The response of *Nicotiana attenuata* plants to herbivory: polymorphism and functions in nature

DISSERTATION

zur Erlangung des akademischen Grades

Doctor rerum naturalium
(Dr. rer. nat.)

vorgelegt

dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

von

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Beginn der Promotion: 28. Februar 2007
Eingereicht am: 13. April 2012
Tag der Verteidigung: 17. Juli 2012

Common sense is the knack of seeing things as they are,
and doing things as they ought to be done.
- Calvin Ellis Stowe

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Introduction

Plants: the trophic base of terrestrial ecosystems

The oxygenic photosynthesis performed by plants and phytoplankton is the primary source of biomass production on earth (Field 1998), and therefore the ultimate source of energy consumed by heterotrophs, which include most fungi, bacteria, and animals. Plants migrated from water to land between 450 and 470 million years ago (Gray *et al.* 1982) and became the primary biomass producers for terrestrial ecosystems. The first insects appeared on land shortly thereafter, around 400 mya (Engel and Grimaldi 2004), and began eating plants (Labandeira 1998). Insects comprise 75% of animal species (Chapman 2009); and if one considers numbers of individuals rather than numbers of species, insects massively dominate the animal kingdom. Insects are thus the most abundant herbivores - as opposed to fungi and bacteria, which are classified as pathogens - and so most anti-herbivore defenses evolved by plants target insects. However, herbivory by other arthropods (e.g. spider mites feeding on solanaceous plants) or browsing mammals (e.g. giraffes feeding on acacias) also exerts selective pressure.

By the time animal herbivory evolved, plants were already practiced in defense against evolutionarily more ancient fungi and bacteria (Staal and Dixelius 2007). The anti-herbivore response shares several characteristics with the immune response against microorganisms. Both are offset by receptor-mediated signal cascades (although the receptors of herbivore elicitors have not yet been identified), and both are mediated by partially overlapping hormonal networks, and have local and systemic components (Howe and Jander 2008; Bonaventure *et al.* 2011). However, in one important way, herbivores present a different problem for plants. As opposed to microbes, which invade individual plant cells or the intercellular space from an infection site, herbivorous animals are mobile feeders, and many are capable of destroying thousands of cells in one bite. The strategy of the immune response to microbes usually involves isolating the invasion site (Pieterse *et al.* 2009). Although herbivores can sometimes be immobilized, they cannot be isolated in the way that microbes can. Herbivores must thus be deterred or actively repelled, if they cannot be killed. Isolation, repellence, deterrence, and mortality are mediated by physical barriers as well as a large number of different plant metabolites (Howe and Jander 2008, Pieterse *et al.* 2009).

Plant defenses- a short history

For a large part of the history of plant science, many metabolites were not thought to function in defense, or anything else. The importance of photosynthetically fixed carbon and the derived compounds necessary for basic metabolism and heterotroph nutrition, also called "**primary metabolism**", was always obvious. However, the plethora of other metabolites which have no clear function in basic metabolism, growth or development, were traditionally considered to be "flotsam and jetsam on the metabolic beach" (Haslam 1986), or biological waste products employed by plants to dispose of surplus carbon and nutrients (discussed in Hartmann 1996).

This view contradicts a basic principle of evolution: that non-adaptive traits are not conserved. It was Gottfried Fraenkel who, in a 1959 essay in *Science* entitled "The raison d'être of secondary plant substances" first suggested the importance of these "**secondary metabolites**" to the communities of chemists, ecologists, entomologists and plant scientists, whose disciplines would merge to form the field of chemical ecology. (Since the first successful transformation of plants in 1983 by Barton and colleagues, molecular biology tools have also been integrated into chemical ecology.) Fraenkel's thesis was that plant "secondary metabolites" were adaptive and had been shaped by coevolution with insect herbivores (Fraenkel 1959).

Fraenkel had discovered a formerly forgotten body of literature from the German scientist Ernst Stahl in the nineteenth century which showed that plant metabolites could determine herbivore feeding choice. Stahl had conducted several feeding choice assays with different species of slugs, and found that slugs which would not feed on specific plants would feed with gusto on the same plants following solvent extraction (Stahl 1888). He hypothesized that the extracted chemicals were responsible for the deterrence of slug feeding. Stahl furthermore suggested that evolutionary pressure exerted by herbivores had shaped plant "secondary" chemistry (Fraenkel 1959).

Because the field of chemical ecology began with the realization that "secondary metabolism" may be primary to plant survival, the terms **general metabolism** and **specialized metabolism** are now sometimes used to differentiate between photosynthate and essential metabolites versus other, often defense-related metabolites.

Discovering the functions of herbivore-induced plant metabolites

As Fraenkel pointed out, and Ehrlich and Raven (1964) elaborated, the interaction between plants and herbivores is a story of coevolution over hundreds of millions of years, although that coevolutionary relationship is likely more complicated than Ehrlich and Raven predicted (Stamp 2003). Herbivore-elicited signaling **induces** a multipronged response in plants that includes toxic, antidigestive, and repellent compounds meant to directly affect herbivores (**direct defenses**); as well as **indirect defense** traits such as extrafloral nectar or **herbivory-induced plant volatiles** (**HIPVs**) meant to indirectly debilitate or remove herbivores by attracting their parasitoids or predators (Kessler and Heil 2011). Like HIPVs, many anti-herbivore defenses are induced by herbivory. There are also **constitutive** defenses which are already present prior to herbivore attack, such as thorns on roses, or xanthotoxin in the leaves of wild parsnips (Wittstock and Gershenzon 2002). There is not a clear divide between constitutive and induced defense: many constitutive defenses have an induced component, meaning that upon herbivore attack their production is upregulated, or they are redistributed in the plant. For example, nicotine is constitutively present in many species of tobacco, but upon damage its production in roots is upregulated, and it is transported from roots to shoots (Baldwin 1999). Furthermore, some defenses, like glucosinolates,

may be constitutively present in an inactive form, but **activated** upon herbivore attack (Wentzell and Kliebenstein 2008).

Herbivores have evolved an astounding variety of mechanisms to tolerate or resist plant defenses (e.g. Wink and Theile 2002), and even sequester them for their own use (Opitz and Müller 2009). Thus plant metabolites produced in response to herbivory may be detrimental to most herbivores, but may even benefit specialist herbivores. As the name suggests, specialists have evolved to complete their life cycle on a restricted group of plants. This entails the abilities to detoxify or avoid dangerous host plant metabolites, and even to adapt those metabolites for the herbivore's own ends. Specialists may, for example, use host plant metabolites as location cues or oviposition stimulants, or sequester toxic metabolites for their own defense (Wittstock and Gershenzon 2002). This tailored adaptation to the chemistry of closely related host plants is possible because many compound classes are both common within, and mostly limited to, one clade of plants. An example is the glucosinolates produced by the order *Brassicales* (but also the genus *Drypetes* in the order *Malphigiales,* Rodman *et al.* 1996). However, other metabolites, such as antidigestive protease inhibitor peptides, are widely distributed throughout the plant kingdom (Hartl *et al.* 2011), and both generalist and specialist herbivores may have evolved adaptations to them.

When evaluating plants' responses to herbivory, it is important to consider that plants have evolved not only under evolutionary pressure from herbivores, but also under both positive and negative pressure from other biotic and abiotic factors. The evolutionary **function** of a metabolite (or other trait) is defined as its positive effect on plant fitness in a specific ecological situation (based on the definition in Karban and Baldwin 1997). Given the number of ecological challenges plants may face simultaneously (e.g., pathogens carried by feeding herbivores, drought and light stress, attracting pollinators while repelling florivores), it may not be surprising that many metabolites seem to have multiple functions. For example, nicotine deters herbivory by generalists in vegetative and reproductive tissue (Steppuhn *et al.* 2004; Kessler *et al.* 2008), but nicotine in floral nectar also increases pollen dispersal and decreases nectar removal (Kessler *et al.* 2008).

Furthermore, the functions of metabolites cannot be determined in isolation, but must take into account the whole-plant background. This is because co-expressed metabolites may interact with each other to modify function. One example from the wild tobacco *Nicotiana attenuata* is the functional combination of the neurotoxin nicotine with antidigestive **trypsin protease inhibitors** (**TPIs**): in the absence of nicotine, generalist lepidopteran larvae respond to TPIs by increasing their intake of plant tissue, thus doing more damage to the plant and meeting their protein requirements; but in the presence of nicotine, generalist larvae are forced to reduce their plant tissue intake, and as a result grow smaller and cause less damage on plants producing nicotine and TPIs than on plants producing only nicotine (Steppuhn and Baldwin 2007). Furthermore, genetically manipulating the expression of nicotine and TPIs in two different native accessions of *N. attenuata* revealed that the same metabolites play different roles in the responses of these two accessions to herbivory (Steppuhn *et al.* 2008).

Studies of natural diversity are important to generate hypotheses about the functions, and functional interactions of plant metabolites. However, these ecological functions are best understood through comparing isogenic lines which vary only in a trait of interest (Bergelson *et al.* 1996), and this is usually only possible using genetically transformed plants (although pleiotropy can still be a problem). A more detailed overview of transgenic techniques, their benefits and drawbacks, and appropriate control measures is given at the beginning of the **Discussion**.

Tests of ecological function have a straightforward evolutionary interpretation when performed with native plants in their natural habitat. In contrast, there is no clear evolutionary interpretation of experiments with cultivated plants, because these plants have been removed from the ecosystems in which they evolved, and have additionally undergone artificial selection to increase yield and decrease content of compounds unpleasant to the human palate. Thus, their native ability to withstand herbivore attack has been altered. However, field research on metabolite function in cultivated plant species is vital to increase yield, generate stress-resistant crops, and design methods which reduce the environmental impact of agriculture (Kos *et al.* 2009).

Finally, it must be kept in mind that plant responses to herbivory are not exclusively defense responses. The ultimate goal of a plant suffering from herbivore attack is to maximize its fitness despite herbivore damage, and this may be achieved not only by defending against herbivores, but also by tolerating or outgrowing herbivory. For example, *N. attenuata* increases its ability to tolerate loss of photosynthetic leaf tissue by shuttling sugar to roots for safe storage, via an SNF1-related kinase-mediated system (Schwachtje *et al.* 2006). Native accessions of *N. attenuata* also differ in their inherent competitive ability, which is inversely correlated to their production of known defense metabolites (Glawe *et al.* 2003).

Fraenkel's (1959) revolutionary *raison d'ĕtre* of specialized plant metabolites might now, 53 years later, be updated to *"raisons" d'ĕtre*. Metabolites should be considered in the context of plant survival strategies, rather than solely in the context of defense against herbivores, and it cannot be assumed that herbivore-induced traits function as anti-herbivore defenses. Furthermore, natural variation within a species is the raw material for evolution, and knowledge of natural variation should inform controlled experiments which demonstrate functions of plant metabolites in nature.

Overview of the dissertation

Chemical ecology concerns itself with much more than plant-herbivore interactions, including plant-microbe, plant-pollinator, insect-insect, and insect-microbe interactions; however, plant-insect interactions are where it all began, and encompass the work presented in this dissertation. The chapters are organized so as to take the reader from an overview of the literature on **plant volatile** (**PV**)-mediated ecological interactions, through an investigation of PVs and other specialized metabolites produced by plants in response to herbivory, and finally to experiments which test the defensive value of specialized metabolites for the plant in its ecological community.

Chapter 1 provides an in-depth introduction to the ecological model system which was used to perform the work presented here: the wild tobacco *N. attenuata* and its defense-related chemistry, its specialist herbivores *Manduca sexta* and *M. quinquemaculata*, the insect predators *Geocoris* spp., and the native ecosystem in the Great Basin Desert of the southwestern USA, in which interactions occur among these players, as well as a number of other naturally-occurring herbivores (**Fig. 1**). **Chapter 1** also introduces the field of HIPV research. HIPVs are the subject of much of the work presented in the remaining chapters. They comprise a large and diverse group of herbivore-induced specialized metabolites which can have both direct and indirect defensive functions. In the 1980's, several groundbreaking studies revealed that damaged plants emit volatile factors which can induce resistance traits in neighbors, attract predators of herbivores, and direct herbivore host plant location (Rhoades 1983; Baldwin and Schultz 1983; Sabelis and Vermaat 1984; Dicke 1986); studies in the 1990's first showed that HIPVs can also repel herbivores

Figure 1 – The *Nicotiana attenuata* system: photographs from *N. attenuata*'s native habitat in the Great Basin Desert of southwestern Utah. Left, from top to bottom, herbivores of *N. attenuata*: the suckfly *Tupiocoris notatus* (A. Kessler), flea beetle *Epitrix* sp. (A. Steppuhn), tomato hornworm *Manduca quinquemaculata* (D. Kessler), tobacco budworm *Heliothis virescens* (A. Steppuhn). *T. notatus*, *Epitrix* sp. and *M. quinquemaculata* (like its sister species *M. sexta*) specialize on solanaceous plants, whereas *H. virescens* feeds on several species in different families. Middle, a native post-fire population of *N. attenuata* in southwestern Utah (D. Kessler). Right, from top, pollinators: an ovipositing *Manduca* moth (D. Kessler), the hummingbird *Archilochus alexandri* (D. Kessler); and bottom, the predatory big-eyed bug *Geocoris* sp. (A. Kessler).

(Dicke and Dijkman 1992), and are sufficient to attract parasitoids of herbivores (Turlings *et al.* 1995). Multiple additional functions have since been attributed to HIPVs and other PVs; these diverse proposed functions, as well as the biosynthetic origins and chemical diversity of the different biosynthetic classes of PVs, are summarized in **Table 1** and discussed in **Chapter 1**. Finally, in **Chapter 1** an experimental approach to testing the ecological function of HIPV emission and its effects in communities is described.

Chapter 2 describes the natural variation in *M. sexta*-induced signaling between two accessions of *N. attenuata* collected from Utah and Arizona in the USA. In these accessions, differences in early signaling events in response to the application of *M. sexta* oral secretions (OS) underlie large differences in the production of herbivore-induced metabolites. In particular, one of the HIPVs most commonly emitted by wild *N. attenuata* plants, *trans*-α-bergamotene, is not emitted by one of the accessions. (In this manuscript, *trans*-α-bergamotene is referred to as *cis*-α-bergamotene: the isomer of α-bergamotene produced by *N. attenuata* had originally been reported to be the *cis*-isomer, but this was corrected in **Chapter 3**.)

Chapter 3 describes large differences in the total volatile profile of native *N. attenuata* accessions from a single population in Utah, after induction by wounding and application of *M. sexta* OS (W+OS), or a jasmonate hormone; the jasmonates are among the main hormonal regulators of herbivore-induced responses. Variation among accessions in the emission of individual volatiles is correlated with levels of herbivory-regulated hormones in order to generate hypotheses about the role of hormone signaling in mediating the observed variation.

The largest class of HIPVs, in terms of numbers of different structures, is the terpenoids (see **Table**

1). Terpenoids also include non-volatile compounds, and together the volatile and non-volatile terpenoids form the largest group of plant metabolites, encompassing both general and specialized metabolites. **Chapter 4** describes an abundant group of terpenoid metabolites in *N. attenuata*, **17-hydroxygeranyllinalool-diterpene glycosides** (**HGL-DTGs**), and the regulation of their metabolism by herbivore elicitors. The HGL-DTGs exemplify several fascinating qualities of plant defense metabolites. They are preferentially allocated to young vegetative and reproductive tissues, in accordance with the **Optimal Defense Hypothesis** (McKey 1974, 1979, Feeny 1975, 1976, Rhoades and Cates 1976, Rhoades 1979, Stamp 2003), which is considered in more detail in the **Discussion**. Although HGL-DTGs are constitutively produced in large amounts, changes in their metabolism are induced by herbivory, and they have a large effect on the performance and feeding preference of specialist herbivores. These effects are demonstrated both in controlled glasshouse experiments, and for plants grown in their natural habitat.

In **Chapter 5**, it is tested whether a specific group of HIPVs, the **green leaf volatiles** (**GLVs**) comprising fatty acid-derived C6 aldehydes, alcohols, and esters, can increase plant fitness in nature by increasing predation of herbivores from plants. GLVs have been shown to attract predators of herbivores, but like most HIPVs, the GLVs have multiple other effects, not all of which benefit plant fitness. The impact on plant reproduction of producing an antidigestive defense, TPIs, is also tested in the plant's native ecosystem. It is not clear why plants should prefer to starve their herbivores using an antidigestive defense than to poison or repel them, especially since the potential protein deficiency caused by ingestion of TPIs can be overcome by adapted herbivores if they simply eat more plant tissue. However, TPIs are shown to have an effect on herbivore behavior which may support indirect defense, and HIPV-mediated indirect defense is shown in **Chapter 5** to indeed increase production of buds and flowers in nature, which correlates to plant Darwinian fitness.

Table 1 – Biosynthetic classes of plant VOCs and their biosynthesis, proposed functions, and structural variety.

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[1]	Number of known structures
Fatty acid derivatives	Methyl jasmonate (MJ)	Jasmonates are oxylipins synthesized from 16:3 and 18:3 fatty acids dioxygenated at C13 by 13-lipoxegynase (13-LOX).[2]	Methyl jasmonate is among the main components of the scent of the jasmine flower, where it was first discovered.[3] It is a volatile plant hormone and may be involved in plant-plant signalling.[4, 5, 6]	302.9	Four stereoisomers.
	Green leaf volatiles (GLVs)	GLVs are synthesized via the cleavage of 13-LOX products by hydroperoxide lyase (HPL) to yield 1-hexanal (from 18:2 fatty acids) or *cis*-3-hexenal (18:3). *cis*-3-Hexenal can be isomerized to *trans*-2-hexenal spontaneously or by an isomerase; the hexenals can be converted to alcohols by alcohol dehydrogenases, and the alcohols can be esterified.[7]	Green leaf volatiles make up the "cut grass" smell typical of wounded plant tissue,[8] contribute to the odour of fruits and flowers,[9] are emitted from roots and are important recognition and flavour components for animal consumers.[10] GLVs may contribute to plant defence as antimicrobials[11] or antifungals,[12] direct[13] and indirect[12] anti-herbivore defences, and between[14, 15]- and within-plant[16] alarm signals.	*cis*-3-hexenal 127.3 *cis*-3-hexenol 156.5 *cis*-3-hexenyl acetate 174.2	At least 32 known from plants: four aldehydes (hexanal, *cis*-3-hexenal, *trans*-2-hexenal, and *trans*-3-hexenal) which provide substrate for four alcohols and at least 24 esters (acetates, propionates, butyrates, isobutyrates, valerates, and isovalerates).
	Nine-carbon volatile aldehydes, alcohols and esters	Nine-carbon derivatives are synthesized from 9-lipoxygenase (9-LOX) products of 18:2 and 18:3 fatty acids cleaved at the 9th carbon by HPL and, like GLVs, include aldehydes, alcohols and esters. Some HPLs specifically cleave 9- or 13-hydroperoxides whereas others cleave both 9- and 13-hydroperoxides. 9-HPL products from 18:2 fatty acids contain one double bond, and those from 18:3 fatty acids contain two double bonds.[17]	9-HPL products and their derivatives are odour and flavour components of flowers and fruits,[13] and may be involved in seed development in almond.[18] 9C aldehydes have antifungal properties.[19]	*trans*, *trans*-3,6-nonadienal 201.8 *trans*, *trans*-3,6-nonadienol 214.7 *trans*, *trans*-3,6-nonadienyl acetate 247.4	At least 15: five aldehydes which can be converted to five alcohols, which can be esterified; only the acetate esters are well represented in literature.

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[1]	Number of known structures
Terpenoids	Terpene hydrocarbons Most volatile terpenoids have a five (hemiterpene)-, ten (monoterpene)- or fifteen (sesquiterpene)-carbon skeleton.	Terpene hydrocarbons are synthesized from the 5-carbon precursors isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) produced via one of two pathways in plants: the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway in plastids or the mevalonic acid (MVA) pathway in the cytosol. Generally, hemiterpenes and monoterpenes are synthesized in the plastid and sesquiterpenes in the cytosol; some sesquiterpenes may be synthesized in the mitochondrion from cytosolic substrate.[20, 21] Emission is usually light-dependent.[22]	Terpene hydrocarbons are components of flower, fruit, green tissue, and root odours. Many are allelopathic[23] or may act in plant defence as antimicrobials or antifungals,[24, 25] direct (cytochrome P450-inducing)[26,] and indirect[27] anti-herbivore defences, or attract pollinators.[28] Most react with atmospheric ozone[29] and could be involved in plant oxidative stress responses.[30]	Isoprene: 34.1 Monoterpenes: ca. 140-180[31] Sesquiterpenes: >200[31]	Isoprene is the only hemiterpene. At least one thousand different mono- and approximately five thousand different sesquiterpenes are known.[32] Most are mono- or polycyclic.
	Some terpenoids have an irregular number of carbons (eight to eighteen); these are called homoterpenes or apocarotenoids depending on their origin, and are derived from the cleavage of larger terpenoids.	The homoterpene *trans*,*trans*-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E*,*E*)-TMTT] (C_{16}) is derived from the diterpene geranyllinalool (C_{20}) in the plastid,[33] and the homoterpene *trans*-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT] (C_{11}) is derived from the sesquiterpene (3*S*)-*trans*-nerolidol (C_{15}) in the cytosol[34] by oxidative degradation, possibly catalyzed by cytochrome P450 enzymes.[9] Apocarotenoids (C_8-C_{18}) are cleaved from carotenoids in the plastid by carotenoid cleavage oxygenases (CCOs).[35, 36]	(*E*,*E*)-TMTT and (*E*)-DMNT are herbivore-induced volatiles in many plants and can attract parasitoids and predators to plants with feeding herbivores.[9] Apocarotenoids are flavour and odour components of flowers, fruit, and green tissue.[37] In flowers, apocarotenoids increase apparency both to pollinators and predators and may be attractive or repellent; in fruit, they are associated with ripening.[38] Some have antifungal properties.[39]	(*E*,*E*)-TMTT 293.2 (*E*)-DMNT 195.6	(*E*,*E*)-TMTT and (*E*)-DMNT are the only homoterpenes known to be widespread in plants. Additionally, three different homoterpenes are emitted from elm leaves following oviposition by the elm leaf beetle *Xanthogaleruca luteola*,[40] and other structures are known in insects.
	Oxidized terpenes and their derivatives	Terpenoid hydrocarbons may be further modified by e.g. cytochrome P450 enzymes, and the products may be oxidized by dehydrogenases, esterified by acyltransferases, or reduced. Some terpene synthase enzymes incorporate a molecule of CO_2 to produce oxidized terpenoids as their initial product.[9] Terpene alcohols may also be glycosylated, but the glycosides are not volatile.	Oxidized terpenes and derivatives are also components of flower, fruit, green tissue and root odours.[41] They have similar ecological and physiological roles to those of terpene hydrocarbons, but are more often directly toxic.[25]	Boiling points are higher than the corresponding terpene hydrocarbons.	Thousands

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[1]	Number of known structures
Phenyl-propanoids and benzenoids	Acid, aldehyde and alcohol derivatives of L-phenylalanine	L-phenylalanine is converted to *trans*-cinnamic acid via L-phenylalanine ammonia lyase (PAL). Further conversion of *trans*-cinnamic acid to other phenylpropanoids is shared with the lignin biosynthetic pathway through the steps of monolignol biosynthesis. Benzenoids originate from the same biosynthetic pathway, but the side chain of *trans*-cinnamic acid is enzymatically shortened by two carbons. L-phenylalanine derivatives with a C_2 side chain compete with phenylpropanoids and benzenoids for substrate and are synthesized via different pathways.[9]	Common in floral scents;[42] source of pungent flavour in black pepper and chili peppers (capsaicinoids). Methyl salicylate is a component of an herbivore-induced blend attractive to some predators and parasitoids.[43, 44]	ca. 180-325[45]	ca. 20% of all known plant volatiles[46]
Amino acid derivatives	Acids, aldehydes, alcohols, esters, nitrogen- and sulfur-containing VOCs derived from amino acids other than L-phenylalanine	Amino acids are deaminated or transaminated to form α-keto acids, which are carboxylated and may subsequently be reduced, oxidated or esterified. Amino acids may also be precursors for acyl coA molecules used in esterification reactions catalyzed by alcohol acyltransferases.[9] Ethylene is derived from methionine and thus belongs in this category.	Branched-chain amino acid (Leu, Ile, Val) derivatives are common in fruit. Amino-acid derived esters are found in flowers and fruits.[9] Putrid sulfur-containing compounds, likely derived from methioinine,[9] may serve as direct defences.[47]	ethylene -103.7 3-methylbutan-2-ol 113.6 butyl acetate 126.6	

[1]Royal Society of Chemistry. acc. July 2010. ChemSpider: http://www.chemspider.com/
[2]Wasternack 2007
[3]Demole *et al*. 1962
[4]Karban *et al*. 2000
[5]Preston *et al*. 2001
[6]Kessler *et al*. 2006
[7]Matsui 2006
[8]Hatanaka *et al*. 1987
[9]Dudareva *et al*. 2006
[10]Halitschke *et al*. 2004
[11]Deng *et al*. 1993
[12]Shiojiri *et al*. 2006
[13]Vancanneyt *et al*. 2001
[14]Baldwin *et al*. 2006
[15]Paschold *et al*. 2006
[16]Frost *et al*. 2008
[17]De Domenico *et al*. 2007
[18]Mita *et al*. 2005
[19]Matsui *et al*. 2006
[20]Rodŕiguez-Concepción 2006
[21]Kappers *et al*. 2005
[22]Lerdau and Grey 2003
[23]Mizutani 1999
[24]Cowan 1999
[25]Khosla and Keasling 2003
[26]Brattsten 1983
[27]Degenhardt *et al*. 2003
[28]Schiestl 2010
[29]Calogirou *et al*. 1999
[30]Vickers *et al*. 2009
[31]Harborne 1973
[32]Seigler 2008
[33]Herde *et al*. 2008
[34]Boland and Gäbler 1989
[35]Auldridge *et al*. 2006
[36]Walter *et al*. 2010
[37]Camara and Bouvier 2004
[38]Bouvier *et al*. 2005
[39]Maffei 2010
[40]Wegener and Schulz 2002
[41]Dudareva *et al*. 2004
[42]Vogt 2009
[43]van Poecke *et al*. 2001
[44]Ament *et al*. 2004
[45]Oyama-Okubo *et al*. 2005
[46]Qualley and Dudareva 2008
[47]Berkov *et al*. 2000

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Overview of Manuscripts

Note: the formatting and terminology used in the original manuscripts has been maintained, and so there are some minor formatting and terminology differences among chapters.

