher than that of larvae fed older rosette leaves. Furthermore, 8 of the 12 hornworms fed young leaves (66.7%) had molted into the 4th larval instar while all hornworms fed old leaves were still in the 2nd instar by day 8.

Nicotine and PPO concentration. Concentration of nicotine ($F_{4, 35} = 20.7$, $P < 0.0001$) and activity of PPOs ($F_{4, 15} = 3.8$, $P = 0.025$) decreased with leaf age (Fig. 4 B, C).

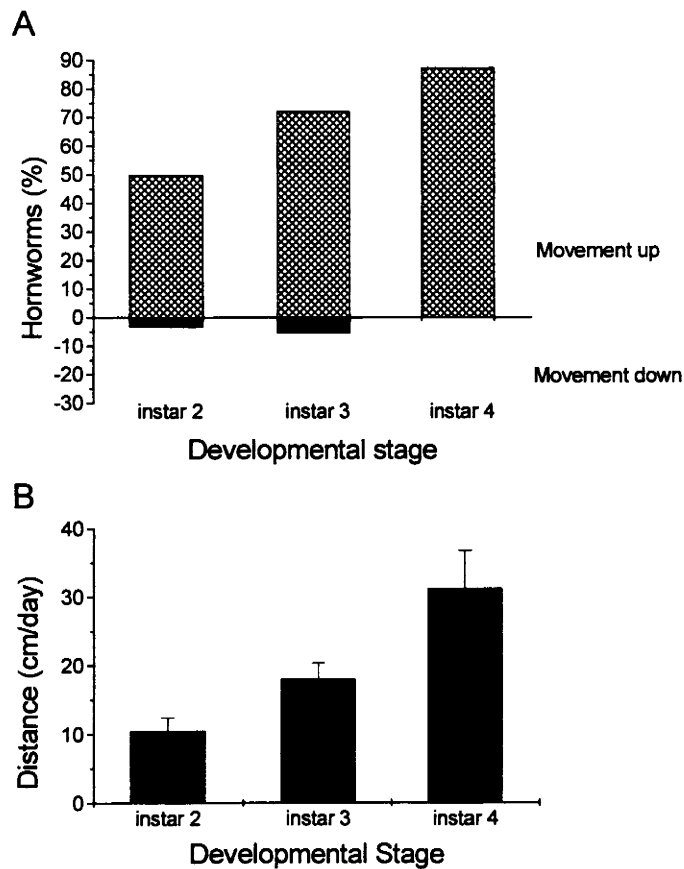


Fig. 2. Hornworm movement in different instars. **A)** Percentages of hornworms moving up or down in different larval instars as a measure of the probability and direction (Black: downward; crosshatched: upward) of hornworm movement. Larvae in the first instar didn't leave the leaf on which they hatched and are therefore excluded. **B)** Mean \pm SEM of the distance (cm) moved per day of different larval instars. First instar larvae were excluded because they didn't move more than 20 mm per day, and 5th instar larvae were excluded because they frequently left the plant and moved distances that were difficult to measure quantitatively.

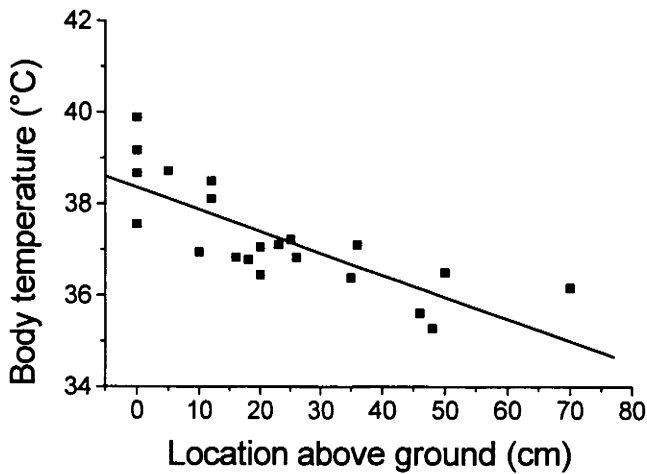


Fig. 3. Hornworm body temperature at different heights above the ground. Body temperatures of 5th instar larvae are negatively correlated ($R^2 = 0.614$, $P < 0.0001$) with the height on the plant on which they were found (air temperature: 38°C, soil temperature: 69°C). Excluding measurements of caterpillars on the ground (0 cm) does not alter the relationship ($R^2 = 0.524$, $P = 0.001$).

Mortality. Hornworm mortality was significantly age-dependent on the censused plants ($H_{4, 153} = 30.763$, $P < 0.0001$). During the observation period, eggs had the highest mortality rate, followed by the 1st and 2nd instars, and mortality of 3rd-4th instars was negligible (Fig. 5A). Throughout the census, *G. pallens*, which typically resides on the ground, was the only observed predator of eggs and 1st-2nd instars. Older hornworms appeared to be too big to be successfully attacked by the bugs. We also observed *G. pallens* attacking leaf bugs, *Tupicoris notatus*, and flea beetles, *Epitrix hirtipennis*. In a separate census, we observed a single Carabidae beetle feeding on a 3rd instar, and 3 Heteropteran stilt bugs feeding on hornworm eggs, but again *G. pallens* was by far the most important predator.

The pattern of *intra*-plant mortality rates of *Manduca* eggs and larvae was highest on rosette leaves and decreased with ascending leaf positions (ANOVA: $F_{2, 18} = 39.5$, $P < 0.0001$; Fig. 5B). The average daily mortality rate ($70.8 \pm 7.1\%$) of the caterpillars on rosette leaves was 2.6-fold and 11.6-fold higher than that of caterpillars on leaves in the middle and the uppermost youngest leaves and flowers, respectively. The same pattern was observed when eggs were manually attached to different leaf positions (Friedman-test: $Chi^2 = 11.375$, $P = 0.0034$).

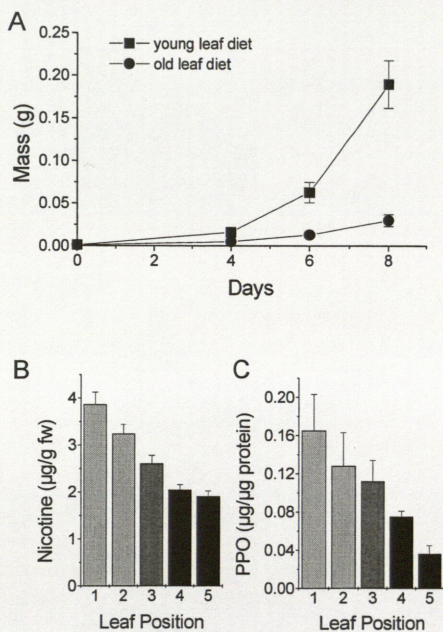


Fig. 4. Hornworm performance and leaf quality. Mean mass \pm SEM of: **A)** *M. quinquemaculata* hornworms just after hatching (day 0) and after 4, 6 and 8 d of feeding on young (squares) and old (circles) leaves freshly excised from naturally growing *N. attenuata* plants; **B)** nicotine content; and **C)** polyphenoloxidase activity (PPO): protein ratio in the following leaf positions of *N. attenuata* rosette plants: 1+2 (light gray): young leaves, 3 (gray): source-sink transition leaf, 4-5 (black): old leaves

Averaging across the 20 replicates, 75% of the eggs or freshly hatched larvae on old rosette leaves were killed within the first 24 h after experimental oviposition. This mortality rate was 2.5-fold and 3-fold higher than that measured at the s3 position in the middle of the plant (Wilcoxon-Sign-Rank, $Z=-2.5$, $P=0.013$) and the young stem leaves of the s8 position (Wilcoxon-Sign-Rank, $Z=-2.9$, $P=0.004$), respectively (Fig. 5C).

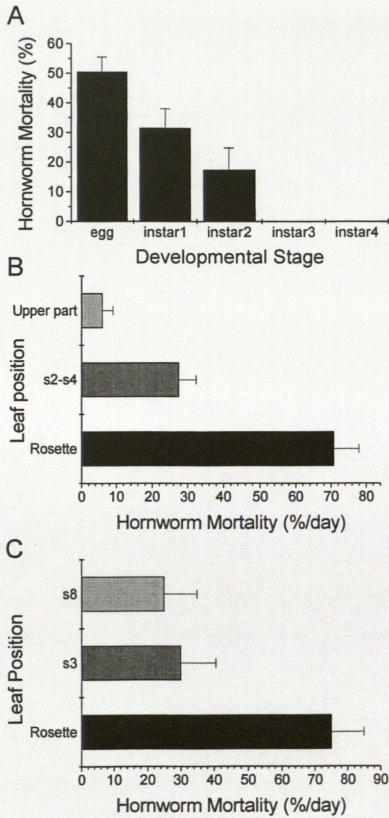


Fig. 5. Age- and position-dependence of hornworm mortality. **A)** Mean \pm SEM mortality of *M. quinquemaculata* in different immature stages (egg, instar 1-4) observed over a 10-d period in a natural population. **B)** Mean \pm SEM daily mortality rate of hornworms on basal rosette leaves (black bar), in the middle stem region (gray bar), leaf position s2-s4, and the top of the plant (light gray bar), s5-s7 and inflorescence. The middle region of the plant (s2-s4) received more than half (60.3%) of all eggs in natural populations (Fig. 1). Mortality data were collected from a repeatedly censused focal population of hornworms in nature. **C)** Daily mortality rate of hornworm eggs glued to different leaf positions in a natural population. Leaf position designations follows Fig. 5B.

Discussion

Our results clearly demonstrate that *M. quinquemaculata* moths prefer to oviposit on elongating plants as compared to plants in the rosette-stage, and select leaves in the middle section of the stem. Within-plant variation in food quality, secondary metabolites, thermal regimes, larval movement and predation risk provide insights into the consequences of the moth's *intra*-plant oviposition preferences for larval growth and survival.

Food quality was examined in bioassays where hornworms fed on leaves of different age. Feeding on younger leaves for *M. quinquemaculata* larvae consistently resulted in faster growth. Preference for younger leaves is known from numerous other lepidopteran herbivores (Scriber and Slansky 1981, Barone 1998) including *M. sexta*, a sister species of *M. quinquemaculata* (data not shown), and is frequently correlated with higher nitrogen and water and lower allelochemical contents in younger leaves (Stamp and Bowers 1990b). In *N. attenuata*, young leaves have higher protein contents, but also higher constitutive concentrations of nicotine, PPOs and PIs (van Dam et al. 2001b). Although both *M. sexta* and *M. quinquemaculata* include non-solanaceous plants in their diet (Mechaber and Hildebrand 2000) they are considered solanaceous specialists and are likely adapted to high titers of defensive compounds in younger leaves (Scriber and Slansky 1981, Appel and Martin 1992). More generalist herbivores on *N. attenuata*, namely noctuid larvae and weevil beetles, usually attack older leaves (pers. obs.) that are lower in nutrients but also nicotine and PI contents (Baldwin and Ohnmeiss 1993, Ohnmeiss and Baldwin 2000, van Dam et al. 2001b). That hornworms clearly perform better on younger leaves and are not dramatically deterred by their high allelochemical concentrations begs the question, why moths did not oviposit on the very youngest leaves on the stem, and the inflorescence with the highest nutritive values and, in the case of the corolla, the lowest nicotine concentration (Euler and Baldwin 1996). And, moreover, why did we not find an oviposition preference for the younger rosette leaves on which caterpillars perform as well as on stem leaves? We propose that the availability of food and the costs of movement when this food is depleted can account for the slight downward oviposition preference (Fig. 1).

Caterpillars moved very little during the first two instars and hence oviposition site determined food choice for these instars. A starvation period during the 1st and 2nd instars was found to reduce *M. sexta*'s larval development more than feeding on fully JA-

induced *N. attenuata* leaves (van Dam et al. 2000). Thus for these larval instars, the costs of movement, which includes increases in starvation and predation risks (Schultz 1983, Bernays 1997) are likely greater than the costs of coping with a plant's induced defenses. Oviposition on the apical leaves and inflorescences would not provide larvae with sufficient biomass to sustain initial growth without a potentially costly move (van Dam et al. 2001a). However, movement costs and food availability are not alone sufficient to explain the observed oviposition pattern (Fig. 1), because the majority of plant biomass is found in the basal rosette leaves (van Dam et al. 2001a), and oviposition on these would afford larvae the opportunity to minimize their subsequent movement requirements. In addition to larval performance, the pattern of mortality from predators provides a second explanation for the upward bias in oviposition preference. The mortality of *M. quinquemaculata* eggs and caterpillars was much higher on the rosette leaves than at higher leaf positions. But these census data could not identify the mechanism(s) responsible for the pattern since egg load differed dramatically within the plant (Fig. 1). In the controlled experiment in which experimental oviposition rates were equalized across leaf positions, all mortality was caused by the generalist, ground-dwelling predator *G. pallens* which preferentially attacked eggs and larvae on lower leaf positions (Fig. 5B, C). Moreover, *G. pallens* are small bugs and do not attack the larger hornworms older than the early 3rd instar, partly due to the hornworms' size but also their more effective defensive behavior (Walters et al. 2001). In summary, predation by *G. pallens* is the most important mortality factor for eggs and early instars of *M. quinquemaculata* in this system (Kessler and Baldwin 2001) for three consecutive seasons (pers. obs.) and can account for the upward bias in oviposition preference.

Thermoregulation may also constrain larval behavior and contribute to the oviposition preferences of the adult moth. The observed correlation of body temperature and feeding site (Fig. 3) and the paraheliotropic orientation of larvae at midday suggests that *M. quinquemaculata* is behaviorally regulating its body temperature on *N. attenuata* (Casey 1976). Such behaviors might minimize evaporative water loss, which increases disproportionately above 35°C (Casey 1977) and allow larvae to maximize their food intake, which is also temperature dependent (Stamp and Bowers 1990a). Thermal constraints may be even more pronounced under high predation pressure, since *M. quinquemaculata* defends against attackers by water-costly spitting (Woods and Bernays 2000, Walters et al. 2001). Larvae under high predation pressure (feeding at lower leaf positions) will defend themselves more often and hence may lose more water than larvae

under lower predation pressures (at higher positions on the plant). As such, predators could influence hornworm behavior in a non-lethal way (Stamp 1997) and presumably, the movement costs for these later instars are offset by the greater costs of defending against attackers.

We have identified 3 environmental factors (temperature, food quality and predation risk) which together can account for the overall pattern of *M. quinquemaculata* oviposition behavior and likely provide the selection pressures that sculpt oviposition behavior. Moreover, the results highlight the overarching importance of predation risk in this system. The plant clearly plays an active role in enhancing the predation risk by releasing VOCs and ovipositing moths clearly respond to these plant signals by altering their oviposition choices (De Moraes et al. 2001, Kessler and Baldwin 2001). Ovipositing moths select the parts of the plant with low predation risks that allow for high larval growth rates and have sufficient biomass to sustain growth until movement costs are minimal. Since the constitutive concentration of phytochemicals is high in these selected young leaves, the larvae may realize some protection against natural enemies. *Cotesia congregata* parasitoids, for example, experience higher mortality when parasitizing *M. sexta* larvae, feeding on high nicotine diets (Barbosa et al. 1991). The importance of plant-derived defenses for larval defense can be evaluated with plants that are unable to synthesize particular defense compounds and such experiments are in progress. Our results demonstrate that oviposition preferences are likely determined by many different factors in addition to the effects of plant quality (Courtney and Kibota 1990). Mothers clearly respond to proximate signals from the plant and its environment to optimize the survival of their offspring. Uncovering these signals will provide valuable tools for pest control.

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Manuscript 3

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**Defensive function of herbivore-induced plant volatile emissions in
nature**

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Running title: Plant volatile emission in nature

Abstract

Herbivore-attack is known to increase the emission of volatiles, which attract predators to herbivore-damaged plants in the laboratory. We quantified volatile emissions from *Nicotiana attenuata* plants growing in natural populations during attack by 3 species of leaf-feeding herbivores and mimicked the release of 5 commonly emitted volatiles individually. Three compounds (*cis*-3-hexen-1-ol, linalool, *cis*- α -bergamotene) increased egg predation rates by a generalist predator; linalool and the complete blend decreased lepidopteran oviposition rates. As a consequence, a plant could reduce the number of herbivores by more than 90% by releasing volatiles. These results confirm that indirect defenses can operate in nature.

Plants defend themselves against herbivores with chemical and physical defenses that directly influence herbivore performance and indirectly through traits that attract the natural enemies of herbivores (1-3). One such indirect defense, the release of volatile organic compounds (VOCs) specifically after herbivory, is known to attract parasitoids and predators to actively feeding larvae in the laboratory (4, 5), and evidence from agricultural systems suggests a role for herbivore-induced VOCs in increasing predation pressure (6-8). However, conclusive evidence has been lacking, and it is not even known whether plants growing in natural populations increase VOC emissions after herbivore attack. VOCs might be able to function as indirect defenses only in simplified agro-ecosystems, in which a single natural enemy species of a herbivore can act as an important bio-control agent on an agricultural plant (9). In contrast, in natural systems herbivore mortality is more commonly mediated by a suite of generalist enemies (10). Moreover, both the qualitative and quantitative characteristics of herbivore-induced plumes of VOCs are known to vary among plant genotypes (11-13); the genetic variation commonly found in natural populations may undermine the reliability of VOCs as a signal for natural enemies, as prior exposure is often needed to associate plant VOCs with the occurrence of a feeding herbivore (5,14,15). Herbivore-induced plant VOCs may also influence herbivore host-location behavior, potentially increasing herbivore attack on plants releasing VOCs (1, 2).

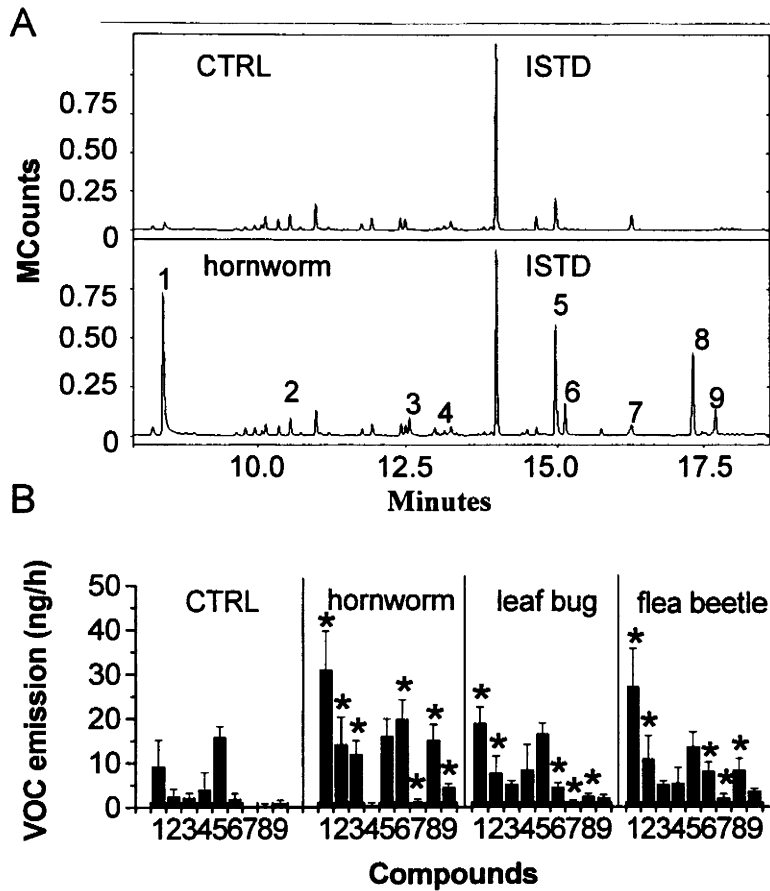


FIG. 1. VOC release in response to herbivory in nature. **A** Representative Total Ion Chromatograms of the headspace of an undamaged leaf from an undamaged plant (CTRL) in comparison to a leaf damaged by a *M. quinquemaculata* larva (hornworm). **B** Comparison of the mean (\pm SEM) emissions of VOCs from undamaged plants (CTRL) and plants damaged by *M. quinquemaculata* larvae (hornworm), *D. minimus* (leaf bug) and *E. hirtipennis* (flea beetle) (with 8 replicate plants per treatment in the same native population). Since herbivore-induced VOCs from *N. attenuata* are emitted with different kinetics depending on the compound (13), data from the 7 h trapping, 24 h after herbivore infestation are presented. Each VOC trap was spiked with 300ng tetraline as an internal standard (ISTD), eluted with 750 μ l dichloromethane and analyzed by GC-MS (21). The labels in both **A** and **B** represent *cis*-3-hexen-1-ol (1), *trans*- β -ocimene (2), *cis*-3-hexenyl acetate (3), linalool (4), terpineol (5), *cis*-3-hexenyl butyrate (6) methyl salicylate (7), *cis*- α -bergamotene (8), *trans*- β -farnesene (9). Stars designate compounds whose emission was significantly [$P < 0.05$; Bonferroni-corrected Fishers protected least significant difference (LSD) test of an (ANOVA)] elevated in comparison with undamaged control plants.

To evaluate the role of herbivore-induced VOCs in nature, we characterized the VOCs released from *Nicotiana attenuata* Torr. ex Wats (Solanaceae) plants growing in a native population (16-18) in the Great Basin desert of southwest Utah, which were under continuous attack by three numerically-dominant folivores: the caterpillars of *Manduca quinquemaculata* (Lepidoptera, Sphingidae), the leaf bug *Dicyphus minimus* (Heteroptera, Miridae) and the flea beetle *Epitrix hirtipennis* (Coleoptera, Chrysomelidae) (19). We used an open-flow trapping design (13) to collect VOCs individually from 32 plants growing in a natural population which each had one leaf attacked by one of the 3 herbivore species, or remained undamaged (13, 20; Fig 1A,B). All plants were growing in a 150 m² portion of the population (18) and were sampled simultaneously for 7 h. GC-MS analysis (21) of the trapped VOCs revealed that all 3 herbivore species elicited increases in the same suite of VOCs, although the odor profiles were not identical (Fig 1B). The pattern and amount of herbivore-induced VOCs trapped from *N. attenuata* growing in the field were very similar to those found in laboratory studies with plants attacked by *M. sexta* larvae (13).

The emitted VOCs common to all three herbivores are derived from three biosynthetic pathways. Green leaf volatiles (*cis*-3-hexene-1-ol, *cis*-3-hexenyl acetate, *cis*-3-hexenyl butyrate; 1,3,6 in Fig 1) derived from the octadecanoid pathway are known to be emitted rapidly after damage (11, 13) but are not specific to plants attacked by herbivores (2). The terpenoids (*trans*- β -ocimene, *cis*- α -bergamotene and *trans*- β -farnesene; 2, 8, 9 in Fig 1) are emitted more slowly, typically 24 h after attack (11, 13). Only 5 of the 24 attacked plants emitted linalool (4 in Fig 1), which is consistent with previous findings that only some *N. attenuata* genotypes are linalool emitters (13). All attacked plants showed significantly elevated emissions of *cis*- α -bergamotene, which is known to be elicited in *N. attenuata* when a suite of 7-8 fatty acid-amino acid conjugates that occur in the oral secretions of both *M. sexta* and *M. quinquemaculata* larvae are introduced to leaf wounds (22) as well as when plants are treated with methyl jasmonate (MeJA; 13). Finally, the emission of the shikimate-derived methyl salicylate (MeSA; 7 in Fig 1) was significantly elevated in the headspace of all attacked plants. Since all of these compounds have been identified in the headspace of other herbivore-infested plants (2, 5, 23, 24), they may function as universal signs of herbivore damage and we hypothesized that increasing the emission of single compounds in the context of the plants' natural background emission should attract natural enemies in nature.

To test this hypothesis, we mimicked the herbivore-induced emission of individual compounds from each biosynthetic class. To mimic the volatile release, we applied 200 μg of either MeJA, MeSA, *cis*-3-hexene-1-ol, *trans*- β -ocimene, *racemic* linalool or *cis*- α -bergamotene in 20 μl of lanolin paste to the stems of flowering plants in a natural population (18). Controls were treated with 20 μl of pure lanolin. To determine the fidelity of the mimicry, we trapped the VOCs from whole plants and found the emission to be very similar to those of herbivore-infested or MeJA-treated plants (Fig 2A: data shown for control, MeJA and *cis*- α -bergamotene treated plants). Application of individual compounds (with the exception of MeJA, which elicited a majority of herbivore-induced VOCs) resulted in increased emissions of only the applied compound, which was released in quantities within the range of emissions observed in herbivore-infested plants (Fig 2A; 25).

We used *M. sexta* eggs to measure predation rates to avoid the confounding influence of direct defenses elicited by herbivore feeding. A single hornworm larva can completely defoliate 1-10 reproductively mature *N. attenuata* plants in the course of its development, and hornworm larvae have been responsible for most of the leaf area lost to insect herbivores in 6 *N. attenuata* populations monitored in SW Utah over the past 2 years. *Manduca* eggs are typically laid singly on plants, but clusters of 4-7 eggs on a single leaf are occasionally found. To measure predation rates, we fixed 5 *M. sexta* eggs to the underside of both the 2nd and 3rd stem leaves (26) of 105 unattacked *N. attenuata* plants, using a neutral α -cellulose glue that was known not to effect changes in VOC emissions (27). Lanolin paste with and without the individual VOCs was placed on the stem between the leaves.

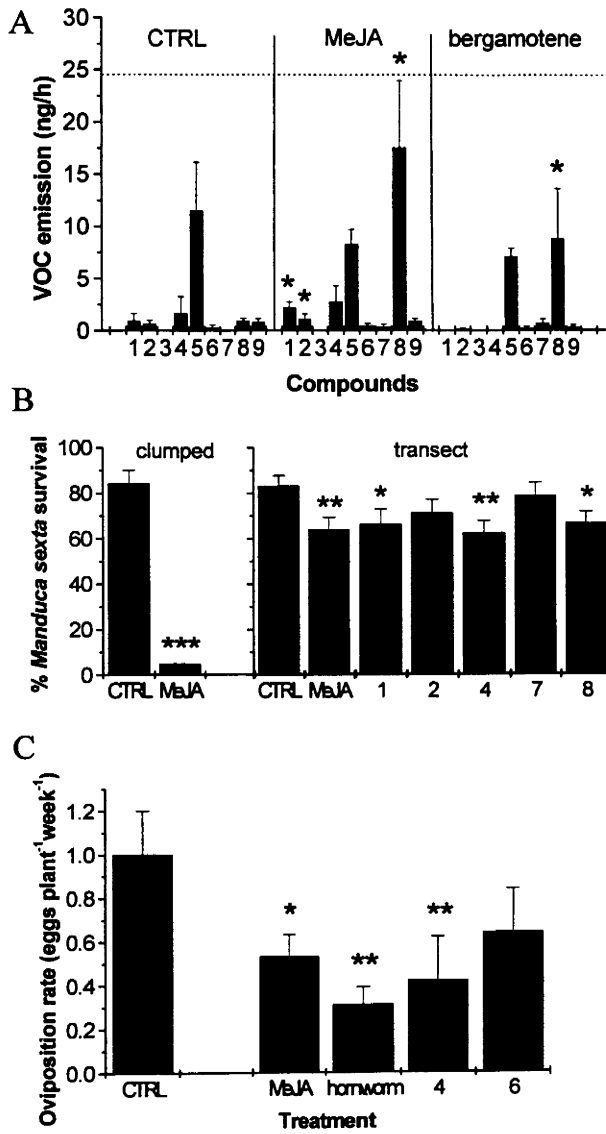


FIG.2. VOCs and their influence on the survivorship of hornworm eggs and oviposition rates of adult *M. quinquemaculata* in nature. **A** Mean (\pm SEM) whole-plant emissions of 9 VOCs from plants (4 per treatment) treated with lanoline paste (CTRL), methyl jasmonate (MeJA) and *cis*- α -bergamotene (bergamotene) 24 h after application. Plants were enclosed in an open 2 L conical plastic chamber, and the headspace air was pulled through a charcoal trap for 7 h at a flow rate of 450 to 500 ml min⁻¹ by a vacuum pump (13). The “bergamotene” treatment (as well as the *cis*-3-hexene-1-ol, *trans*- β -ocimene, linalool, methyl salicylate treatments; 25) only increased the emission of the applied compound. The dotted line denotes the highest natural *cis*- α -bergamotene emission rate measured from a plant damaged by a hornworm in this study. **B** Mean (\pm SEM) % survival of *M. sexta* eggs on plants releasing elevated amounts of single VOCs: *cis*-3-hexene-1-ol

(1), *trans*- β -ocimene (2), linalool (4), methyl salicylate (7), *cis*- α -bergamotene (8). In the “clumped” experimental design, plants treated with the same VOC were within 3-5 m of each other, while in the “transect” experimental design, similarly treated plants were within 21-35 m of each other. C Mean (\pm SEM) *M. quinquemaculata* oviposition rates on MeJA-treated plants, plants under previous attack by hornworms, and plants treated with *cis*-3-hexenyl butyrate (6) and linalool (4). Stars represent significant differences from control (CTRL) plants (* P <0.05, ** P <0.01, *** P <0.001) as determined by Fisher’s protected LSD test of an ANOVA.

Plants were of the same size (30-40 cm), unattacked and 3-5 m apart in a linear 500 m transect across a population of more than 100,000 plants. Treatments were applied so that no 2 plants of the same treatment were within 21-35 m from each other. This distance was sufficient to prevent the plumes from similarly treated plants from interacting and provided a conservative measure of the ecological function of VOC emission. In another experiment in the same population of plants, in which distances between similarly treated plants were only 3-5 m of each other, predation rates were 13-fold higher (Fig 2B; data for MeJA and control treatments shown), probably because of the lack of independence of replicates within a treatment.

During the experiment, only one predator (*Geocoris pallens*; Heteroptera, Geocoridae) was observed feeding on the eggs and hatching larvae. Attacked eggs are emptied and hence easy to distinguish. Moreover, this predator was repeatedly observed preying on leaf bugs and flea beetles. During the previous field season, *G. pallens* was responsible for 95% of the *M. quinquemaculata* and *M. sexta* larvae mortality (26); no *Manduca* eggs on *N. attenuata* were parasitized. Survival of eggs and neonatant larvae was monitored for 48h. After 24h the mortality was already significantly higher on plants treated with MeJA ($36 \pm 5.4\%$), *cis*-3-hexene-1-ol ($33.8 \pm 6.7\%$), linalool ($37.5 \pm 5.3\%$) and *cis*- α -bergamotene ($33.3 \pm 5.1\%$), while mortality on plants treated with MeSA ($21.2 \pm 5.8\%$) and *trans*- β -ocimene ($28.7 \pm 5.8\%$) did not differ significantly from mortality on untreated control plants ($16.7 \pm 4.4\%$; Fig 2B).

To determine if the herbivore-induced release of VOCs influenced the oviposition behavior of native adult *Manduca* moths, we established a new transect with unattacked plants across the same populations with the following treatments (28): 1-4 foraging *M. quinquemaculata* 1st to 3rd instar larvae per plant; MeJA to elicit a majority of the herbivore-induced VOCs; one green leaf VOC (*cis*-3-hexenyl butyrate) and one terpenoid

(linalool); and a control lanolin treatment. At the time of the experiment, *M. quinquemaculata* adults were ovipositing, and the number of eggs deposited on each plant were counted and removed every 2nd day for 14 days. Lanolin treatments were refreshed every 48 h to maintain VOC emissions. Over a one-week period, moths laid fewer eggs per plant on plants under attack by caterpillars (0.31 ± 0.08), plants treated with MeJA (0.53 ± 0.1) and on plants treated with and releasing linalool (0.42 ± 0.2). Plants treated with *cis*-3-hexenyl butyrate (0.64 ± 0.2) received the same number of eggs as control plants (1.0 ± 0.2) (Fig 2C).

By releasing VOCs after herbivore attack, a plant can profoundly influence both oviposition and predation rates in nature and thereby influence both “bottom-up” as well as “top-down” control over its herbivore populations. The emission of linalool alone caused a 2.4 fold reduction in oviposition rate. Daily predation rates on plants releasing VOCs, extrapolated to 1 week, were 4.9-7.5 times higher than those observed on control plants, hence the top-down effects in this experiment were more strongly influenced by VOC emissions. The cumulative effect, calculated from both the bottom-up and the top-down components of this indirect defense (25) could reduce the numbers of the plant’s most significant insect folivore, *M. quinquemaculata*, by 91.7 (MeJA treatment) and 94.5% (linalool treatment). Herbivores as well as predators appear to use the same volatile signals, suggesting that plants are under strong selection to release them.

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18. *N. attenuata* is a post-fire annual plant that can dominate the pioneer community 1-3 years after a fire destroys the dominant vegetation and stimulates germination from a long-lived seed bank. Experiments were performed during the 2000 growing season (April-August) with a large population growing on a 2.72 km² area (T43SR17W section 32) west of St. George, Utah, which burned for 7 days after a lightning strike on June 17, 1999 (fire number W246).
19. Quantitative census of the herbivorous arthropod community residing on 250 *N. attenuata* plants at each of 2 burns during the 2000 field season revealed the following percentage representations: *D. minimus* (52.9%), *E. hirtipennis* (18.3%), *Geocoris*

- spp.* (9.6%), *M. quinquemaculata* (9.2%), *M. sexta* (3.3%), others (5.8%). While both *Manduca* species co-occurred on *N. attenuata* plants, *M. quinquemaculata* was more abundant earlier in the season.
20. Previous laboratory experiments with *N. attenuata* revealed that whole-plant VOC emissions were quantitatively related to the amount of herbivore-specific leaf damage. Hence we infested plants with the following numbers of the 3 herbivore species to release comparable amounts of VOCs: 8 adult *D. minimus*; 5 adult *E. hirtipennis*; one 3rd instar *M. quinquemaculata* larva (A. Keßler unpublished results). To restrict insects to a single leaf and to trap volatiles from this leaf, leaf and insects were enclosed in 400 ml polystyrene chambers fitted with holes at both ends. Air was pulled through the chamber at 450-500 ml min⁻¹ (measured by a mass flow meter: Aalborg Instruments, Orangeburg, NY, USA) and subsequently through a charcoal air sampling trap (ORBOTM-32; SUPELCO, State College, PA, USA) by a portable 12 V DC vacuum pump (Gast Mfg, Benton Harbor, MI, USA). Measurements in- and outside of the chambers revealed no temperature differences throughout the two days of 14 h sampling.
 21. Analysis was performed at the field sites with a Shimadzu (Model 5000) quadrupole GC-MS programmed as follows: injector 225°C, initial column temperature held at 45°C for 6 min, increased at 10°C min⁻¹ to 130°C, increased at 5°C min⁻¹ to 180°C, increased at 20°C min⁻¹ to 230°C, held for 5 min, increased at 20°C min⁻¹ to 250°C and held for 5 min. Helium carrier gas flow was set to 1 ml min⁻¹ with an electronic pressure control unit. Spectra were collected at -70eV and compounds were identified by comparison of retention times and mass spectra with those of authentic standards.
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 25. A comparison of natural VOC emission with emissions from plants treated with individual VOCs as well as a detailed summary of observed and calculated values of top-down and bottom-up effects are available as supplementary data at *Science Online* at www.sciencemag.org/cgi/content/full/291/5511/2141/DC1.
 26. We chose to attach *M. sexta* eggs on the underside of 2nd and 3rd stem leaves (measured from the bottom of the plant) to measure predation rates because an

extensive study during the 1999 field season of mortality factors of both *Manduca* species revealed that predation rates depended on leaf position. The probability of predation for eggs and 1st to 3rd instar hornworms on rosette leaves was 2.6 times higher than that on the 2nd-4th stem leaves and 11.8 times higher than that on the 5th or younger stem leaves (A. Keßler unpublished results). We followed the fate of 174 naturally oviposited hornworm eggs and could ascribe approximately 95% of the mortality to *G. pallens* predation. The decreasing predation rates of eggs and larvae feeding at higher leaf positions was likely due to the ground-dwelling behavior of *G. pallens*. We monitored the mortality of 559 naturally oviposited eggs in the 1999 field season, of which 71.75% were laid on 1st to 4th leaf position. Approximately half of the mortality occurred at the egg stage (50.5%), 31.5% to 1st instar larvae and 17.4% to 2nd instar larvae (A. Keßler unpublished results).

27. Quantities of VOCs trapped for 7 h from whole plants 24 h after treatment with the α -cellulose glue used to fix eggs to leaves in the field experiment did not significantly increase as compared to water-treated control plants (all P's for compounds 1-7 > 0.88). Laboratory experiments also demonstrated that naturally oviposited *M. sexta* eggs did not elicit a detectable plant VOC release in comparison to control plants without eggs (A. Keßler, unpublished results).
28. We used 40-50 cm tall flowering plants, growing 3-5 m apart in a 400 m linear transect across the population.
29. Supported by Max-Planck-Gesellschaft. We thank Drs. P. Feeny, J. Gershenzon, M. Hilker, J. McNeil, F. Roces, A. Roda, E. Wheeler and 3 anonymous reviewers for helpful comments, Dr. B. Krock and H. Thomas for purifying the *cis*- α -bergamotene, Dr. R. Baumann for assistance with species determinations, and Brigham Young University for use of their awesome Lytle Preserve as a field station.

Supplementary data

(Science Online at www.sciencemag.org/cgi/content/full/291/5511/2141/DC1).

Supplemental Table 1: Mean emission rate \pm SEM of *cis*-3-hexen-1-ol (1), *trans*- β -ocimene (2), linalool (4), *cis*-3-hexenyl butyrate (6), methyl salicylate (7) and *cis*- α -bergamotene (8) measured without damage (CTRL), after damage by various herbivores, and after application of methyl jasmonate (MeJA) and single VOCs (1-8). The maximum measured emission of a single compound induced by herbivore damage is given as "Max value".

	Compounds (ng h ⁻¹)					
	1	2	4	6	7	8
Herbivore damage						
CTRL	9.22 \pm 5.93	2.46 \pm 1.64	3.94 \pm 3.94	1.91 \pm 1.2	0 \pm 0	0.42 \pm 0.42
Hornworm	31.03 \pm 8.75	14.22 \pm 6.17	0.5 \pm 0.5	19.88 \pm 4.44	1.17 \pm 0.65	15.13 \pm 3.59
Leaf bug	18.97 \pm 6.64	7.65 \pm 3.86	8.41 \pm 5.71	4.51 \pm 1.01	1.14 \pm 0.44	2.44 \pm 0.59
Flea beetle	27.12 \pm 8.62	10.84 \pm 5.24	5.4 \pm 3.56	8.09 \pm 2.02	2.08 \pm 0.82	8.36 \pm 2.63
Max. value	74.22	43.36	41.78	48.19	5.34	35.78
Compound application						
MeJA	2.2 \pm 0.54	1.1 \pm 0.49	2.76 \pm 1.54	0.46 \pm 0.2	0.3 \pm 0.3	17.522 \pm 6.46
1	6.52 \pm 3.42	0.43 \pm 0.26	2.46 \pm 2.46	0.21 \pm 0.21	0 \pm 0	0.64 \pm 0.64
2	1.69 \pm 1.65	20.47 \pm 0.7	1.23 \pm 0.66	0.34 \pm 0.13	0 \pm 0	0 \pm 0
4	1.12 \pm 0.65	0.49 \pm 0.49	18.75 \pm 4.45	0.16 \pm 0.16	0 \pm 0	0.84 \pm 0.32
7	1.81 \pm 0.34	1.19 \pm 0.72	0.44 \pm 0.44	0 \pm 0	6.88 \pm 3.53	2.82 \pm 1.53
8	0 \pm 0	0.13 \pm 0.08	0 \pm 0	0.18 \pm 0.18	0.66 \pm 0.41	8.8 \pm 4.8

Supplemental Table 2. Effects of increased volatile emission on oviposition rate (bottom-up) and mortality rate (top-down) on *M. quinquemaculata* herbivore loads (cumulative effect). The experimentally measured mean values \pm SEM for oviposition (OR) and mortality/ survival (MR/SR) rates in the MeJA and linalool treatments were standardized to the corresponding controls (CTRL) to estimate the relative treatment effects (ROR and RSR, respectively). The cumulative effect (CE) was calculated from the herbivore load (HL) of hornworm eggs after the plant was treated with various VOCs. (ND = not determined). These calculations assume constant oviposition and mortality rates for a week. The oviposition data is derived from a two-week measurement, so this assumption is in line with the empirical measures. The measures of mortality rates are likely to be conservative. As demonstrated in the “clumped” experiment (see Fig 2 in manuscript), *Geocoris* converge on emitting plants that are spatially clumped and can inflict much higher mortality rates in one day. We used the data from our linear transects which likely underestimates the cumulative mortality effects of VOC emission. We chose a 7-day time interval for the calculation, for during this time, eggs can develop into 1st instar larvae, which are also very vulnerable to *Geocoris* predators (26).