Manuscript 1

Asking the ecosystem if herbivory-inducible plant volatiles (HIPVs) have defensive functions

Meredith C. Schuman and Ian T. Baldwin

In press in *The ecology of plant secondary metabolites: genes to global processes* (Chapter 15), G. R. Iason, M. Dicke and S. E. Hartley (Eds.), in the series *Ecological Reviews*. Cambridge University Press, Cambridge, UK (2012).

In Manuscript 1, I reviewed the literature on the many functions of herbivore-induced plant volatiles (HIPVs). I described the *Nicotiana attenuata - Manduca - Geocoris* ecological model system used to test ecological hypotheses about plant responses to herbivory. I summarized the knowledge which has been gained from this system, and the approach of synthesizing molecular biology and analytical chemistry with field ecology, which permits the manipulation of specific traits to test their metabolic consequences and ecological functions. I also described the " ask-the-plant" approach to this model system, in which the researcher attempts to conduct an unbiased analysis of transformed versus wild-type plant phenotypes in the plant's native habitat, in order to discover the ecological functions of specific genes. At the end of this chapter, I outlined an example experiment which exemplifies the ask-the-plant approach, and which will comprise my first postdoctoral work, should I successfully defend this doctoral thesis.

Meredith Schuman and Professor Ian T. Baldwin reviewed the literature, designed the proposed experiment and wrote the manuscript.

Manuscript 2

A comparison of two *Nicotiana attenuata* accessions reveals large differences in signaling induced by oral secretions of the specialist herbivore *Manduca sexta*

Jianqiang Wu, Christian Hettenhausen, Meredith C. Schuman and Ian T. Baldwin

Published in *Plant Physiology*, doi:10.1104/pp.107.114785 (2008)

In Manuscript 2, we analyzed changes in mitogen-activated protein kinase (MAPK) regulation and hormone production which comprise the divergent signaling response to *M. sexta* oral secretions (OS) in two accessions of *N. attenuata*, and measured the resulting differences in metabolite production in these two accessions. I analyzed the OS- and hormone-induced emissions of (*E*)-?-bergamotene, an HIPV known to attract predators of herbivores and thereby reduce the herbivore load of *N. attenuata* plants in nature. One of the two accessions produced lower levels of jasmonate hormones following OS treatment, but previous work has shown that jasmonate levels are strongly correlated to *trans*-α-bergamotene emission in the other accession. Treatment with jasmonate could not restore *trans*-α-bergamotene emission in the first accession, indicating that the cause of polymorphism in (*trans*-α-bergamotene emission is downstream of differences in MAPK and jasmonate signaling. (In this manuscript, *trans*-α-bergamotene is referred to as *cis*-α-bergamotene: the isomer of α-bergamotene produced by *N. attenuata* had originally been reported to be the *cis*-isomer, but this was corrected in manuscript 3.)

Jianqiang Wu, Christian Hettenhausen, and Meredith Schuman planned and performed the experiments, analyzed the data and wrote the manuscript. Professor Ian T. Baldwin designed and coordinated the study and wrote the manuscript.

Manuscript 3

Polymorphism in jasmonate signaling partially accounts for the variety of volatiles produced by *Nicotiana attenuata* plants in a native population

Meredith C. Schuman, Nicolas Heinzel, Emmanuel Gaquerel, Ales Svatos, and Ian T. Baldwin

Published in *New Phytologist*, doi:10.1111/j.1469-8137.2009.02894.x (2009)

In Manuscript 3, I analyzed the herbivory- and jasmonate-elicited volatile emissions of four *N. attenuata* accessions originating from seed collections of neighboring plants in a native population, in comparison with the well-characterized "UT" inbred line of *N. attenuata*. Experiments were conducted in a common garden design in the glasshouse, in order to reveal differences independent of environmental variation. The four most abundant volatiles were analyzed by one-dimensional gas chromatography-mass spectrometry (GC-MS) and total detectable volatiles were analyzed in parallel by more sensitive two-dimensional GCxGC-MS. Accessions varied significantly in their emission of all four abundant and an additional 17 detectable volatiles after simulated herbivory, and these volatiles represented all three major biosynthetic classes (fatty acid derivatives, terpenoids, benzenoids) as well as compounds of uknown biosynthetic origin. Among the four abundant volatiles, we identified a sesquiterpene previously uncharacterized in *N. attenuata*, α-duprezianene, and identified the sesquiterpene α-bergamotene as the *trans*-, and not the *cis*-isomer which was previously reported. Differences among accessions in the emission of *trans*-α-bergamotene and a less abundant unidentified sesquiterpene correlated positively with differences in their herbivory-induced accumulation of jasmonates and jasmonate-regulated gene

transcripts, whereas differences in the emission of methyl salicylate were negatively correlated to differences in jasmonic acid levels; the latter reflected a general negative correlation between salicylic and jasmonic acid in accessions, which supports data from other plants showing that these two phytohormones act antagonistically. Differences in the emission of the remaining 18 volatiles were independent of differences in jasmonate signaling. Furthermore, differences in volatile emission were not reduced by treatment with synthetic jasmonate hormone, which instead resulted in a greater number of volatiles (43) differing significantly among accessions. However, jasmonate treatment reduced differences in *trans*-α-bergamotene emission. Thus we concluded that most variation in *N. attenuata*'s volatile emission after herbivory occurs through processes downstream of herbivore-induced jasmonate signaling.

Meredith Schuman and Dr. Nicolas Heinzel planned and performed experiments, analyzed data, identified the *trans*-isomer of α-bergamotene, and wrote the manuscript. Dr. Emmanuel Gaquerel analyzed the GCxGC-MS data and wrote the manuscript. Dr. Ales Svatos performed hydrogenation and hydrolysis reactions and MS experiments with samples by which he identified α-duprezianene, and contributed those methods and identification data to the manuscript. Prof. Ian T. Baldwin designed and coordinated the study and wrote the manuscript.

Manuscript 4

Jasmonate and ppHsystemin regulate key malonylation steps in the biosynthesis of 17-hydroxygeranyllinalool diterpene glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*

Sven Heiling*, Meredith C. Schuman*, Matthias Schöttner, Purba Mukerjee, Beatrice Berger, Bernd Schneider, Amir Jassbi, and Ian T. Baldwin

*These authors contributed equally to the manuscript

Published in *The Plant Cell*, doi:10.1105/tpc.109.071449 (2010)

In Manuscript 4, we characterized the structural diversity and regulation of 17-17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) in *N. attenuata*. We identified 11 compounds, of which seven were novel structures. Absolute quantification of HGL-DTGs revealed they were present in amounts equivalent to starch (mg per g FM) and thus much more abundant than most specialized plant metabolites. HGL-DTGs were especially abundant in young and reproductive tissues. We generated stable RNAi lines silenced in the enzyme providing the precursor to the HGL-DTG diterpene skeleton (GERANYLGERANYL DIPHOSPHATE SYNTHASE, GGPPS), and thus deficient in total HGL-DTGs. I conducted field and glasshouse bioassays with *GGPPS* RNAi lines which revealed that total HGL-DTGs significantly reduce the performance of the specialist *Manduca sexta* and feeding damage by the specialist mirid *Tupiocoris notatus*; *M. sexta* larval mass was reduced more by HGL-DTGs (10-fold) than by any other defense metabolite yet identified in *N. attenuata*. Herbivore elicitation altered the biosynthetic dynamics of HGL-DTGs and upregulated the synthesis of singly and multiply malonylated structures. Using isogenic RNAi lines deficient in jasmonate biosynthesis and perception, or in the *N. attenuata* Hyp-rich glycopeptide systemin precursor (ppHsystemin), we demonstrated that these biosynthetic dynamics depended on herbivore-induced jasmonate signaling, and were modified by ppHsystemin, which had not previously been shown to have a defensive function in *N. attenuata*.

Sven Heiling and Meredith Schuman planned and performed experiments, analyzed data and wrote the manuscript. Matthias Schöttner, Purba Mukerjee, and Beatrice Berger planned and performed experiments and analyzed data. Bernd Schneider performed NMR analysis of HGL-DTGs and assisted with the NMR data analysis. Amir Jassbi contributed the idea that the HGL-DTGs could be malonylated and that malonylated HGL-DTGs should be sought and their regulation analyzed in *N. attenuata* . Professor Ian T. Baldwin planned and performed experiments, designed and coordinated the study and wrote the manuscript.

Manuscript 5

Herbivory-induced volatiles function as defenses increasing plant fitness in nature

Meredith C. Schuman, Kathleen Barthel and Ian T. Baldwin

This manuscript has been revised following review at *Science*, and has been resubmitted to *Science*.

Note: following the acceptance of this dissertation, a revised version of this manuscript was accepted at eLife, *and is currently in press.*

In Manuscript 5, I demonstrated that herbivore-induced plant volatiles (HIPVs) can increase plant growth and reproduction in nature by attracting predators of herbivores, thereby reducing the herbivore load of the plant. Previous work had demonstrated that HIPVs can attract predators and parasitoids of herbivores, but HIPVs have many other effects (as reviewed in manuscript 1) which may benefit or harm plant fitness, and their effect on plant fitness had not been demonstrated. We chose to manipulate a subset of HIPVs: green leaf volatiles (GLVs: fatty acid-derived C6 aldehydes, alcohols and esters). GLVs have been shown to attract both herbivores and their predators, to influence pollinating and ovipositing *Manduca* moths, and to have antimicrobial properties, and thus reflect the panoply of potential HIPV functions. In *N. attenuata*, GLVs can be silenced independently of other HIPVs and total plant defenses via RNAi of a single biosynthetic enzyme. In a two-year field study, GLV-emitting plants grew larger and had greater reproductive output than GLV-deficient plants, but only in the presence of predatory *Geocoris* insects, which preferentially predated herbivores from GLV-emitters. We also manipulated the production of a growth-retardant anti-herbivore defense, trypsin protease inhibitors (TPIs). Although TPIs have been shown previously to increase *N. attenuata*'s reproduction under herbivore attack in glasshouse studies, TPI-producing plants did not outperform TPI-deficient plants in the field, indicating that the direct benefit to the plant was small. However, TPIs significantly altered the defensive behavior of *Manduca* larvae in response to mimicked predator attack, and thus may function to support indirect defense in nature.

Meredith Schuman planned and performed experiments, analyzed data and wrote the manuscript. Kathleen Barthel planned and performed experiments and analyzed data. Professor Ian T. Baldwin planned and performed experiments, designed and coordinated the study and wrote the manuscript.

CHAPTER 1

Asking the ecosystem if herbivory-inducible plant volatiles (HIPVs) have defensive functions

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Chapter 15 in **The ecology of plant secondary metabolites : genes to global processes.** Eds. Glenn R. Iason, Marcel Dicke and Susan E. Hartley. Ecological Reviews **10**. Cambridge University Press, Cambridge, UK (2012)

1.1 Introduction

Plant volatiles (PVs) comprise cues exchanged among plants and members of their ecological communities, including other plants, microorganisms and insects. Moreover, some PVs may protect plants against oxidative and thermal damage. PVs that are specifically herbivory-inducible (HIPVs) can betray the location of feeding herbivores to their natural enemies, and some HIPVs may defend plants by repelling herbivores or attracting natural enemies. However, the fitness benefits of HIPVs have not been clearly demonstrated in any plant system, so it remains unclear whether they function as indirect defences (Allison and Hare, 2009; Dicke and Baldwin, 2010). Indeed, HIPVs can be detrimental to plants, causing them to be more apparent to and attract herbivores as well as non-beneficial natural enemies that may interfere with other mutualistic interactors, such as pollinators (Halitschke *et al.*, 2008; Kessler and Halitschke, 2009). And it is not clear whether the carnivores found in native plant populations can cope with variability in HIPV emissions. Within single populations of a species, there can be significant variation in the

production of PVs among individuals (e.g. Skoula *et al.*, 2000; Delphia *et al.*, 2009), also after herbivore attack (Schuman *et al.*, 2009), raising the question of whether HIPVs are reliable indicators. Do natural enemies learn which compounds are relevant in each population, or are they innately programmed to respond to certain HIPVs? Do plants which emit different or greater amounts of HIPVs than their neighbours risk making themselves more apparent to herbivores and other detrimental visitors, or benefit from greater apparency to beneficial natural enemies? The best way to answer these questions is to ask the ecosystem in which the plant evolved; however, plant volatile research has a history of anthropomorphic metaphors and utilitarian motivations which we suggest may prevent researchers from placing their experiments in the proper ecological context. This chapter will describe an approach which attempts to "phytopomorphize" the researcher by using field experiments with wild-type (WT) and appropriate transformed lines of the wild tobacco *Nicotiana attenuata*, in its native ecosystem, to direct the project.

1.2 Challenges in elucidating the functions of PVs

1.2.1 The thousand and one functions of PVs?

Most PVs are derived from fatty acids, amino acids (primarily L-phenylalanine), or the terpenoid precursor isopentenyl diphosphate. Figure 1 depicts biosynthesis of fatty acid-derived and terpenoid PVs in *N. attenuata*, which reflects what is known about these biosynthetic pathways in other plants. Biosynthesis of amino acid-derived PVs is more complex and less well characterized; see Dudareva *et al.* (2006) for a review. Lower vapor pressure compounds whose primary function is not thought to be mediated by volatilization, such as the alkaloid nicotine (Figure 2), also contribute to plant volatile bouquets at sufficiently high temperatures. Perhaps the most abundant HIPV, methanol released from pectin by pectin methylesterase (von Dahl *et al.* 2006, Körner *et al.* 2009), does not fit into the three main PV biosynthetic categories. PV biosynthesis and storage are reviewed in Dudareva *et al.* (2006) and Baldwin (2010).

PVs are emitted from vegetative, reproductive and root tissues as well as from specialized structures such as glandular trichomes or resin ducts which produce so-called "essential oils" or resins (Mahmoud and Croteau 2002). Root emissions have so far been poorly characterized (Wenke *et al.*, 2010). PV emission may be controlled developmentally and diurnally as well as via induced signalling following wounding, herbivory, abiotic stress and other elicitation events (Karban and Baldwin 1997 Table 4.4; Arimura *et al.*, 2005; Holopainen and Gershenzon 2010; Loreto and Schnitzler, 2010; Niinemets 2010; Peñuelas and Staudt 2010). As the ubiquity of their emission suggests, PVs are thought to function in nearly every aspect of plants' biotic and abiotic interactions, including:

- alerting pollinators, herbivores and predators and parasitoids of herbivores to the plant's presence (attraction or apparency) (Raguso 2008; Unsicker *et al.*, 2009),
- alerting plants to a neighbouring plant's presence or condition (plant-plant communication or the "talking plants" phenomenon) (Baldwin *et al.*, 2006; Heil and Karban 2010);

- alerting predators and parasitoids to the presence of herbivores, and/or deterring herbivores from colonizing a plant (indirect defence or "crying for help") (Turlings and Wäckers 2004; Heil 2008);

- alerting herbivores to the presence of each other (Dicke 1986; Dicke and van Loon 2000; Kessler and Baldwin 2001; De Moraes *et al.*, 2001);

- alerting neighbouring plants or remote tissues of the same plant to the presence of herbivores (systemic signalling) (Arimura *et al.*, 2000; Kost and Heil 2006; Frost *et al.*, 2008; Heil and Ton 2008);

- increasing or decreasing the competitive ability of neighbouring plants (allelopathy) (Mizutani 1999; Singh *et al.*, 2003);

- increasing or decreasing the palatability of plant tissue to herbivores (feeding stimulant or antifeedant) (Halitschke *et al.*, 2004; Laothawornkitkul *et al.*, 2008);

- reducing growth of, or colonization by microbes (antimicrobial) (Deng *et al.*, 1993; Cowan 1999; Khosla and Keasling 2003; Shiojiri *et al.*, 2006); and

- alleviating oxidative stress due to drought, high UV exposure, high ozone levels, heat, or other stresses that impair metabolism in photosynthetic tissues (antioxidant) (Vickers *et al.*, 2009; Loreto and Schnitzler 2010).

This chapter will focus on the possible functions of HIPVs, but first we will review what is known about the functions of PVs in general. For other topics, the reader is directed to the above references.

In order for a metabolite to have an evolutionary function, by definition, it must increase plant fitness when employed by a plant in its natural setting (Karban and Baldwin 1997). Beneficial or detrimental effects to the community interacting with the plant are only relevant to the evolved function of PVs insofar as they influence plant fitness. Yet there is little data showing any effects of HIPV emissions on plants' evolutionary fitness (Allison and Hare 2009; Dicke and Baldwin 2010) despite repeated calls to the community to demonstrate exactly this, beginning with the first discussion of tritrophic plant-herbivore-carnivore/parasitoid interactions by Price *et al.* (1980).

All areas of scientific research move forward by developing tools which provide advanced resolution and control over the systems studied. PV research is no exception. The field has amassed a significant body of knowledge about mechanisms of biosynthesis and emission, and possible roles of PVs in mediating signalling and ecological interactions, but our ability to demonstrate the proposed functions of PVs is limited by our current toolbox. We need a set of tools which allows us to *discover* the functions of PVs in native plants, in the context of their evolved ecologies.

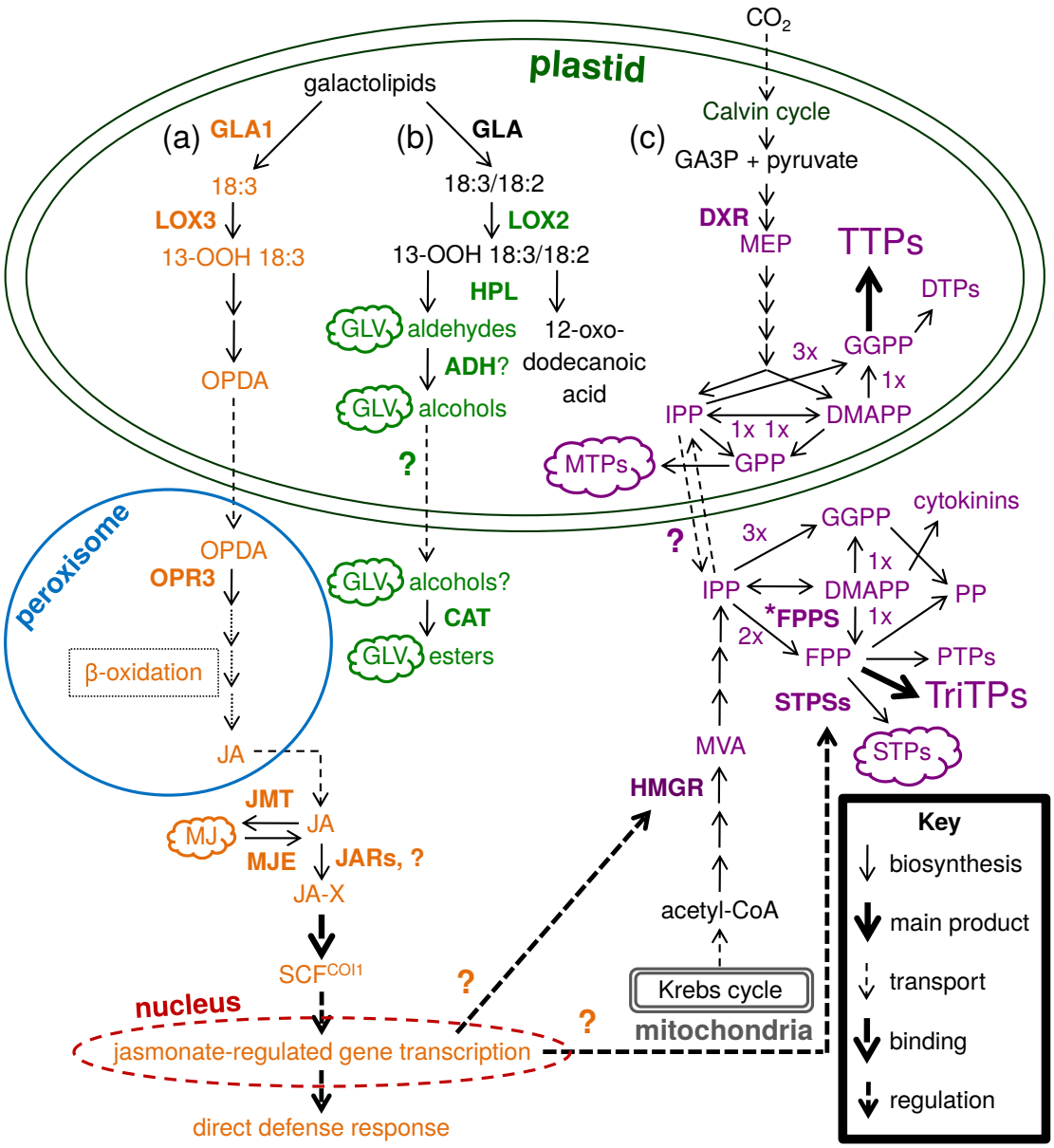
plastid
galactolipids
(a) GLA1
18:3
LOX3
13-OOH 18:3
OPDA
(b) GLA
18:3/18:2
LOX2
13-OOH 18:3/18:2
HPL
GLV aldehydes
ADH?
GLV alcohols
12-oxo-dodecanoic acid
?
GLV alcohols?
CAT
GLV esters
CO2
Calvin cycle
(c)
GA3P + pyruvate
DXR
MEP
TTPs
DTPs
GGPP
3x
1x
IPP
DMAPP
1x 1x
GPP
MTPs
peroxisome
OPDA
OPR3
β-oxidation
JA
JMT
MJ
JA
MJE
JARs, ?
JA-X
SCFCOI1
nucleus
jasmonate-regulated gene transcription
direct defense response
?
?
?
cytokinins
GGPP
3x
1x
IPP
DMAPP
PP
*FPPS
1x
2x
FPP
PTPs
STPSs
TriTPs
STPs
MVA
HMGR
acetyl-CoA
Krebs cycle
mitochondria
Key
biosynthesis
main product
transport
binding
regulation

Figure 1 – The biosynthetic pathways in *N. attenuata* for jasmonates (orange), GLVs (green) and terpenoids (purple), and the putative mechanisms for the influence of jasmonate signaling on sesquiterpene emission. Enzymes and products in black are not specific for a particular pathway. VOCs are marked by clouds.
(a) Jasmonate biosynthesis: 18:3 polyunsaturated fatty acids are released from the plastid membrane by wound- or herbivory-induced lipase activity, most likely by glycolipase 1 (GLA1). The free fatty acids are peroxidated by lipoxygenase 3 (LOX3) and converted to 12-oxy-phytodienoic acid (OPDA) in two additional steps. OPDA is transported to the peroxisome (likely as a CoA conjugate), where it is reduced and undergoes three rounds of beta-oxidation to yield the 12-carbon jasmonic acid (JA). JA moves to the cytosol and is modified, for example by methylation (by jasmonate methyl transferase, JMT) to produce methyl jasmonate (MJ), which can be demethylated back to JA by methyl jasmonate esterase, MJE; or conjugation to amino acids by JA-amino acid conjugate synthase (JAR) enzymes. Jasmonate conjugates (JA-X) bind to the SCF^{COI1} ubiquitination complex which marks Jasmonate ZIM-domain (JAZ) repressors of the jasmonate response for degradation. Only JA-Ile has been shown to actively bind to SCF^{COI1}, but herbivore-induced terpene emission is probably elicited by the interaction of a different conjugate with SCF^{COI1}. Degradation of JAZ repressors permits the transcription of jasmonate-regulated genes and the activation of defense responses.
(b) GLV biosynthesis: A different GLA cleaves 18:3 and 18:2 polyunsaturated fatty acids from the plastid membrane and these are peroxidated by LOX2. The resulting 18C 13-peroxides are cleaved by hydroperoxide lyase (HPL) into *cis*-3-hexenal (18:3) or 1-hexanal (18:2) and 12-oxo-dodecanoic acid, the precursor of traumatin. *cis*-3-Hexenal can be isomerized to *trans*-2-hexenal. The aldehydes are converted by alcohol dehydrogenase (ADH) in the plastid or in the cytosol to the corresponding alcohols; the alcohols are esterified in the cytosol by CoA-acetyltransferase (CAT).
(c) Terpenoid biosynthesis: There are two terpenoid biosynthetic pathways in plants. The plastidial pathway takes its substrate glyceraldehyde-3-phosphate (GA3P) and pyruvate directly from photosynthesis. The first committed product is methylerythritol phosphate (MEP), produced from the immediate precursor deoxyxylulose 5-phosphate (DXP) by the rate-limiting enzyme DXP reductoisomerase (DXR). MEP is converted in five steps to the 5C terpenoid building blocks isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which can be interconverted by an isomerase. One molecule of IPP and one of DMAPP are combined by geranyl pyrophosphate synthase (GPPS) to create 10C GPP, the precursor for 10C monoterpenes (MTPs) and their derivatives, or three IPP and one DMAPP are combined by geranylgeranyl pyrophosphate synthase (GGPPS) to create 20C GGPP, the substrate both for 20C diterpenes (DTPs) and derivatives including gibberellins and plastoquinone, and for the most abundant products of the plastidial pathway: 40C tetraterpenes (TTPs) and derivatives including phylloquinones, chlorophylls, tocopherols, carotenoids, ABA, and volatile carotenoid derivatives. There is thought to be an exchange of IPP between the cytosolic and plastidial pathways, but the mechanism and regulation are not well understood. The cytosolic pathway takes acetyl-CoA from the Krebs cycle to form the first committed product, mevalonic acid (MVA), synthesized from the immediate precursor hydroxymethylglutyryl coenzyme A (HMG-CoA) by the rate-limiting enzyme HMG-CoA reductase (HMGR). MVA is converted in three steps to IPP, which can be isomerized to DMAPP. All or part of the cytosolic pathway may take place in vesicles or peroxisomes, and HMGR in *Arabidopsis thaliana* is anchored in ER-derived vesicles with the active site facing the cytosol. Two IPP and one DMAPP from the cytosolic pathway are combined by farnesyl pyrophosphate synthase (FPPS) to form 15C FPP, which is used as substrate for polyterpenes (>40C, PTPs, including dolichol), for triterpenes and derivatives (TriTPs, including sterols, the most abundant products of the cytosolic pathway), and for sesquiterpene synthases (STPSs) to make 15C sesquiterpenes (STPs) (see Figure 4). FPP is also synthesized in the mitochondria from cytosolic substrate; some sesqui- and homoterpenes (nerolidol, DMNT) and ubiquinone are produced there. The jasmonate signaling pathway may upregulate STP emission after herbivore attack either by increasing HMGR activity, STPS activity, or both. Cytosolic GGPPS provides GGPP for protein prenylation (PP); FPP is also a substrate for prenylation of proteins. DMAPP is also used for cytokinin biosynthesis.