Treatment	CTRL	MeJA	Linalool (4)	1	2	7	8
Bottom-up							
Oviposition rate, OR (eggs plant ⁻¹ week ⁻¹)	1.00 \pm 0.2	0.53 \pm 0.1	0.42 \pm 0.2	ND	ND	ND	ND
Relative oviposition rate, ROR for OR _{CTRL} = 1	1	0.53	0.42				
Top-down							
Daily Mortality Rate, MR _{day}	0.17 \pm 0.04	0.36 \pm 0.05	0.38 \pm 0.05	0.34 \pm 0.07	0.29 \pm 0.06	0.21 \pm 0.06	0.33 \pm 0.05
Daily Survival Rate, SR _{day}	0.83 \pm 0.04	0.64 \pm 0.05	0.63 \pm 0.05	0.66 \pm 0.07	0.71 \pm 0.06	0.79 \pm 0.06	0.67 \pm 0.05
Weekly Survival Rate, SR _{week} = SR _{day} ⁷	0.28	0.043	0.037	0.054	0.091	0.19	0.061
Relative Weekly Survival Rate, RSR for SR _{CTRL} = 1	1	0.15	0.13	0.19	0.32	0.67	0.22
Cumulative Effect							
Relative Herbivore Load, HL = ROR x RSR	1	0.081	0.056				
Relative Cumulative Effect (%), CE = (1 - HL)100		91.9	94.5				

Manuscript 4

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Plant vaccination against herbivore attack in nature

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Running title: Plant vaccination against herbivory

Abstract

Plants respond to herbivore attack with direct and indirect defenses that can be tailored to different herbivore species and can mediate competitive interactions between herbivores. With experiments using natural populations in SW Utah, we demonstrated that wild tobacco plants, *Nicotiana attenuata*, attacked by the mirid (*Tupiocoris notatus*), received 75% fewer tomato hornworm (*Manduca quinquemaculata*) eggs; sustained 85% slower growth of hornworms; and were 61% more attractive to the predator *Geocoris pallens*, which decreased hornworm survival. Hence prior attack by mirids diminishes the plant's attractiveness to the hornworm. The vaccinating effect of initial *T. notatus* feeding prevents the 40.7% fitness loss that hornworm-attacked plants experience. We conclude that plants can benefit from competitive interactions within their herbivore community.

Plant responses to certain pathogens and herbivores may result in resistance to subsequent attack from the same organisms as well as to different organisms (1). Such a cross-resistance, called immunization (2) or vaccination (1), can benefit the plant if the fitness consequences of attack from the initial attacker are lower than those from subsequent attackers. Vaccination has been commercially used to protect crop plants by inoculating them with less harmful pathogens or by applying pathogen-derived elicitors, both of which induce defense reactions against subsequent attackers with high impact (3).

Competitive interactions between arthropod herbivores may also result from such plant-mediated cross-resistance (1, 4). For example, phytophagous Willamette mites (*Eotetranychus willamettei*) induced resistance in Zinfandel grapevines against the economically more damaging Pacific spider mite (*Tetranychus pacificus*), a vaccinating effect that controlled Pacific spider mite populations and increased sugar concentrations of the harvested grapes (5). The apparent asymmetric competition between these two phytophagous mite species was amplified by the introduction of a shared predator (6). Despite the apparent success of this study, vaccinating plants against losses to herbivores by inducing resistance with benign herbivores has not gained acceptance in agriculture (1), nor is it known to occur in natural populations, probably because the requirements are onerous. Two are essential: the more competitive herbivore species should be the less damaging one (asymmetric competition); and the plant should realize a fitness benefit from the first challenge (resistance). While competitive interactions between herbivores are often but not always (7) plant mediated and asymmetric (8), the fitness consequences

of the interaction for the plants remain unclear and are rarely examined. The only study that examines fitness effects of prior herbivore attack (9) did not demonstrate asymmetric competition and used a herbivore that was not part of the plant's natural community in this study (10). Here we report an apparent competitive interaction between the tomato hornworm, *Manduca quinquemaculata*, and a mirid, *Tupiocoris notatus*, on the wild tobacco plant, *Nicotiana attenuata*, which results when both the plant's direct defenses slow the growth of hornworms and its indirect defenses attract the generalist predator, *Geocoris pallens*. Finally, we evaluate the consequences for plant fitness resulting from these interactions.

For wild tobacco, both direct and indirect defenses are known to increase the fitness of plants under attack in natural populations (11, 12). This species increases its production of secondary metabolites (nicotine, proteinase inhibitors, phenolics, volatile terpenoids, etc.) after attack, which both diminishes the plant's palatability and attracts generalist predators (12-17). Moreover, the plant appears to recognize attack from particular herbivores by the introduction of fatty acid-amino acid conjugates from the herbivore saliva into plant wounds and is able to tailor its defense responses accordingly (18).

N. attenuata occurs ephemerally in large populations after fires in desert habitats in southwestern USA and germinates from long-lived seed banks in response to factors in wood smoke (19). This 'fire-chasing' behavior forces the plant's arthropod community to re-establish itself with every new population. While studying the herbivore community of many populations of plants, we noticed that two of the most abundant herbivores, the tomato hornworm and the mirid, tend not to overlap spatially, even in adjoining populations. In particular, plant populations with established mirid populations are rarely found supporting large hornworm populations.

Our formal investigation of the interactions of *N. attenuata*'s arthropod community in the Great Basin desert in southwestern Utah started in June 1999. We censused 30-40 cm tall plants in linear transects (4 to 6 m distance between plants) at two different burns (20) and recorded the occurrence and dynamics of the two most abundant herbivores over two weeks in two-day intervals. The daily mortality rate of hornworms was significantly higher in the "HI91"-population than in the "Pahcoon"-population (Table 1). Density-dependent mortality factors for the hornworms could not account for the difference, because the moth oviposition rate was similar in both populations (Table 1). As in previous observations, the most remarkable difference was the presence of

mirids, which were nearly absent at the “Pahcoon”-population but abundant in the “HI91”-population. The opposite was true for hornworm larvae and eggs (Table 1). These observations led us to hypothesize that the presence and/or feeding activity of the mirids may either directly influence the performance or indirectly increase the predation rate and thus the mortality of hornworm larvae.

Table 1. Mirid density and *Manduca quinquemaculata* hornworm mortality and adult oviposition rate in two *Nicotiana attenuata* populations.

Population	Plants with Mirids (%)		Oviposition rate (eggs plant ⁻¹ day ⁻¹) ± SEM		Hornworm mortality (% day ⁻¹) ± SEM	
	n	n	n	n	n	n
Pahcoon	36	2.7	66	0.53 ± 0.06	36	13.4 ± 5.2
HI 91	26	84.6	30	0.49 ± 0.06	26	34.4 ± 8.8

To test the direct effect of mirid attack on hornworm performance on *N. attenuata* plants, we conducted a no-choice feeding experiment. We collected the third stem leaves of elongated *N. attenuata* plants, which had either been previously attacked by 5 to 10 mirid individuals or had not been attacked at all. Twelve damaged and 12 undamaged leaves were placed in individual 1 L PE boxes and infested with one hornworm egg, freshly collected from the field. Caterpillars were weighed just after hatching and at 4, 6, and 8 days thereafter. To control for environmental and genetic factors that may influence host-plant susceptibility, we chose developmentally similar leaves from plants in the same developmental stage and population. The leaves were replaced every other day by developmentally similar leaves from new plants to avoid any influence of leaf removal. Larvae on undamaged control plants had an up to 85% higher weight gain (day 8) and reached the next larval instar 2 days earlier (Fig. 1A; Repeated measure ANOVA $F_{1,62} = 7.972$, $P = 0.0064$). The lower susceptibility of mirid-damaged leaves to hornworms is correlated with dramatic changes in secondary metabolite content [Supplementary Table 1 (21)], suggesting a mirid-induced resistance of the plant to subsequent attack by hornworms.

A laboratory experiment with the sibling species *M. sexta*, in which mirids were experimentally assigned to plants, produced a similar effect on hornworm growth and

performance, and excluded the possibility that mirids in the field had selected plants of low quality for hornworm larvae. In this experiment, we measured an up to 45.3% greater weight gain (day 5) in hornworms feeding on undamaged control plants than in hornworms feeding on mirid-damaged plants [Supplementary Fig. 1 (21)], which is comparable to results from similar experiments with *N. attenuata* genotypes differing in trypsin protease inhibitor production (22), and to plants elicited with methyl jasmonate (15).

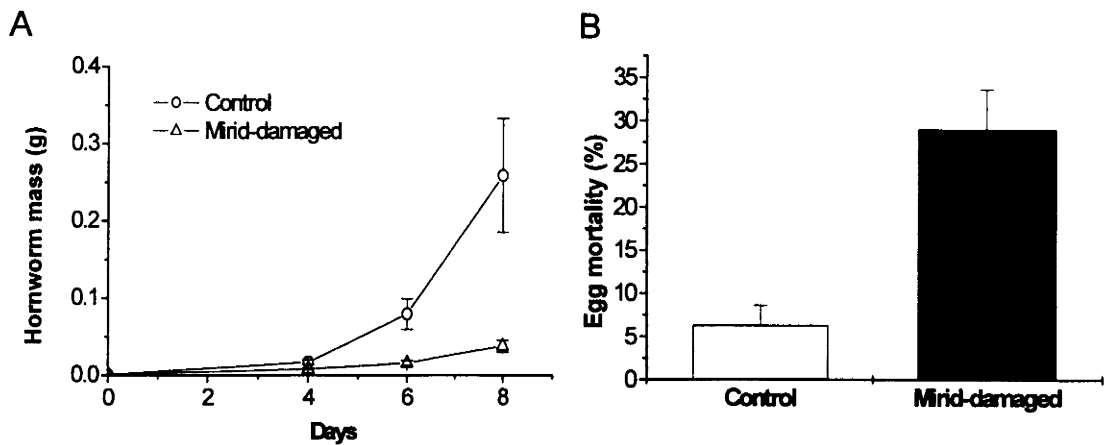


FIG. 1. Performance and mortality of *M. quinquemaculata* hornworms was reduced on field collected *T. notatus* (mirid) damaged plants. **A** Comparison of the mean (\pm SEM) hornworm mass 0, 4, 6, and 8 days after feeding on control leaves from undamaged plants (open circles) vs. leaves of plants previously damaged by the mirid *T. notatus* (open triangles). **B** Mean (\pm SEM) mortality of hornworm eggs experimentally applied to undamaged control plants and plants under *T. notatus* attack.

Slower growth does not necessarily result in higher mortality but may increase predation risk if predators are present (23, 24). In the *N. attenuata*-*Manduca* system, the generalist predator, *G. pallens*, which is responsible for up to 95 % of the mortality of hornworm eggs and young larvae, also attacks mirids in all developmental stages (12). Moreover, mirid-damage induces the release of volatile organic compounds, some of which attract foraging *G. pallens* (12). To test whether previous mirid damage influences the predation pressure on hornworms that arrive subsequently, we conducted a field experiment in June 2001 in a population of about 100,000 plants (20). We chose 40 elongated but not yet flowering plants of the same developmental stage (30-40 cm tall) along a linear transect across the population and infested every second plant ($n=20$) with

10 mirid adults. The minimum distance between two plants of the same treatment was 6 m. Two days after the infestation, we glued 5 hornworm eggs on the second and 5 eggs on the third leaf positions of each of the 40 plants using an α -cellulose glue (12). Predation on the eggs was monitored over two days, until hatching. We used eggs rather than larvae in this experiment to avoid eliciting inducible responses to the hornworm feeding. Because *G. pallens* pierces and empties eggs, leaving the eggshells nearly intact, it is easy to distinguish between predation and other mortality sources. On the plants damaged by mirids, we found an average 61% higher predation rate than on undamaged plants (Fig. 1B; Mann-Whitney U-test, $z = -3.667$, $P = 0.0002$). The mirids do not feed on hornworm eggs. We tested this possibility by offering hornworm eggs (without leaf material) to 15 mirids. None of the 20 eggs were damaged and after 2 days all larvae had hatched. This suggests that the egg mortality was caused by *G. pallens*, the only predator observed during the experiment.

Manduca females avoid ovipositing on plants that have been previously damaged by conspecific larvae, or that have been treated with compounds such as methyl jasmonate or linalool, which are typically released after herbivore damage (12). If previous mirid feeding significantly affects hornworm performance and predation, we predict higher fitness and thus natural selection for moths that also avoid ovipositing on plants damaged by mirids. In a field experiment in May 2000 (20), we infested 20 plants each with 10 *T. notatus* adults, and compared moth oviposition on these plants with those on 20 uninfested plants. The 40 plants in this experiment were elongated but not yet flowering and were chosen along a transect across the population. The plants were examined for eggs every second day over a 2-week period. All freshly laid eggs were removed to avoid their potential repelling effects on moths searching for an oviposition site. Oviposition by itself does not elicit VOC release; larvae must hatch and begin feeding (12). The oviposition rate on plants without mirid damage was 75% higher than that on damaged plants (Fig. 2; $t = -2.47$, $P = 0.018$). Thus moths avoid ovipositing on plants infested by mirids. The colonization of undamaged versus hornworm-damaged plants by the mirids is variable and correlates with different mirid densities [Supplementary Fig. 2 (21)]. In field experiments in two different populations with an experimental design similar to that described above, one population of plants previously attacked by hornworms (Apex mine 2000) was clearly avoided by mirids, but another population (HI91-2001) was not.

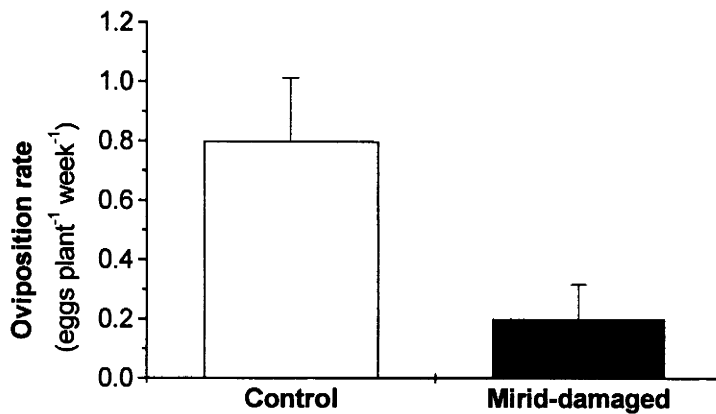


FIG. 2. *Manduca* oviposition on mirid damaged plants. Comparison between the mean (\pm SEM) weekly oviposition rate of *Manduca* moths on undamaged *N. attenuata* plants and on plants previously damaged by the mirid *T. notatus*.

To determine the fitness consequences of the reduced hornworm performance and oviposition on mirid-attacked plants, we chose 20 triplicates of undamaged, elongating *N. attenuata* plants of the same phenology and size, and added either no herbivore, 10 mirids (25) or one hornworm to one of the three plants to simulate the characteristic herbivore densities found in nature. The study was performed from June to July 2001 in a natural population and the plants' damage levels and herbivore loads were monitored every second day during a 4-week period. We removed naturally oviposited hornworm eggs and mirid colonizers from control plants. From the plants infested with hornworms, we removed mirid colonizers to exclusively observe hornworm effects (26). None of the mirid-treated plants received naturally oviposited hornworm eggs, supporting the hypothesis that mirid damage repels ovipositing moths. By adding a new caterpillar of the respective instar when a previous caterpillar died, we ensured that the hornworm-treated plants were under continuous attack until the hornworms reached the stage at which they naturally emigrate from the plant. The experiment did not include plants under continuous attack from both herbivores, because the hornworms have a very low survival rate on mirid-attacked plants (27). Moreover, such co-infestation is rarely observed in nature (see above). On July 7, when plants had stopped growing and senesced, we harvested, counted and measured all seed capsules. Capsule size (28) and the seed number were significantly correlated ($n=19$, $R^2=0.81$, $P<0.0001$). Because capsule size did not vary between the

treatments (ANOVA, $F_{2,57} = 0.319$, $P = 0.73$), the capsule number remained a reliable measure for the fitness of *N. attenuata*, as it has been in earlier experiments (11). Although the capsule number did not differ between control plants and mirid-damaged plants (Fisher's PLSD of ANOVA, $P = 0.75$), it was significantly reduced in hornworm-damaged plants (ANOVA, $F_{2,57} = 4.74$, $P = 0.0125$; Fishers PLSD, $P = 0.0067$). Plants tolerated mirid attack without fitness consequences, yet hornworm damage reduced their lifetime seed production by 40.7% (Fig. 3). Moreover, capsule number was negatively correlated with the amount of damage inflicted by hornworms [Supplementary Fig. 3 (21)].

Our results demonstrate that initial mirid feeding "vaccinates" wild tobacco plants by repelling *Manduca* oviposition and reducing hornworm survival. To our knowledge this is the first reported case in which a negative interaction between two herbivores results in a fitness benefit for the plant. Our study emphasizes the central role of induced plant defenses in structuring arthropod communities and confirms earlier studies (4, 8,

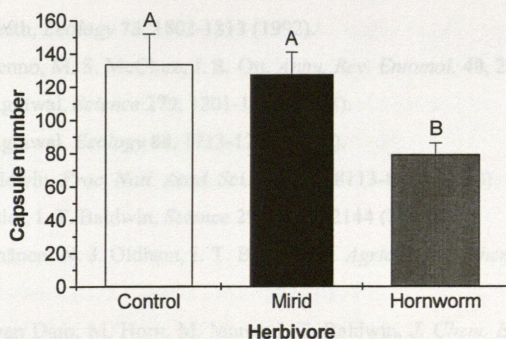


FIG. 3. Seed capsule production of *N. attenuata* attacked by *M. quinquemaculata* and *T. notatus*. Comparison of the seed capsule number produced by undamaged plants (Control), plants damaged by mirids, and plants under continuous attack of *M. quinquemaculata* hornworms. Letters designate treatments that significantly differ in the capsule number ($P < 0.05$; Bonferroni-corrected Fishers protected LSD test of an ANOVA).

29, 30). The fact that under certain circumstances (top-down control of the herbivore community, prior vaccination by the less harmful herbivore etc.) these ecological

interactions can produce a fitness benefit for the plant, suggests that herbivore-vaccination may find application in agriculture.

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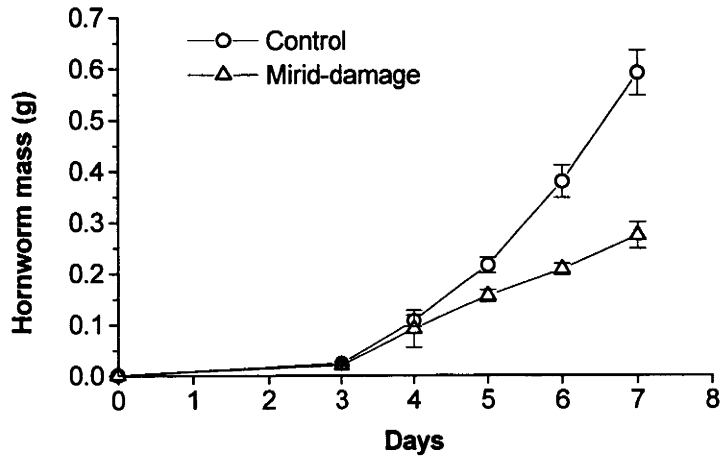
20. Plant populations in this study. Season 1999: Pahcoon Spring Burn (June 1998; R310; T41S, R18W) 2588.5 ha, 1×10^6 plants; HI 91 Burn (June 1998; R313; T41S, R18W) 189.8 ha, 50, 000 plants. Season 2000: Apex mine (June 1999; W246; T43S, R17W) 272 ha, 150, 000 plants. Season 2001: HI 91-2001 (June 2000; T42S, R18W) 150 ha, 100,000 plants.
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25. A census of 100 plants in the population showed that plants attacked by mirids hosted between 3 and 16 individuals at the time the experiment started.
26. Mirids colonized hornworm-damaged plants with the same frequency as control plants. The mean numbers of mirids per plant removed over the course of the experiment did not differ between control (0.98 ± 0.35 , $N= 2$) and hornworm damaged plants (1.7 ± 0.39 , $N= 20$; Student's t-test, $t= -1.39$, $P= 0.17$).
27. An alternative experimental design to reduce high mortality rates of hornworms on mirid-infested plants would exclude the highly mobile predator *G. pallens*. Cages completely enclosing the plants effectively exclude this predator, but because they influence the microclimatic conditions and the plant's ability to produce direct defenses, they confound the analysis.
28. Determined by measuring length and width of the capsules and calculating their volume (mm^3) by treating them as cylinders.
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Supplementary data

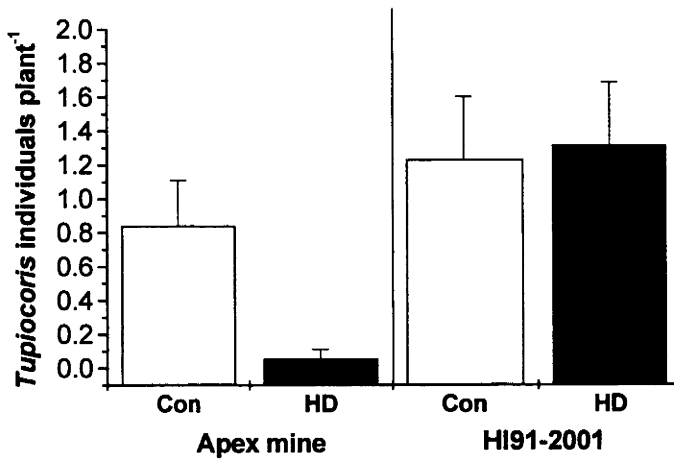
Supplementary Table 1. Concentrations^a of secondary metabolites after mirid damage on *Nicotiana attenuata* plants. Sixteen hydroponically-grown *N. attenuata* plants in the rosette stage of growth were placed in insect cages. Eight plants were infested, each with 10 mirid adults. Eight control plants were placed in insect-free cages. Seven days later, leaves that were two leaf positions younger than the source-sink transition leaf were harvested and analyzed for secondary metabolites with an HPLC-based screen described by Keinänen et al. (13).

Compound	Control ^b	Mirid-damage ^b	increase	P ^c
Nicotine	324.1± 20.4	337.5± 43.5		Ns
Caffeoylputrescine	3.8± 0.6	8.7± 2.0	2.2x	*
Chlorogenic acid	6.1± 0.6	13.8± 2.5	2.3x	*
Cryptochlorogenic acid	5.3± 1.1	11.8± 2.3	2.2x	*
Rutin	404.1± 71.9	533.3± 87.7		Ns
Unknown RT 10.2	5.7± 1.4	29.7± 6.6	5.2x	**
Unknown RT12.9	13.5± 7.2	72.1± 21	5.3x	*
Unknown RT 13.2	7.0± 2.0	50.8± 14.7	7.3x	*
DTG 8	550.9± 96.4	1047.8± 132.3	1.9x	**
DTG 9	2059.5± 375.4	3643.6± 466.2	1.8x	*

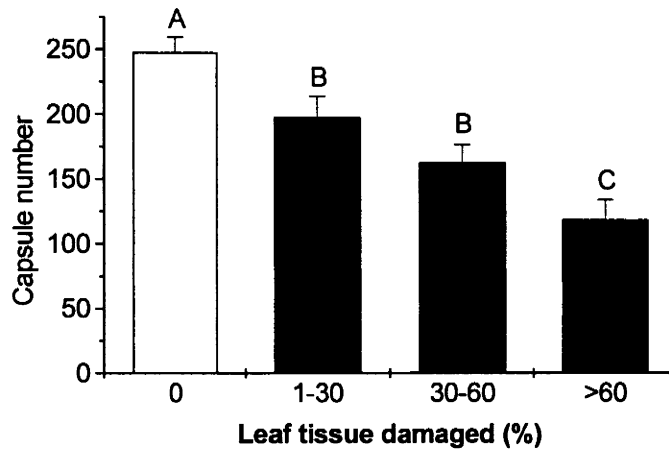
^a Expressed as $\mu\text{g g}^{-1}$ fresh mass; caffeoylputrescine and cryptochlorogenic acid are expressed as chlorogenic acid; unknown peaks and diterpene glycosides (DTG) are expressed as peak areas at 210 nm mg^{-1} fresh mass. ^b Data are means \pm SE, $n=8$. ^c The comparisons are performed with Student's t-test; * $P<0.05$, ** $P<0.01$, Ns- not significant.



Supplementary Fig. 1. Mean (\pm SEM) mass of *M. sexta* hornworms on mirid-damaged and undamaged control plants. In a laboratory experiment, 20 hydroponically grown *N. attenuata* plants were infested, each with 10 mirids, that were allowed to feed for 3 days. Another set of 20 plants remained undamaged. Freshly hatched hornworms were applied singly to the plants and their mass was measured on days 0, 3, 4, 5, 6, and 7. Hornworms on undamaged plants had a significant faster weight gain than hornworms on mirid-damaged plants (repeated measures ANOVA: $F_{1, 238} = 6.95$; $P = 0.009$).



Supplementary Fig. 2. Mean number (\pm SEM) of mirids (*Tupiocoris notatus*) colonizing undamaged (Con) and hornworm damaged (HD) *Nicotiana attenuata* plants on two different burns: “Apex mine” and “HI91-2001”. On Apex mine we selected 38 elongated plants along a transect bisecting a 272 ha population [Apex mine burn (June 1999; W246; T43S, R17W)] of about 150,000 plants. Half of the plants were infested with 1-4 first to third instar *Manduca quinquemaculata* caterpillars and all plants were censused for *T. notatus* every second day over a 14-day interval. *T. notatus* colonized control plants more heavily than hornworm damaged plants ($t = -2.8$, $P = 0.008$). Moreover, 50% of the control plants harbored *T. notatus*, while only 5.5% of the caterpillar-damaged plants also hosted mirids (two-way contingency test: Pearson $\chi^2 = 7.13$, $P = 0.008$). With the same experimental design in a population on HI91-2001 ($n = 40$), we found no differences in the colonization of control plants and hornworm-damaged plants by *T. notatus* (Student’s t -test, $t = -0.037$, $P = 0.9705$). This difference in mirid colonization is most likely caused by population size differences and thus differences in intraspecific competitive interactions, so that at low densities, mirids prefer uninfested plants, but at high densities they do not. On Apex mine, 36.7% of 98 censused plants (on a linear transect across the population) were infested by mirids, while on HI91-2001, 72.9% of 59 censused plants hosted *Tupiocoris* (Two way contingency test: Pearson $\chi^2 = 19.25$, $P < 0.0001$).



Supplementary Fig. 3. Mean capsule number (\pm SEM) of *N. attenuata* plants after different levels of *M. quinquemaculata* damage. One hundred flowering plants of similar phenology and size in a natural population [Supplementary Figure 1 (21)] were categorized by the tissue loss they received from hornworms. Eight plants were excluded from the analysis, because they were damaged by the flea beetle *Epitrix hirtipennis*, 57 plants remained undamaged (0%), 22 plants had leaf damage that only involved parts of the laminae (1-30% tissue loss), 7 plants were damaged so that complete leaves, including the midrib were missing (30-60% tissue loss) and 6 plants received damage to the apical meristem and the stem (>60% tissue loss). Lifetime capsule production strongly depends on the amount of hornworm damage that the plants received (ANOVA, $F_{3,88} = 7.014$, $P = 0.0003$). Different letters designate damage levels that result in significantly different capsule numbers ($P < 0.05$; Bonferroni-corrected Fisher's protected LSD test of an ANOVA).

Manuscript 5

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Plant responses to insect herbivory: The emerging molecular analysis

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Running titel: Plant responses to insect herbivory

Abstract Plants respond to herbivore attack with a bewildering array of responses, broadly categorized as direct and indirect defenses, and tolerance. Plant-herbivore interactions are played out on spatial scales that include the cellular responses, well-studied in plant-pathogen interactions, as well as responses that function at whole-plant and community levels. The plant's wound response plays a central role but is frequently altered by insect-specific elicitors, giving plants the potential to optimize their defenses. In this review, we emphasize studies that advance the molecular understanding of elicited direct and indirect defenses and include verifications with insect bioassays. Large-scale transcriptional changes accompany insect-induced resistance, which is organized into specific temporal and spatial patterns and points to the existence of herbivore-specific *trans*-activating elements orchestrating the responses. Such organizational elements could help elucidate the molecular control over the diversity of responses elicited by herbivore attack.

Key Words plant-arthropod interaction, insect elicitors, direct defense, indirect defense

WHY PLANT RESPONSES TO PATHOGENS AND HERBIVORES FREQUENTLY DIFFER

Autotrophs require sophisticated defenses if they are to survive in a world full of heterotrophs. Attacks from heterotrophs occur on spatial scales ranging from microbes to moose, and plants require defenses that are effective at all levels. Defenses against microbes can be highly effective on small spatial scales; the hypersensitive response (HR), in which cells immediately surrounding the infection site rapidly die and fill with antimicrobial compounds to prevent the spread of the pathogen, is the best-studied example (58). Although the HR is an extremely effective defense, this cellular suicide cannot be used without large costs and requires a sophisticated recognition system to avoid inappropriate deployment. Hence the HR, and the burst of reactive oxygen species (ROS) (114) that frequently precedes the HR and may contribute to apoptosis, are activated when a plant's surveillance system (*R*-genes, encoding receptor proteins) binds

various elicitors from the attacking pathogen (proteins, peptides, lipids, polysaccharides) (58). Although the HR can be effective against sedentary herbivores that attack particular tissues [such as phloem-feeding aphids (73)], it is not effective against most free-living herbivores, which avoid an HR by simply moving to another feeding site. This autonomy and the resulting physiological independence of herbivores from their host plants profoundly expand the spatial scale of the plant-herbivore interaction to include not only whole-plant responses but also the community in which the plant lives (**Figure 1**).

Most insect herbivores arrive at a plant after their devoted mothers have carefully selected and, in some cases, manipulated [by microbial inoculations: e.g., bark beetles (88); or altering source-sink relationships: e.g., aphids (60)] the host plant and endowed the young herbivore nutritionally and developmentally, enabling it to launch its first attack. The young herbivore is fully equipped with mandibles and other feeding apparatus to force its way through the plant's protective covering, an efficient digestive tract in which plant parts can be digested and assimilated in a milieu controlled by the herbivore (112), and mobility and sensory systems that allow it to move in response to heterogeneity in plant suitability (90). This physiological and behavioral autonomy can account for overarching differences between how plants respond to herbivores and pathogens and in how an attack is perceived.

In contrast to pathogen attack, herbivore attack is frequently associated with wounding, and the "recognition" of herbivore attack frequently involves modifications of a plant's wound response (6, 56, 118). Moreover, the physiological and behavioral autonomy of herbivores also allows plants to use defenses that would be ineffective against pathogens. For example, plants use secondary metabolites that are specifically targeted against organ systems unique to herbivores [nervous, digestive, endocrine, etc. (96)] and use higher trophic-level interactions defensively by providing information or nutritional encouragement to the predators of herbivores. The recruitment of the natural enemies of herbivores drastically increases the spatial scale of the interaction (21).

Plant-herbivore interactions are described in an enormous, largely ecological and entomological literature into which molecular techniques have only recently been injected. To assist molecularly oriented readers in coming to grips with this vast literature, we select recent reviews of different aspects of the interactions (**Figure 1**) and present a primer of the main concepts and terms used to describe the interaction. We follow this with a review of recent literature on advances in the molecular understanding

of the “recognition” of herbivore attack by plants and the resistance mechanisms that have been verified with insect bioassays.

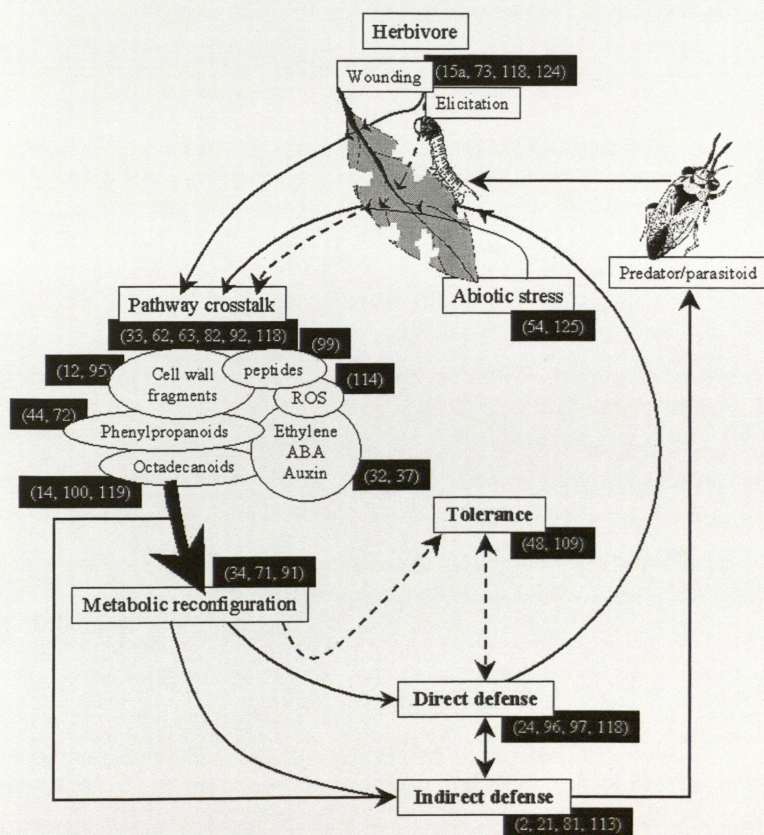


Figure 1 The arena of plant-induced resistance to arthropods. An attacking herbivore wounds the plant and applies or injects elicitors. Wound- and herbivore-specific elicitors in combination with abiotic stresses differentially activate various signaling pathways. These signal cascades interact (pathway crosstalk) to either directly produce volatile signals that function as indirect defenses or effect a fine-tuned metabolic reconfiguration and the expression of defense-related genes. As a consequence of these changes, resources are allocated to regrowth (tolerance) or the production of compounds that

directly affect the attacking herbivore (direct defenses: toxic, antinutritive, and antidigestive compounds) or indirectly (indirect defenses) by attracting natural enemies. Recent reviews summarizing the knowledge of particular parts of the arena are listed in black boxes. Evidence is emerging that arthropod-induced resistance results from a coordinated production of specific direct and indirect defenses that complement the existing constitutive defenses of the plant.

A PRIMER IN PLANT-HERBIVORE INTERACTION TERMINOLOGY

Fitness-Based Evaluations of Resistance Traits

Much of the interest in plant-herbivore interactions among ecologists stems from a seminal paper by Ehrlich & Raven (25), which coined the term “coevolution” and stimulated entomological studies of how plants and insects influence each other’s evolutionary trajectories. As a result of this evolutionary focus, the functional analysis of plant traits is frequently evaluated at higher-level integrations of plant performance, namely the correlates of Darwinian fitness (production of seeds, pollen, tubers, etc.). The adjective “defensive” is usually reserved for traits that increase plant fitness correlates when plants are under attack (50). This evolutionary emphasis and fitness-based analysis of plant traits have focused interest on the factors that contribute to maintaining the variability in resistance so frequently observed in nature. A central thrust has been to understand why plants are not always resistant, when it has such clear fitness benefits. This emphasis on understanding variability has focused attention on the fitness costs of different types of resistance: their modes of expression (constitutive or induced) and the environmental, evolutionary, and developmental constraints on them (42, 96, 97). Resistance traits can be broadly categorized into three defense strategies: direct and indirect defenses and tolerance (50). Plants are either constitutively resistant as a result of preformed resistance traits, or they become resistant after an attack as a result of herbivore-induced changes that are either localized to the tissues adjacent to an attack site or systemically expressed throughout the plant.

Costs of Defense

Just as the indiscriminate deployment of the HR would likely severely compromise a plant's performance, the production of resistance traits when they are not needed is likely to be costly for a number of reasons. First, resistance traits can be costly to produce if fitness-limiting resources (such as nitrogen) are invested (5) or if the traits are also toxic to the plant. However, resistance costs can also arise from higher-level ecological processes. For example, specialized herbivores may sequester a plant's defenses and use them for their own defense against predators, or compounds that provide defense against generalist herbivores may attract specialist herbivores, which use them as host-location signals (113). Moreover, the defenses may disrupt important mutualistic interactions, such as pollination, which are also mediated by insects (1). These fitness costs probably provide the selection pressure behind the evolution of inducible resistance, if inducible expression allows plants to forego these fitness costs when the defense is not needed.

Direct Defenses

Direct defenses are any plant traits (e.g., thorns, silica, trichomes, primary and secondary metabolites) that by themselves affect the susceptibility to and/or the performance of attacking arthropods and thus increase plant fitness in environments with herbivores. Defensive secondary metabolites are categorized by their mode of action (24). Proteinase inhibitors (PI) (antidigestive proteins) are inducible by wounding and herbivory and influence herbivore performance by inhibiting insect digestive enzymes (55, 110). Polyphenol oxidases are antinutritive enzymes that decrease the nutritive value of the wounded plant by cross-linking proteins or catalyzing the oxidation of phenolic secondary metabolites to reactive and polymerizing quinones. Toxic compounds (e.g., alkaloids, terpenoids, phenolics) poison generalist herbivores, forcing specialists to invest resources in detoxification mechanisms that in turn incur growth and development costs.

Indirect Defenses

Indirect defenses are plant traits that attract predators and parasitoids of herbivores and increase the carnivore's foraging success and thereby facilitate top-down control of herbivore populations (50). Volatile organic compounds (VOCs) released by herbivore-attacked plants are known to be attractive to arthropod predators and parasitoids in

laboratory experiments on agricultural plants (21) and have recently been shown to function defensively under natural conditions (52). The VOC response can be highly specific; parasitic wasps often use this specificity to locate particular hosts or even the particular instars of their hosts (113). However, generalist predators are also attracted by single components of the VOC bouquet, which are commonly emitted after attack from a diverse set of herbivore species (52). In addition to attracting natural enemies of the herbivores, the VOC release can function as a direct defense by repelling the ovipositing herbivores (19, 52). Finally, it may be involved in plant-plant interactions (27). In addition to supplying information to natural enemies about the location and activity of foraging herbivores, plants also provide food and shelter to the enemies of herbivores (3). Extrafloral nectaries increase their rate of nectar secretion after herbivore attack, and these carbohydrates and proteins (from various food bodies or even pollen) provide nutritional encouragement for predators to increase their foraging rate in certain areas of a plant (40). Shelter is provided as specialized structures (leaf domatia) or modifications of existing structures (hollow thorns, stems).

Tolerance

Tolerance decreases the fitness consequences of herbivore attack for a plant. A plant genotype is termed tolerant if it can sustain tissue loss with little or no decrease in fitness relative to that in the undamaged state (109). Although genotypes clearly vary in their tolerance, the mechanisms underlying such variation are not understood. Certain morphological traits, such as meristem sequestration and reactivation, as well as photoassimilate storage in below-ground and stem structures, in addition to physiological responses, such as herbivore-induced increases in photosynthetic capacities, and nutrient uptake, are correlated with compensatory growth following herbivore attack (109). However, the functionally mysterious transcriptional reconfiguration that follows herbivore attack is most likely to hold the key to a more detailed mechanistic understanding of tolerance responses.

ELICITORS FROM HERBIVORES

Any compound that comes from herbivores and interacts with the plant on a cellular level is a potential elicitor. So far, herbivore-specific elicitors have been isolated from oral

secretions of lepidopteran species and the oviposition fluid of weevil beetles---the two insect fluids that regularly come in contact with plant wounds (Figure 2). Additionally, some evidence suggests that microbes present in the digestive organs of herbivores are involved in the production of elicitors found in oral secretions (107).

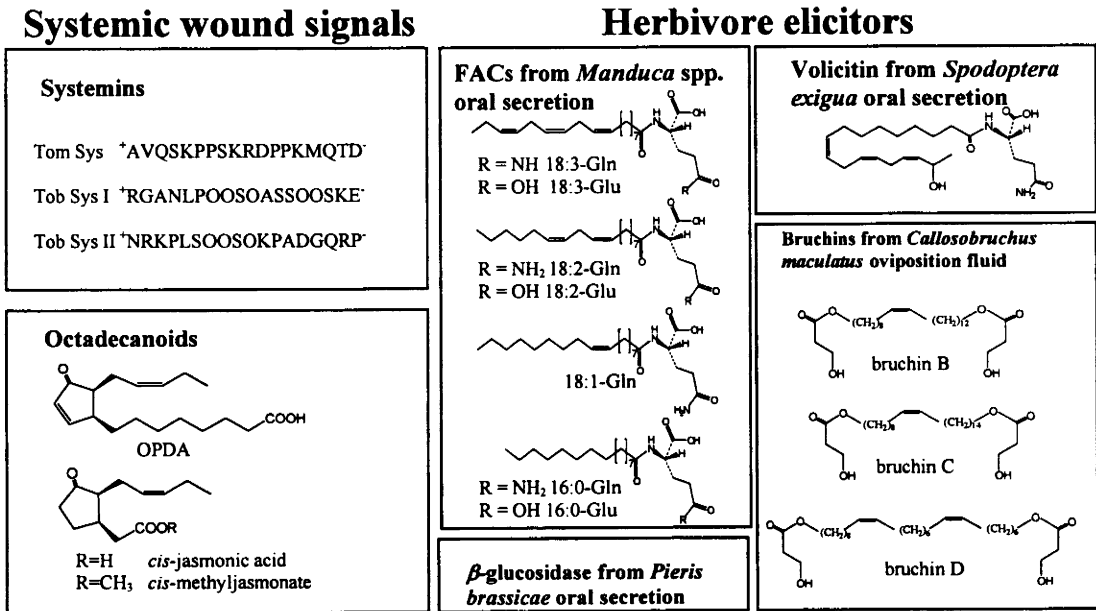


Figure 2 Examples of systemic wound- and herbivore-specific signals demonstrated to elicit either direct or indirect defenses in plants. Systemins are polypeptide systemic wound signals from solanaceous plants, such as tomato (Tom Sys) and tobacco (Tob Sys I and II), which activate the octadecanoid pathway but are also inducible by various oxylipins (83, 99). Oxylipins, as illustrated by the octadecanoids, 12-oxophytodienoic acid (OPDA), jasmonic acid, and methyl jasmonate, elicit defense gene expression, numerous secondary metabolites, and insect resistance (14). Herbivore-specific elicitors have been identified in insect oral secretions and oviposition fluids, the two fluids that commonly come into contact with the wounded plant tissue. *Manduca* and *Spodoptera exigua* oral secretions contain the class of fatty-acid-amino-acid conjugates (FACs) found to actively elicit volatile organic compounds (VOCs), which function as indirect defenses (36, 113). The FACs from *Manduca* also elicit other herbivore-specific changes (Figure 3). *Pieris brassicae* oral secretion contains the enzyme β -glucosidase that also elicits VOC emission (66). The novel class of elicitors from cowpea weevil oviposition fluid (bruchins) elicits neoplastic tissue formation in peas, which expels the oviposited egg from the pea leaf tissue (23).