Some of the first field studies which set out to demonstrate defensive functions of HIPV emissions used agricultural plants in agricultural fields, rather than native plants in a natural setting (e.g. de Moraes *et al.*, 1998; Thaler 1999; Rasmann *et al.*, 2005) and thus are difficult to interpret as showing evolved functions. The first field studies on wild plants (e.g. Rhoades 1983) lacked the required controls to conclusively attribute functions to individual compounds or groups of compounds. Genetic and chemical tools to manipulate HIPV emission have allowed us to move beyond foundational studies correlating plant damage or HIPV profiles to observed effects. Studies with transgenic plants have demonstrated effects of specific HIPVs and groups of HIPVs on herbivores (e.g. Aharoni *et al.*, 2003; Halitschke *et al.*, 2004), and their predators and parasitoids (e.g. Kappers *et al.*, 2005; Schnee *et al.*, 2006; Degenhardt *et al.*, 2009), but most do so in an agricultural or laboratory setting and thus again, the interactions demonstrated cannot be clearly interpreted as evolved functions of HIPVs. Many of these studies also use transgenic plants engineered to constitutively emit HIPVs through ectopic expression of HIPV biosynthetic enzymes. Although ectopic constitutive expression is a useful instrument to manipulate PV emission, it removes herbivore inducibility from the information usually conveyed by HIPVs; controlling the exposure of animals to constitutively emitting plants can perhaps mimic induction. Finally, and most importantly, very few studies attempt to quantify fitness effects of HIPV emission. Van Loon *et al.*, (2000) and Hoballah and Turlings (2001) are frequently miscited as demonstrating fitness effects of HIPVs, but both studies only examine the effect of parasitzed versus unparasitized larvae on plant fitness: HIPVs played no role in either study. To our knowledge only one study has quantified the effect of specific endogenous PVs on the fitness of a plant in its native environment (Kessler *et al.*, 2008).

1.2.2 What do we know about PV function?

Now that tools have been developed to manipulate *in planta* PV emissions in increasingly realistic ways, tool development must focus on manipulating the ecological context of HIPV-mediated interactions. Tests of HIPV function require simplified models, but we must be aware of the information we are omitting from our models, and the impact these omissions have on our ability to draw conclusions about natural interactions. HIPV-mediated interactions demonstrated in simplified settings consisting of one plant, one herbivore, and one parasitoid or predator cannot be assumed to occur in nature, nor to significantly affect plant fitness in a natural setting where additional interactions within and among trophic levels may either amplify or negate the effect of a particular tritrophic interaction (Dicke and Baldwin 2010). Constitutive patterns of PV emission, engineered by ectopic expression of genetic elements are not an adequate model of the inducible, tissue- and/or temporally specific emissions seen in native plants, in which the timing of emission both conveys additional information and limits its potential recipients. Ideally, simplification in model-building is driven by an intimate understanding of which details are most important in a natural setting, and this should guide the development of the next generation of tools to advance the field of PV functional research.

We lack molecular and chemical tools necessary to attribute functions to PVs in field studies, and thus to discover strategies employed by plants and their interactors in nature which may not be obvious to us. These necessary tools include genetic mutants *of native plants*, deficient or enhanced in the production, release, or perception of specific compounds, which can be compared to otherwise identical wild-type (WT) or empty vector control (EVC) plants *in their native ecologies*;

combined with transgenic or chemical manipulation of the same compounds in order to recreate a WT phenotype in mutants, or a mutant phenotype in WT plants. To correctly interpret roles of different PVs, we must also conduct unbiased, comprehensive analyses of PV profiles from WT and genetically modified plants. For a discussion of unbiased approaches to PV analysis, see van Dam and Poppy (2008).

The current large body of knowledge regarding PV biosynthesis and regulation provides hints to the functions of PVs in nature and thus to the design of the tools for our new toolbox. What has been well-demonstrated in previous studies?

- That the production of PVs may be controlled via changes to enzyme abundance and activity or substrate flux (Arimura *et al.*, 2000; Dudareva and Negre 2005; Arimura *et al.*, 2008),
- That the emission of many terpenoid PVs (Figure 1) is strongly diurnal and may be closely connected to diurnal patterns of primary metabolism (Halitschke *et al.*, 2000; Rodríguez-Concepción 2006; Arimura *et al.*, 2008),
- That some PVs are emitted exclusively, or in much greater amounts after herbivore damage (Pare and Tumlinson 1999) or oviposition (Hilker and Meiners 2006) and that the changes in emission are elicited by chemicals associated with these events (Alborn *et al.*, 1997, 2003; Gaquerel *et al.*, 2009),
- That feeding or oviposition (Hilker *et al.*, 2002; Fatouros 2009) of an herbivore on plant tissue can attract or arrest predators or parasitoids,
- That many specialist parasitoids innately respond to HIPVs induced by feeding or oviposition of their prey, although this is not always the case (Allison and Hare, 2010), and generalists can quickly learn to respond to prey-associated HIPVs (Lewis and Tumlinson 1988; Dukas and Duan 2000; de Boer and Dicke 2005),
- That different herbivores may either cause similar (Kessler and Baldwin 2004) or different (de Moraes *et al.*, 1998) HIPV emission profiles, which has consequences for herbivore, predator and parasitoid visitation;
- That competition among predators and parasitoids may interfere with the effects of HIPVs on herbivore parasitization or predation (Rosenheim 1998; Finke and Denno 2004);
- That HIPVs can also attract other herbivores (Bolter *et al.*, 1997; Carroll *et al.*, 2006; Halitschke *et al.*, 2008) or mediate competition among herbivores (Denno *et al.*, 1995; Kaplan and Denno 2007);
- That there is considerable variation among individual plants (e.g. Skoula *et al.*, 2000; Gouinguene *et al.*, 2001; Hare 2007; Delphia *et al.*, 2009; Schuman *et al.*, 2009) or populations in PV emission and that these differences may be associated with differences in biosynthesis (Tholl *et al.*, 2005), signalling (Wu *et al.*, 2008) or environmental conditions (Takabayashi *et al.*, 1994);
- That plants engineered to constitutively produce PVs which are usually induced by herbivory can attract previously trained predators or parasitoids in situations of forced interaction (e.g. Kappers *et al.*, 2005; Schnee *et al.*, 2006),

- That herbivory can also induce changes to floral phenotypes (Kessler and Halitschke 2009) and PV profiles, for example causing a change from herbivorous to non-herbivorous pollinator attraction (Kessler *et al.*, 2010).

However, there are few studies demonstrating the natural functions of PVs, in particular:

- Whether plants derive an overall fitness benefit from producing particular floral and vegetative PVs under natural conditions, or whether there are other reasons for the emission of some PVs in wild and in cultivated plants (Allison and Hare 2009; Dicke and Baldwin 2010).
- Whether the production patterns of PVs (if emission is induced, when and by what) and their coordination with the production of other defences enhance plant fitness (Euler and Baldwin 1996; Halitschke *et al.*, 2000; Kahl *et al.*, 2000; Gierl and Frey 2001).
- How predators, parasitoids, floral visitors and herbivores develop responses to particular PVs (Allison and Hare 2009).
- The extent to which floral visitors are affected by vegetative PVs, and vegetative visitors are affected by floral PVs (Heil 2008).
- Consequences of diverse PV profiles within single populations and between populations or ecotypes (Steppuhn, Schuman and Baldwin 2008; Schuman *et al.*, 2009).
- Whether plants benefit or suffer from emitting different amounts of PVs than their neighbours. For example, does a plant that emits much greater levels of HIPVs benefit from attracting more predators or attracting predators away from its neighbours, or do its neighbours benefit from the attraction of too many predators for the actual prey reward (Shiorishi *et al.*, 2010)?
- Whether plants which constitutively emit an HIPV can "fool" predators into thinking they have been induced by herbivores. If so, what is the evolutionarily stable balance of these "cry wolf" plants with "honest" inducible emitters (a question which has been initiated and developed by M. Sabelis)?

To address these open questions, we need a toolbox which allows us to precisely and rigorously manipulate PV emissions in a natural setting, and experimental designs which take plants' ecological communities into account.

1.3 The ask-the-ecosystem approach

1.3.1 *Nicotiana attenuata* and its community as an ecological model system

Our group has developed methods which allow us to ask plants and their interactors about functions of PV emission without making prior assumptions about what the answers might be, by using transgenic and chemical techniques to manipulate PV emission and then comparing wild-type (WT) with manipulated plants in the plant's native ecosystem. This approach allows us to

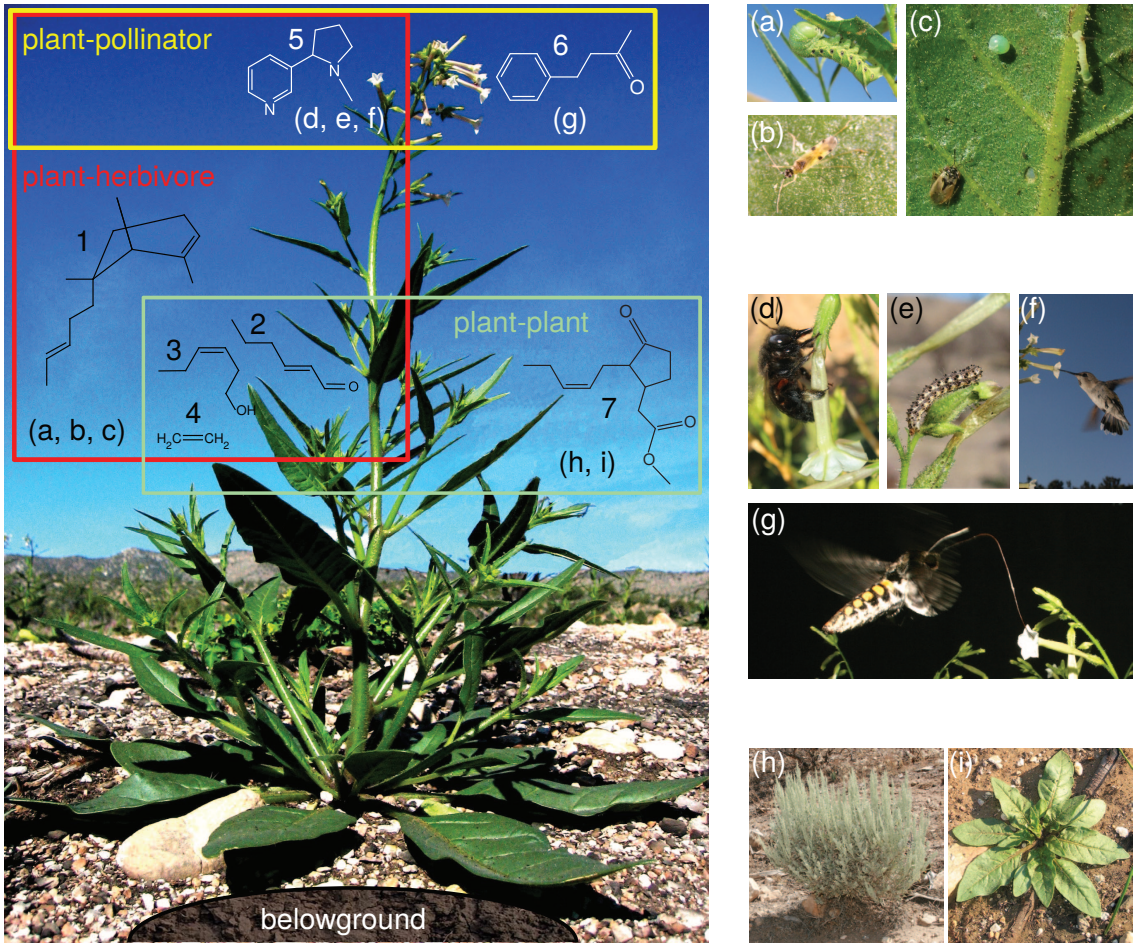

Figure 2 – Interactions mediated by VOCs in the *Nicotiana attenuata* system.
VOCs: 1, *trans*-α-bergamotene (TAB); 2, *trans*-2-hexenal; 3, *cis*-3-hexenol; 4, ethylene; 5, nicotine; 6, benzylacetone; 7, methyl jasmonate (MJ)
Feeding by either (a) larvae of the specialist *Manduca* spp. (Lepidoptera, Sphingidae; shown is *M. quinquemaculata*) or (b) solanaceous specialist mirids (species *Tupiocoris notatus*) induces herbivory-specific VOCs comprising particular mixtures of (1) TAB and other terpenes, and GLVs of which the most abundant are (2) *trans*-2-hexenal and (3) *cis*-3-hexenol. *Geocoris* spp. (Hemiptera, Lygaeidae) predate herbivores on *N. attenuata* and respond to herbivore-induced VOCs (1-3); shown is (c) a *G. pseudopallens* adult on a leaf with a *Manduca* egg and neonate larva. The gaseous hormone (4) ethylene, which is also elicited by some herbivores, plays a role in regulating both (5) nicotine production and plant-plant competition. (5) Nicotine, found both in vegetative and reproductive tissue, repels (d) nectar-robbing carpenter bees (*Xylocopa spp.*) and defends against generalist herbivores including (e) the budworm *Heliothis virescens*. (f) Hummingbirds (here, *Archilochus alexandri*) are daytime pollinators of *N. attenuata* and dislike (5) nicotine, which reduces the amount of nectar they remove per flower visited, and forces them to visit more flowers. (g) *Manduca* adults (here, *M. sexta*) are also pollinators and are attracted to *N. attenuata* flowers by (6) benzylacetone. The hormone (7) MJ is released in large amounts by (h) damaged sagebrush (*Artemisia tridentata*) and has been shown to increase defenses in *N. attenuata* growing nearby; GLVs released from damaged *N. attenuata* (2, 3) influence gene transcription in (i) conspecifics. To date, little is known about belowground interactions among *N. attenuata*, conspecifics, other plant species, herbivores, and microbes, but root volatiles are expected to play a role. *N. attenuata* plants © C. von Dahl (big), R. Halitschke (small); animals and *A. tridentata* © D. Kessler.

learn about both expected and unexpected evolved interactions. The knowledge gained is prerequisite to understanding how PV-mediated interactions function in the real world, and that understanding is important for employing these mechanisms successfully in human-made systems (Degenhardt *et al.*, 2003).

We began by selecting an ecological model plant, the wild coyote tobacco *Nicotiana attenuata* (Solanaceae) (Figure 2). *N. attenuata* is a fire-chaser which forms monocultures in nitrogen-rich post-fire soils, similar to agricultural plants in artificially fertilized fields. Its relationship to cultivated solanaceous species (*N. tabacum, Lycopersicon esculentum, Solanum tuberosum*) and the wild tobacco *N. sylvestris* allows us to benefit from the ecological, chemical, and molecular genetic tools developed in these systems. By closely studying the plant, its ecosystem, and its natural diversity, we can learn what questions can best be addressed in this system, and which traits seem the most important for plants' evolutionary success. Figure 2 presents a summary of *N. attenuata*'s HIPV-mediated interactions. Interactions with floral visitors are included because these can be affected by herbivory: changes to floral PV profiles are also part of herbivory-induced (HI) changes to PVs (Kessler *et al.* 2010).

The jasmonate phytohormones are particularly important in moderating defence responses against herbivory, which is likely the environmental stress which exerts the greatest selective pressure on *N. attenuata*. Research in our system showed that *N. attenuata* plants employ and benefit from jasmonate-mediated defences when attacked by herbivores, both in nature and in the laboratory (Baldwin, 1998; van Dam *et al.*, 2000; Stork *et al.*, 2009), and yet that plants demonstrate significant genetic and defence-related phenotypic variation within and between populations (Halitschke *et al.*, 2000; Glawe *et al.*, 2003; Bahulikar *et al.*, 2004; Schuman *et al.*, 2009). Jasmonates control the elicitation of both direct and indirect defences in *N. attenuata*, and in combination with salicylic acid and the volatile phytohormone ethylene (see Figure 2), orchestrate specific responses to attacking specialist and generalist herbivores (Kahl *et al.*, 2000; Diezel *et al.*, 2009). In particular, upon attack by the nicotine-resistant solanaceous specialist *Manduca sexta*, *N. attenuata* down-regulates its production of nicotine and up-regulates the emission of putative indirect defence compounds such as the sesquiterpene *trans*-α-bergamotene (TAB). Unlike nicotine accumulation, TAB emission is not down-regulated by ethylene or inhibitors of jasmonate biosynthesis, although emission is jasmonate-dependent (Halitschke *et al.*, 2000; Kahl *et al.*, 2000; Halitschke *et al.*, 2008).

In order to dissect variation in, and regulation of direct and indirect defences, we have generated plants altered in the biosynthesis or perception of individual signalling- and defence-related compounds (Krügel *et al.*, 2002, and references above). Comparing altered plants to the corresponding WT or EVC plants (Schwachtje *et al.*, 2008) allows us to determine the function of the manipulated trait(s) in a single genetic background. Altering the same trait in multiple genetic backgrounds allows us to study the influence of genetic background on trait function, and thus explore the consequences of natural variation for plant defence (Steppuhn, Schuman and Baldwin 2008). We have additionally built up a toolkit of techniques and chemical elicitors including standardized procedures for wounding and for application of herbivore oral secretions (OS), supplementation of methyl jasmonate (MJ) or perfuming with specific PVs which allow us to reproducibly manipulate plants' behavior and "recover" some of the phenotypes "lost" from transformants, thus robustly demonstrating that the manipulated genes and their products are responsible for observed phenotypic effects.

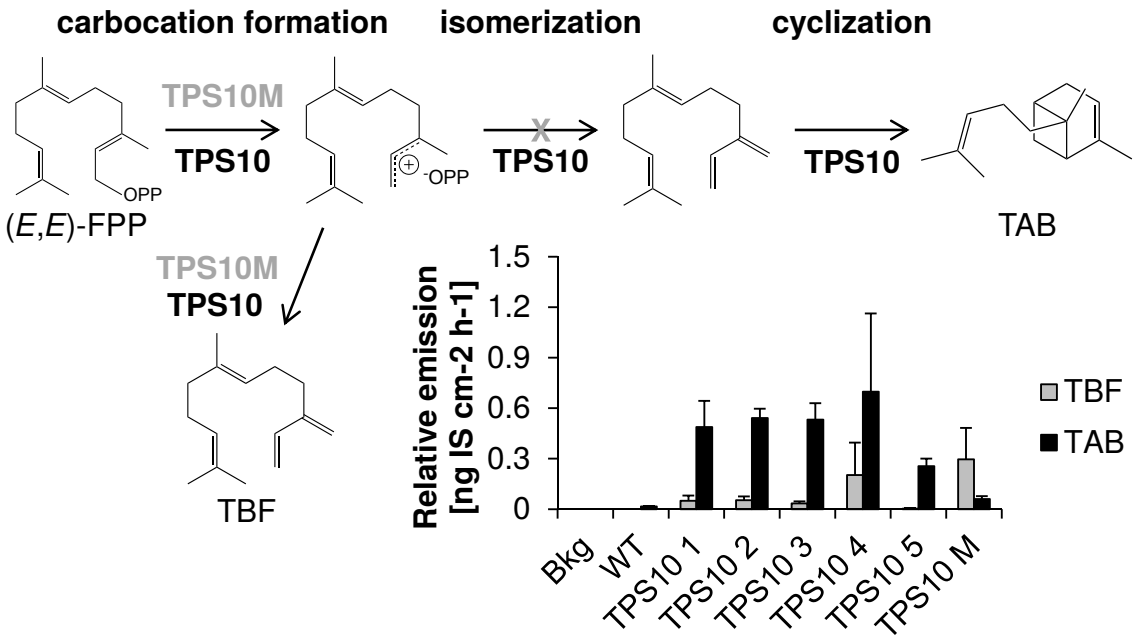

Figure 3 – Mechanism of TBF and TAB biosynthesis by the sesquiterpene synthase ZmTPS10 and the point mutant ZmTPS10M, and constitutive TBF and TAB emission from wild-type (WT) and multiple lines transformed with 35S::*TPS10* or 35S::*TPS10M* constructs. Bkg = background control (ambient air). Scale is relative to ng of tetralin internal standard (IS). Biosynthetic pathway simplified from Figure 4 of Köllner *et al.* (2009).

1.3.2 *N. attenuata*'s HIPV-mediated interactions

We discovered a great diversity of volatile emission following treatment with wounding plus *M. sexta* OS, or supplementation with MJ in populations of *N. attenuata* (Schuman *et al.*, 2009). Nevertheless, several studies by our group have indicated the importance of particular HIPVs in defence and apparency to pollinators, florivores, nectar robbers, herbivores and predators (Figure 2; Kessler and Baldwin 2001; Halitschke *et al.*, 2008; Kessler *et al.*, 2008). We have evidence that generalist *Geocoris* predatory insects in *N. attenuata*'s habitat (Figure 2c) must learn to respond to HIPVs (S. Allmann and I.T. Baldwin, unpublished data), yet we see that *Geocoris* consistently respond to the same groups of compounds: GLVs which are characteristic of leaf damage in all plants and can carry an herbivory-specific signature (Allmann and Baldwin, 2010), and induced sesquiterpenes which are emitted most strongly beginning 1 d after herbivore attack (Skibbe *et al.*, 2008); TAB is thought to be the most important of *N. attenuata*'s sesquiterpenes for *Geocoris* (Halitschke *et al.*, 2000). However, a naturally-occurring genotype of *N. attenuata* deficient in TAB was able to attract predators just as well as a TAB-producing genotype in an area where TAB production should be common (Steppuhn, Schuman and Baldwin 2008). Thus native *Geocoris* predators seem to be telling us they will learn to respond to HIPVs when they are associated with prey. Many questions remain open in our understanding of *N. attenuata*'s HIPV-mediated interactions, in particular those introduced at the end of section 1.2.2 regarding the influence of particular HIPVs on plant fitness and the community effects of HIPV emissions for plants in populations.

1.4 Discovering HIPV functions in a natural system

As an example of the ask-the-ecosystem approach applied to HIPVs, we present an experimental set-up designed to separate the effects of TAB emission from the emission of GLVs and total HIPVs and from the induction of direct defences. For this experiment, we have generated lines of *N. attenuata* manipulated in three biosynthetic genes: lipoxygenase 3 (*lox3*) which provides substrate for jasmonates (Figure 1a), *lox2* which provides substrate for GLVs (Figure 1b), and *TPS10*, a sesquiterpene synthase (STPS, Figure 1c) from *Zea mays* which produces TAB and its linear precursor, *trans*β-farnesene (TBF) (Figure 3; Köllner *et al.* 2009). Silencing *lox3* produces plants deficient in jasmonate signalling which cannot induce direct defences and jasmonate-mediated HIPVs, including TAB. GLV emission during herbivore feeding is not regulated by jasmonate signalling, so in order to reduce total HIPVs and provide a "passive" background for engineered *TPS10* terpene emission, we have additionally silenced *lox2*. (IR*lox2/3* plants have been characterized together with S. Allmann.) To manipulate TAB emission independently from endogenous signalling, in collaboration with J. Gershenzon, we have chosen to ectopically overexpress either *TPS10* or a point mutant version of the enzyme (*TPS10M*) which cannot cyclize TBF to TAB (Figure 3 Köllner *et al.* 2009), under the control of a 35S promotor. We predict that this will allow us to manipulate TAB without altering *N. attenuata*'s native VOC emissions, because the ectopically-expressed enzyme will not be part of the regulatory framework that governs the expression and activity of native enzymes controlling HI-VOC emissions.. Comparing plants expressing *TPS10* with those expressing *TPS10M* allows us to separate the fitness effects of emitting TAB, an endogenous HIPV, from those of emitting TBF, a structurally related PV emitted at very low or undetectable levels from WT *N. attenuata*. Preliminary data from these transformed lines (Figure 3) confirms that 35S::TPS10 and 35S::TPS10M lines produce the product profile that we predicted, with little or no effect on the emission of other VOCs (data not shown).