Two classes of elicitors have been isolated from the oral secretions of lepidopteran larvae; both elicit indirect defense responses. The first class includes lytic enzymes, such as β -glucosidase that was isolated from *Pieris brassicae* and elicits the release of terpenoid volatiles from cabbage leaves (66). Other lytic enzymes have been found in the saliva of other lepidopteran species, such as glucose oxidases in *Helicoverpa zea* saliva (29); in piercing insects, such as alkaline phosphatase in whitefly (*Bemisia tabaci*) saliva (31); and in a wide array of watery digestive enzymes from aphid saliva (73); but their roles as elicitors of defense responses have not yet been established.

The second class of elicitors comprises fatty-acid-amino-acid conjugates (FACs), which have been found in the regurgitant of larval Sphingidae (36), Noctuidae, and Geometridae (4, 86). The FAC volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine], from *Spodoptera exigua*, induces excised maize seedlings to release the same odor blend of volatile terpenoids and indole that is released when they are damaged by caterpillar feeding (4). Unfortunately a recent study with intact maize seedlings found volicitin to only elicit VOC release when applied to plants at midnight and that the release from excised seedlings was much greater than that from intact plants. Moreover, volicitin was less effective than JA in eliciting VOC release (103a). At last it is interesting to note that none of the separated enantiomers of volicitin were active in lima bean, which is also a host plant of the beet armyworm (108), suggesting that volicitin as an elicitor deserves additional research..

Recently, another class of herbivore-specific elicitors has been determined to induce a novel type of defense response in peas. These elicitors, long chain diols that are mono and diesterified with 3-hydroxypropanoic acid, are called bruchins because they are excreted with the oviposition fluid of pea and cowpea weevils (Bruchidae) (**Figure 2**). In certain genotypes of peas, bruchins elicit neoplastic growth on pods, which lifts the egg out of the oviposition site and impedes larval entry into the pod (23). Because the neoplastic growth exposes the larvae to predators, parasites, and desiccation, it may function as an indirect defense. Similarly, the oviposition of elm leaf beetles, *Xanthogaleruca luteola*, elicits the emission of specific VOCs from the field elm host trees. The oviposition-induced VOC release attracts parasitic wasps that attack eggs (70), but the chemical basis of this elicitation remains unknown.

The early steps in the herbivore elicitation process remain to be elucidated. No receptors comparable to the *R*-gene products for pathogen recognition have been found

for herbivore elicitors. Glucose oxidases may increase H_2O_2 production at the site of attack and may potentiate elicitation by forming ROS (29). Another mechanism has been suggested by Engelberth et al. (26), who found that the channel-forming peptide alamethicin, produced by the parasitic fungus *Trichoderma viridae*, elicited the release of volatiles in lima bean that are comparable to those elicited when the jasmonic acid (JA) cascade is antagonized by the salicylic acid (SA) cascade. However, such channel-forming peptides have not yet been found in the saliva of insect herbivores.

The only system so far to demonstrate a link from the elicitor to subsequent signaling steps is the *Nicotiana attenuata*-*Manduca* system (**Figure 3**) (see Crosstalk below). FACs in the oral secretions of two *Manduca* species elicit JA and ethylene bursts, which are involved in the alterations of the *N. attenuata* wound responses (36, 103, 121).

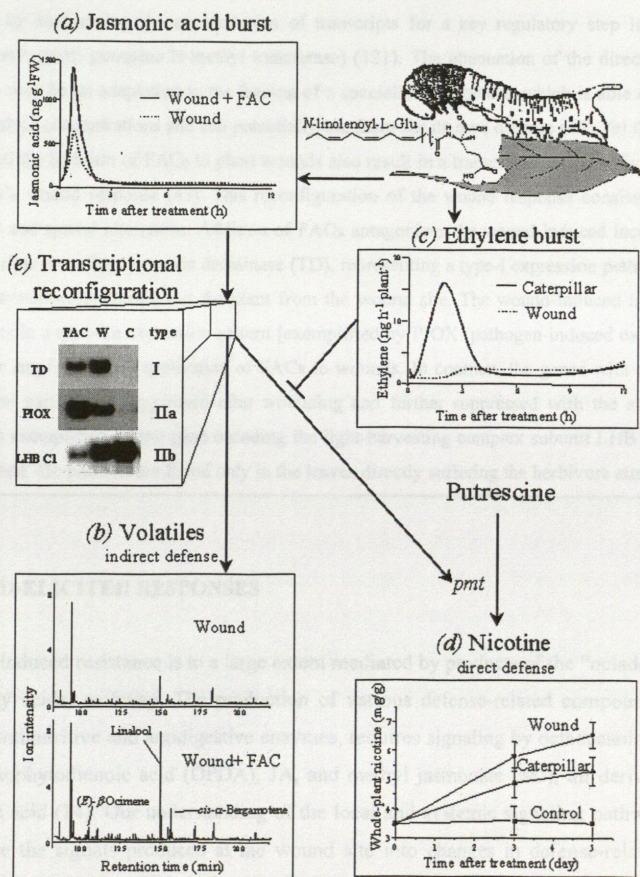


Figure 3 Alteration of the wound response of wild tobacco plants (*Nicotiana attenuata*) by *Manduca* caterpillar feeding. (a) Wounding of the leaf tissue results in a JA burst, which is amplified by caterpillar feeding and the application of FACs from larval oral secretions to the wound (36, 103). (b) Caterpillar attack, or the application of FACs to wounds, but not wounding alone induces the production of volatiles that function as predator attractants in the plant's indirect

defense (35, 36). (c) Caterpillar feeding and the application of their oral secretions to wounds cause an ethylene burst (49), which (d) attenuates the wound- and JA-induced accumulation of nicotine by suppressing the accumulation of transcripts for a key regulatory step in nicotine biosynthesis (pmt: putresine N-methyl transferase) (121). The attenuation of the direct defense, nicotine, may be an adaptation to the feeding of a specialized herbivore, which is able to tolerate high alkaloid concentrations and can potentially use them for its own defense (49). (e) Caterpillar attack and the addition of FACs to plant wounds also result in a transcriptional reconfiguration of the plant's wound response (43). This reconfiguration of the wound response consists of three temporal and spatial alterations. Addition of FACs antagonizes the wound-induced increase (W) of transcripts encoding threonine deaminase (TD), representing a type-I expression pattern, which spreads systemically throughout the plant from the wound site. The wound-induced increase in transcripts in a type-IIa expression pattern [exemplified by PIOX (pathogen-induced oxygenase)] is further amplified after application of FACs to wounds. In contrast, the genes with a type-IIb expression pattern are suppressed after wounding and further suppressed with the addition of FACs, as exemplified by the gene encoding the light-harvesting complex subunit LHB C1. Both type-IIa and -IIb patterns are found only in the leaves directly suffering the herbivore attack (102).

WOUND-ELICITED RESPONSES

Wound-induced resistance is to a large extent mediated by products of the “octadecanoid” (C₁₈-fatty acids) pathway. The production of various defense-related compounds, e.g., toxins, antinutritive and antidigestive enzymes, requires signaling by octadecanoids, such as 12-oxophytodienoic acid (OPDA), JA, and methyl jasmonate (MJ), all derived from linolenic acid (14). Our understanding of the local and systemic signaling pathways that transduce the signals produced at the wound site into changes in defense-related gene expression throughout the plant and of how herbivore-specific elicitors modify these signaling pathways is still sketchy. An encouraging trend has been the increase in studies using herbivore bioassays to evaluate the consequences of manipulations, in particular signal transduction chains, as well as those using insect attack and transcriptional profiling to identify new signaling pathways.

Wound Signals

Both herbivore feeding and mechanical damage induce systemic responses that are rapidly propagated throughout the plant as well as responses that are restricted to the wound site. Systemic responses require mobile signals; these could be electrical (41), hydraulic (65), and chemical (99). In tomato, PIs are induced by signals transported in both xylem and phloem tissues. Steam girdling the petiole, which kills the phloem but leaves the xylem intact, does not prevent systemic induction of PIs after severe wounding, suggesting a xylem-transported signal. Small crushing wounds do not, however, induce a xylem-mediated signal; rather, they elicit PI production in organs distal to the wound and with intact phloem connections (94). Moreover, *Pin2* (PI II) gene expression can be induced by electrical signals that are associated with wounding. The response and propagation of wound-induced electrical signals apparently requires intact abscisic acid (ABA) signaling pathways, because they both are lacking in (ABA)-deficient tomato mutants (41).

Immediately after wounding, plants transiently produce ROS, such as the superoxide anion, locally in the damaged tissue and H₂O₂ both locally and systemically throughout the plant (114). Because wound-induced oligogalacturonides transiently elicit ROS production, they are thought to be a primary signal of tissue damage (62). Moreover, both oligogalacturonides and fungal-derived chitosan can elicit ROS and the expression of wound-inducible PI genes. However, oligogalacturonides have limited mobility and are also induced by systemins (**Figure 2**), and they probably represent a local, intermediate step in signaling following systemin production, rather than a mobile primary signal (62).

Systemin, the first described oligopeptide with phytohormonal function, is thought to represent the primary wound signal in some solanaceous plants (tomato, pepper, black nightshade, tobacco). Cleaved from a 200-amino acid precursor called prosystemin, and probably transported in the phloem, it is active at femtomolar levels and is the best-verified mobile signal (99). Transformed tomato plants expressing prosystemin cDNA in an antisense orientation do not express PI I and II after wounding and are more susceptible to attacking *Manduca sexta* larvae (80). Plants transformed to overexpress the prosystemin gene, in turn, exhibit constitutively activated wound responses and PI transcript expression (69). Systemin-binding proteins in the plasma membrane of tomatoes and on the surface of *Lycopersicon peruvianum* suspension cultured cells initiate a complex wound cascade after binding systemin. The wound cascade concludes

with the activation of a phospholipase A₂, which releases linolenic acid from the plasma membrane, supplying the substrate for the initial step in the octadecanoid pathway (99). The systemin-mediated wound cascade is likely to be the main cascade that is subsequently directly modified by herbivore-specific elicitors or indirectly through the recruitment of other signaling cascades. With the recognition that C₁₆-fatty acids and other lipids can be used in the production of JA and other potential signal molecules, the term "octadecanoids" (C₁₈-fatty acids) should be broadened to "oxylipins" (28). However, because the major advances in the endogenous manipulation of wound- and herbivore-induced oxylipins are derived from C₁₈- fatty acid substrates, we retain the term octadecanoids in the following discussion.

Octadecanoids

The importance of the signaling function of octadecanoids for plant-insect interactions has recently been demonstrated by manipulating three enzymes in the pathway: lipoxygenase (LOX), hydroperoxide lyase (HPL), and JA carboxyl methyltransferase (JMT).

LOX, the nonheme iron-containing dioxygenase, catalyzes the oxygenation of linolenic acid to the 9- and 13-hydroperoxides, which are in turn converted to aldehydes and oxoacids. Products from 13-hydroperoxy linolenic acid can be further elaborated by enzymatic cyclization, reduction, and β -oxidation to produce JA. The LOX proteins play roles in plant growth and development, in maturation and senescence, and in the metabolic responses to pathogen attack and wounding that are thought to be mediated by their role in the biosynthesis of oxylipin signals such as JA and OPDA (14, 100).

A number of studies suggest that different LOX isoforms have different functions. In *A. thaliana*, cosuppression-mediated depletion of a LOX isoform led to a decrease in the wound-induced JA levels but did not affect basal JA levels (9). Different LOX isoforms are encoded by multigene families, of which at least three have been found in potato. Transgenic potato plants devoid of one 13-LOX isoform (LOX-H3) through antisense-mediated depletion of the LOX mRNA do not accumulate PIs in response to wounding. Moreover, when Colorado potato beetle larvae and beet armyworm larvae fed on antisense plants, they grew significantly larger than those fed on wild-type plants. More puzzling, however, was the observation that the effect of LOX-H3 on resistance was apparently not through its involvement in the wound-induced increase of JA. JA levels

after wounding were similar in both antisense and wild-type plants, and the exogenous application of JA was not able to recover wild-type PI levels in LOX-H3 antisense plants (98). These results suggest that LOX-H3 is producing a JA-independent signal by one of several possible mechanisms: LOX-H3 may directly affect a *pin* gene response; it may be involved in a regulatory network that influences the expression of other LOX genes; or it may affect the wound-responsive production of ethylene that is required, together with JA, for maximal *pin2* expression in tomato (79). It is interesting to note that LOX-H3 antisense potato plants produced more flowers and on average 20% more tuber mass than did wild-type plants, suggesting that the resistance mechanisms mediated by this isoform of LOX incur fitness costs when they are not needed (98).

One of the LOX-catalyzed products, 13(*S*)-HPOT, is a substrate for several other enzymes that are thought to be important in plant-insect interactions, including HPL, which cleaves the 13-hydroperoxide into C₆-aldehydes and C₁₂-oxoacids (100). The C₆-aldehydes are thought to protect the wound site from microbial infection and to function as a direct defense against some herbivores, whereas the C₁₂-products, which include traumatin and traumatic acid, may be involved in wound healing (78). More recently, the C₆-aldehydes have been suggested to play a role in directly eliciting defense-related gene expression (8), systemin-based signaling (106), and signaling between plants (27).

Recent experiments with HPL-depleted potato plants showed that HPL gene expression is developmentally controlled and the activity levels are posttranscriptionally regulated (115), complementing results from tomato studies (45). Moreover, normal wound-induced gene (*AOS*, *LOX-H1*, *LOX-H3*, *prosystemin*, *Pin2*, *LAP*) expression was found in these plants, despite diminished hexenal/ 3-hexenal production, suggesting that these metabolites do not play a signaling role. It is interesting to note that *Myzus persicae* aphids performed better on HPL-depleted plants than they did on wild-type plants, suggesting that the C₆-aldehydes produced by HPL may play an important role in direct defenses (115).

If the 13(*S*)-HPOT produced by LOX is not metabolized by HPL, it can become a substrate for allene oxide synthase (AOS), which produces the unstable allene oxide; this, in turn, is cyclized by allene oxide cyclase (AOC) to form enantiomerically pure 9*S*,13*S*-OPDA (100). A subsequent reduction and β -oxidation of OPDA produces JA. Both OPDA and JA actively regulate defense gene expression and elicit resistance (14). Most of the genes that encode enzymes in the JA biosynthetic pathway are activated by

wounding, and some (e.g., *LOX*, *AOS*, *OPDA reductase*) are also upregulated by exogenous JA application, suggesting positive feedback control (62). Overexpression of flax *AOS* in potato increased constitutive JA concentrations but did not influence the expression of *pin2* genes (38), which suggests a JA-independent signal for *pin*-gene expression. However, the resistance of the *AOS* overexpressing potato lines against insect attack was not reported.

Although most of the octadecanoids are involved in systemic responses, their function as a mobile signal has yet to be conclusively demonstrated. Applications of ^{14}C labeled JA to leaves of *Nicotiana sylvestris* plants provided evidence for shoot-to-root transport with a kinetic that was identical to the appearance of a transient increase in endogenous JA concentrations in the roots of plants after leaf wounding, suggesting that JA was transported from wounded leaves to roots in these plants (123). Glucuronidase (GUS) reporter gene fusion experiments with the *AOS* promoter from *A. thaliana* and *N. tabacum* found *AOS* activation locally and systemically upon wounding. In contrast to the findings from *N. sylvestris*, this induction remained restricted to the application site of JA or OPDA, suggesting that these two octadecanoids were not systemically transported in the transformed plants (57). However, recently the gene for *S*-adenosyl-L-methionine-JMT from *A. thaliana*, which catalyzes the formation of MJ from JA, was cloned (104). JMT transcripts were induced both locally and systemically after wounding and MJ treatments. Transgenic plants overexpressing *JMT* had elevated MJ levels and constitutively expressed JA responsive genes, such as *VSP* and *PDF1.2*, but they exhibited wild-type-JA contents. The expression of defense genes may have contributed to enhanced levels of resistance against the pathogen *Botrytis cinerea* (104). These results suggest that MJ may regulate defensive gene expression and have the potential for mobile intra-and interplant signal function. Earlier work had proposed that MJ was an airborne signal that functioned in interplant communication for defense responses (27, 51).

Crosstalk

Although it is becoming increasingly clear that single signal cascades, as illustrated by the oxylipins, can produce a bewildering array of potential secondary signal molecules with potentially different functions (14, 28, 119), it has also become apparent that herbivore attack frequently involves the recruitment of several signal cascades, the interaction

(“crosstalk”) among which may explain the specificity of the responses. Reymond & Farmer (92) proposed a tunable dial as a model for the regulation of defensive gene expression, based on the crosstalk of the three signal pathways for JA, ethylene, and SA. According to this model, a plant tailors its defensive responses to a specific attacker by eliciting signal molecules from the three pathways to different degrees. The elicitation of multiple pathways after attack is likely common and may be necessary if plants are to tailor their responses adaptively to the diverse herbivore species that attack them (118). Herbivores frequently attack in guilds, specifically or opportunistically vectoring microbes into the resulting wounds. These interactions of herbivore and pathogen attack and the resulting signal crosstalk may compromise a plant’s responses. Some attackers may induce a pathway (e.g., pathogens activating primary SA responsive genes) that influences the activity of another (e.g., chewing herbivores activating JA responsive genes), thereby compromising the plant’s defensive reaction against one or both of the enemies (63, 82).

How these responses are fine-tuned to optimize the defense against a particular herbivore species or guild is the subject of recent investigations and reviews (11, 118). One of the clearest differences in defense responses to herbivores exists between chewing caterpillars and phloem-sap-sucking whiteflies or aphids. That attack from *Manduca sexta* caterpillars is “recognized” by the plant as evidenced by a JA burst far greater than that produced by the wounding that herbivores’ feeding behavior causes (68, 103) (Figure 3). This JA burst is associated with expression of both wound-responsive genes and a set of novel JA-independent genes. The introduction of oral secretions from the feeding caterpillar into the wound site can account for the differences (36, 56, 102). Aphid feeding, in contrast, induces the expression of pathogen-responsive, SA-, and wound-responsive, JA-regulated genes (118). Green peach aphids, *Myzus persicae*, induced SA-dependent transcription of *PR-1* and *BGL2* in wild-type plants but not in *npr1* mutant plants, which are deficient in SA signaling (76). In addition, plants attacked by aphids had higher mRNA levels of *PDF1.2* (encoding defensin) and *LOX2* (encoding lipoxygenase), both of which are wound inducible and involved in the JA signaling cascade. SA- and JA-dependent genes have also been induced in plants attacked by phloem-feeding whiteflies. These whiteflies induce the expression of genes involved in lignin production, SA biosynthesis, oxidative burst, as well as in pathogenesis-related and JA-responsive PR proteins (118). Moreover, silverleaf whitefly feeding on squash induces accumulation of transcripts encoding *SLW3*, a gene that is not responsive to any known

wound or defense signal. This suggests that there are other defense signal cascades waiting to be discovered (116).

The observation that whitefly and aphid feeding elicit both JA- and SA-induced genes runs counter to well-described observations that SA and other cyclooxygenase inhibitors can effectively inhibit wound-induced JA production and JA-elicited gene expression (7, 84). Moreover, pathogen-inducible SA, such as is elicited by TMV infection, inhibits wound-inducible JA accumulation and secondary metabolite accumulation (87). The stimulation of SAR (systemic acquired resistance) with the SA mimic, BTH (benzothiadiazole), attenuated the JA-induced expression of polyphenol oxidase in tomato plants and increased the performance of *Spodoptera exigua* caterpillars, suggesting compromised defense responses. Other examples further undermine the belief that SA exclusively mediates pathogen responses and JA exclusively mediates herbivore responses. *Pseudomonas syringae*, a tomato pathogen, induces responses typically associated with SAR and SA signaling, such as PR-protein expression, but also expression of PI genes, which are normally considered to be JA induced. This activation of different pathways may underlie the observed crossresistance of *P. syringae*-attacked tomato plants against both the pathogen and subsequently feeding noctuid larvae (11).

Crosstalk between ethylene and octadecanoid pathways can be either synergistic or antagonistic. Synergistic effects of JA and ethylene have been reported from *A. thaliana*, in the expression of defensive genes [e.g., *PDF1.2* (85)]; from tomato, for maximal induction of *PI* gene expression (79); and from cultivated tobacco, *Nicotiana tabacum*, for the expression of two *PR* genes encoding PR1b and osmotin (122). In contrast, in wild tobacco plants (*N. attenuata*), ethylene antagonizes JA-induced transcript accumulation after herbivore damage (Figure 3). In this species, wounding and mammalian herbivore attack increase the production of a potent defense metabolite, nicotine, which in turn is activated by proportional changes in endogenous JA production (7) as well as exogenous JA applications (5). Attack by the tobacco hornworm, *M. sexta*, a solanaceous specialist, or application of its regurgitant to wounds results in a JA burst (36, 103) and reduces induced nicotine production (68). The attenuation of nicotine accumulation results from an ethylene burst after hornworm feeding (49), which antagonizes the wound-induced transcriptional increase in the nicotine biosynthetic genes *NaPMT1* and *NaPMT2* (121).

Genoud & Metraux (33) summarized examples of the crosstalk between different signal cascades and modeled them as Boolean networks with logical gates and circuits.

This model complements that of Reymond & Farmer (92) and makes concrete predictions regarding the outcome of the crosstalk between pathways. The utility of this approach is limited by our incomplete understanding of all the cascades that are involved. Also lacking from the model is how crosstalk translates to ecological interactions among players on the second and third trophic levels and how compromised plant defense responses translate into plant fitness. An understanding of the functional consequences of crosstalk requires a sophisticated understanding of whole-plant function, which the *Manduca-Nicotiana* interaction illustrates (Figure 3).

In this natural interaction, JA and ethylene bursts are both induced when larval-specific elicitors (FACs) (Figure 2) are introduced to the feeding sites and result in reduced nicotine induction. As such, the crosstalk appears to benefit the herbivore and suggests that *Manduca* is feeding in a “stealthy” fashion, reducing its dietary intake of nicotine by suppressing the nicotine (68). However, parallel to the nicotine attenuation, *Manduca* feeding and application of oral secretions or FACs to the wound induce the emission of a suite of VOCs (35) that function as an indirect defense by attracting predators to the feeding herbivore (52). With the downregulation of a direct defense and the parallel upregulation of an indirect defense, *N. attenuata* may be optimizing the defensive function of its volatile release by suppressing nicotine production, which could be sequestered by the herbivore and used against predators attracted by the volatile release. Plant defense compounds are commonly sequestered by adapted herbivores for their own defense, and thus induced nicotine production may wreak havoc with the plant’s ability to use “top-down” processes as a defense (113).

MOLECULAR ADVANCES IN DIRECT DEFENSES

Almost any plant trait can be manipulated to function as a direct defense; however, most of the research has focused on the veritable arsenal of secondary metabolites that function as poisons, digestibility reducers, and repellants. Although many potential direct defenses have been identified, definitive proof of the defensive function of a particular metabolite is in large part limited to the few examples in which the molecular basis of production is sufficiently understood to allow its expression to be manipulated. We limit our review to those studies that demonstrably influence a plant-insect interaction or contribute to an understanding of how variability in direct-defense profiles is generated. In the

evolutionary arms race between plants and insects, plants are expected to be under strong selection to evolve new defenses as insects evolve resistance to the initial suite of defenses (25). The mechanisms responsible for the generation of variability in direct defenses are therefore of particular interest. Because a majority of the molecular efforts have focused on model solanaceous and brassicaceous plant systems, these are the systems with the most advances.

The defensive function of PIs was first described in solanaceous plants and is now one of the best-verified groups of direct defenses. PIs are expressed in seeds and tubers and also in vegetative tissue after wounding. Wound-induced PIs have been shown to enhance plants' resistance to insects by inhibiting the proteolytic enzymes of the attacking insect. A majority of the described plant PIs are inhibitors of trypsin and chymotrypsin (55), but recent work with maize (110) describes an herbivore-induced PI that inhibits both elastase and chymotrypsin in the midgut of *Spodoptera littoralis* larvae. The defensive effectiveness of PIs depends on their affinity and specificity for the midgut proteinases of the attacking insect and the ability of the insect to alter its proteinase profile and overexpress proteinases, which are PI insensitive after ingestion of PI-laced food (55). Hence a PI that is able to inhibit two types of insect proteinases may be particularly difficult for the herbivore to counter and may be useful in engineering durable resistance in crops.

Because PIs and other defensive proteins are direct gene products, their defensive effects have been tested by genetic transformation in a number of plant species. A recent example is the transformation of white poplar with an *A. thaliana* cystein proteinase inhibitor gene (*Atcys*), which conferred resistance to a major insect pest, *Chrysomela populi*, by inhibiting most of the digestive proteinase activity of this chrysomelid beetle (18). However, when novel PIs are expressed in a host plant, resistance to all of a plant's herbivores is rarely achieved, and the degree of resistance is in part determined by the insect's counter responses. A study that compared the performance of three generalist lepidopteran herbivores on each of three different host plants (tobacco, *Arabidopsis*, and oilseed rape), each transformed to express the mustard trypsin inhibitor MTI-2, demonstrated that the chemical milieu in which the PI is expressed influenced its defensive function (17). To keep one step ahead of rapidly adapting herbivores, plants have evolved mechanisms to produce many new active PIs. Some of these may be able to retain their defensive function in the constantly changing chemical environments of a plant as it matures and senesces. The multidomain structure of some PIs may allow a

plant to produce inhibitors against a broad spectrum of proteases that retain their defensive function in different chemical environments.

The serine proteinase inhibitors of the potato type-II inhibitor family consist mostly of two repeated domains (124), whereas in the stigmas of *Nicotiana glauca* flowers, the PIs are produced from a precursor protein with six repeat domains (61) that after proteolytic processing eventually produces six single domain PIs (four trypsin and two chymotrypsin PIs) and a novel two-domain PI. A four-domain PI from the stigmas of the same plant species has recently been discovered (74). These multidomain structures probably allow plants to target a large number of different proteases within a relatively short period of time (74). Over evolutionary time, the reactive sites in the PI genes have accumulated a larger number of mutations, which we would expect to be the signature of an evolutionary arms race to diversify PI properties (39).

Arabidopsis produces many different glucosinolates that can protect plants against generalist herbivores and pathogens but also function as feeding and/or oviposition attractants for Brassicaceae specialists (13). Heterogeneous selection pressures may therefore maintain the variation in metabolite profiles within populations. A model for how the qualitative and quantitative diversity of glucosinolate profiles is maintained was recently proposed by Kliebenstein et al. (53). They examined 39 *Arabidopsis* ecotypes and found that polymorphisms at only five loci, each coding for different branch points in the glucosinolate biosynthetic pathway, were sufficient to generate 14 qualitatively different leaf glucosinolate profiles, including 34 different structures, most of which are derived from methionine by chain elongation. Moreover, a single locus appeared to control a majority (nearly 75%) of the observed quantitative variation.

Transformation has been used to examine the importance of both qualitative and quantitative variation in glucosinolate profiles of *A. thaliana*. For example, the *CYP79A1* gene from *Sorghum bicolor*, which encodes an enzyme that converts *p*-hydroxyphenylacetaldoxime to *p*-hydroxybenzylglucosinolate, sinalbin (a glucosinolate not normally found in *A. thaliana*), was transferred to *A. thaliana* plants (77). The expression of this gene caused a fourfold increase in total glucosinolate levels (largely owing to increased sinalbin production) but did not alter the acceptance of the plants by two brassicaceous specialist flea beetles (*Phyllotreta nemorum* and *P. cruciferae*) in choice tests. This demonstrates that the plant is unlikely to realize lower herbivore loads from these specialists even with an enormous increase in investment in glucosinolate production.

For a plant to rid itself of its specialist herbivore community might require the evolution of an entirely new defense system. Advances in the ability to transform plants with all of the enzymes required for an entire secondary metabolite pathway have allowed researchers to recreate exactly such an evolutionary event. Cyanogenic glucosides, such as dhurrin, are not normally found in any brassicaceous plants, but recently the complete biosynthetic pathway for dhurrin was transferred to *A. thaliana* by expressing two multifunctional microsomal P450 enzymes (CYP79A1 and CYP1E1) and a soluble UDPG-glucosyltransferase (sbHMNGT) (111). It is remarkable that these three enzymes self-organized into a functional complex that efficiently transferred reaction products so that transformed plants were able to produce and store large amounts of dhurrin. Transformed plants were completely resistant to the Brassicaceae-specialist *P. nemorum*. The ability to transfer entire secondary metabolite pathways between species will allow researchers to test a fundamental tenet of the theory by Ehrlich & Raven (25) of plant-herbivore coevolution: that the diversity of secondary metabolites represents constraints on the evolution of herbivore-host selection.

Resistance to herbivores in *Arabidopsis* is correlated with glucosinolate production and breakdown (myrosinase) and the presence of trichomes (67). However, a recent QTL analysis revealed a locus that did not map to any locus of the previously known resistance traits. Jander et al. (47) crossed two commonly studied *Arabidopsis* ecotypes that differed in their susceptibility to the larvae of *Trichoplusia ni* (a generalist noctuid), the Landsberg *erecta* ecotype, and the Columbia ecotype, which is considerably more resistant. Susceptibility mapped to the *TASTY* locus on chromosome 1, which was distinct from genes that affect trichome density, disease resistance, glucosinolate content, and flowering time (47), but close to a recently discovered locus, *ESP*, which causes the formation of epithionitriles during the hydrolysis of glucosinolates instead of isothiocyanates (59). Glucosinolates are hydrolyzed during wounding and herbivore attack, and this finding underscores the importance of studying direct defenses not only *in planta*, but also in the insect digestive system.

Because herbivores take plant material into an environment that they chemically control (112) and adapt their digestive and detoxification systems to neutralize the effects of direct defenses (90), studying these mechanisms and the chemical dynamics that occur in insect guts might provide important insights into the plant traits that provide direct defense. However, it is also clear that the spatial scale needs to be broadened beyond the insect gut to include insect behavior and the natural enemies of insects. For example,

when *Brassica napus* plants were transformed with the gene coding for a potato PI, diamondback moth larvae compensated for their decreased digestive efficiency by eating more leaf material (120). Such compensatory responses demonstrate that many direct defenses, particularly those that slow herbivore growth but do not kill them, may not function as defenses if they are not expressed in concert with indirect defenses, namely plant traits that increase the foraging efficiency of the natural enemies of herbivores (75). A combination of defenses that slow the growth of herbivores and increase the probability of their mortality before they become reproductively mature is likely to strongly suppress the growth of herbivore populations and represent a particularly effective defense.

MOLECULAR ADVANCES IN INDIRECT DEFENSES

Analysis of the costs of resistance suggests that certain direct defenses can incur substantial metabolic loads and decrease plant fitness when other demands are made on a plant, such as when they are grown with competitors (5). Perhaps as a consequence of these costs and other constraints on the use and effectiveness of direct defenses, plants have evolved defensive mutualisms in which, for a small investment in information-containing VOC releases or nutritional rewards, insects from higher trophic levels are recruited for a plant's defensive needs. Natural selection should favor both plant genotypes that use traits that enhance effectiveness of natural enemies on one hand and predator genotypes that are able to use such plant traits on the other hand (21). Many plant species express these indirect defense traits when they are attacked by herbivores, and many predators clearly use these traits to increase their foraging efficiency (Figure 1). Although evidence for the effectiveness of these defenses in nature is mounting, much remains to be discovered about the mechanisms responsible for expression of these traits. In the following section, we review the molecular advances in two indirect defenses: One is widespread among plants, herbivore-induced VOC emissions, and one is limited to a few taxa, the induction of extrafloral nectar production.

Volatile Organic Compounds

The VOC emission of more than 15 plant species involved in plant-spider mite-predatory mite, plant-caterpillar-parasitoid (21), plant-leaf beetle-egg parasitoid (70), and plant-caterpillar-predatory bug interactions (52) has been examined. In all systems, the host

plant releases wound- and herbivory-inducible volatiles that function as signals in tritrophic interactions. Detailed analysis of the released volatile bouquets has identified many signals that are common to many different plant species, but there are also many compounds that are species specific and are elicited by herbivore-specific cues (21, 36).

The volatiles originate from at least three biosynthetic pathways. First, the so-called green-leaf volatiles, C₆-alcohols and -aldehydes, are produced from α -linolenic acid and linoleic acid via their respective hydroperoxides (78). Some of the green-leaf volatiles may function as direct defenses, as was elegantly demonstrated in a study of transgenic potato plants with depleted HPL (see Section Octadecanoids), which exhibit lower resistance to aphids (115). Green-leaf volatiles also play a role as infochemicals (signals with information content). *Cis*-3-hexen-1-ol is commonly found in the headspace of plants after herbivore attack (35, 113), and enhancing its release from plants in a field study attracted a generalist predator (52). *Trans*-2-hexenal, another green-leaf volatile with biocide effects (15) and commonly emitted after herbivore wounding (19), elicits the accumulation of sesquiterpenoid phytoalexins in wounded cotton and *Arabidopsis*, suggesting a potential role in intra- and interplant signaling. The electrophile α,β -unsaturated carbonyl group of many green-leaf volatiles may confer the ability to induce stress and defense responses in plants (8, 27), but these potential functions need to be verified.

Second are the terpenes derived from the two (mevalonate and nonmevalonate) isoprenoid pathways. Both mono- and sesquiterpenes play a major role as infochemicals in attracting predators and parasitoids to attacked plants (113) as well as functioning as phytoalexins (46). Elicitors present in the insect regurgitant induce the release of mono- and sesquiterpenes after herbivore attack (4, 21, 36). Some of the terpenoids induced in lima beans after herbivore damage [homoterpenes 3*E*-4,8-dimethyl-1,3,7-nonatriene (DMNT) and 3*E*,7*E*-4,8,12-trimethyl-1,3,7,11-tridecatetraene] are synthesized *de novo* in response to herbivore attack (22), and DMNT emission has recently been shown to depend on the herbivore-specific expression of (*E*)-neridol synthase, which catalyzes the synthesis of the sesquiterpene precursor (3*S*)-(i>E)-neridol (16). Many terpenoids are emitted transiently and systemically after arthropod damage (21, 35, 113), and initial evidence suggests that the release of some compounds may be under transcriptional regulation (105).

Two regulatory enzymes in terpenoid biosynthesis are hydroxymethyl glutaryl-CoA reductase (HMGR), which catalyzes the first committed step to the mevalonate terpenoid pathway, and the family of terpenoid synthases, which catalyzes isomerizations and cyclizations of prenyl diphosphates into mono- and sesquiterpenes. The plastidial monoterpene synthases and the cytosolic sesquiterpene synthases share a high degree of sequence similarity and reaction mechanisms (10), which has made functional knockouts difficult to generate. Although the accumulation of HMGR transcripts is induced rapidly by wounding and amplified by herbivore regurgitants (56) and C₆ aldehydes (8), no mutants are currently available for this gene. A first cyclase mutant identified from maize (105) carried an *Ac* (transposition mutation) insertion in the sesquiterpene cyclase, *stc1*, which is normally induced 15- to 30-fold by insect damage, insect oral secretion, and purified volicitin (**Figure 2**) in wild-type plants. In contrast, *stc1* was not induced in mutant plants, and an analysis of volatiles revealed that *stc1* encodes a synthase for a naphthalene-based sesquiterpene (105). Naphthalene is unfortunately not a major component of the VOC mixture of maize plants, and its function in indirect defense is still unknown.

The third pathway involves a group of volatile compounds emitted by herbivore-damaged plants that are derived from shikimate. This pathway links metabolism of carbohydrates to the biosynthesis of aromatic compounds in microorganisms and plants (44). Methyl salicylate, derived from this pathway, is emitted after herbivore damage but not after mechanical wounding by lima beans and wild tobacco. When applied to lima beans, methyl salicylate was attractive to foraging predatory mites (20) but was not attractive to predatory bugs foraging on wild tobacco in a field experiment (52). Another shikimate-derived metabolite is indole, which is released from maize seedlings after damage by beet armyworm caterpillars but not after mechanical damage. The blend of terpenoids and indole released from maize is attractive to the endoparasitic wasp *Cotesia marginiventris*, which attacks larvae of several Lepidoptera species (113). The enzyme indole-3-glycerol phosphate lyase catalyzes the formation of free indole and is, like naphthalene-containing terpenoids selectively activated by volicitin (**Figure 2**), an elicitor derived from caterpillar regurgitant (30).

The ecological function of VOCs---namely attracting predators and parasitoids and reducing the herbivore load of the plant---has been demonstrated in nature (52), but direct proof that plants that rely on this indirect defense experience fitness benefits is still lacking. Understanding the mechanisms responsible for the herbivore-induced VOC

release and manipulating these mechanisms under field conditions will provide such proof and, additionally, evaluate the agricultural utility of this defense mechanism. Crops that release volatile signals in response to herbivore attack could provide the basis for a new era in sustainable biological control of agricultural pests.

Extrafloral Nectar

In addition to VOC releases, plants use bait to attract the natural enemies of herbivores. These have been particularly elaborated in ant plants, which produce extrafloral nectar as well as Pearl and Mullerian food bodies to attract ants (3). These ant mutualists provide defense, which is so effective that, through evolutionary time, ant-housing acacias have apparently lost their chemical defenses [e.g., cyanogenic glucosides (89)]. Extrafloral nectaries are found in at least 66 families, and several studies have documented the defensive role they play for the plants by attracting wasps and ants that attack herbivorous insects and hence reduce damage from herbivores (3). Several studies have shown that the density of extrafloral nectaries and amino acid concentration of the nectar increases after herbivore attack, but until recently, nothing was known about the underlying mechanisms of elicitation. In *Macaranga tanarius* trees, extrafloral nectar secretion increased after herbivory, mechanical leaf damage, and exogenous JA application (40). In addition, phenidone, an antagonist of endogenous JA biosynthesis, inhibited wound-induced extrafloral nectar secretion, and both the transient increase in JA as well as the wound-induced nectar flow were strongly correlated with the amount of damage. Higher nectar secretion resulted in higher numbers of visitors and defenders, which significantly reduced herbivory. The JA cascade is involved in this indirect defense, as it is in herbivore-induced VOC release.

Ecological research into indirect defenses that are mediated by plant traits has demonstrated that the defensive value of the VOC release and nectar production lies principally in the spatial information these traits provide to the predators. Specifically, plants are helping small predators locate actively foraging herbivores in the vastly larger spatial dimensions of host plants. As a result, if indirect defenses are to be successfully applied in agricultural crops, the spatial information content of the signal must be preserved. If crops were engineered to constitutively release VOCs by, for example, a terpene synthase under control of a constitutive promoter, predators and parasites would

rapidly learn to ignore these signals or, worse, to associate them with hunger and thus avoid emitting plants. Therefore the identification of herbivory-responsive regulatory elements will likely be important for the use of these defenses in biotechnology applications.

COMPLEXITY AND COORDINATION OF INSECT-INDUCED RESPONSES

The changes in plant metabolism in response to herbivore or pathogen attack are probably orchestrated by complex transcriptional changes that include genes coding for both primary and secondary metabolism. Procedures for the analysis of differential expression (microarrays, subtractive libraries, AFLP-cDNA display, and DDRT-PCR) allow researchers to study changes in the “transcriptome,” which are elicited in response to herbivore attack or to identify differences in expression between genotypes that differ in resistance. The first results of these techniques, which are just beginning to be applied to plant-insect interactions, suggest that the changes elicited by herbivore attack are comparable in scope and magnitude to those elicited by pathogen attack (64, 101).

The first microarray study of plant-insect interactions analyzed the timing, dynamics, and regulation of the expression of 150 wound-induced genes in *A. thaliana* (93). A time-course analysis of responses elicited by wounding identified groups of genes with similar behaviors, one of which was correlated with the appearance of signals from the jasmonate cascade: OPDA, dnOPDA (dinoroxophytodienoic acid), and JA. But not all genes in this group depended on the JA signaling, as revealed by the comparison of responses from the coronatine-insensitive *coi1-1* mutant (which is JA insensitive) with wild-type plants. Moreover, a comparison of expression patterns in mechanically wounded and *Pieris*-caterpillar wounded plants revealed very different transcript profiles particularly in the expression of the water-stress-induced genes, which were reduced in insect-attacked plants. However, the timing and the magnitude of damage caused by insect feeding were not mimicked in the mechanical wound treatment, so it is difficult to know whether the lack of drought-associated expression was a specific response to *Pieris* feeding (93). This microarray study examined a plant-insect interaction with a “boutique” chip consisting of a preselected group of genes. With the availability of microarrays that cover the complete genome of *A. thaliana*, we look forward to the first truly unbiased analyses of the transcriptional changes induced by herbivore attack in this model plant.