We use this set of transformed lines to ask *N. attenuata* and its ecological community about the effects of altering TAB emission, whether the effect of TAB emission depends on the action of jasmonate-mediated defences and on the total PV background, and to what extent the quality and effects of HIPV emission depend on the presence, and phenotypes of neighbour plants (see below). Including plants impaired in inducible direct defences and HIPVs grants us an additional level of sensitivity: TAB emission may have a stronger effect in the absence of other defences and other HIPVs; and impaired plants are likely to experience larger neighbour-dependent effects on their levels of herbivore damage, and perhaps on their fitness, than are WT plants.

Neighbour-dependent effects are the effects of the presence and identity of neighbour plants on growth, HIPV emission, and interactions with herbivores and predators. Neighbour-dependent effects may be mediated by competition, plant-plant communication, and the herbivore- and predator-attractive or repellent properties of neighbours. To discover these effects, we compare populations (both mixed- and monocultures) versus individual plants. Individuals and populations are randomly distributed across a field plot in *N. attenuata*'s native habitat. The random order is modified to ensure that no two populations or individuals of the same type are directly adjacent, so that an animal finding itself at any point in the experimental plot may always choose among different types.

Our set of transformed lines (Table 1.1) gives us all of the comparisons we need in order to dissect the effects of TAB and TBF emission in the context of a WT HIPV and defence background, or in

Populations (**p**)

#	Type	Emitter	Monitor
1	Mono	WT	WT
2	Mixed	35S::TPS10	WT
3	Mixed	35S::TPS10M	WT
4	Mono	35S::TPS10	35S::TPS10
5	Mono	35S::TPS10M	35S::TPS10M
6	Mono	IRlox2/3	IRlox2/3
7	Mixed	35S::TPS10 xIRlox2/3	IRlox2/3
8	Mixed	35S::TPS10M xIRlox2/3	IRlox2/3
9	Mono	35S::TPS10 xIRlox2/3	35S::TPS10 xIRlox2/3
10	Mono	35S::TPS10M xIRlox2/3	35S::TPS10M xIRlox2/3

Individuals (i)

#	Line
1	WT
2	35S::TPS10
3	35S::TPS10M
4	IRlox2/3
5	35S::TPS10xIRlox2/3
6	35S::TPS10MxIRlox2/3

p		**6**		**9**		**2**		**1**	
p p	**5**	**9**	**8**	**2**	**10**	**5**	**3**	**8**	**4**
p i	**7**	6	**7**	1	**4**	4	**1**	2	**3**
i **p**	4	**6**	5	**2**	2	**6**	3	**8**	5
p i	**10**	1	**9**	1	**1**	5	**5**	4	**3**
i **p**	3	**5**	6	**6**	2	**9**	6	**1**	3
p p	**10**	**8**	**7**	**3**	**10**	**5**	**4**	**3**	**2**
p i	**4**	3	**1**	1	**7**	4	**6**	1	**7**
i **p**	5	**2**	6	**10**	2	**1**	6	**8**	3
p i	**4**	4	**8**	4	**4**	3	**6**	5	**10**
i **p**	5	**3**	2	**9**	1	**9**	6	**5**	2
p p	**8**	**9**	**2**	**4**	**7**	**3**	**1**	**2**	**10**
p i	**5**	4	**6**	6	**2**	3	**7**	5	**7**
i **p**	1	**10**	5	**4**	2	**8**	4	**5**	2
p i	**1**	3	**3**	4	**9**	1	**6**	5	**10**
i **p**	6	**6**	1	**8**	2	**1**	6	**4**	3
p p	**9**	**3**	**5**	**4**	**3**	**9**	**2**	**8**	**7**
p i	**7**	1	**2**	2	**2**	6	**6**	3	**1**
i **p**	5	**1**	6	**10**	3	**10**	4	**5**	4
p i	**5**	2	**4**	4	**3**	5	**6**	6	**9**
i **p**	1	**8**	5	**10**	1	**8**	3	**2**	2
p p	**1**	**4**	**6**	**7**	**5**	**9**	**7**		**3**
p	R1	R2	R3	R4	R5	R6	R7	R8	R9

Figure 4 – Planting scheme for the proposed experiment in a field plot on Lytle Preserve in the Great Basin Desert in southwestern Utah. Twelve replicates each of ten different population types and six different individuals are planted in a randomized design such that populations and individuals are interspersed and distributed across the field plot, and no replicate populations or individuals are neighbours. Populations are in bold, individuals in normal type. Populations consist of five plants: four monitors at the corners of a 0.4 x 0.4 m square and one emitter in the center. All units (populations or individuals) are planted 1.25 m apart (measured from the edge of a population) down a row (R) between two irrigation lines; each row is separated by ca. 20 cm from the neighbouring row and is ca. 1 m wide. Units are centered on top of the row. The planting is distributed across nine rows.

"passive" backgrounds lacking WT HIPV and defence profiles. Taking these lines to *N. attenuata*'s native environment, we can ask the plant and its community what the evolved functions of TAB, GLVs and other HIPVs might be. Once we have observed effects in the field, we can confirm them under more controlled conditions in the laboratory and glasshouse. Although this is an open and exploratory experiment, we can propose several hypotheses based on ideas about HIPV function. These hypotheses (Table 1.2) guided our choice of phenotypes and planting design.

1.5 Conclusions and outlook

Since the first reports in the 1980's of "talking trees" and plants "crying for help", continuing through to today's growing concern with the interaction of PVs and climate change (Peñuelas 2008), PVs have been a hot topic which intrigues scientists and non-scientists alike. Humans love to anthropomorphize, and what better way to anthropomorphize plants than through stories in which they talk, listen, sniff each other out, and cry for help? While the delightful metaphors which have been created by the field of PV research have inspired effective PR campaigns – which is both good and important for research science – perhaps they have negatively influenced the focus of the research. This is because in order to understand what plants are doing and why, and in order to create proper simplified models for experiments, plant researchers should not be anthropomorphizing their subjects, but rather phytopomorphizing themselves.

Table 1.1 introduces a set of different HIPV emission/defence phenotypes used as ecological molecular tools in an experiment aimed at discovering natural functions of HIPVs. The potential to move from an anthropomorphic towards a phytopomorphic understanding lies in choosing a set of plant phenotypes that lets us test the accuracy of our metaphors. As we gather data on the functions of the traits we have manipulated to create each phenotype, the metaphors which grant us "intuitive access" to the phenotypes will likely change.

It is also important to keep in mind that a large portion of *N. attenuata*'s HIPV emission is not manipulated in our example experiment. This portion includes methanol, ethylene, the floral compounds benzylacetone and nicotine (Figure 12) (nicotine is also volatilized from leaf tissue), and several mono- and sesquiterpenes which are constitutively emitted and not regulated by *N. attenuata* after herbivory. We are not assuming that the compounds we have manipulated, namely GLVs, TAB and total HIPVs, are the most important PVs for *N. attenuata*'s ecological interactions, but we are using our toolbox to ask *how* important they are.

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Table 1 – Transformants used to investigate effects of enhancing terpenoid emission in the presence or absence of other HIPVs and defences. Note that we introduce metaphors in the Phenotype column; these are meant to help the reader develop an intuitive understanding of our chosen experimental phenotypes, but our experiment is designed to allow us to test the accuracy of these metaphors. A distinction is made between constitutive (*C*) and herbivore-inducible (*I*) metabolite levels. "Odoriferous" 35S::TPS plants constitutively emit similar amounts of terpenoids as herbivore-induced WT plants, and may be able to induce even greater emission upon induction (see Figure 2). "Unresponsive" IR*lox2/3* plants cannot induce direct or indirect defences upon herbivore attack and have lower constitutive defence levels; everything but GLV emission can be "restored" to a WT phenotype by MJ application. IR*lox2/3* x 35S::TPS "odoriferous unresponsive" plants constitutively emit TAB and TBF in an IR*lox2/3* phenotypic background.

Genotype	Phenotype	(HI)PVs								Signalling		Direct defence	
		GLVs		Terpenoids						Jasmonates			
				Total		TAB		TBF					
Constitutive/Inducible		*C*	*I*	*C*	*I*	*C*	*I*	*C*	*I*	*C*	*I*	*C*	*I*
WT	Wild-type	n.d.	+	o	+	o	+	o	+	o	+	o	+
35S::*TPS10*	"Odoriferous" plants: ectopic TAB and TBF emission	n.d.	+	+	+++	++	+++	+	++	o	+	o	+
35S::*TPS10M*		n.d.	+	+	+++	+	++	++	+++	o	+	o	+
IR*lox2/3*	"Unresponsive" plants: lacking induced defence including HIPVs	n.d.	n.d.	-	- (+)	-	- (+)	-	- (+)	-	- (+)	-	- (+)
IR*lox2/3* x 35S::*TPS10*	"Odoriferous unresponsive" plants: ectopic TAB and TBF emission in the absence of induced defence and HIPVs	n.d.	n.d.	++	++ (+++)	++	++ (+++)	+	+ (++)	-	- (+)	-	- (+)
IR*lox2/3* x 35S::*TPS10M*		n.d.	n.d.	++	++ (+++)	+	+ (++)	++	++ (+++)	-	- (+)	-	- (+)

Key

o	Untreated WT levels	+	Greater than untreated WT levels, ++ > + > o
-	Less than corresponding WT levels, -- < -	n.d.	Not detectable
(+)	Recovered by MJ application to level indicated in parentheses ()		

Table 2 – Hypotheses and predicted outcomes for the example field experiment.

Hypothesis	Prediction
Plant-plant interactions	
Growth in competition with other plants affects VOC emissions.	The same genotype will have different PV profiles when it growing in a population versus alone.
GLV, TAB and TBF emission enhance/decrease competitive ability.	35S::*TPS10* will grow best/worst and be fittest/least fit in competition with other genotypes; IR*lox2/3* will grow worst/best and be least fit/fittest in competition with other genotypes.
Plant-herbivore interactions	
Herbivores are repelled by indirect defences.	Plant phenotypes which attract the most *Geocoris* will attract the fewest herbivores and have greater fitness than phenotypes with the same direct defence background.
Herbivores are most attracted to plants producing the greatest amount of PVs.	Number of herbivores: 35S::*TPS* > IR*lox2/3* x 35S::TPS > WT > IR*lox2/3*.
High terpene emission deters herbivores from feeding.	Amount of damage per herbivore attack: IR*lox2/3* > WT > IR*lox2/3* x 35S::*TPS* > 35S::*TPS*.
Low GLV emission deters herbivores from feeding.	IR*lox2/3* and IR*lox2/3* x 35S::*TPS* treated with MJ will suffer the least damage per herbivore attack. If GLV emission is more important than direct defence, then plants with an IR*lox2/3* background will suffer less damage even without MJ treatment.
Herbivores cause most damage on plants with the least direct defence.	IR*lox2/3* and IR*lox2/3* x 35S::*TPS* will suffer the most damage per herbivore attack.
Herbivores cause most damage on plants with the least indirect defence.	Plant phenotypes which attract the fewest *Geocoris* will accumulate the most herbivore damage: IR*lox2/3* > IR*lox2/3* x 35S::*TPS* > IR*lox2/3* x 35S::*TPS10* > 35S::*TPS10M* > WT or 35S::*TPS10*. This will be reflected in realized plant fitness, and will not be affected by MJ treatment.
Plant-predator interactions	
Geocoris predators learn to respond to HIPVs.	If a change in emission is sufficient for a learned association, then *Geocoris* will be most attracted to plants producing high levels of TAB and WT levels of GLVs, which will be the most common herbivore-inducible VOCs in the experiment: herbivore-induced 35S::*TPS10* plants. If presence/absence of an HIPV is necessary for associative learning, *Geocoris* will respond to GLVs and GLV-emitting genotypes, but may not respond to TAB; they will be most attracted to induced WT plants.
Geocoris shows an innate response to GLVS and TAB.	*Geocoris* will be most attracted to herbivore-induced 35S::*TPS10* plants.

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CHAPTER 2

A comparison of two *Nicotiana attenuata* accessions reveals large differences in *Manduca sexta* oral secretions-induced signaling events

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Published in *Plant Physiology*, doi:10.1104/pp.107.114785 (2008)

Journal research area: Plants interacting with other organisms

Focus Issue: Plant Interaction with Arthropod Herbivores

2.1 Abstract

Genetic variation within and among populations provides the raw material for evolution. Although many studies describe inter- and intraspecific variation of defensive metabolites, little is known about variation among plant populations within early signaling responses elicited either by herbivore attack, or by herbivore oral secretions (OS) introduced into wounds during feeding. We compared the OS-elicited early responses as well as the anti-herbivore defensive metabolites in two *Nicotiana attenuata* accessions and show that, compared with an accession collected from Utah (UT, USA), an Arizona (AZ, USA) accession has lower activity of herbivore-elicited salicylic acid-induced protein kinase (SIPK), an important MAP kinase involved in herbivore resistance. These differences in SIPK activity were associated with substantially different levels of OS-elicited jasmonic acid (JA), JA-isoleucine conjugate (JA-Ile), and ethylene bursts. Gene expression level polymorphism (ELP) determines phenotypic variation among populations, and we found the two

accessions to have significantly different ELPs in the genes involved in early signaling responses to herbivory. In addition, differences in the concentrations of several secondary metabolites that contribute to *N. attenuata*'s direct and indirect defenses were found between the UT and the AZ accessions. This study demonstrates significant natural variation in regulatory elements that mediate plant responses to herbivore attack, highlighting the role of ELP in producing a diversity of plant defense phenotypes.

2.2 Introduction

How organisms adapt to their ever-changing environment remains a fundamental and challenging question in biology. Within a species, heritable phenotypic variation among populations, or even individuals within populations, reflect genetic differences – a central requirement for evolutionary change. In recent years, with the completion of several genome sequences, large-scale analyses have revealed substantial intraspecific genetic variation in humans (Morley *et al.*, 2004), yeast (Brem *et al.*, 2002), *Drosophila* (Nuzhdin *et al.*, 2004), *Caenorhabditis elegans* (Denver *et al.*, 2004), and *Arabidopsis thaliana* (Koornneef *et al.*, 2004; Schmid *et al.*, 2003; Nordborg *et al.*, 2005; Schmid *et al.*, 2005; Clark *et al.*, 2007), the model organism of choice for plant genetics.

Variations in the transcription of particular genes, called expression level polymorphism (ELP), is a hallmark (and perhaps often the cause) of diversified phenotypic traits (Doerge, 2002). ELP has been shown in many genes among different *Arabidopsis* accessions by their distinct expression levels in response to exogenously applied salicylic acid and jasmonic acid (JA) (Kliebenstein *et al.*, 2006; Traw *et al.*, 2003; van Leeuwen *et al.*, 2007). In different populations, ELPs in particular genes have been shown to produce variation in pathogen resistance (Grant *et al.*, 1995; Gassmann *et al.*, 1999), flowering time (Johanson *et al.*, 2000; Lempe *et al.*, 2005; Werner *et al.*, 2005), herbivore resistance, and secondary metabolite production (Kliebenstein *et al.*, 2001; Lambrix *et al.*, 2001; Kliebenstein *et al.*, 2002). However little is known about the influence of ELPs in diversifying herbivore-induced early signaling events.

Nicotiana attenuata, a wild tobacco plant whose range in western North America stretches from southern Canada to northern Mexico, has been intensively studied with regard to how it responds to *Manduca sexta*, its major insect herbivore (Baldwin, 2001). The moment *M. sexta* begins to feed, a cascade of signaling events lead to increased defense levels (Fig. 1): *N. attenuata* plants recognize herbivory-specific elicitors derived from *M. sexta* oral secretions (OS), namely, fatty acid-amino acid conjugates (FACs), and quickly activate MAP kinase (MAPK) signaling and, subsequently, JA- and ethylene (ET)-mediated defense responses (Kahl *et al.*, 2000; Halitschke and Baldwin, 2003; Wu *et al.*, 2007a). These include 1) a highly reconfigured transcriptome that includes changes in transcript levels of defense- and growth-related genes; 2) the release of volatile organic compounds (VOCs) which function as an indirect defense; and 3) the accumulation of herbivore-deterrent or -damaging secondary metabolites, e.g. nicotine and trypsin proteinase inhibitors (TPIs) (Reymond and Farmer, 1998; Hermsmeier *et al.*, 2001; Kessler and Baldwin, 2001; Zavala *et al.*, 2004b).

TPIs are a class of important direct defense compounds in *Nicotiana* (as in many other plants): by inhibiting digestive proteinases in the *M. sexta* midgut, they slow growth and increase mortality of insect larvae (Glawe *et al.*, 2003; Zavala *et al.*, 2004b). *N. attenuata* accessions collected from

Southwestern Utah and Flagstaff Arizona (USA) have been previously found to have distinct TPI levels: the Utah accession (UT) has herbivore-inducible TPI activity, whereas the Arizona accession (AZ) has no detectable TPI activity or mRNA expression, resulting from a deletion mutation in the *TPI* coding sequence which leads to the degradation of *TPI* mRNA by nonsense-mediated mRNA decay (NMD) (Wu *et al.*, 2007b). Nevertheless, a study by Steppuhn *et al.* (in review) has shown that AZ plants have an effective system of JA-mediated direct defense against *M. sexta* , and that these plants are fitter than UT plants when planted into the UT native environment, and suffer less attack from the most voracious native herbivores, when both accessions are transformed to make them equivalent in nicotine and TPI production. This implies that AZ has reconfigured its system of direct defenses to compensate for the lack of TPI.

It is generally accepted that genetic changes in both *cis*- and *trans*-regulatory elements contribute remarkably more than changes in functional genes to phenotypic variation (Doebley and Lukens, 1998; Carroll, 2000; Gilad *et al.*, 2006). Among the regulatory elements that account for herbivore resistance in plants, those involved in early responses are particularly interesting, because many play key roles in transducing responses to various environmental stresses into changes in downstream gene expression and eventually into changes in phenotypic traits. Using the *N. attenuata* UT and AZ accessions, we examined the phenotypic variation in the early responses to herbivory; furthermore, we analyzed a number of genes that are involved in these early responses to characterize how these genes' ELP may account for the phenotypic variation.

We show that, in addition to the established difference in expression of the *TPI* gene, many other genes involved in anti-herbivore defense display ELP in these two accessions. Compared with UT, AZ shows lower levels of herbivory-induced salicylic acid-induced protein kinase (SIPK) activity, an important MAPK related to herbivore defense in plants; furthermore, *SIPK* and wound-induced protein kinase (*WIPK*), as well as two other MAPK genes in AZ, have different transcript levels after wounding or herbivory. Compared with UT, transcripts of genes involved in phytohormone biosynthesis, as well as two other transcription factors, have distinct levels of abundance in AZ; more importantly, the herbivore-induced production of JA, JA-Ile and ET are partly impaired in AZ. Concentrations of several secondary metabolites involved in herbivore resistance also differed considerably between these two accessions. This work illustrates the high level of phenotypic and genetic variation in herbivore resistance traits among natural populations, and provides insight into how variation in regulatory elements may contribute to phenotypic diversity, and thus to plants' adaptation to their environments.

2.3 Results

2.3.1 Herbivory activates lower levels of SIPK activity in AZ than in UT plants

After wounding or herbivore attack, *N. attenuata* rapidly activates SIPK and WIPK. Activation of SIPK and WIPK, upstream signal transduction components that modulate downstream phytohormonal and transcriptional changes in plants, is crucial for the plants' ability to initiate herbivore resistance (Zhang and Klessig, 2001; Liu and Zhang, 2004; Wu *et al.*, 2007a).

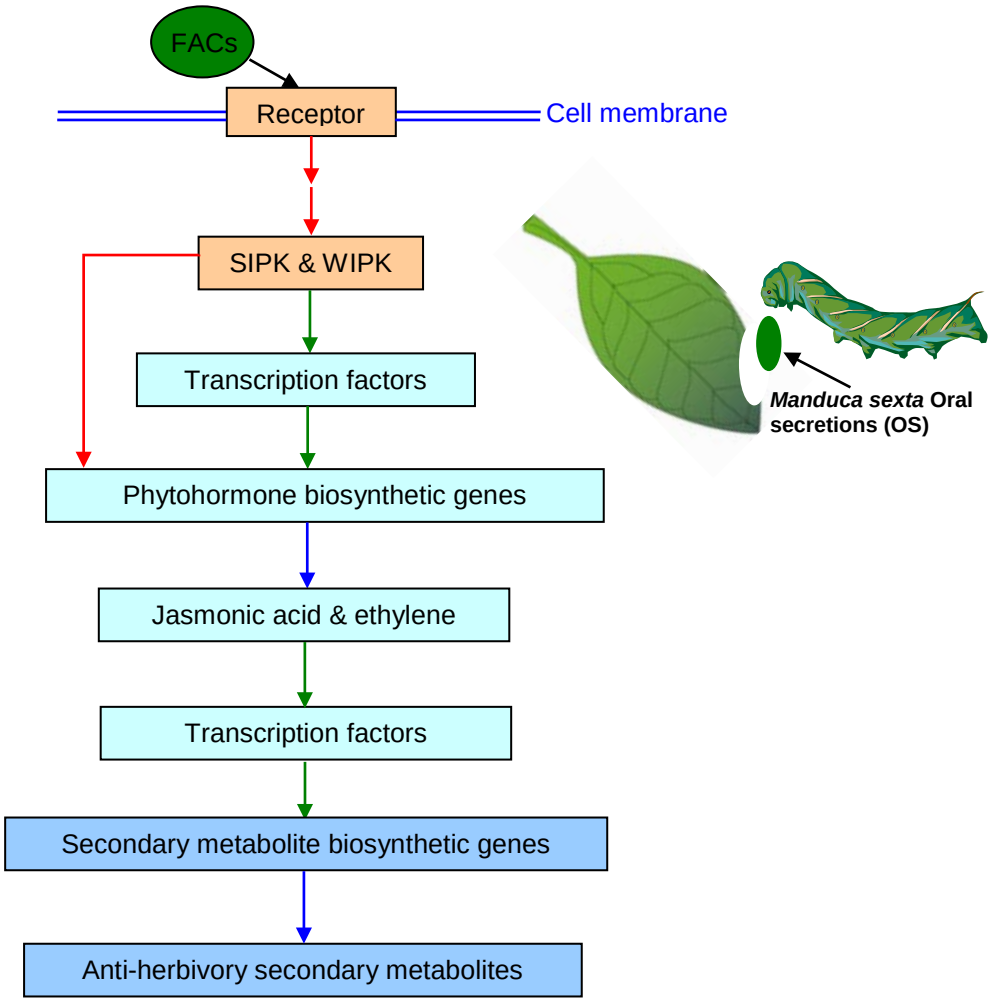

Figure 1 – Molecular events in *Nicotiana attenuata* after attack from *Manduca sexta*. Putative receptors in *N. attenuata* bind fatty acid-amino acid conjugates (FACs) present in the oral secretions (OS) of *M. sexta* larvae. SIPK and WIPK, two MAP kinases, are activated within minutes of the initiation of feeding. SIPK and WIPK regulate transcript levels (TLs) of a number of transcription factors which, in turn, mediate the TLs of genes encoding phytohormone biosynthetic enzymes, or directly regulate the stability or activity of the enzymes by phosphorylation. The increased levels of phytohormones lead to changes in the TLs of transcription factors which regulate the TLs of genes involved in secondary metabolite biosyntheses. Red arrows represent direct interactions, e.g. phosphorylation; green arrows indicate transcriptional regulation; blue arrows indicate biosynthesis pathways.

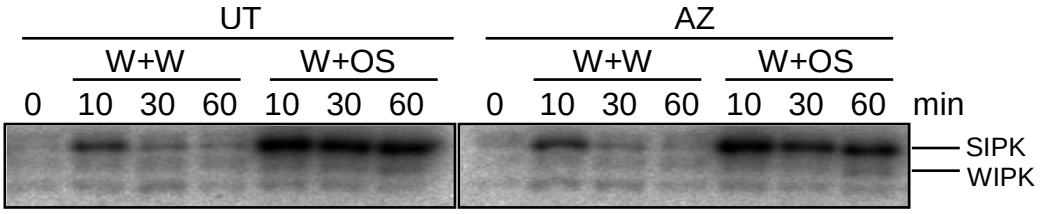

Figure 2 – *Nicotiana attenuata* plants of the Arizona (AZ) accession have lower levels of *Manduca sexta* oral secretions-elicited SIPK activity than do plants of the Utah (UT) accession. *N. attenuata* UT and AZ plants were grown under identical conditions. Rosette-stage leaves (node +1) were wounded with a pattern wheel; 20 µL of either water (W+W) or *M. sexta* oral secretions (W+OS) was applied to the wounds. Leaves from 5 replicate plants were harvested at the indicated times after elicitation and pooled. Kinase activity was analyzed by an in-gel kinase assay using myelin basic protein as the substrate.

To investigate whether MAPKs are activated differently between these two accessions, we wounded both UT and AZ leaves with a pattern wheel (leaves at node +1) and immediately applied *M. sexta* oral secretions (OS) (W+OS) or water (W+W). Samples were collected at different times and their MAPK activity was examined by an in-gel kinase assay (Fig. 2). After W+W treatment, UT and AZ plants showed the same levels of SIPK activity. W+OS induced much higher and longer-lasting SIPK activity than did W+W. Although 10 min after W+OS treatment, both accessions had the same levels of SIPK activity, W+OS-induced SIPK activity declined faster in AZ: after 30 and 60 min, SIPK in AZ showed less activity than in UT. Similar results were obtained in an independently repeated experiment (Figure S1), as well as in another experiment in which synthetic FACs, the elicitors in *M. sexta* OS (Wu *et al.*, 2007a), were used (Figure S2). Activity levels of WIPK are much lower than those of SIPK (Wu *et al.*, 2007a) and show a very weak band in the in-gel kinase assay (Fig. 2); no difference between UT and AZ was detected.

The in-gel kinase assay indicates that both AZ and UT plants can perceive FACs from *M. sexta* OS and, in response, activate SIPK and WIPK. However, a negative regulation system in AZ plants deactivates SIPK more quickly than it does in UT plants.

2.3.2 MAP kinase genes have different transcript levels in UT and AZ plants

Recent studies of MAPKs other than SIPK and WIPK have pointed to their critical roles in mediating plant stress responses (Tena *et al.*, 2001; Zhang and Klessig, 2001; Pedley and Martin, 2005). We chose several MAPK genes that have been shown to be involved in early responses within the *N. attenuata-M. sexta* interaction and measured their transcript levels in both UT and AZ using quantitative real time-PCR (qRT-PCR).

After 1.5 h, W+OS treatment induced higher levels of *SIPK* transcript than did W+W treatment in UT. Neither treatment changed the levels of *SIPK* in AZ, and after 1.5 h, AZ had lower levels of W+OS-induced *SIPK* than did UT (Fig. 3A). Both treatments greatly induced *WIPK* in UT and AZ, with higher levels after W+OS treatment. Overall, *WIPK* transcript levels in UT and AZ

did not differ from one another, except that *WIPK* levels in AZ declined faster 1.5 h after W+OS treatment (Fig. 3B).

Naf4 is a close homologue of *SIPK*, which might be derived from a recent gene duplication event (Ren *et al*., 2006; Wu *et al*., 2007a). Remarkably, qRT-PCR showed that even when UT and AZ plants were not induced (Fig. 3C), AZ had 25 fold higher levels of *Naf4* transcript than did UT. After induction, a distinct transcript profile of *Naf4* was also detected. In UT, treatments with both W+W and W+OS elevated the transcript levels of *Naf4*, with higher levels after W+OS treatment. Conversely, *Naf4* transcript levels in AZ rapidly decreased several-fold after each treatment (Fig. 3C).

MPK4 is another MAPK involved at least in pathogen- and wounding-induced responses (Petersen *et al*., 2000; Gomi *et al*., 2005). We measured *MPK4* transcript levels before and after W+W and W+OS treatments (Fig. 3D). Prior to treatment, AZ and UT showed similar levels of *MPK4* transcript. In UT, neither treatment changed the levels of *MPK4*; in contrast, W+OS significantly increased *MPK4* levels in AZ by 1.5 h (P = 0.0007, unpaired *t*-test).

The ELP of these upstream kinases may result in different levels of their activity in UT and AZ, which may produce different downstream responses.

2.3.3 AZ has lower herbivore-induced transcript levels of *WRKY* transcription factors than UT

Transcription factors play critical roles in regulating gene transcript levels. WRKYs, which are plant-specific transcription factors, regulate various aspects of plant development and stress responses (Eulgem *et al*., 2000; Ulker and Somssich, 2004). It is well known that transcription factors are the main targets of MAPKs (Hazzalin and Mahadevan, 2002; Yang *et al*., 2003). To investigate whether there is any ELP of *WRKY* genes between OS-elicited UT and AZ plants, which might result from the ELP of MAPKs, we measured the transcript abundance of three *WRKY* genes: *WRKY3*, *WRKY6*, and *SubD48*. WRKY6 has been shown to be an important signaling transduction component contributing to *N. attenuata*'s responses to herbivory (Qu *et al*., unpublished data) and is located downstream of SIPK and WIPK (Wu *et al*., 2007a). *SubD48* is also implicated in herbivore resistance, as W+OS treatment induced higher levels of *SubD48* transcript than did W+W (Wu *et al*., 2007a).

When not treated, both accessions had similar levels of *WRKY6* transcript (P = 0.62, unpaired *t*-test). Transcript abundance of *WRKY6* reached maximum levels after only 0.5 h, increasing 450 and 3250 fold after W+W and W+OS treatment, respectively, in UT (Fig. 4A). AZ had remarkably lower *WRKY6* transcript levels after both treatments, although both accessions showed similar induction patterns in that W+OS induced much higher levels of *WRKY6* than did W+W. Although *WRKY3* did not show greater induction by W+OS, W+W-induced *WRKY3* transcript levels decreased faster than those induced by W+OS after reaching maximum levels in both accessions at 0.5 h. No statistical difference in *WRKY3* transcript levels was detected between UT and AZ before or after either induction (Fig. 4B). Transcript levels of *SubD48* were the same in UT and AZ when plants were not induced, and W+OS induced higher levels of *SubD48* transcript than did W+W in both accessions (Fig. 4C). Nevertheless, *SubD48* levels were higher in W+OS-treated AZ than in UT plants.

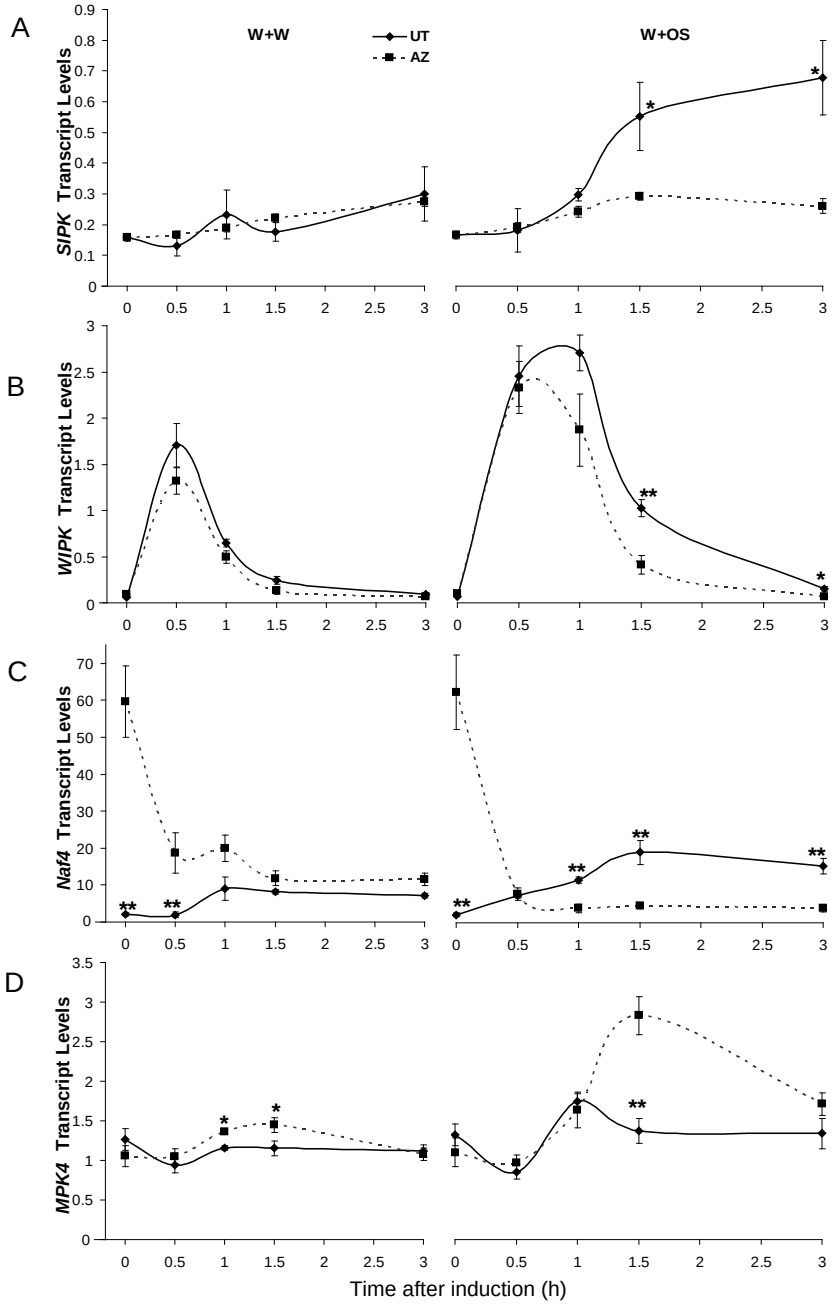

Figure 3 – MAP kinase genes in *Nicotiana attenuata* Utah (UT) and Arizona (AZ) accessions have different levels of transcript accumulation. *N. attenuata* UT and AZ plants were grown under identical conditions. Leaves at node +1 were wounded with a pattern wheel; 20 µL of either water (W+W) or *Manduca sexta* oral secretions (W+OS) was applied to the wounds. Individual leaves from 5 replicate plants were harvested at the indicated times after elicitation. The mean (± SE) expression levels of *SIPK* (A), *WIPK* (B), *Naf4* (C), and *MPK4* (D) were measured with qRT-PCR. Asterisks represent significantly different transcript levels between UT and the AZ accessions at the indicated times (unpaired *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N = 5).

These data indicate that UT and AZ plants have different expression levels of transcription factors that are involved in herbivore resistance. The ELP of these *trans*-regulatory elements may also contribute to further variation in downstream gene transcript levels.

2.4 AZ has lower levels of herbivore-induced JA, JA-Ile and ET than does UT

JA, JA-Ile, and ET play critical roles in mediating plants' defense against herbivores (Reymond and Farmer, 1998; Halitschke and Baldwin, 2003; Staswick and Tiryaki, 2004; Kang *et al*., 2006; von Dahl *et al*., 2007). The evidence is increasing that SIPK and WIPK play central roles in mediating plants' JA and ET biosynthesis (Seo *et al*., 1995; Liu and Zhang, 2004; Wu *et al*., 2007a). To investigate whether the different levels of SIPK activity and ELP in many early signaling genes led to variation of phytohormone production in these two *N. attenuata* accessions, we measured both W+W- and W+OS-induced JA, JA-Ile, and ET levels.

Without treatment, both accessions had similar low levels of JA (6 ng/g fresh weight (FW), P = 0.28, unpaired *t*-test). In UT, W+OS treatment induced higher levels of JA than did W+W treatment (Fig. 5A, top), indicating that UT plants recognize herbivory and deploy herbivore-specific defenses. This JA burst was much smaller in AZ: W+OS induced only 40% of the JA levels in W+OS-treated UT (Fig. 5A, top). To examine whether the ELP of genes involved in JA biosynthesis contributed to this phenotype, we measured the transcript levels of *lipoxygenase 3* (*LOX3*), which encodes an important enzyme in JA biosynthesis (Fig. 5A, bottom) (Halitschke and Baldwin, 2003; Wasternack, 2007). When not induced, UT and AZ plants had the same levels of *LOX3* transcript (P = 0.55, unpaired *t* -test). Both W+W and W+OS greatly induced *LOX3*, which declined faster in AZ: at 3 h, W+W- and W+OS-treated AZ had as little as 7% and 0.2%, respectively, of the *LOX3* levels found in UT.

Similar results were obtained for measurements of JA-Ile levels. W+OS treatment induced higher levels of JA-Ile than did W+W treatment in both accessions (Fig. 5B, top). 1 h after either treatment, UT showed about 70% higher levels of JA-Ile than did AZ. *JAR4* and *JAR6* have been identified as the enzymes that conjugate JA and Ile to form JA-Ile (Wang et al., 2007); we measured the transcript levels of both genes (Fig. 5B, middle and bottom). AZ had basal transcript levels of both *JAR4* and *JAR6* that were almost 10-fold lower than those in UT (P = 0.038 and 0.005, respectively, unpaired *t*-test). However, 1 to1.5 h after induction, *JAR4* and *JAR6* transcript levels were similar in both accessions. The difference between JA-Ile levels in UT and AZ may have resulted from the different levels of both JA a substrate for the conjugation reaction and JAR activity, since the basal levels of JAR protein might also be considerably lower in AZ.

W+W treatment did not induce a detectable amount of ET in UT or AZ (data not shown). After W+OS treatment, 25 nL ET was produced for every gram of UT leaf tissue (Fig. 5C, top); AZ produced only one-third as much. We measured the transcript profiles of two genes involved in ET biosynthesis, *ACC oxidase 1* (*ACO1*) and *ACC synthase 3a* (*ACS3a*) in both accessions (Wang *et al*., 2002; von Dahl *et al*., 2007). Unexpectedly, levels of *ACO1* transcript in untreated AZ plants were about 6-fold higher than in untreated UT plants (Fig. 5C, middle). In UT, W+OS-induced *ACO1* transcript levels were no greater than those induced by W+W. In contrast, W+OS induced much higher levels of *ACO1* than did W+W in AZ. Furthermore, AZ had much higher transcript levels of

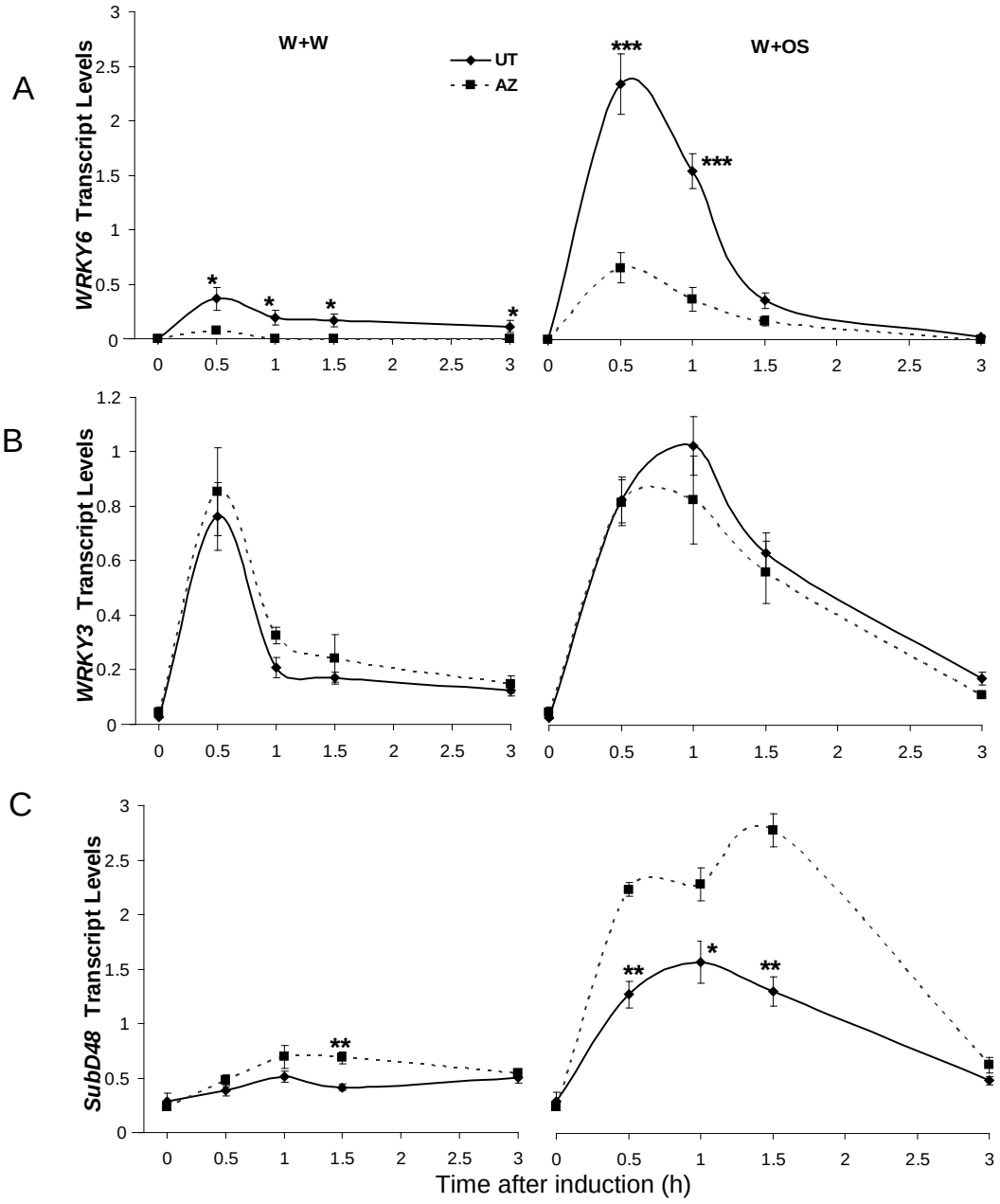

Figure 4 – Transcript levels of different *WRKY* transcription factors in *Nicotiana attenuata* Utah (UT) and Arizona (AZ) accessions. *N. attenuata* UT and AZ plants were grown under identical conditions. Rosette-stage leaves (+1 position) were wounded with a pattern wheel; 20 µL of either water (W+W) or *Manduca sexta* oral secretions (W+OS) was applied to the wounds. Individual leaves from 5 replicate accessions were harvested at the indicated times after elicitation. The mean (± SE) transcript levels of *WRKY6* (A), *WRKY3* (B), and *SubD48* (C) were measured with qRT-PCR. Asterisks represent significantly different transcript levels between UT and the AZ accessions at the indicated times (unpaired *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, N = 5).

ACO1 than did UT under all conditions (Fig. 5C, middle). When untreated, UT and AZ had very similar *ACS3a* transcript levels (P = 0.48, unpaired *t*-test), and W+W treatment induced similar *ACS3a* levels in both accessions (P $>$ 0.064, unpaired *t*-test); however, after W+OS treatment, *ACS3a* levels were several-fold higher in AZ than in UT (Fig. 5C, bottom).

These data show that UT and AZ have distinct herbivore-induced JA, JA-Ile, and ET responses. The ELP of phytohormone biosynthetic genes may contribute to the phenotypic variation between these accessions, but the differential regulation of ET production between the two accessions is clearly more complex.

2.5 UT and AZ produce different levels of anti-herbivory secondary metabolites

Herbivore-induced kinase activation, and changes in phytohormones and the transcriptome, alter the accumulation of secondary metabolites to raise plant defenses against herbivore attackers. In *N. attenuata*, these herbivore-induced defenses include nicotine and TPI (Steppuhn *et al.*, 2004; Zavala *et al.*, 2004b). Because AZ plants have lost their TPI defense mechanism (Wu *et al.*, 2007b), we analyzed the levels of nicotine as well as two phenolic compounds, chlorogenic acid and rutin, which are putative anti-herbivore defenses (Isman and Duffey, 1982; Bi *et al.*, 1997), and may constitute part of *N. attenuata*'s direct defense.

Four days after plants were treated with W+W or W+OS, when levels of nicotine were at their highest (Baldwin *et al.*, 1998), samples were harvested and analyzed by HPLC (Fig. 6A). Without treatment, AZ had 50% more nicotine than did UT. Nicotine concentrations increased in both UT and AZ after treatment; nevertheless, AZ still contained 35% and 20% more nicotine compared to UT plants identically treated with W+OS and W+W, respectively.

Compared with UT, more chlorogenic acid and rutin were detected in AZ (Fig. 6B, C). Under normal conditions, AZ contained 1.3-fold more chlorogenic acid than did UT. After W+W and W+OS treatments, AZ still contained higher levels of chlorogenic acid. Rutin levels were not changed after either W+W or W+OS treatment; similarly to chlorogenic acid, concentrations of rutin in AZ were about 50% higher than in UT.

In addition to direct defenses, *N. attenuata* deploys indirect defenses to attract predators of herbivores. One of the most important indirect defense compounds is *cis*-α-bergamotene (CAB), an herbivory-inducible VOC (Kessler and Baldwin, 2001). We found that W+OS treatment enhanced CAB emission in UT 7 fold; however, neither W+W nor W+OS induced higher levels of CAB emission in AZ (Fig. 6D). Since CAB biosynthesis is regulated by JA (Halitschke *et al.*, 2000), we examined whether the decreased JA levels in AZ after W+OS treatment were associated with diminished CAB levels. We applied 150 µg of methyl jasmonate (MeJA) dissolved in lanolin paste or pure lanolin paste (lanolin) as a control to both accessions and measured CAB emission. In both accessions, CAB emission was highly induced but the MeJA treatment didn't restore the deficiency of CAB production in AZ; levels were still significantly lower than those in UT (Fig. 6E). This suggests that during evolution, AZ may have lost or reconfigured *cis*- or *trans*-elements involved in the regulation of CAB biosynthesis.

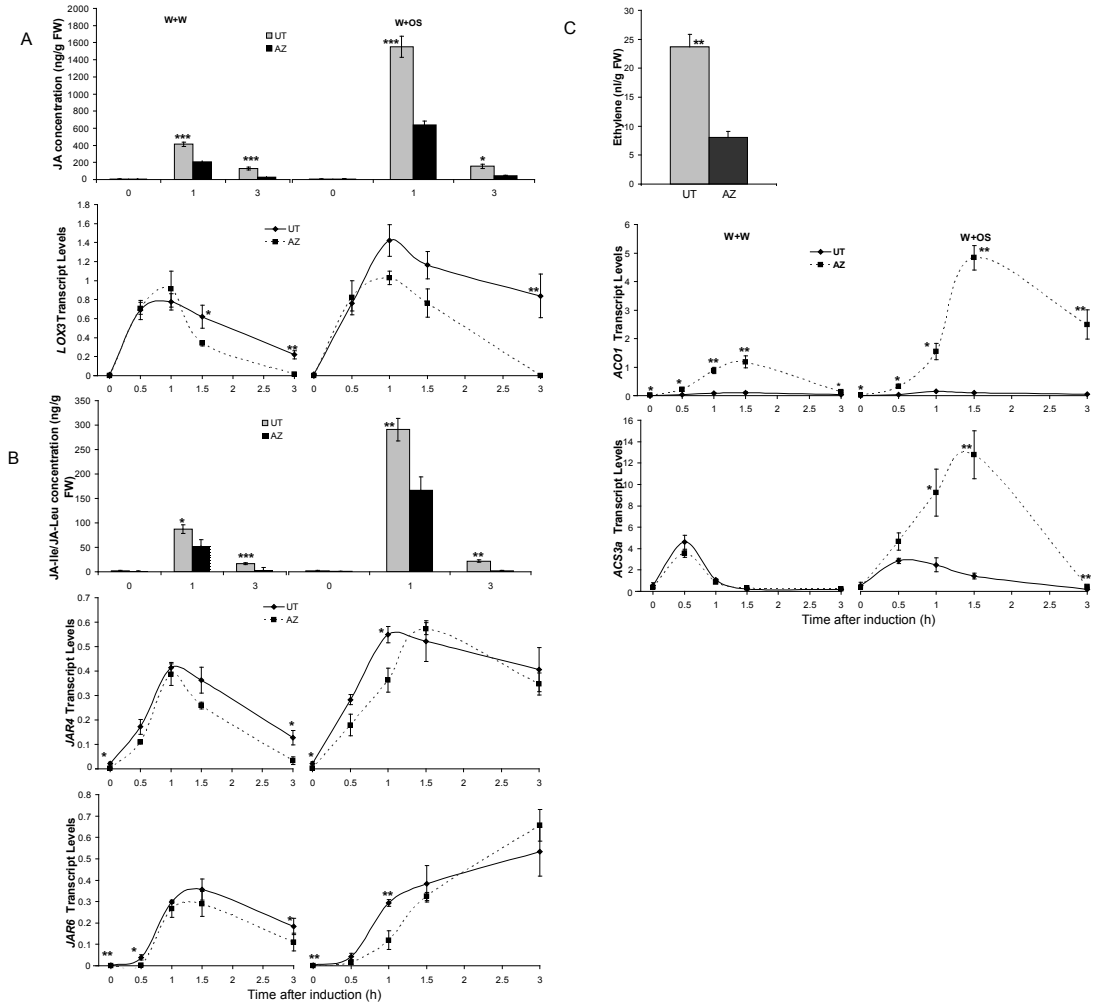

Figure 5 – *Nicotiana attenuata* Utah (UT) and Arizona (AZ) accessions have different levels of wound- and OS-elicited phytohormones. *N. attenuata* UT and AZ plants were grown under identical conditions. Rosette-stage leaves (+1 position) were wounded with a pattern wheel; 20 µL of either water (W+W) or *Manduca sexta* oral secretions (W+OS) was applied to the wounds. A, Mean (± SE) JA concentrations in leaves harvested at indicated times were measured with HPLC-MS/MS (top panel); the mean (± SE) transcript levels of *LOX3* were measured with qRT-PCR (bottom panel). B, Mean (± SE) JA-Ile/JA-Leu concentrations in leaves harvested at indicated times were measured with HPLC-MS/MS (top panel); the mean (± SE) transcript levels of *JAR4*, *JAR6* were measured with qRT-PCR (middle and bottom panel, respectively). C, Mean (± SE) ethylene accumulated (top panel) from treated leaves enclosed in 250-mL flasks and measured after 5 h with a photoacoustic laser spectrometer (N = 3). The mean (± SE) transcript levels of *ACO1* and *ACS3a* in leaves harvested at indicated times were measured with qRT-PCR (middle and bottom panel, respectively). Asterisks represent significantly different transcript levels between UT and the AZ accessions at the indicated times (unpaired *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, N = 5).