For studies with other plant systems that are not supported by genome sequencing projects, other less-expensive approaches provide unbiased analyses of insect-induced transcriptional changes. In one such analysis, differential display (DDRT-PCR) was used to analyze the transcriptional changes in *N. attenuata* after damage by the specialist herbivore *Manduca sexta*. The putative functions of induced and repressed transcripts could be crudely categorized as being involved in photosynthesis, electron transport, cytoskeleton, carbon and nitrogen metabolism, and pathogen response (43). Transcripts involved in photosynthesis were strongly downregulated, whereas transcripts responding to stress, wounding, and invasion of pathogens or involved in shifting carbon and nitrogen were upregulated. From this study, it was estimated that more than 500 genes responded to the attack of this specialized herbivore. To separate the wound-induced changes from the changes elicited by the *M. sexta* oral secretion and regurgitant, a subset of the differentially expressed transcripts was analyzed, and three discrete patterns of expression were identified (102). Regurgitant modified the wound-induced responses by suppressing wound-induced transcripts systemically in the plant (type I) or amplifying the wound response in the attacked leaves (type II). This amplification was either a downregulation of wound-suppressed transcripts (type IIb) or an upregulation of wound-increased transcripts (type IIa) (Figure 3). It is interesting to note that all three patterns of *Manduca*-induced transcriptional changes of the wound response of *N. attenuata* could be fully mimicked by adding minute amounts of FACs to wounds (36) (Figure 3). The amounts of FACs required are so small that they may be transferred to the plant during normal feeding (102).

These two studies demonstrate that herbivore attack causes a coordinated transcriptional reorganization of the plant, which, in turn, points to the existence of herbivore- and wound-specific *trans*-acting factors that mediate the coordinated changes. Although nothing is known about the identity of such transcription factors, the recently discovered ORCA3, a JA-responsive APETALA2 (AP2)-domain transcription factor from *Catharanthus roseus* that regulates the expression of genes from both primary and secondary metabolism required for the production of terpenoid indole alkaloids (117), provides support for the concept. Identification of such regulatory factors will represent a major advance in understanding the bewilderingly complex transcriptional and phenotypic changes that are elicited after herbivore attack.

CONCLUSION

Plant-herbivore interactions, in contrast to plant-pathogen interactions, are characterized by greater physiological independence of the actors, which has two important consequences for future work. First, the physiological independence of insect herbivores means that for many plant-insect interactions, the wound response will play a prominent role. The seminal work of Ryan and colleagues (99) in understanding the wound response provides an important foundation from which to understand how elicitors from herbivores modify these responses. Second, the arena in which the interaction is played out is clearly very large and includes not only the whole plant but its surrounding biotic community. These larger-scale interactions have been extensively studied by ecologists, and whole-organism entomologists and molecular biologists interested in understanding the function of the transcriptional changes observed after insect attack will benefit from establishing collaborations with these research communities.

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2. Discussion

Plant-herbivore interactions are played out in an arena that is much bigger than the plant itself (Baldwin et al. 2001). Research under natural circumstances provides the strongest data for understanding the mechanisms that determine the rules of the “games” in this arena. Much of the bewildering diversity of plant responses to wounding and herbivory can be understood only by using bioassays and field experiments (Karban and Baldwin 1997). As the examples from this collection of papers demonstrate, the plant’s chemical changes influence the behavior of herbivores and predators and generate complex ecological interactions, which in turn can influence its reproductive success. The herbivore inducible emission of VOCs deserves special attention, because VOCs can serve as reliable signals that provide (in addition to resistance against e.g. fungi) information about herbivore and pathogen attack and thus can have an indirect defensive function by facilitating top-down control through plant-benign enemies of the herbivores (Dicke and Grostal 2001).

2.1 Herbivore induced VOC emission in *N. attenuata*

To study functional aspects of a certain plant defense trait it is necessary to understand its underlying physiological mechanisms. The groundwork to understand the mechanisms of VOC emission in *N. attenuata* was laid in laboratory experiments (Manuscript 1), the results of which are presented first, before their ecological consequences are discussed (Manuscript 3 and 4). The analysis of herbivore-induced VOCs in *N. attenuata* revealed numerous parallels with other plant species. For example, the most abundantly released volatiles are systemically released with a diurnal pattern of emission. (*E*)- β -ocimene, linalool and *cis*- α -bergamotene are terpenoids that have also been reported from herbivore-elicited cotton, tobacco, lima bean and corn plants (Loughrin et al. 1995, Dicke and van Loon 2000, De Moraes et al. 2001). In contrast to wound-induced nicotine production, the release of these terpenoids is induced by herbivore feeding or application of oral secretions, but not by mechanical wounding. The plant recognizes wounding, as evidenced by an endogenous jasmonate burst (McCloud and Baldwin 1997), which is propagated throughout the plant (Schittko et al. 2000). The production of nicotine is strongly correlated with the amount of wounding and thus with the amount of endogenous

jasmonate (Baldwin and Ohnmeiss 1993). However external application of methyljasmonate elicits nicotine production (direct defenses) (Baldwin 1996) as well as increased terpenoid emission (indirect defense), both in proportion to the applied dose of MeJA. Interestingly inhibitors of endogenous wound-induced JA production and subsequent nicotine production (salicylates and auxins) do not inhibit the volatile release when *M. sexta* oral secretion is applied to wounds. On one hand, these results suggest that the endogenous JA response to wounding is necessary and sufficient for the elicitation of the wound induced nicotine response, but on the other hand the herbivore-specific induction of terpenoid emission points to the existence of at least one other herbivore-induced signal. Additional support for the role of herbivores in eliciting alteration of the wound response in *N. attenuata* comes from mechanistic studies of the nicotine production. *Manduca* feeding or application of its oral secretions to the wound causes a much higher endogenous JA induction than does wounding alone (McCloud and Baldwin 1997). Because nicotine production increases with increased endogenous and exogenous JA concentrations, we would expect higher nicotine levels in *Manduca*-damaged plants. But, in addition to the amplified JA burst, hornworm damaged and regurgitant-treated plants exhibit an ethylene burst (Kahl et al. 2000) which antagonizes the wound-induced transcriptional increase in the nicotine biosynthetic genes *NaPMT1* (*N. attenuata Putrescine N-methyl transferase 1*) and *NaPMT2*, causing lower nicotine concentrations in these plants (Winz and Baldwin 2001).

Substances in the oral secretions activate the signals that alter the wound responses. Herbivores, while feeding, not only wound the plant tissue but also inject (sap sucking insects) or apply (chewing insects) oral secretions. Insect-specific elicitors have been found in oral secretions of several herbivore species and belong to two classes of compounds: lytic enzymes and fatty acid-amino acid conjugates (FAC). FACs have been found in the regurgitant of larval Sphingidae (Halitschke et al. 2001), Noctuidae and Geometridae (Alborn et al. 1997, Pohnert et al. 1999). The mechanism of action and signaling remains to be discovered, but it has been established that wounding and the application of FACs are necessary and sufficient for the induction of terpenoid emission and the ethylene burst in *N. attenuata* (Halitschke et al. 2001). Moreover, in *N. attenuata* plants a transcriptional reconfiguration was observed after herbivore feeding that involves the expression of genes not only of the secondary metabolism but also of primary metabolism (Hermsmeier et al. 2001). Plants appear to fine-tune and alter their wound response according to the attacking herbivore, but how plants cope with multiple

herbivore attack and how plants react to commonly observed damage by more than one herbivore species remain challenging questions (Paul et al. 2000). Additionally, environmental and genetic factors alter the wound response and possibly compromise appropriate plant responses to herbivory (Gouinguene et al. 2001, Schmelz et al. 2001).

For example, in *N. attenuata* the herbivore-induced VOC emission differs depending on the genotypes examined, which is consistent with experiments from other plant species (Loughrin et al. 1995, Gouinguene et al. 2001). Additionally, although the VOC emission is a systemic response, the amount of emitted compounds depends on the position of the damaged leave on the plant, with the highest emissions resulting from the treatment of an older stem leaf. These findings raise the question of whether VOCs can function as reliable signals for predators or parasitoids of the herbivores and thus as an indirect defense. Previous studies were performed on relatively homogeneous laboratory and agricultural systems, and it was not known whether plants show herbivore inducible VOC emission under natural conditions (van der Meijden and Klinkhamer 2000).

Novel methods allowed collecting of VOCs under field circumstances and testing their relevance as an indirect defense for the plant, thereby closing the missing link between mechanisms causing VOC emission and their function in nature (Manuscript 3). Wild *N. attenuata* plants emitted the same set of volatiles as plants in laboratory experiments after damage by *Manduca* larvae. In addition it became clear that damage by two other herbivore species, the leaf bug *Tupiocoris notatus* (*Dicyphus minimus*) and the flea beetle *Epitrix hirtipennis*, also elicits the same set of volatiles as *Manduca* chewing. Alone, the emission pattern and thus the ratios between compounds differed. As a potential signal with kairomonal effect the VOC plume from *N. attenuata* combines specificity in the different emission patterns and generality by emitting compounds that simply signal herbivore damage.

2.2 VOCs as infochemicals

The studies on native *N. attenuata* revealed at least three major assumptions required for herbivore-induced VOCs to function as indirect defenses: A) The volatile signal should be sufficiently specific to allow a potential enemy of the herbivores to locate its prey/host effectively. B) The herbivore population has to be top-down controlled, so that predators/parasitoids using the signal significantly reduce herbivore load and thereby increase plant fitness. C) The positive fitness effect of attracting predators/parasitoids

must be greater than the potential negative effect of attracting additional herbivores with the same signals.

The first assumption is met in wild *N. attenuata* due to the two types of information contained in the VOC signal: a) the generality that a herbivore is feeding (sufficient for a generalist predator) and b) the specificity a specialized parasitoid needs to find the exact right host. Previous studies on other plant species, attracting parasitoid wasps to the damaging herbivores, reported very specific host selection, and even discrimination of different host instars using the VOC signal that the herbivore feeding had elicited in the plant (Turlings and Benrey 1998).

The second assumption of top-down control is also met in the *N. attenuata* system. The Geocoridae bug *Geocoris pallens* is a generalist predator that attacks all three major insect herbivore species (*T. notatus*, *E. hirtipennis*, *M. quinquemaculata*) on *N. attenuata* and a number of other herbivores on other host plants (<http://www.biohaven.com/geocb.htm>). It causes an instar dependent mortality pattern among *Manduca* larvae, with highest mortality on eggs and lowest in the older instars, because hornworms older than 2nd instar are too large to be killed by the bug. Additionally, *G. pallens* usually resides on the ground, causing a higher mortality rate on eggs and larvae situated lower in the plant (Manuscript 2). In summary, this predatory bug is responsible for about 95% of the hornworm mortality, which results in significant top-down control over the *Manduca* population on *N. attenuata*. Similar effects have been reported from spider-mite predatory mite systems on lima bean, where predatory mites use the VOC signal to find their prey. Like the predatory mites searching for spider mite prey (Dicke et al. 1998) and parasitoid wasps searching for a host caterpillar (Turlings and Benrey 1998), *G. pallens* is attracted by volatiles emitted by the herbivore-damaged wild tobacco plant, and single herbivory-associated volatiles out of the complete emitted spectrum seem to be sufficient to increase bug predation on *Manduca* eggs and younger larvae. In contrast to the other examples, *G. pallens* is not a specialized predator but rather a generalist using the general information of the VOC signal.

In contrast to the third assumption, an increased VOC emission after herbivore feeding may attract not only enemies of the herbivores but also additional herbivores because they are searching for mating couples, or because the plant is more susceptible after initial damage or the plant is just easier to detect by emitting an amplified signal. For example, the leaf beetle *Oreina caraliae* (Kalberer et al. 2001) and the scarabid beetle *Popillia japonica* (Loughrin et al. 1997, Loughrin et al. 1998) are both attracted by the

volatiles released from their damaged host plants. In the *N. attenuata* system herbivore attraction to damaged plants has not been observed so far. In fact, with *M. quinquemaculata*, the most important herbivore of *N. attenuata*, we find just the opposite effect of VOC emission. *Manduca* adults usually oviposit only one egg per plant, which is most likely an adaptation for two important factors. First, to reach the pupal state a *Manduca* hornworm consumes three to five mature wild tobacco plants and the hornworm rarely leaves the plant of birth until the 4th larval instar. More than one hornworm per plant would therefore mean a strong intraspecific competition for food, which could be prevented by dispersing eggs across plants. Second, plants already damaged by a caterpillar are much more attractive for foraging *G. pallens* than undamaged plants and eggs oviposited after others had already hatched would experience very high predation pressure. Given these circumstances, it is not surprising that *Manduca* moths avoided ovipositing on plants that were already damaged and were repelled in their oviposition choices by the same volatile signals that the predatory bugs used to locate their prey. A similar repellent effect of the herbivore induced VOC signal was also observed with *Heliothis virescens* on *Nicotiana tabacum* (De Moraes et al. 2001) and adds a new dimension to the understanding of the role of volatile plant cues in mediating tritrophic interactions.

According to these findings, by releasing VOCs after herbivore attack, a plant can facilitate both bottom-up (herbivore repellence) and top-down (predator attraction) control over its herbivore population. For *N. attenuata* the calculated multiplicative effect of both ecological consequences of VOC emission could reduce the numbers of the plant's most significant insect folivore, *M. quinquemaculata*, by more than 90%. Such a high number

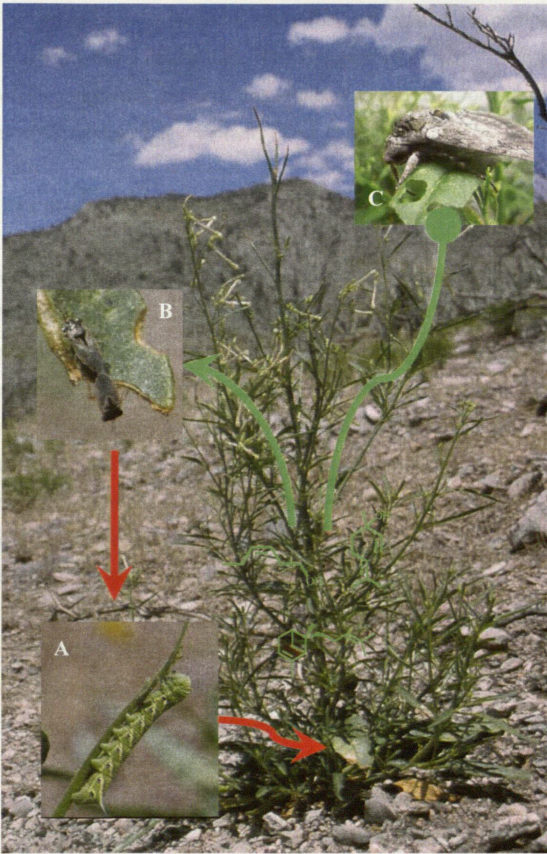


FIG. 3. If a wild tobacco plant (*Nicotiana attenuata*) gets damaged by *Manduca quinquemaculata* (A) it responds by releasing volatile chemical compounds into the environment. These compounds function as signals that help predatory bugs (*Geocoris pallens*) (B) finding their prey, the hawkmoth larva, and deter egg-laying adult moths (C). The plant's "call for help" therefore functions as a double edged sword, reducing the number of herbivores by decreasing their recruitment and increasing their mortality.

suggests a high impact of indirect defense on the plant's reproductive success (Fig. 3). In a study on maize cultivars Hoballah and Turlings (Hoballah and Turlings 2001) measured

fitness variables and found a 30% higher reproductive success among plants, which were damaged by parasitized *Spodoptera littoralis* caterpillars in comparison to plants damaged by unparasitized caterpillars. The endoparasitic wasps, *Cotesia marginiventris* and *Camponotus sonorensis*, in turn, are attracted to the *S. littoralis* caterpillars by the VOC signal of the damaged maize plants (Rose et al. 1998).

2.3 VOCs and biocontrol in agriculture

What can we learn from the findings under natural conditions? In view of the significant effects of VOC emission as indirect defenses, it is intriguing to consider their agricultural applications (Agrawal 2000). At present there is a great variety of predators and parasitoids which are already being used or planned to be used for biocontrol (<http://www.biohaven.com>), but the knowledge about the information flow in tritrophic systems can help to make the “enemies of the plants’ enemies” more effective. Therefore, it is important to review the three requirements for the effective use of VOC as indirect defense trait in an agricultural context.

First, the crop plant has to give off a sufficient and reliable VOC signal after herbivore damage. In the process of domestication plants may have lost the ability to defend themselves by either direct or indirect means. Loughrin et al. (Loughrin et al. 1995) for example found wild varieties of cotton which emitted 10-fold higher amounts of herbivore inducible VOCs than did their domesticated counterparts. Moreover, substantial variability in induced volatile signals was observed among maize cultivars as well as among wild relatives of maize, both in terms of quantity and quality of volatile emissions (Gouinguene et al. 2001). Consequently a successful application of indirect defense in crop plants requires detailed tests of the target cultivar. Alternatively, if high-emitting cultivars are to be engineered, they must be transformed with constructs that result in VOC emissions only when they are attacked by a herbivore. In other words the VOC signal must present reliable information about the location and state of a herbivore for the predator, or for other ovipositing herbivores. It has to be associated with low food quality by the herbivore and with prey or host by the predator or parasitoid, respectively.

Second, there must be a predator or parasitoid, which uses the signal and is capable of controlling the herbivore population. A generalist predator has the benefit that its population may be maintained on other prey species and it is already in situ if the target herbivore appears. This is the situation we find in the *N. attenuata*-*M.*

quinquemaculat-G. pallens system. However, it is also possible that a generalist predator prefers another prey to the target herbivore and therefore the signal wouldn't help the plant. A specialized predator or parasitoid must be brought into the system when needed, and, under natural conditions, it would require time to build up a population of sufficient size to control the herbivore population. Finally, as has been recently shown for predatory mites, interactions, such as competition, intraguild predation and cannibalism, affect the development and coexistence of predator populations and can have significance for biological control of pest organisms (Schausberger and Walzer 2001).

Third, the herbivore behavior to VOC emission (repellent or attractant) has to be considered not only for target organisms but also for other potential pests of a particular crop plant, because the attraction of additional herbivores compromises the biological control by means of indirect defense.

2.4 Plant vaccination

Another mechanism based on plant-mediated interspecific interaction of the arthropod community has already been used in agriculture and promises similar success in agricultural biocontrol. Plant responses to certain pathogens and herbivores may result in cross-resistance to subsequent attackers, a mechanism called immunization (Kuc 1982) or vaccination (Karban and Baldwin 1997). Such a vaccination can benefit the plant if the fitness consequences of attack from the initial attacker are lower than those from subsequent attackers. Vaccinations have been commercially used to protect crop plants against pathogen attackers with high impact by previously inoculating the plant with less harmful pathogens or by applying pathogen-derived elicitors (Moffat 2001). There are only very few recorded examples where less harmful herbivores induce resistance against more onerous herbivores (Agrawal 1998). Phytophagous Willamette mites (*Eotetranychus willamettei*), for example, induced resistance in Zinfandel grapevines against the economical more damaging Pacific spider mite (*Tetranychus pacificus*) and thereby increased the sugar concentration of the harvested grapes (Karban et al. 1994, Karban et al. 1997).

The initial damage of *N. attenuata* by the mirid *T. notatus* vaccinates the plant against subsequently arriving *M. quinquemaculata* larvae. Two factors cause this plant-beneficial effect. First, hornworm performance is strongly reduced on mirid-damaged plants, which is due to the plant's reduced nutritional value (mirids feed on cell content)

and the synchronously increased concentrations of induced defensive compounds in the leaf tissue. Second, the presence and feeding activity attracts the generalist predator *G. pallens*, which preys on both species the mirids and the hornworms. *Manduca* on a mirid-damaged plant therefore experiences a relatively high predation pressure from the egg-state on. The combination of both plant mediated factors give plants with initial mirid-damage and subsequent hornworm attack a significant fitness benefit over plants which are attacked by hornworms only. The generalist predator *G. pallens* amplifies the vaccinating effect, a mechanism that has also been observed in the Willamet mite-Zinfandel vine-system (Karban et al. 1994). The study with wild tobacco emphasizes the central role of induced plant defenses in structuring arthropod communities and thereby confirms earlier studies.