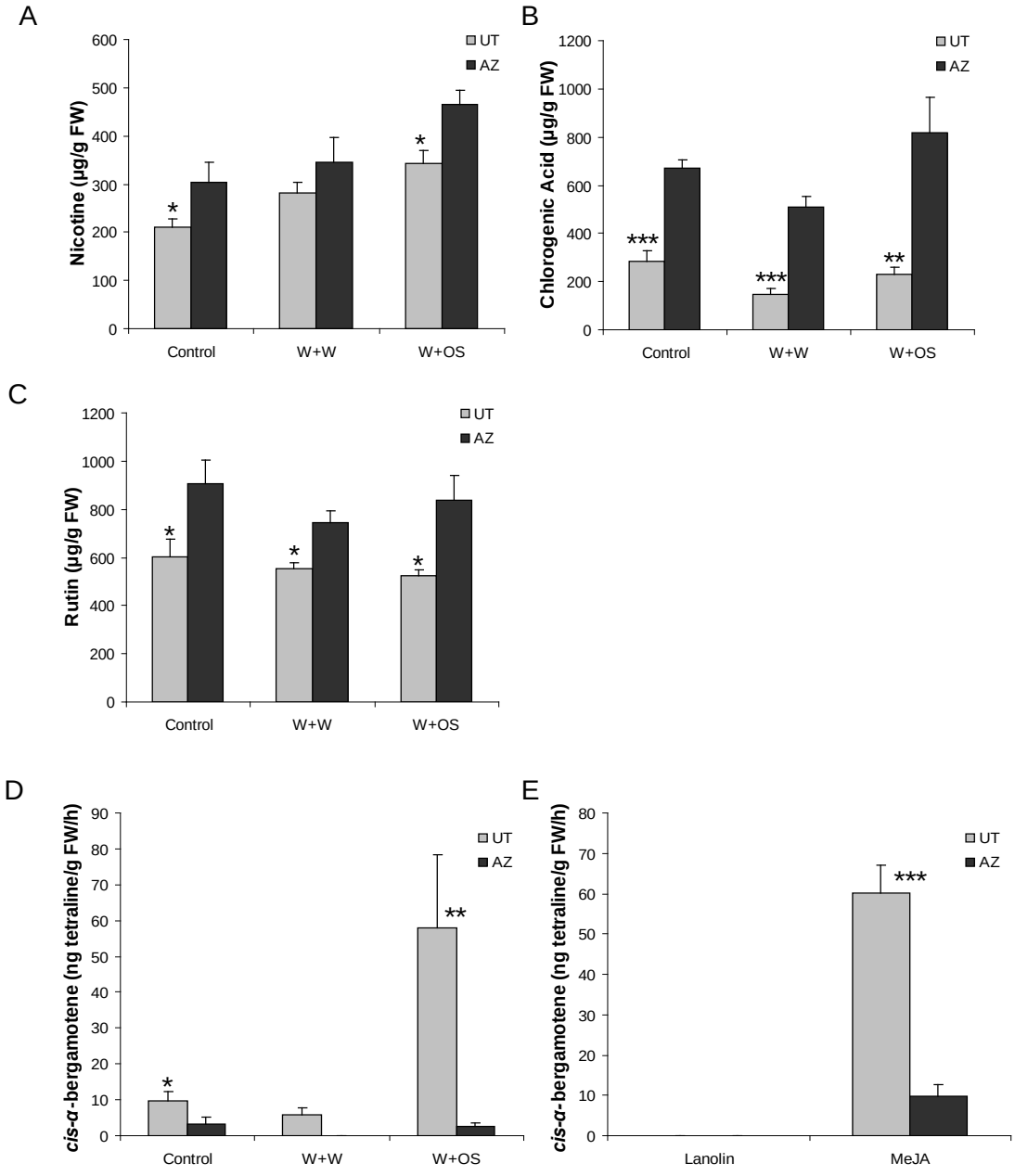

Figure 6 – *Nicotiana attenuata* Utah (UT) and Arizona (AZ) accessions produce different amounts of secondary metabolites that function as direct and indirect defenses. *N. attenuata* UT and AZ plants were grown under identical conditions. A to C, Rosette-stage leaves (+1 position) were wounded with a pattern wheel; 20 µL of either water (W+W) or *Manduca sexta* oral secretions (W+OS) was applied to the wounds. Non-treated plants served as control. Four days after treatments, plant tissue samples were harvested; nicotine (A), chlorogenic acid (B), and , rutin (C) concentrations were analyzed using HPLC. The emission of *cis*-α-bergamotene was measured in from treated leaves (+2 position) at 24 to 32 h post-treatment with either W+W or W+OS, with no treatment as control (D) or 150 µg MeJA in lanolin paste, with lanolin as control (E). Absolute emission is not comparable between D and E as these were two separate experiments. (unpaired *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, $N = 5$)

2.6 Discussion

In this study, we examined the early herbivore-induced responses in two *N. attenuata* accessions collected from Utah and Arizona, USA. We measured 1) the activity of two MAPKs, SIPK and WIPK; 2) the levels of the herbivore-induced phytohormones JA, JA-Ile, and ET; 3) differential transcript accumulation of several genes encoding MAPKs, transcription factors, phytohormone biosynthesis enzymes, which we took to be indicative of ELPs in these genes, and furthermore, 4) the resulting variation in concentrations of direct and indirect defense-related secondary metabolites.

2.6.1 Natural variation in herbivore-induced signaling events

MAPK signaling has been shown to play a critical role in mediating plants' resistance to herbivores (Kandoth *et al.*, 2007; Wu *et al.*, 2007a). FACs from *M. sexta* OS are introduced into plant cells through wounds generated by herbivore feeding. These FACs bind to putative receptors in *N. attenuata*, enabling plants to perceive herbivory. This perception results in the activation of MAPKs, most importantly SIPK and WIPK (Wu *et al.*, 2007a). We examined kinase activity and kinase gene transcript levels in both UT and AZ. Activity of SIPK and WIPK reached the same maximum levels in both accessions, indicating that both are equally able to perceive herbivory and initiate herbivory-induced signal cascades. Nevertheless, the speed with which SIPK activity levels decrease in AZ following simulated herbivory (W+OS) suggests that the negative regulation of SIPK is stronger in AZ. A recent study in *Arabidopsis* has shown that a phosphatase, AP2C1, might be responsible for this negative regulation (Schweighofer *et al.*, 2007).

Both SIPK and WIPK modulate herbivory-induced JA biosynthesis (Wu *et al.*, 2007a). AZ plants accumulate lower levels of JA after simulated herbivory, consistent with rapidly decreasing levels of herbivore-induced SIPK activity. Compared with JA biosynthesis, ET biosynthesis is simpler: only 2 enzymes, ACO and ACS, are responsible for converting *S*-adenosylmethionine to ET (Wang *et al.*, 2002). SIPK directly phosphorylates certain ACSs (but not all), which dramatically increases their stability (Liu and Zhang, 2004). Longer-lasting herbivore-induced SIPK activity levels may partly account for the higher amount of ET produced in UT plants than in AZ plants. However, the large differences of herbivore-induced JA and ET levels between UT and AZ plants are unlikely solely resulted from the difference of SIPK activity. Variation of many genes involved in JA and ET biosynthesis probably also contributed to the difference of these 2 phytohormones in UT and AZ plants.

In addition to *SIPK* and *WIPK*, we found ELP in two other MAPK genes: *Naf4* and *MPK4*. *Naf4* shows high sequence homology to *SIPK* and thus may have a similar function (Ren *et al.*, 2006). *MPK4* is known to be involved in pathogen resistance and wound-induced responses, but as yet has no known function in herbivore resistance (Petersen *et al.*, 2000; Gomi *et al.*, 2005). Since kinases are upstream regulatory elements that influence the transcription of a wide rage of genes, variation in their constitutive and/or induced levels is likely to produce large sets of ELPs.

Most MAPK targets are transcription factors (Hazzalin and Mahadevan, 2002). Two WRKY transcription factors, *WRKY6* and *SubD48*, displayed ELP between UT and AZ; however, there was

no ELP in a third WRKY (*WRKY3*). Transcript levels of *WRKY6* have been shown to be positively regulated by both SIPK and WIPK (Wu *et al*., 2007a). Thus, we can assume that the different herbivory-induced SIPK activity in UT and AZ may have resulted in the observed ELP of *WRKY6*. MAPKs, JA, and ET are well known to regulate expression of a wide array of genes. Therefore, many other transcription factors downstream of herbivory-induced MAPKs, JA and ET, are likely to also show ELP between UT and AZ.

The large and sometimes unpredictable variation between these two *N. attenuata* accessions in their herbivore-induced early responses is indicative not only the complexity of the signaling networks, but also of the cross-talk and feedback regulation that characterize the relationships among their components, which include receptors, kinases, phytohormones, and transcription factors, with each group of molecules potentially participating in multiple layers of regulation. Studying the molecular mechanisms that underlie the phenotypic changes in these early signaling events will eventually provide us with a detailed understanding of how plants fine-tune their signaling systems, as well as how evolutionary forces have shaped genetic variation among natural populations.

2.6.2 Natural variation of secondary metabolites

Plants' reconfiguration of their transcriptomes and proteomes following herbivore attack results in the deployment of the last layer of the defense system, which comprises various anti-herbivore secondary metabolites (Baldwin, 2001). We found that AZ produces greater amounts of both nicotine and phenolic compounds (chlorogenic acid and rutin) than does UT. Nicotine is a potent chemical defense that has a strong negative effect on *M. sexta* and on many other herbivores and which has been previously shown to be a critical component of JA-mediated direct defense in AZ as well as in UT (Steppuhn *et al*., 2004). Nicotine biosynthesis is both upregulated by JA and downregulated by OS-induced ET (Baldwin *et al*., 1994; von Dahl *et al*., 2007; Voelckel *et al*., 2001). Thus, higher nicotine levels in AZ could indicate that decreased negative regulation by ET plays a greater role than decreased positive regulation by JA in nicotine accumulation after herbivory. Alternately, it could reflect a greater availability of nitrogen for nicotine biosynthesis due to the lack of nitrogen-intensive TPI production in AZ. However, given that AZ produces constitutively higher amounts of nicotine, whereas ET and JA can only influence induced nicotine production; and given that such high constitutive nicotine production was not seen in a line of the UT accession which was silenced for TPI (Zavala et al., 2004a), we assume that changes in certain *trans*- or *cis*-element(s) in the regulation of nicotine biosynthesis have enabled AZ to produce more nicotine than UT.

The low level of CAB production in AZ illustrates the complex changes in its regulatory system: that AZ is able to produce CAB, but releases less than 10% as much as UT after OS-elicitation indicates that the biosynthesis machinery is intact, but the regulatory system is constrained. That the decrease in production cannot be recovered with MeJA application shows that the bottleneck in regulation is downstream of the JA. Little is known about how CAB and other VOCs in *N. attenuata*, which may also act as indirect defense compounds, are synthesized and regulated. The synthesis of phenolic compounds in *N. attenuata* and their possible influence on *M. sexta* performance also remains to be characterized; some studies have revealed potential anti-herbivore functions of phenolic compounds (Isman and Duffey, 1982; Bi *et al*., 1997). Further biochemical

and genetic studies are needed to elucidate the ELPs in biosynthetic genes which may underlie the differential regulation of these compounds.

2.6.3 Ecological and evolutionary significance

Understanding the mechanisms responsible for the phenotypic variation in herbivore defense systems between these two *N. attenuata* accessions provide insights into their evolution. The *TPI* gene has been identified as a null allele in AZ (Zavala *et al.*, 2004b; Wu *et al.*, 2007b). In this study, we show that despite (or perhaps due to?) the loss of its TPI defense mechanism, AZ produces greater amounts of nicotine and phenolics. The higher concentrations of these compounds in AZ may be a result of natural selection following the event of *TPI* mutation, enabling AZ to compensate for the loss of TPI. How the *TPI* null allele was fixed in AZ is unclear, since this mutation could have considerably lowered the ability of AZ to survive under herbivory pressure: using *TPI*-silenced transgenic plants, Zavala *et al.* (2004a) demonstrated that TPI production is costly under normal conditions, because plants having lower TPI levels grow faster and have higher fitness due to the re-allocation of nitrogen; however, under selection pressure from herbivory, TPI-containing plants realize greater fitness due to the effectiveness of this defense (Zavala *et al.*, 2004a, b). One hypothesis is that after the *TPI* mutation event in AZ, there was very low selection pressure from herbivores; therefore, this null *TPI* allele was quickly fixed, since it provided plants with higher fitness. An alternate scenario which could lead to the fixation of the null *TPI* allele would be genetic drift and small original population size. In either case, over time, (re-imposed) selection pressure from herbivory could have led to higher concentrations of other anti-herbivore compounds, i.e., nicotine and phenolics. In nature, indirect defense compounds (e.g. CAB) are also highly effective in protecting plants from herbivory (Kessler and Baldwin, 2001). It is unclear why AZ has lost most of its CAB production. Whether in its natural habitat, AZ depends more on direct defense, instead of indirect defense, against herbivory, or whether the predators in this habitat respond to other volatile signals remains to be studied.

Plants recollected in 2005 from the same region where the original AZ accession was collected in 1996 (near Flagstaff, Arizona) had the same null mutation in *TPI* (Baldwin and Schuman, unpublished data). This suggests that plants of the AZ accession have adapted to persist in their native environment via as-yet unidentified defense or tolerance mechanisms. Coevolution has long been considered to have shaped the diversity of both plants and herbivores (Ehrlich and Raven, 1964; Mauricio, 2001; Zangerl and Berenbaum, 2005). Further ecological studies could provide the necessary data to connect diversity in herbivore-induced plant responses to differences in native herbivore population structures and their dynamics, and to determine whether different selection forces imposed by diverse insect populations could have caused polymorphisms in defense compound production between native plant populations.

Recent studies of *Arabidopsis* populations have revealed considerable variation on both the genomic and the transcriptomic level (Kliebenstein *et al.*, 2006; Clark*et al.*, 2007; Kim *et al.*, 2007). It is very unlikely that only one or a few genetic changes differentiate AZ and UT. Between these two accessions, we predict that thousands of genes have variations on sequence and/or expression levels. These variations definitely complicate the interpretation of the changes of many phenotypic traits, since most of them are polygenic, as illustrated by the great difference of herbivore-induced JA and ET production in UT and AZ accessions. Nevertheless, ever-increasing theoretical and

practical advances have provided us with the ability to unravel the complex genetic basis that is responsible for the variations we have detected (Kliebenstein *et al.*, 2001; Kliebenstein *et al.*, 2002; Wentzell *et al.*, 2007). Using biochemical, genetic, and population genetic tools, we expect to see variations in many loci that contribute to the phenotypic variation between UT and AZ; it won't be surprising if these loci turn out to be mostly regulatory elements: transcription factor binding sites in promoters (*cis*-elements), genes coding for transcription factors (*trans*-elements), or even genes involved in modifying transcription factors, such as their binding activity, stability, and localization.

2.7 Materials and Methods

2.7.1 Plant material and experimental conditions

Seed collections of *Nicotiana attenuata* Utah (UT) and Arizona (AZ) accessions are described in Glawe *et al.* (2003). Nearly homozygous UT and the AZ accessions were obtained by inbreeding for 17 and 7 generations in the glasshouse, respectively. For this, in each generation, 1 randomly selected plant was used for seeds collection. No artifical selection was conducted.

Seeds were germinated on agar plates containing Gamborg B5 media (Duchefa, `http://www.duchefa.com`). Plants were grown in the glasshouse in 1 L individual pots at 26 to 28°C under 16 h of light. All treatments were performed with plants in the rosette stage of growth. The second fully elongated (+1 position) leaves were used, unless otherwise noted. For W+W treatment, 20 µL of deionized water were immediately applied to wounds generated by a pattern wheel; similarly, for W+OS treatment 20 µL of *Manduca sexta* oral secretions (OS) (diluted 1:5 in water, v/v) were applied to wounds. For methyl jasmonate (MeJA) treatment, 20 µL methyl jasmonate dissolved in lanolin (7.5 µg/µL) were rubbed on the base of the leaf with a small spatula; as a control, pure lanolin was used (lanolin). Samples from untreated plants were used as controls. Unless otherwise noted, 5 replicate plants were used. After specific time periods, leaves were excised, flash frozen in liquid nitrogen, and stored at -80°C until use.

2.7.2 Quantitative real time-PCR (qRT-PCR) analyses

Total RNA was extracted from 100 mg leaf tissue using the Trizol reagent (Invitrogen, `http://www.invitrogen.com`). The qRT-PCR procedure is described in detail in Wu *et al.* (2007a). All RNA samples were reverse transcribed using oligo(dT) and the Superscript II enzyme (Invitrogen). qRT-PCR was performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems, `http://www.appliedbiosystems.com`), following conditions recommended by the manufacturer. In all analyses, a *N. attenuata actin* gene was used to normalize the concentrations of cDNA samples. The primers and probes used for the Taqman-based analysis, as well as the primers used for SYBR Green-based analyses are listed in Tables S1 & S2, respectively.

2.7.3 Phytohormone analyses

Jasmonic acid (JA) and JA-isoleucine conjugate (JA-Ile) were analyzed using a HPLC-MS/MS method. Leaf tissue (200 mg) was ground in liquid nitrogen and extracted with ethyl acetate containing 200 ng of $^{13}C_2$-JA and *para*-coumaric acid as internal standard for JA and JA-Ile/JA-leucine (JA-Leu), respectively. After centrifuging, supernatants were dried in a concentrator (Eppendorf, `http://www.eppendorf.com`). The pellet from each sample was further extracted with 500 µL of 70% methanol (v/v). The supernatants were directly injected onto a 1200L HPLC-MS/MS (Varian, `http://www.varianinc.com`), and the levels of JA and JA-Ile/JA-Leu were quantified by comparing their peak areas with those from internal standards (JA-Ile and JA-Leu couldn't be quantified separately, due to their similar chemical properties and identical molecular weights).

Ethylene (ET) was measured on a photoacoustic spectrometer (INVIVO GmbH, `http://www.invivo-gmbh.de`). Three freshly detached leaves were treated either with W+W or W+OS, immediately sealed in a three-neck 250-mL flask with a round bottom, and kept in the glasshouse for 5 h. The headspace was flushed into a photoacoustic laser spectrometer with hydrocarbon-free clean air, and the ET concentration was quantified by comparing ET peak areas with peak areas generated by a standard ET gas.

2.7.4 Analysis of nicotine, chlorogenic acid, and rutin

Nicotine, chlorogenic acid, and rutin were analyzed using an HPLC-DAD method (Keinanen *et al.*, 2001). About 100 mg leaf tissue was extracted with 1 mL extraction solution (40% methanol, 0.5% acetic acid). After centrifuging, the supernatants were collected and injected into an Agilent 1100 HPLC-DAD (Agilent, `http://www.agilent.com`) installed with an Inertsil ODS-3 column (Phenomenex, `http://www.phenomenex.com`). Nicotine, rutin, and chlorogenic acid in different concentrations were used as external standards.

2.7.5 Measurement of *cis*-α-bergamotene (CAB)

Volatiles were collected from plants following treatment to the +2 leaf with lanolin, MeJA in lanolin, W+W, or W+OS as described, or no treatment. Two separate sets of plants were used: one for lanolin or MeJA treatment, and one for control, W+W, or W+OS treatment. Plants were placed at a maximum distance from each other on a table in the glasshouse. At 24 h after treatment, treated leaves were enclosed in two 50 mL food-quality plastic containers secured with miniature claw-style hair clips. Ambient air flowed into the cage primarily through a trimmed P1000 pipette tip inserted into the bottom container, and was pulled out through a self-packed glass tube containing glass wool and 20 mg of SuperQ (Alltech, `http://www.discoverysciences.com`), secured in a second trimmed P1000 pipette tip inserted into the top container. Airflow was powered by a manifold vacuum pump as described in Halitschke *et al.* (2000). Background volatiles present in ambient air were also collected using empty trapping containers; CAB was not present in quantifiable levels in background controls. Trapping was stopped after 8 h and traps were either immediately eluted by spiking each trap with 400 ng tetraline as an internal standard (IS) and pushing through 250 µL dichloromethane into a GC vial, or stored at -20°C until elution. Flow rates through each trap were measured and included as correlates in statistical analyses.

Eluents were separated on a DB-5 column (Agilent, http://www.agilent.com) in a Varian CP-3800 GC coupled with a Varian Saturn 4000 ion trap MS in EI mode (Varian, http://www.varianinc.com). Relative emission of CAB was expressed in ng tetraline/g FW/h. Data were log-transformed prior to analysis to meet requirements of normality and homogeneity of variance; 1 was added to all values prior to transformation to accommodate 0 values.

2.7.6 Statistical analysis

Statistics were done using the StatView software (SAS Institute, http://www.sas.com).

2.7.7 In-gel kinase assay

An in-gel kinase assay was performed following Zhang & Klessig (1997). Proteins were extracted from 100 mg tissue with 250 µL of extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 10 mM DTT, and complete proteinase inhibitor cocktail tablets [Roche, http://www.roche-applied-science.com]). Protein samples containing 10 µg of total protein were separated on a 10% SDS-polyacrylamide gel embedded with 0.2 mg/mL myelin basic protein (Sigma, http://www.sigmaaldrich.com). After electrophoresis, the gel was washed 3 times for 30 min each in a washing buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg/mL BSA, and 0.1% Triton X-100 [v/v]) at room temperature. Then, kinases in the gel were renatured in 25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3 VO_4, and 5 mM NaF at 4°C overnight, with three changes of buffer. At room temperature, the gel was then incubated in a reaction solution (25mM Tris, pH 7.5, 2mM EGTA, 12mM $MgCl_2$, 1mMDTT, and 0.1mM Na_3 VO_4) with 0.2 mM ATP plus 50 µCi of γ-^{32}P-ATP (3000 Ci/mmol) for 1 h. The reaction was stopped by a fixation solution (5% trichloroacetic acid and 1% sodium pyrophosphate [w/v]). After 6 h of washing with five changes, the gel was dried on a gel dryer (Bio-Rad, http://www.bio-rad.com), and image was obtained on an FLA-3000 phosphor imager system (FujiFilm Life Science, http://www.fujifilm.com).

Acknowledgments

We thank Eva Rothe & Dr. Matthias Schöttner for their help with the phytohormone and HPLC-DAD analysis, Stefan Meldau for the in-gel kinase assay, Emily Wheeler for editorial assistance and the Max Planck Society for funding.

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Supplemental

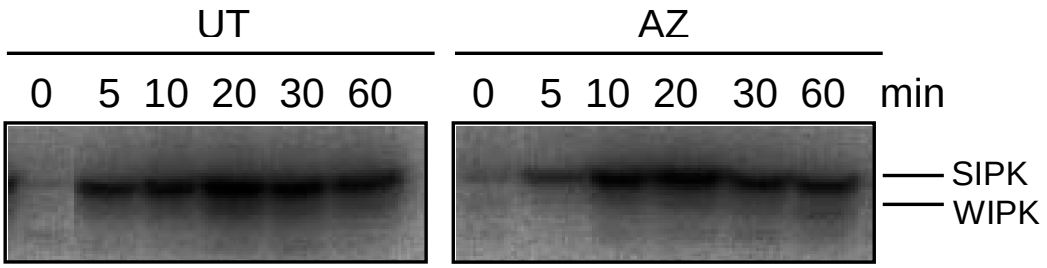

Figure S1 – *Nicotiana attenuata* plants of the Arizona (AZ) accession have lower levels of *Manduca sexta* oral secretions-elicited SIPK activity than do plants of the Utah (UT) accession. *N. attenuata* UT and AZ plants were grown under identical conditions. Rosette-stage leaves (+1 position) were wounded with a pattern wheel; 20 µL of *Manduca sexta* OS were applied to the wounds. Leaves from 4 replicate plants were harvested at the indicated times after elicitation and pooled. Kinase activity was analyzed by an in-gel kinase assay using myelin basic protein as the substrate.

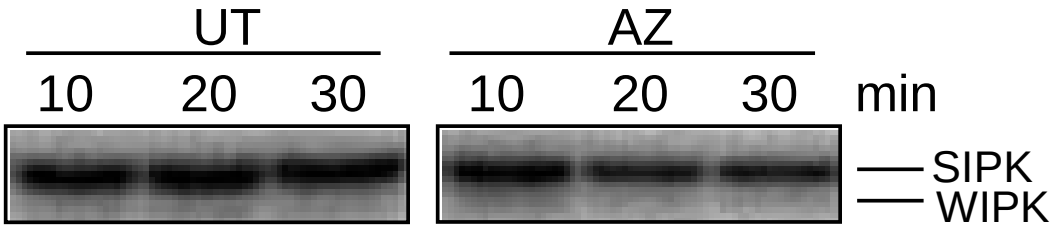

Figure S2 – *Nicotiana attenuata* plants of the Arizona (AZ) accession have lower levels of fatty acid-amino acid conjugates (FACs)-elicited SIPK activity than do plants of the Utah (UT) accession. *N. attenuata* UT and AZ plants were grown under identical conditions. Rosette-stage leaves (+1 position) were wounded with a pattern wheel; 20 µL of synthetic FACs (*N*-linolenoyl-l-Gln, *N*-linolenoyl-l-Glu, *N*-linoleoyl-l-Gln, and *N*-linoleoyl-l-Glu; 0.2 mM each) were applied to the wounds. Leaves from 4 replicate plants were harvested at the indicated times after elicitation and pooled. Kinase activity was analyzed by an in-gel kinase assay using myelin basic protein as the substrate.

Table S1 – Primers and probes used for Taqman-based qRT-PCR

Genes	Primer 1 (5'-3')	Primer 2 (5'-3')	Taqman probe (5'-3')	Reporter	Quencher
WIPK	GGTTTATCAGAGGATCACTGCCA	AACATTCGCGGAATGTATGTATTTT	CTTCATGTATCAGCTCCTCCGTGGCC	FAM	BHQI
MPK4	TAGGAGCAACTCCGGTGCC	GCAAGGACAACATCTGAGACAGAT	CCACCGTGTTACGACATACTCCGTCATG	FAM	BHQI
LOX3	GGCAGTGAAATTCAAAGTAAGAGC	CCCAAAATTTGAATCCACAACA	CAGTGAGGAACAAGAACAAGGAAGATCTGAAG	FAM	TAMRA
ACO1	CTATTGAATCTGATGTCAAGCTG	TATGTAGTAGGGACACACGCTT	CAACTGCATAGATCCAAATTCAAGAGTACTAAAG	FAM	BHQI
ACS3a	ATCCTTCAAATCCATTAGGCAC	AACACTGATGAATTTCGGCTG	TGCGACGAAATTTATGCTGCTACTGTC	FAM	BHQI
WRKY6	ACAAAACAAAGATGAAGTTCCAAAG	GGAGAAGCTGGTGATGAAGATG	AAGTCATTTCCACCTTGTTCTTTGCCA	FAM	TAMRA
WRKY3	CAGGATATGCAAATTCAGAGGATTC	ATTCAATTCAGCAGAGCAATGTG	TGTCATCTCGAGGCTCGTCCTTTGCT	FAM	TAMRA
AOS	GACGGCAAGAGTTTTCCCAC	TAACCGCCGGTGAGTTCAGT	CTTCACCGGAACTTTCATGCCGTCG	FAM	TAMRA
Actin	GGTCGTACCACCGGTATTGTG	GTCAAGACGGAGAATGGCATG	TCAGCCACACCGTCCCAATTTATGAGG	Yakima Yellow	BHQI
JAR4	ATGCCAGTCGGTCTAACTGAA	TGCCATTGTGGAATCCTTTTAT	CAGGTCTGTATCGCTATAGGCTCGGTGATGT	FAM	BHQI
JAR6	TGGAGTAAACGTTAACCCGAAA	AGAATTTGCTTGCTCAATGCCA	TGCCCCCTGAGCTAGTCACTTATGCA	FAM	BHQI

Table S2 – Primers used for SYBR-Green-based qRT-PCR

Genes	Primer 1 (5'-3')	Primer 2 (5'-3')
SIPK	GTTGACGAATTTTCCAAAACAAAGT	CCGGAATATTATCCATACCGGCC
SubD48	AGCCGTAGCTGATGTTGCTGT	TAGAAATTGGGCCTTTACGAAATC
Naf4	CACATACAGCAACTGAATACAAAAAG	CCGGAATGTTATCGATTCCGGAA

CHAPTER 3

Polymorphism in jasmonate signaling partially accounts for the variety of volatiles produced by *Nicotiana attenuata* plants in a native population

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Published in *New Phytologist*, doi:10.1111/j.1469-8137.2009.02894.x (2009)

3.1 Summary

- Herbivore- and jasmonate-induced volatile organic compounds (VOCs) - which mediate indirect defense - must provide reliable information for predators that frequently learn to associate their release with feeding herbivores. Yet little is known about variation of these cues within populations of native plants, on a scale encountered by predators.

- We examined variation in herbivore-elicited VOC emissions and patterns of herbivore-induced jasmonate signaling from accessions of *Nicotiana attenuata* co-occurring in a native population. Herbivore oral secretions (OS)- and methyl jasmonate (MJ)-elicited VOC emissions were characterized using GC-MS, high resolution two-dimensional gas chromatography-time of flight mass spectrometry (GCxGC-ToF-MS) and micro-hydrolysis and -hydrogenation reactions.

- Accessions varied in emissions of abundant (*trans*-α-bergamotene, α-duprezianene, *trans*-β-ocimene, and *cis*-3-hexenol) and total detectable VOCs, as well as the accumulation of jasmonates, the jasmonate antagonist salicylic acid (SA), abscisic acid (ABA) and jasmonate signaling-related transcripts after OS elicitation. Yet MJ treatment exacerbated differences in VOC emission, suggesting that much variation in VOC emission is caused by processes downstream of jasmonate signaling.

- Co-occurring *N. attenuata* plants emit different VOCs following simulated herbivore elicitation due to in part to differences in jasmonate production and responsiveness, which could reduce the effectiveness of induced indirect defense.

3.2 Introduction

Wild plants use many strategies to survive herbivore attack including the constitutive or inducible production of toxic and antidigestive compounds (direct defenses); attraction, via volatile compounds or rewards such as extrafloral nectar, of predators and parasitoids of herbivores (indirect defenses); physical barriers to herbivore feeding; and enhanced growth or sugar reallocation, to ensure sufficient energy stores for reproduction regardless of tissue loss to herbivores (Constabel, 1999; Schwachtje & Baldwin, 2008). Indirect defenses mediated by induced volatile signaling can be a particularly efficient strategy, because the investment of fitness-limiting resources to produce volatile compounds is likely less than that required to activate direct defenses (Gershenzon, 1994; Halitschke *et al*., 2000). The protection afforded by indirect defenses, which can completely remove or disable herbivores at an early stage of attack, can be greater than that resulting from the deployment of direct defenses, which affects herbivore growth and preference but usually does not cause adapted herbivores to leave the plant (Kessler & Baldwin, 2001; Steppuhn *et al*., 2004).

Plants of the native tobacco *Nicotiana attenuata* Torrey ex Watson (*Solanaceae*) germinate after fires from long-lived seed banks to form monocultures in the Great Basin desert, where they are attacked by an unpredictable herbivore community. Feeding by larvae of *Manduca sexta* and *M. quinquemaculata* (*Lepidoptera*, Sphingidae), however, frequently accounts for most of the leaf area removed in a given season (Kessler & Baldwin, 2004). Plants employ an effective indirect defense strategy against *Manduca* via the jasmonate-mediated release of terpenoid and fatty acid-derived VOCs that begin to attract native predatory insects of the genus *Geocoris* (Hemiptera, Lygaeidae) by the day following herbivore elicitation (Halitschke *et al*., 2008; Skibbe *et al*., 2008).

Monocultures of *N. attenuata* can be highly diverse: plants within a population are likely to be genetically less similar than plants from different populations (Bahulikar *et al*., 2004). Nevertheless, phytohormone signaling in response to herbivore attack appears to be consistent in native populations. Wild *N. attenuata* plants reliably produce a wounding-induced jasmonate burst which is amplified by fatty acid-amino acid conjugates (FACs) in the oral secretions (OS) from *M. sexta* larvae (Kahl *et al*., 2000; Halitschke *et al*., 2001; Schittko *et al*., 2001; Stork *et al*. 2009), and jasmonate-mediated responses provide a significant benefit to plants in wild populations (Baldwin, 1998). Yet it is not known how similar the magnitude or quality of these responses is among plants in a population.

VOC emissions can vary greatly among genotypes of cultivated and wild plants (see, for example, Takabayashi *et al.*, 1994; Gouinguene *et al.*, 2001; Köllner *et al.*, 2008), including the solanaceous wild plant *Datura wrightii* (Hare, 2007). Exceedingly few studies have addressed within-population variation in native plants (Delphia *et al* ., 2009), and these have focused on underlying genetic mechanisms (Skoula *et al.*, 2000). Halitschke *et al.* (2000) sampled the headspace of multiple *N. attenuata* plants growing in wild populations and found that some VOCs were more consistently emitted than others, but did not quantify the variation in VOC emissions or examine its origins in plant physiology.

For herbivore-induced VOC emission to function as an effective indirect defense, insects must learn to associate bouquets of (variable) scents with their prey which must indicate the presence or absence of prey feeding on the correct host plant species (Dicke *et al.*, 2003; van Dam & Poppy, 2008). The *Geocoris* predators native to *N. attenuata*'s habitat might be capable of such adaptive learning, as has been shown for other predators and parasitoids (Takabayashi, 1994; Drukker *et al.*, 2000; de Boer & Dicke, 2006; Tamò *et al.*, 2006). To address mechanisms of predator adaptive learning, we must first understand the variation in the VOC signals that single *Geocoris* individuals could encounter in *N. attenuata* populations, and in what ways these signals might be reliable indicators of herbivore presence.

We therefore asked: How do VOC emissions vary among co-occurring accessions of *N. attenuata* after simulated herbivore attack, and to what extent does variation correspond to differences in herbivore-induced signaling? We collected seeds from native *N. attenuata* plants in a wild population (accessions 1, 2, 3, and 4, F0) on a spatial scale that might be encountered by an individual predator, and grew them in a controlled environment together with an inbred line (UT, F22) to reveal genetically, rather than environmentally controlled, variation among individual plants and accessions. We measured the emission of VOCs 24-32 h following treatment of plants with wounding plus *M. sexta* oral secretions (W+OS) and again of the same plants with methyl jasmonate (MJ). For each accession, we also determined constitutive (control), wounding (W)-induced, and W+OS-induced production of hormones known to be involved in herbivore-responsive signaling: jasmonic acid (JA) and the jasmonoyl-isoleucine conjugate (JA-Ile), salicylic acid (SA) and abscisic acid (ABA); as well as transcript accumulation of genes involved in OS perception, jasmonate biosynthesis and signaling, to better understand the sources of variation in JA and VOC signaling.

3.3 Materials and Methods

3.3.1 Plant material and growth conditions

Seeds were collected from four plants growing in a native population of *N. attenuata* at a burn site near Santa Clara, UT, in July 2007. Parent plants of accessions 1, 2, and 3 were growing within 1 m of each other near a burned juniper tree (N37 04.594 W113 49.994); parent plant of accession 4 was located ca. 65 m downhill (N37 04.559 W113 49.979). The well-characterized inbred line " UT" which we used as a control comparison was collected from southwestern UT in 1996 (population U in Glawe *et al.*, 2003, ca. 6.5 km west of the 2007 population) and had been self-fertilized in

the glasshouse in Jena, Germany for 22 generations. Seed germination and glasshouse growth conditions are described in Krügel *et al.* (2002).

3.3.2 Analyses of signaling gene transcripts and phytohormones

Plant treatments and sampling were randomized temporally among accessions, and plants were spatially randomized prior to elicitation and sampling. To determine herbivore-induced transcriptional and phytohormone signaling responses (N=5), plants were either left untreated (control), or wounded by using a pattern wheel which produced 3 rows of puncture wounds on either side of the midvein of the first fully-expanded leaf (position +1). Puncture wounds were immediate treated 20 µL of either distilled water (W) or oral secretions (OS) from *M. sexta* larvae diluted 1:5 with distilled water (W+OS), referred to as "W+OS elicitation". Water or OS was pipetted onto the wounded leaf and gently dispersed across the surface with a gloved finger. Gloves were changed between treatments. The +1 leaves of treated plants were removed after 1 h and divided lengthwise along each side of the midvein, and each half was separately flash-frozen in liquid nitrogen and stored at -80°C until extraction. Harvesting of +1 leaves in the same manner from control plants was interspersed throughout the treatment and harvesting of W- and W+OS-treated leaves.

For phytohormone analysis, we extracted tissue from the right halves of leaves (as seen from the petiole) in ethyl acetate spiked with 100 ng each of 9,10-dideutero-9,10-dihydro-jasmonic acid (JA-D_2), jasmonoyl isoleucine* (JA-Ile-$^{13}C_6$), 3,4,5,6-tetradeutero salicylic acid (SA-D_4), and hexadeutero abscisic acid (ABA-D_6) as internal standards [IS], re-suspended extracts in 70% methanol and analyzed phytohormone content by HPLC-MS/MS as described in Wang *et al.* (2007a). Phytohormones were quantified as ng IS g^{-1} fresh mass (FM).

RNA was extracted from the left halves of leaves with Tri Reagent (Chomczynski, 1993), checked on an agarose gel and quantified on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Synthesis of cDNA from 0.5 µg RNA per sample and qPCR TaqMan (*Nalox3,* Halitschke & Baldwin, 2003; *Nawipk,* Wu *et al.*, 2007; Applied Biosystems, Foster, CA, USA) and SYBR (*Najaz1,* homologous to *N. tabacum jaz1* [Shoji *et al.*, 2007], I. Galis, unpublished data, forward primer ACTTACCGCAGTTTTGAACC, reverse primer TGTGCCTTTTCTGGTTCAGA; Invitrogen, Carlsbad, CA, USA) were conducted as in Wu *et al.*(2007). Expression was quantified relative to *N. attenuata actin*.

3.3.3 Collection of plant VOCs

Plant treatments and sampling were randomized temporally among accessions, and plants were spatially randomized prior to elicitation and sampling. Eight rosette-stage plants per accession were W+OS-elicited on the second fully-expanded (+2) leaf and placed ca. 0.3 m apart on a single table in the glasshouse. Leaves near the source-sink transition leaf respond the most to treatment (van Dam *et al.*, 2001), but VOC emission may be greatest after treatment of position +2 (Halitschke *et al.*, 2000). From 24-32 h after treatment, treated leaves were enclosed in two shallow 50 mL plastic cups (Huhtamaki, Espoo, Finland) secured with miniature claw-style hair clips, and headspace VOCs were collected on 20 mg of SuperQ (Alltech, Deerfield, Il, USA) by drawing ambient air through these clip cages as described in Wu *et al.* (2008) and Halitschke *et al.* (2000) for

8 h. Background VOCs present in ambient air were collected using empty trapping containers. SuperQ-packed filters were either immediately spiked with 400 ng tetraline as an internal standard (IS) and eluted with 250 µL dichloromethane into a GC vial containing a glass insert, or stored at -20°C until elution.

The same 8 plants per accession were allowed to recover for 1 week, at which point plants were elongated but not flowering. To determine the average maximum jasmonate-elicited VOC response of each accession, plants were treated with 150 µg methyl jasmonate (MJ; Aldrich, 95%, mixture of isomers) dissolved in 20 µL lanolin paste, which was gently spread across the base of the upper leaf surface (position +2, now the oldest stem leaf) using a blunt spatula. We again collected VOCs from 24-32 h from the treated leaf.

To have an indication of developmental differences in VOC emission, an additional group of UT plants was treated in the rosette stage with W+OS, MJ, or no treatment (control), N = 6, and VOCs were collected from 24-32 h after treatment from the treated leaf (+2). The plants were allowed to rest for a week, after which time elongated W+OS- treated and control plants were elicited with MJ, and VOCs were collected from 24-32 h from the treated leaf (+2).

3.3.4 Analysis of VOC emissions

For GC-ion trap MS analysis, analytes in plant total VOC samples were separated on a nonpolar FactorFour VF-5ms column (30 m x 0.25 mm i.d. x 0.25 µm, Varian Inc., Lake Forest CA, USA) in a Varian CP-3800 GC (Varian, inc., Palo Alto, CA, USA) equipped with a CP-8400 auto-injector operated in splitless mode, and analyzed on a coupled Varian Saturn 4000 ion trap mass spectrometer: GC, helium carrier gas, 1µl 30s splitless injection at 250°C, initial temperature 40°C for 5 min, increased at 5°C/min to 185°C, 30°C/min to 300°C 10 min hold; MS, transfer line at 230°C, trap temperature 180°C, scan range from 40 to 399 *m/z* at 1 spectra/s. The most abundant analytes across all samples (*trans*-α-bergamotene, α-duprezianene, *cis*-3-hexenol, and *trans*-β-ocimene) were identified for further analysis. Individual VOCs were quantified relative to the peak area of the tetraline IS in each sample as the relative amount of VOC in ng IS per g FM of the trapped leaf.

For the GCxGC-ToF analysis, the same VOC samples were run on an Agilent 6890N gas chromatograph equipped with an Agilent 7683 auto-injector (Agilent Technologies, Santa Clara, CA, USA) coupled with a LECO Pegasus III time-of-flight mass spectrometer with a 4D thermal modulator upgrade (LECO, St. Joseph, MI, USA). Injected samples were separated first on a nonpolar column (C1 RTX-5MS, 20 m x 0.25 mm i.d. x 0.5 µm, Restek, Bellefonte, PA, USA) and every 6 s (modulation time) transferred to a midpolar column (DB-17, 0.890 m x 0.10 mm i.d. x 0.1 µm Agilent Technologies, Santa Clara, CA, USA) for the second separation. Chromatography and analysis conditions as well as deconvolution, alignment, and integration of VOC analyte peaks are described in Gaquerel *et al.* (2009). During peak table alignment using the comparison feature imbedded in the ChromaToF software (LECO, St. Joseph, MI, USA), mass spectra alignment was accepted at a similarity threshold of 500/1000.

A C9-C24 n-alkane series was used to determine the Kovats retention index (Kovats, 1965). Linear RTs on the GCxGC-ToF were obtained for RI calculation from the sum of the RT values of each compound on C1 and C2, as previously reported (Kusano *et al.*, 2007). All identifications

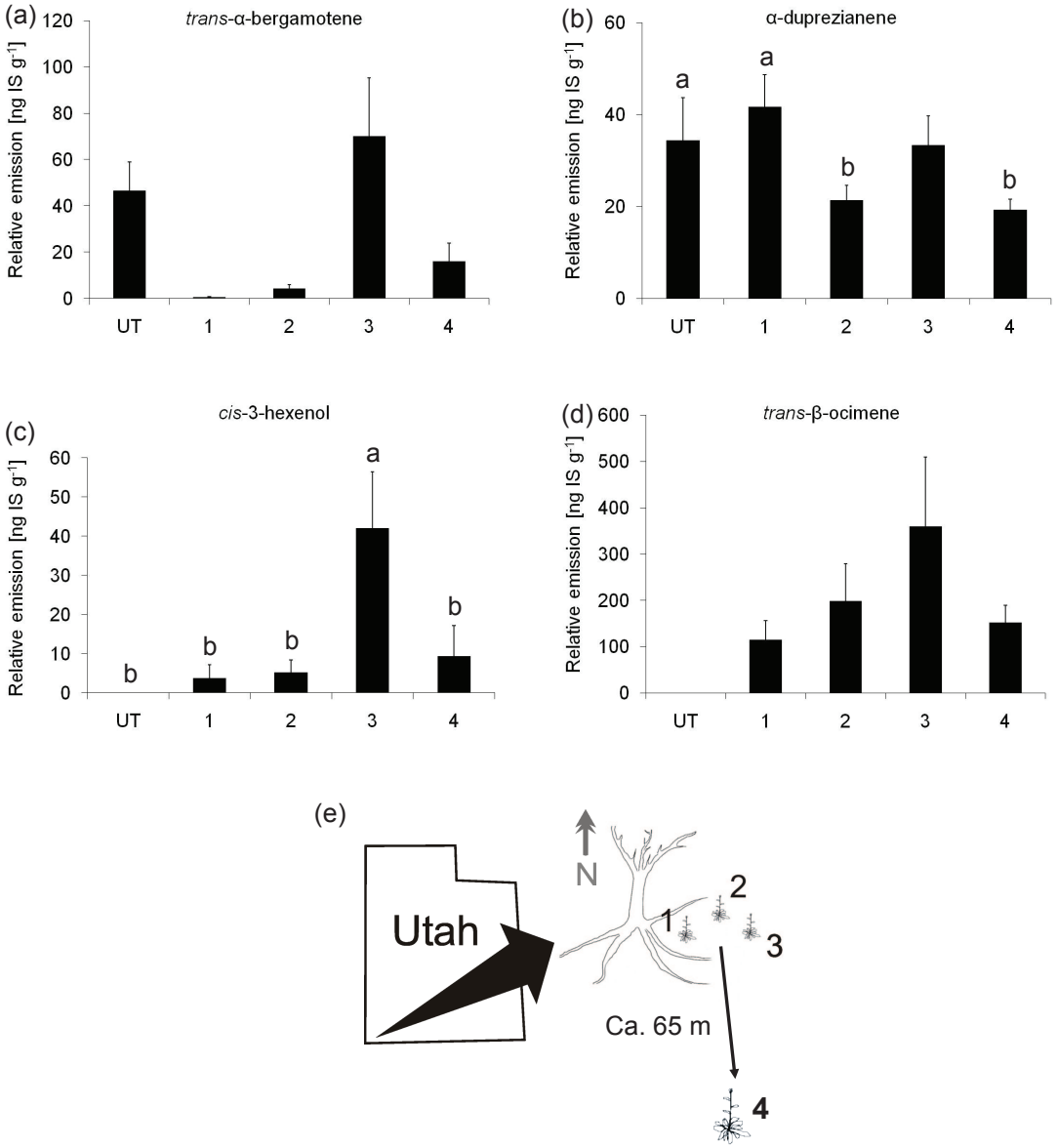


Figure 1 – Emission of the four globally most abundant volatile organic compounds (VOCs) measured 24-32 h following treatment with wounding and *Manduca sexta* oral secretions (W+OS) in five accessions of *Nicotiana attenuata*. These are the sesquiterpenes (a) *trans*-α-bergamotene and (b) α-duprezianene, the monoterpene (c) *trans*-β-ocimene, and the fatty acid derivative (d) *cis*-3-hexenol (detectable, but very low levels in UT). UT = 22x inbred line originating from a post-fire population near the DI Ranch in southwestern UT, USA; (e) 1, 2, 3, and 4 are F_0 plants from seeds gathered from a single post-fire population in southwestern UT near the Lytle Ranch Preserve. Plants 1, 2, and 3 were in a single cluster less than 1 m apart from each other, and 4 was in a second cluster of plants downhill from the cluster containing 1, 2, and 3 and separated by ca. 65 m which was free of other *N. attenuata* plants. Different letters (a, b, c) indicate statistically significant differences in emission among accessions in Scheffe's post-hoc tests ($P < 0.05$) following a significant effect of accession in ANOVAs across all accessions for each VOC; MANOVA analysis also show significant variation across accessions when the individual VOC concentrations were considered to be a multivariate data set (Table S7). n.d. = not detected

were based on mass spectra and retention times compared to authentic reference compounds. When identification was not possible, we tried to determine the class of VOC by typical fragmentation patterns combined with a retention time similar to standard compounds of the same class; otherwise the compound was listed as unknown.

3.3.5 Identification of α-duprezianene by high resolution GC-MS of crude and hydrogenated plant volatile extract

GC-MS experiments were performed using a HP 8570 series gas chromatograph (Agilent, USA) connected to a MasSpec sector-field mass spectrometer (Micromass, Manchester, UK). A nonpolar DB-5 MS column (30 m long, 0.25 mm i.d., phase thickness 0.25 µm, Agilent J & W Scientific) was used for separations. The injector was operated in splitless mode at 220°C. The detector temperature was set to 200°C; standard 70eV spectra were recorded at 1 scan/sec. The temperature of the GC oven was programmed as: 60°C, hold for 2 min; 10°C/min to 320°C and hold at 320°C for 10 min. Helium was used as a carrier gas at constant flow of 0.7ml/min. Data were analyzed using the OPUS software and Wiley ver. 6 and NIST libraries were used for spectra data searches. High-resolution MS (EI) data were obtained using a MasSpec 2 instrument (Micromass, UK) in positive ion mode using 70eV ionization energy (see supplemental data). Perfluorokerosine mixture was used as an internal standard.

Dichloromethane from a plant volatile trapping extract was evaporated and ethylacetate (0.5 mL) was added. The catalyst Pt/C (10%) was added (ca 2 mg), and the reaction vial was evacuated and purged with hydrogen three times. Micro scale hydrogenation (Marques *et al.*, 2004) was performed under vigorous stirring at normal pressure for 1 h. The black suspension was filtered over cotton wool in a Pasteur pipette and the clear solution was analyzed on GC-MS as described above.

Spectra and retention time in the crude plant volatile extract were compared to a sample of α-duprezianene kindly provided by Dr. A. F. Barrero run sequentially on both a nonpolar and polar GC column, and by co-injection.

3.3.6 Statistical analyses

Levene tests for homogeneity of variance were performed on untransformed data. To meet requirements of normality and homogeneity of variance, and to include 0 values, 1 was added to the raw areas of all VOC analytes from 1D and 2D analyses, and, after normalization to the IS, data were log2-transformed; transcript and phytohormone levels were also -log2-(transcripts) or log2 transformed. Differentially emitted VOCs were tested by one-way ANOVAs ($P < 0.05$) across all accessions using, and those which showed significant differences were then tested by MANOVA (Supplemental Table S7), to determine whether the significance remained when the individual VOC concentrations were considered to be a multivariate data set; because of the number of detected VOCs it was not possible to test all by MANOVA. Scheffe's post-hoc tests were conducted for the four abundant VOCs. Transcripts and phytohormones were analyzed by 2-way ANOVAs across treatments (Control, W, W+OS) and accessions followed by Scheffe's post-hoc tests. Pearson's product-moment correlation analyses were performed using R (R Development Core Team,

2005) on untransformed data. When samples were excluded from analysis, this was always due to problems arising during sampling or processing (very poor signal quality for all compounds measured or from internal standard(s), loss of sample during extraction). ANOVAs were calculated in TIGR MeV 3.1 software (ToF measurements) or in StatView (version 5.0, SAS institute) and MANOVAs and the correlation table was calculated in StatView.