2.5 Future perspectives

The application of plant defensive strategies such as VOCs as indirect defense or herbivore induced vaccination in agricultural crops to biologically control pests is an interesting and potentially fruitful aim (Agrawal 2000). But, in addition, plant response to herbivory is one of the best examples for phenotypic plasticity and therefore provides an especially promising research field for ecologist and evolutionary biologists (Karban and Baldwin 1997). Presumably the questions to ask are quite similar because understanding a mechanism opens the path to application.

Plant-herbivore interactions are played out in an arena that is much bigger than the plant itself. Therefore it is necessary to study herbivore-induced transcriptional and physiological changes in light of their ecological consequences. The recent merge of molecular biology and ecology takes credit to this need (Baldwin 2001, Baldwin et al. 2001). The application of modern molecular methods such as cDNA microarrays and DDRT-PCR has expanded the studies on herbivore-induced gene expression to include large-scale investigations of the herbivore-responsive transcriptome, providing information about the regulation of metabolic changes in the plant after herbivore attack. The first microarray study of plant-insect interactions probed 150 *Arabidopsis* genes for their response to *Pieris rapae* larval feeding (Reymond et al. 2000). Many of the genes were induced by both mechanical wounding and herbivore feeding, but the study also found differential transcript accumulation, complementing earlier reports on herbivore-specific secondary metabolite accumulation. For example, many putatively drought-

implicated transcripts of *Arabidopsis* were not induced as dramatically by *P. rapae* feeding damage as by mechanical wounding. Moreover it became clear that both mechanical wounding and herbivore damage induce transcriptional changes not only in defense related genes but also in genes of the primary metabolism. DDRT-PCR has been used in *N. attenuata* to gain an unbiased view of the transcriptional changes induced by the specialist herbivore *M. sexta*. Of an estimated 500 herbivore-responsive genes, 27 were analyzed in detail. They could be crudely classified as being involved in photosynthesis, electron transport, cytoskeleton formation, carbon and nitrogen metabolism, signaling and response to stress, wounding or pathogen attack (Hermsmeier et al. 2001, Schittko et al. 2001). Such coordinated changes in metabolic reconfiguration have also been shown following pathogen attack and point to the existence of basal herbivore-induced regulators of metabolism. The recently discovered transcription factor ORCA-3 (Octadecanoid-Responsive-*Catharanthus*-APETALA2 [AP2]-domain protein-3) most likely represents such a central transcriptional regulator because it directs metabolic fluxes into the biosynthesis of terpenoid indole alkaloid by regulating four genes in the alkaloid metabolic pathway (van der Fits and Memelink 2000, Memelink et al. 2001).

In addition to coordinating plant response to herbivore attack, the arena in which plant herbivore interactions are played out involves the consequences of these responses for the herbivore community or tritrophic interactions. As the examples presented in this thesis show, the interactions between plants, herbivores and predators of the herbivores can be highly complex and presumably cannot be explained by simple gene-for-gene evolution, as plant-pathogen interactions suggest (Lam et al. 2001). The vaccinating effect of initial mirid damage to *Manduca* hornworms on wild tobacco plants is amplified by the action of a shared generalist predator, which in turn is attracted by plant VOCs induced by both herbivore species. A trait defensive against one herbivore is not necessarily valid against another herbivore. Moreover, the effectiveness of a defensive trait may depend on the chemical and nutritional environment in which it is expressed. Recent studies try to approximate these sorts of problems by using mutant plants that lack, overexpress or additionally express defensive genes and by testing these plants in insect bioassays. Thereby researchers depend on either existing natural variation or engineered transformed plants (Manuscript 5).

In contrast to pathogens, herbivores are physiologically independent. This independence gives another clue for future research on plant-herbivore interactions. Until

recently only very few herbivore specific elicitors have been discovered, but these substances, derived from oral secretions or oviposition fluids, alter the wound response and cause specific reactions in plants (Manuscript 5). Manipulating the production of elicitors in the insect and finding the signaling pathways responsible for the alteration of the wound response in the plant will provide tantalizing hints to how the plant's herbivore responses are regulated.

Plants have multiple ways to cope with their enemies. The examples from *N. attenuata* presented in this thesis show the complexity of interaction generated by the plant's inducible responses to herbivory. At the same time, the need to search for additional study systems that may help to understand the coevolutionary process of plant herbivore interactions becomes obvious. The emerging molecular analysis of the ecological phenomenon may then help to find answers to Stahl's (Stahl 1888) question: "How much of what we call a 'plant' is generated by its interactions with other organisms?"

3. Conclusion

3.1 Conclusion (English)

Plants evolved many different ways of coping with their enemies. Their responses to herbivory are broadly categorized as direct and indirect defense, and tolerance. The consequences of herbivore induced plant responses to herbivory are rarely studied in natural plant populations.

The wild tobacco plant *Nicotiana attenuata* is known to increase the production of several secondary metabolites (nicotine) and enzymes (polyphenole oxidase, proteinase inhibitors) in response to herbivore feeding. In addition to these direct defenses the plant releases volatile organic compounds (VOCs) in response to herbivore feeding or application of their oral secretion to the wound. VOCs can play a role in the plant's indirect defense. *N. attenuata*'s clear expression of direct and indirect defense traits and its special ecology as dominating species in dessert plant communities after fires, qualifies it as an ideal system to study plant-herbivore interactions.

The presented collection of studies examines the consequences of herbivore-induced plant-responses for the arthropod community of *N. attenuata*, in their native habitats in the Great Basin desert of southwest Utah/ USA. In particular the phenology and ontogeny of VOC emission were examined in laboratory experiments (A). The results of these experiments and additional field studies examining the oviposition behavior of the most important herbivore, *Manduca quinquemaculata* (B) in relation to predation risk and nutritional needs of developing larvae, were utilized to design field experiments that allowed evaluating the ecological relevance of VOC emission as an indirect defense (C). Moreover, the plant vaccinating effect of leaf bug (*Tupiocoris notatus*) damage to subsequent *M. quinquemaculata* attack and the altering factors were studied on native tobacco plants (D). Lastly the future perspectives in plant-herbivore interaction research are reviewed (E).

A) *N. attenuata* plants show increased emission of particular VOCs within 24 h after application of methyl jasmonate (MeJA), caterpillar feeding, and the treatment of mechanical wounds with larval oral secretions (OS), but not by mechanical damage alone. Plants from different geographic locations produced volatile blends

that differed in composition. The volatile release was strongly light dependent and varied with the amount of damage with OS or MeJA applied to the plant. Inhibitors of wound-induced jasmonate accumulation (salicylates and auxins) did not inhibit the release of volatiles. The individual induction of different leaf positions with OS and, on other plants, the excision of these leaves after induction, demonstrated that the emission is largely a systemic, whole-plant response, which is maximally triggered when a fully expanded leaf is induced. Resource requirements and elicitation of VOC emission are discussed in comparison to nicotine production.

- B) ***Intra-plant oviposition preferences of *M. quinquemaculata* on *N. attenuata* optimize predation risk and nutritional needs of developing larvae and eggs.*** Nearly 70% of the *M. quinquemaculata* eggs were oviposited on young central stem leaves of elongated *N. attenuata* plants. *Intra-plant* movement was very rare in the 1st and common in the 2nd to 4th larval instars. The oviposition preference for, and larval movement towards, younger leaves was correlated with a 40% lower predation risk and a 6.3-fold greater weight gain, suggesting higher nutritive value despite 2.1-fold higher nicotine concentrations and 4.6-fold higher polyphenol oxidase activities. The predatory bug, *Geocoris pallens*, which consumed eggs and larvae with instar- and leaf position-specific preferences, was responsible for the vast majority of *M. quinquemaculata* mortality and may shape the moth's oviposition preference.
- C) **VOCs function as indirect defense in nature.** I quantified volatile emissions from *Nicotiana attenuata* plants growing in natural populations during attack by 3 species of leaf-feeding herbivores and mimicked the release of 5 commonly emitted volatiles individually. Three compounds (*cis*-3-hexen-1-ol, linalool, *cis*- α -bergamotene) increased egg predation rates by a generalist predator; linalool and the complete blend decreased lepidopteran oviposition rates. As a consequence, a plant could reduce the number of herbivores by more than 90% by releasing volatiles.
- D) **Attack from the mirid *Tupiocoris notatus* induces vaccination to *M. quinquemaculata* in *N. attenuata* plants.** With experiments using natural populations in SW Utah, I demonstrated that wild tobacco plants, *Nicotiana attenuata*, attacked by the mirid, received 75% fewer tomato hornworm (*M. quinquemaculata*) eggs; sustained 85% slower growth of hornworms; and were 61% more attractive to the predator *Geocoris pallens*, which decreased hornworm survival. Hence prior attack by mirids diminished the plant's attractiveness to the hornworm. The vaccinating effect

of initial *T. notatus* feeding prevented the 40.7% fitness loss that hornworm-attacked plants experienced. I concluded that plants mediate competitive interactions within their herbivore community and can benefit from them.

- E) As the examples from this thesis demonstrate **plant-herbivore interactions, in contrast to plant-pathogen interactions, are characterized by greater physiological independence of the actors**, which has two important consequences for future work. First, the physiological independence of insect herbivores means that for many plant-insect interactions, the wound response will play a prominent role, but is frequently altered by herbivore-specific elicitors. Second, the arena in which the interaction is played out is clearly very large and includes not only the whole plant but its surrounding biotic community. These larger-scale interactions have been extensively studied by ecologists, and whole-organism entomologists and molecular biologists interested in understanding the function of the transcriptional changes observed after insect attack will benefit from establishing collaborations with these research communities.

3.2 Zusammenfassung (Deutsch)

Pflanzen entwickelten im Lauf der Evolution eine große Vielfalt an Strategien, um sich gegen ihre Feinde, wie Herbivore und Pathogene zu wehren. Die unterschiedlichen Strategien können in direkte und indirekte Abwehr, sowie Toleranz eingeteilt werden. Die ökologischen Konsequenzen der induzierbaren Feindabwehr von Pflanzen unter natürlichen Bedingungen sind nur schlecht untersucht.

In wilden Tabakpflanzen (*Nicotiana attenuata*) beobachtet man nach Herbivorenbefall eine vermehrte Produktion und Anreicherung von Sekundärmetaboliten, (z.B. Nikotin) oder Enzymen (z.B. Polyphenoloxidase und Proteinaseinhibitoren) im Pflanzengewebe. Zusätzlich zu diesen als direkte Abwehr wirkenden Substanzen geben die Pflanzen gasförmige organische Verbindungen (Duftstoffe) in die umgebende Atmosphäre ab. Solche Substanzen können eine Rolle bei der indirekten Abwehr der Pflanze spielen. Die klare Ausprägung von direkten und indirekten Abwehrmechanismen, sowie die interessante Ökologie als Brandkeimer qualifizieren *N. attenuata* als ideales Modellsystem zur Untersuchung von Pflanzen-Herbivoren-Interaktionen.

In der vorgelegten Zusammenstellung von Manuskripten wurden die Auswirkungen von Herbivoren-induzierten Reaktionen der Pflanze auf die Arthropodengemeinschaft von *N. attenuata* untersucht. Laborstudien zu phänologischen und ontogenetischen Besonderheiten der Duftstoffemission (A) legten die Grundlage für spätere Feldexperimente in natürlichen Populationen von *N. attenuata* in der Great Basin Wüste im Südwesten Utahs (USA). Einleitende Felduntersuchungen zum Eiablageverhalten des Tomatenschwärmers (*Manduca quinquemaculata*), dem für *N. attenuata* bedeutendsten pflanzenfressenden Insekt (B), waren bei der Planung von Experimenten, die sich mit der Bewertung der ökologischen Relevanz von Duftstoffemissionen beschäftigten (C), wertvoll. Zusätzlich wurde der pflanzen-immunisierende Effekt des Fraßes von Blattwanzen (*Tupiocoris notatus*) auf die Entwicklung von und den Räuberdruck auf sekundär schädigende *M. quinquemaculata*-Larven studiert (D). Im letzten vorgelegten Manuskript wurden Perspektiven für die Erforschung von Pflanzen-Herbivoren-Interaktionen diskutiert (E).

A) Bei *N. attenuata* beobachtet man eine Erhöhung der Duftstoffemission innerhalb der ersten 24 h nach Behandlung der Pflanze mit Methyljasmonat (MeJA),

mechanischer Verwundung mit Applikation von Raupenspeichel (OS) und Raupenfraß. Diese spezifische Reaktion der Pflanze war nicht durch rein mechanische Verwundung induzierbar. Die Zusammensetzung der von herbivorengeschädigten Pflanzen emittierten Duftstoffe war in Pflanzen aus verschiedenen Populationen unterschiedlich, variierte mit den Lichtverhältnissen und war vom Grad der Schädigung und der Menge von appliziertem MeJA oder OS abhängig. Inhibitoren der Jasmonsäure-Biosynthese konnten die Emission von Duftstoffen nicht hemmen. Die Duftstoffemission wurde systemisch in der gesamten Pflanze induziert, und maximal angeregt, wenn ein bereits voll entwickeltes Blatt beschädigt wurde. Ressourcenbedarf und Besonderheiten der Induktion von Duftstoffemissionen wurden diskutiert.

- B) Das Eiablageverhalten von *M. quinquemaculata* an *N. attenuata* ist optimiert in Bezug auf Nährstoffbedarf der sich entwickelnden Raupen und Räuberdruck durch die räuberische Wanze *Geocoris pallens*.** Nahezu 70% der *M. quinquemaculata*-Eier wurden an jüngeren und mittleren Stengelblättern abgelegt. Die Eiablagepräferenzen der adulten und die Bewegungen der larvalen Tomatenschwärmer auf der Pflanze korrelierten mit einem um 40% niedrigeren Predationsrisiko und mit einer bis zu 6,3-fach höheren Gewichtszunahme der Raupen. Junge Blätter hatten einen höheren Nährwert als alte, obwohl die Nikotin-, wie auch die Polyphenoloxidasekonzentration in jungen Blättern am höchsten war. Einen besonders großen Einfluß auf das Eiablageverhalten von *M. quinquemaculata* hatte die räuberische Wanze *G. pallens*, welche Eier und Larven attackiert.
- C) Flüchtige organische Stoffe spielen eine Rolle bei der indirekten Feindabwehr von wildem Tabak.** Qualitative und quantitative Charakteristika der Duftstoffemission von *N. attenuata* nach Beschädigung durch drei verschiedene herbivore Insektenarten wurden im Freiland experimentell erfaßt und verglichen. Fünf Substanzen aus dem gesamten Duftstoffspektrum der Pflanze wurden verwendet, um deren Signalwirkung auf die räuberische Wanze *G. pallens* und die adulten Tomatenschwärmer zu testen. Die Behandlung der wilden Tabakpflanzen mit drei dieser Substanzen (*cis*-3-Hexen-1-ol, Linalool, *cis*- α -Bergamoten) bewirkte eine erhöhte Predationsrate an *Manduca*-Eiern durch *G. pallens*. Linalool und das gesamte Duftstoffspektrum reduzierten die Eiablage rate von *M. quinquemaculata*. Die induzierbare Duftstoffemission von *N. attenuata* wehrte Herbivoren ab und lockte

Predatoren an. Dadurch wurde die Zahl der Herbivoren an der Pflanze um über 90% reduziert.

- D) Die Beschädigung von *N. attenuata* durch die Blattwanze *Tupiocoris notatus* bewirkt eine „Immunisierung“ der Pflanze gegen *M. quinquemaculata*. Von *T. notatus* geschädigte Tabakpflanzen empfangen 75% weniger Schwärmer-Eier, erlaubten ein bis zu 85% langsames Wachstum der Schwärmer-Larven und waren 61% attraktiver für die räuberische Wanze *G. pallens* als Pflanzen ohne vorherige Beschädigung durch die Blattwanze. Die somit verringerte Attraktivität vorher geschädigter Pflanzen verhinderte einen 40,7%-igen Fitnessverlust, den *Manduca*-geschädigte Pflanzen erfahren hätten. Diese Studie zeigte, dass Pflanzen neben multitrophischen Interaktionen auch Interaktionen innerhalb der sie bewohnenden Herbivoren-Gemeinschaften vermitteln und selbst davon profitieren.
- E) Wie die Fallbeispiele dieser Dissertation zeigten, sind Pflanzen-Herbivoren-Interaktionen, anders als Pflanzen-Pathogen-Interaktionen, durch eine größere physiologische Unabhängigkeit der Gegenspieler gekennzeichnet. Das hat zwei wichtige Konsequenzen für zukünftige Arbeiten. Zum einen spielt bei der Beschädigung der Pflanze durch herbivore Insekten die Verwundung des Pflanzengewebes eine überragende Rolle, die Reaktion der Pflanze darauf ist jedoch zusätzlich durch herbivoren-spezifische Faktoren (z. B. Substanzen im Raupenspeichel) beeinflusst. Zum anderen werden Pflanzen-Herbivoren-Interaktionen in einer Arena ausgetragen, die die Dimensionen der Pflanze bei weitem übersteigt, da die biotische Umwelt der Pflanze einbezogen ist. Die Zusammenarbeit von Ökologen und Molekularbiologen kann helfen, die Funktion der beobachteten induzierbaren Reaktionen der Pflanzen auf Herbivorenfraß zu verstehen.

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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 bei der Auswahl und Auswertung des Materials und bei der Herstellung der Manuskripte behilflich waren sind am Beginn eines jeden Manuskripts angegeben.

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Jena, den 06. Februar 2002

**The consequences of herbivore-induced plant-responses for the arthropod
community of the native tobacco *Nicotiana attenuata***

Dissertation: André Keßler

The authors contribution to the manuscripts

Manuscript 1

Eco-physiological comparison of direct and indirect defenses in *Nicotiana attenuata*

Rayko Halitschke, André Keßler, Johannes Kahl, Andreas Lorenz and Ian T. Baldwin

(Published: *Oecologia* 2000. **124**: 408-417)

This study builds the basis for all following manuscripts and was therefore included in the thesis. Manuscript 1 is a joint effort by all authors, whereby Rayko Halitschke and I, under the guidance of Ian T Baldwin, shared responsibility for planning, realization and analysis of all experiments except the experiment on the diurnal patterns of VOC emission and the single leaf measurements, which were designed by Andreas Lorenz, Johannes Kahl and Rayko Halitschke. In particular I was responsible for planning, data analysis and statistics of the following experiments: “Emissions increase with increasing MeJA and OS treatments”, “Systemic volatile emission is highly dependent on the developmental stage of the treated leaf” and to 50% for “Inhibitors of wound-induced JA accumulation do not suppress the OS-induced volatile release”.

Rayko Halitschke

André Keßler

Johannes Kahl

Andreas Lorenz

Ian T. Baldwin

Manuscript 2

***Manduca quinquemaculata*'s optimization of *intra*-plant oviposition to predation, food quality and thermal constraints**

André Kessler and Ian T. Baldwin

(in press: Ecology 2002)

Manuscript 2 is based on my research ideas, planning, data collection, chemical and statistical analysis, while compilation of the manuscript was a joint effort by both authors (I wrote the first draft, which was optimized after discussions with the co-author).

Ian T. Baldwin

André Keßler

Manuscript 3

Defensive function of herbivore-induced plant volatile emissions in nature

André Keßler and Ian T. Baldwin

(Published: Science 2001. **291**: 2141-2144)

This paper was based on research ideas and planning of both authors. The idea for the experiments with VOC application came from Ian Baldwin, and I am responsible for the experimental design and realization, the chemical and statistical analysis of the data and the compilation of the manuscript (I wrote the first draft, which was optimized after discussions with the co-author). Moreover I developed the VOC trapping method for field experiments.

Ian T. Baldwin

André Keßler

Manuscript 4

Plant vaccination against herbivore attack in nature

André Kessler and Ian T. Baldwin

(Submitted: Science 2001)

I discovered the phenomenon that in two different plant populations the survival rate of *Manduca* larvae depends on the occurrence of *Tupiocoris notatus* and the predation pressure from *Geocoris pallens*. I am responsible for planning and realization of the subsequent supportive experiments (designs were optimized by interaction with the co-author), the chemical and statistical analysis of the data and the compilation of the manuscript (I wrote the first draft, which was optimized after discussions with the co-author).

Ian T. Baldwin

André Keßler

Manuscript 5

Plant responses to insect herbivory: The emerging molecular analysis

André Kessler and Ian T. Baldwin

(Published: Annu. Rev. Plant Biol. 2002. 53: 299-328)

Manuscript 5 was a joint effort of both authors. I am responsible for the sections: “A primer in plant-herbivore interaction”, “Elicitors from herbivores”, “Wound-elicited responses” and “Molecular advances in indirect defenses”.

Ian T. Baldwin

André Keßler

Lebenslauf

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