3.4 Results

3.4.1 Identification of α-duprezianane

A previously unidentified peak at 11.45 min showed a typical bi- or tricyclic sequiterpene mass spectrum [abundant M+• at 204 ($C_{15}H_{24}$), and 161, 119, 93, 69 fragments] with a prominent m/z 148: presumably a neutral loss of butene (C_4H_8) (see supplemental data). The number of double bonds was determined by hydrogenation over Pt/C (10%) providing evidence for one unsaturation (peak at 11.50 min, m/z 206). Notably, a more intense m/z 206 peak at a later RT (12.08 min) was detected showing two dominant neutral losses of C_2H_4 at m/z 178 and C_4H_8 at m/z 150. The double bond in the original $C_{15}H_{24}$ alkene was not reduced, as the C_4H_8 loss was still apparent, but rather one of the rings was hydrogenolyzed. The unidentified sesquiterpene was tentatively identified as a tricyclic alkene. Based on structures in literature (Barrero *et al.*, 1996; Barrero *et al.*, 2000) this compound was determined to be α-duprezianane. This assignment was fully supported by co-injections with authentic standard from Dr. A. F. Barrero.

3.4.2 Emission of VOCs differs significantly among accessions when plants are elicited with M. sexta *OS*

The most abundant VOCs detected in emissions from accessions after W+OS were two sesquiterpenes, the monoterpene *trans*-β-ocimene, and the fatty acid derivative *cis*-3-hexenol. The sesquiterpenes were subsequently identified as *trans*-α-bergamotene (rather than the *cis* isomer previously reported in *N. attenuata*) and α-duprezianene. The corrected identification of the α-bergamotene isomer does not influence previous work in this system, because the isomer of α-bergamotene purified for use in former experiments was also determined to be *trans*- rather than *cis*-α-bergamotene (N. Heinzel, unpublished data).

The emission of the four most abundant VOCs (Fig. 1, Supplemental Table S7) differed significantly among accessions following W+OS treatment; however, *trans*-β-ocimene emission did not differ significantly among the accessions producing this VOC (1, 2, 3, and 4, but not UT) (one-way ANOVAs; final N for each accession: 1, N = 8; 2, N = 8; 3, N = 4; 4, N = 8; UT, N = 7; *trans*-α-bergamotene: $F_{4,30} = 9.17$, $P < 0.0001$; *trans*-β-ocimene: $F_{4,30} = 34.3$, $P < 0.0001$; *cis*-3-hexenol: $F_{4,30} = 2.91$, $P = 0.038$; α-duprezianene: $F_{4,30} = 2.76$, $P = 0.046$). A Pearson's correlation matrix showed that emission levels were unrelated except for *trans*-β-ocimene and *cis* -3-hexenol which were marginally correlated (Supplemental Table S8). On average, accessions from neighboring plants (1, 2, and 3) were no more similar to each other in their emission of these VOCs than they were to an accession collected from a different location in the same population (4) (Fig. 1). Individual plants within each field-collected accession did not differ more from sibling plants in their VOC

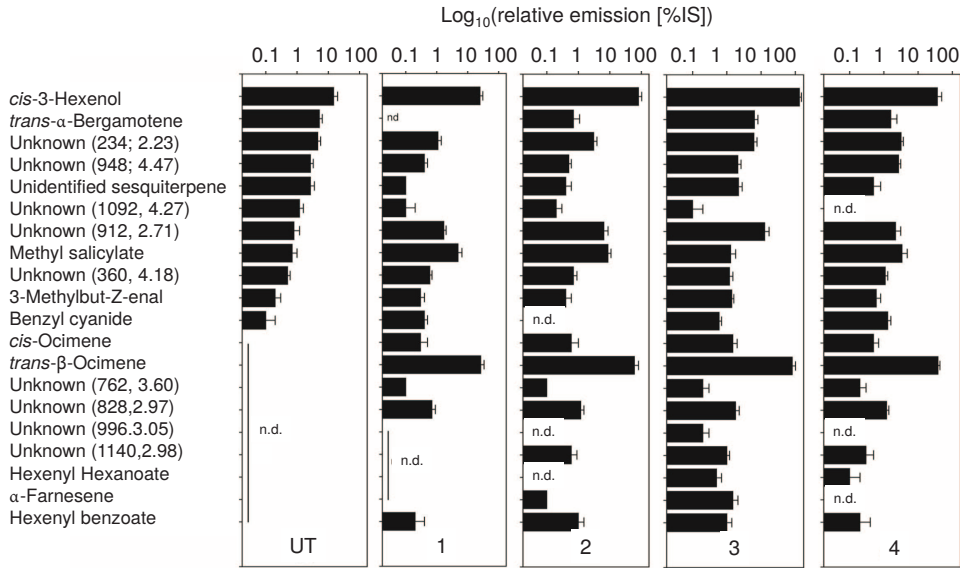


Figure 2 – Diagram of the twenty VOCs out of 391 detected analytes which are differentially emitted by accessions of *N. attenuata* 24-32 h after W+OS treatment as detected by GCxGC-ToF analysis. Differences in emission are significant ($P < 0.05$) as determined by individual ANOVAs across all accessions for each VOC (Supplemental Table S1, S7). n.d. = not detected

emissions than did plants of the F22 UT line: Levene's test for homogeneity of variance revealed nonsignificant differences in the variance of VOC emission within each accession after W+OS treatment, with the exception of *trans*-α-bergamotene, which was due to the fact that only one plant of accession 1 emitted *trans*-α-bergamotene (*trans*-α-bergamotene: $F_{4,30} = 2.89$, $P = 0.039$; *trans*-β-ocimene: $F_{4,30} = 2.37$, $P = 0.075$; *cis*-3-hexenol: $F_{4,30} = 1.00$, $P = 0.422$; α-duprezianene: $F_{4,30} = 1.38$, $P = 0.27$). When accession 1 was removed from analysis the P-value was no longer significant ($F_{3,23} = 1.84$, $P = 0.168$).

We extended our investigation to the large-scale profiling of VOC blends emitted by each accession. Because plant VOCs are highly structurally diverse (Holopainen, 2004) and emitted in a large range of concentrations, we analyzed the same volatile extracts by two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-ToF-MS) to fully resolve these complex mixtures. Seventeen additional volatiles and *trans*-α-bergamotene, *trans*-β-ocimene and *cis*-3-hexenol were detected as statistically different among accessions (ANOVAs for each compound; Fig. 2, Supplemental Tables S1 and S7).

3.4.3 Accumulation of phytohormones and transcripts of signaling genes amongst accessions

We tested the hypothesis that differences in VOC emissions from W+OS-treated plants correlated with differences in W+OS-induced signaling. Control, W, and OS-induced levels of phytohormones known to mediate plant-herbivore interactions (JA, JA-Ile, SA, ABA) differed significantly among accessions ($P \leq 0.05$ in 2-way ANOVAs with accession and treatment as factors; Supplemental Table S2). Maximum concentrations of JA and JA-Ile occur at approximately 1 h after

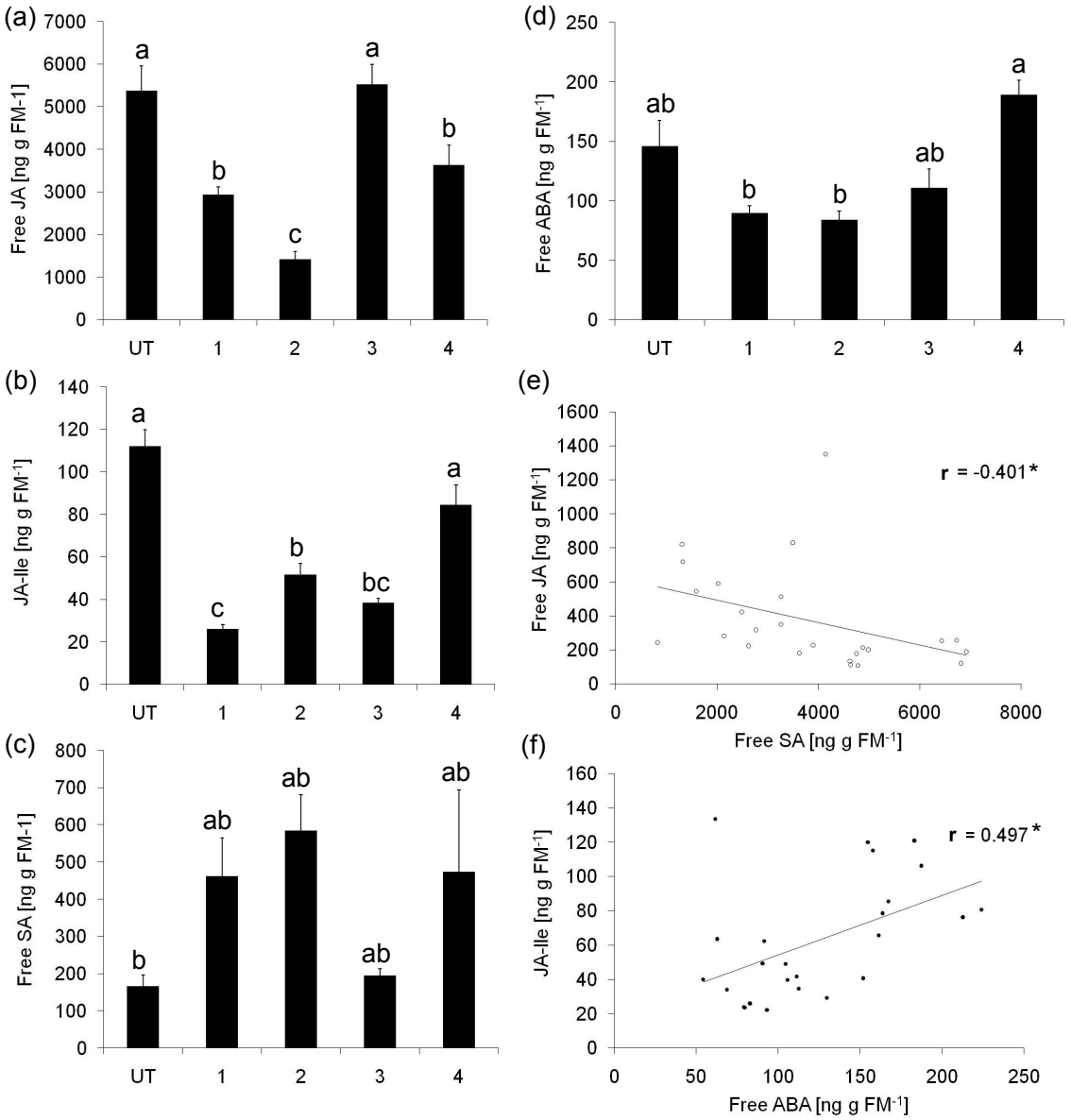


Figure 3 – Levels of defense-related phytohormones elicited after 1 h in five accessions of *N. attenuata*. JA and JA-Ile reach maximum levels at ca. 1 h after W or W+OS elicitation in *N. attenuata*; this has been shown in both the inbred UT line (see for example Wang *et al* ., 2007a) and in wild plants. Shown are levels at 1 h following W+OS treatment of (a) Jasmonic acid (JA), (b) JA-Ile conjugate, (c) salicylic acid (SA) and (d) abscisic acid (ABA). JA and JA-Ile are significantly elicited in all accessions both by wounding (W) and, additionally, by the application of OS to wounds as determined in a 2-way ANOVA ($P < 0.05$) with factors accession and treatment (Supplemental table S2); however, the magnitude of the treatment effect varies by accession for JA-Ile. SA levels are not significantly affected by treatment, and treatment effects on ABA levels vary greatly by accession (Supplemental table S2). (e) There is a significant negative correlation between levels of JA and SA and (f) a significant positive correlation between levels of JA-Ile and ABA after W+OS treatment across all accessions, but no correlation between levels of JA and JA-Ile, JA-Ile and SA, or ABA and SA. Different letters (a, b, c) indicate statistically significant differences ($P < 0.05$) among accessions (UT, 1, 2, 3, 4) at 1 h after W+OS treatment as determined by Scheffe's post-hoc tests. *$P < 0.05$ in a Pearson's correlation test.

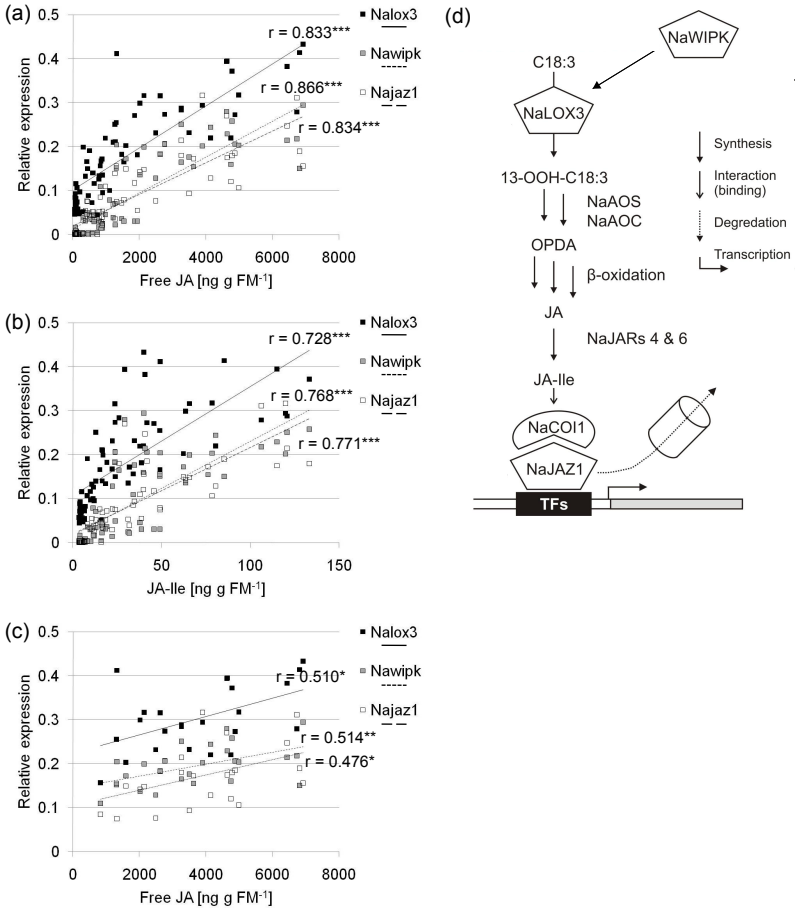


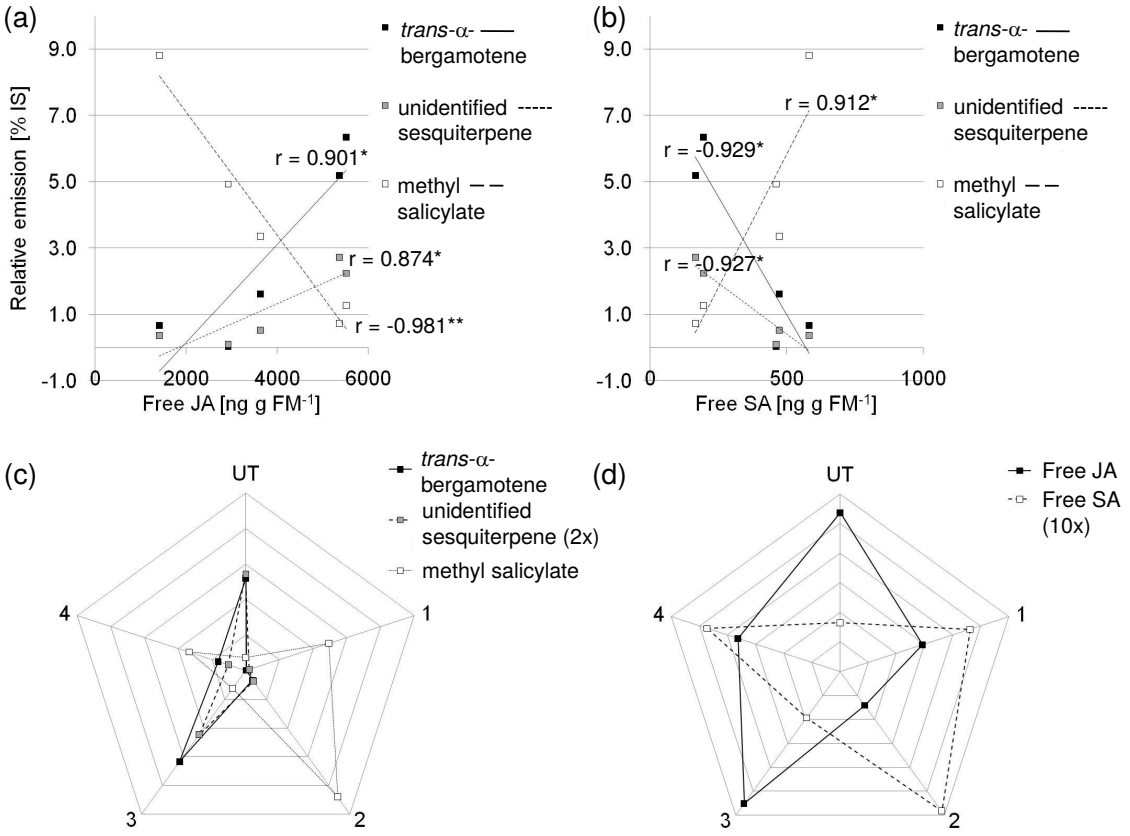
Figure 4 – Transcript abundance of genes involved in the jasmonate response to herbivore elicitors. Transcript levels of genes involved in the jasmonate response to herbivory are significantly and positively correlated to (a) JA and (b) JA-Ile across control, W and W+OS treatments, but only to (c) JA in W+OS-treated leaves. Shown are levels at 1 h post-treatment of phytohormones correlated to the mitogen-activated protein kinase (MAPK) gene *Nawipk* which is transcriptionally upregulated by *M. sexta* OS and is essential for the plant's defense response (Wu *et al.*, 2007); lipoxygenase 3 (*Nalox3*) which converts α-linolenic acid (C18:3) to 13-hydroperoxylinolenic acid (13-OOH-C18:3) in the first step of jasmonate biosynthesis (Halitschke and Baldwin 2003); and the *N. attenuata* homologue to the *N. tabacum* JAZ1 gene, *Najaz1* (I. Galis, unpublished data). JAZ proteins are degraded by the COI1-SCF complex to permit the transcription of jasmonate-elicited genes, and transcripts are elicited by jasmonates as part of the negative feedback regulation of jasmonate signaling (Chini *et al.*, 2007; Thines *et al.*, 2007). Expression is relative to *Naactin*. *$P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$ in a Pearson's correlation test. Differences among accessions in control, W- and W+OS-treated phytohormone and transcript levels were tested in 2-way ANOVAs (see Supplemental Tables S2 and S3). (d) The current model of *M. sexta*-elicited jasmonate signaling in *N. attenuata*. W+OS treatment causes a signaling cascade which activates the MAPKs NaWIPK and NaSIPK (NaSIPK is not shown because it is regulated at the protein level) within 5 minutes after OS contacts the leaf. MAPK activity leads to the activation and/or transcription of enzymes involved in jasmonic acid (JA) biosynthesis and signaling, which begins when α-linolenic acid is converted to 13-hydroperoxylinolenic acid by NaLOX3 in plastids. Allene oxide synthase (NaAOS) converts 13-hydroperoxylinolenic acid to allene oxide, which is cyclized to 12-oxy-phytodienoic acid (OPDA) by allene oxide cyclase (NaAOC). OPDA undergoes three cycles of β-oxidation in peroxisomes to form JA. The enzymes JAR4 and JAR6 catalyze the formation of a variety of JA-amino acid conjugates; JA-Ile has been shown to interact with the F-box protein COI1 in a complex which binds to and degrades JAZ proteins, including NaJAZ1.

wounding and are amplified by OS treatment of the puncture wounds in the inbred UT line and in native accessions of *N. attenuata* (Wang *et al.*, 2007a; Stork *et al.*, 2009). In contrast, concentrations of SA increase within 1 h of wounding and are not further elevated by treatment with *M. sexta* OS (C. Diezel, C. von Dahl, R. Halitschke, I. T. Baldwin, unpublished data). Thus we found no significant effect of W+OS treatment on SA levels at 1 h. For all three phytohormones, the greatest differences among accessions were seen after W+OS elicitation (Table S2, Fig. 3), suggesting different abilities to transduce OS perception into phytohormone production among accessions (Fig. 4d). W+OS-induced phytohormone values were similar among plants within each accession (Levene's test of variance; JA: $F_{4,20} = 0.988$, $P = 0.437$; JA-Ile: $F_{4,20} = 0.987$, $P = 0.437$; SA: $F_{4,20} = 0.870$, $P = 0.500$; ABA: $F_{4,20} = 0.548$, $P = 0.702$).

JA and SA levels in the same leaves were strongly and negatively correlated across all accessions after W+OS, whereas JA-Ile and ABA levels in the same leaves were positively correlated after W+OS (Fig. 3e,f; Pearson's product-moment correlations; JA vs. SA $r = -0.401$, $P = 0.047$; JA-Ile vs. ABA $r = 0.497$, $P = 0.012$). Interestingly, only JA-Ile and ABA showed significantly different patterns of induction by W and W+OS across accessions (Table S2). There was no correlation between JA and JA-Ile, JA-Ile and SA, or SA and ABA levels.

W and OS-elicited accumulation of signaling gene transcripts were analyzed in the same leaves used for phytohormone analysis. We tested for differences among accessions in their induction of gene transcripts involved in OS perception (*Nawipk*), JA biosynthesis (*Nalox3*), and JA perception (*Najaz1*) ($P \leq 0.05$ in 2-way ANOVAs with accession and treatment as factors; Supplemental Table S3). Transcripts of these genes are known to be up-regulated within 1 h of W or W+OS treatment (Skibbe *et al.*, 2008; Meldau *et al.*, 2009; I. Galis, unpublished data). Transcripts were significantly elevated in response to wounding and amplified by OS in all accessions, but overall transcript accumulation of *Nalox3* and *Najaz1* differed significantly, and accessions differed in their treatment response for *Nawipk* (Table S3). Levene's tests of variance revealed that individuals from field-collected seed did not differ from each other more than individuals of the inbred line UT (Nawipk: $F_{4,20} = 0.502$, $P = 0.735$; Nalox3: $F_{4,20} = 0.805$, $P = 0.537$; Najaz1: $F_{4,20} = 0.348$, $P = 0.842$).

Elicited levels of JA and JA-Ile were highly correlated with transcript accumulation as predicted by the current model of herbivore-responsive JA signaling in *N. attenuata* (Fig. 4a,b,d): all Pearson's correlation coefficients were ≥ 0.728 and highly significant ($P < 0.0001$). Thus the model applies to genetically variable native plants as well as to the inbred line UT in which it has been elucidated (see for example Wu *et al.*, 2007; Wang *et al.*, 2007a,b; Paschold *et al.*, 2008). There was also a significant but weak correlation between transcript accumulation and ABA levels after W+OS across accessions (Pearson's product-moment correlations; ABA vs. Nalox3 $r = 0.264$, $P = 0.025$; ABA vs. Nawipk $r = 0.241$, $P = 0.042$; ABA vs. Najaz1 $r = 0.339$, $P = 0.004$) However, in W+OS-elicited samples, only differences in JA, not differences in JA-Ile or ABA, were correlated to differences in transcript accumulation (Fig. 4c).



(d)

	***trans*-α-bergamotene**		**unidentified sesquiterpene**		**Methyl salicylate**	
	r	*P*	r	*P*	r	*P*
Free JA	0.901	0.037	0.874	0.053	-0.981	0.003
Free SA	-0.929	0.023	-0.927	0.014	0.912	0.031

Figure 5 – Correlation between endogenous JA and SA levels and VOCs which were differentially emitted by accessions at 24-32 h following treatment with W+OS; (a) and (b) share a Y-axis. (a) Average relative emission of *trans*-α-bergamotene is significantly and positively correlated to average JA levels across accessions; an unidentified sesquiterpene follows the same pattern, but the correlation is marginally significant ($P < 0.06$); methyl salicylate is significantly and negatively correlated to JA. (b) The two VOCs which are positively correlated to JA levels are significantly and negatively correlated to average SA levels, whereas emission of methyl salicylate is significantly and positively correlated to SA. *$P < 0.05$ or ** $P < 0.01$ in a Pearson's correlation test. No other differentially emitted VOCs correlate significantly to JA or SA levels, and none correlate significantly to JA-Ile or ABA levels. In all cases, correlations are weaker and not significant for control- or wounding plus water (W)-elicited levels of phytohormones, except that MeSA emission after W+OS is also significantly ($P < 0.01$) correlated to control levels of SA (not shown). (c) and (d) provide an alternate visualization of the comparison between average emission of these three VOCs after W+OS treatment, and average levels of JA and SA after W+OS treatment. Distance from the center of the axes corresponds to either magnitude of average relative emission [ng IS g FM^{-1}] (c) or average concentration [ng g FM-1] in W+OS-treated tissue (d). Note that values for the unidentified sesquiterpene in (c) and SA in (d) have been scaled for improved visualization.

3.4.4 Correlation between W+OS-induced endogenous jasmonate signaling and VOC emissions

Only average emissions of *trans*-α-bergamotene, an unidentified sesquiterpene, and methyl salicylate showed strong correlations across accessions to average JA and SA levels after W+OS: *trans* -α-bergamotene and the unidentified sesquiterpene were positively correlated to JA and negatively correlated to SA, whereas methyl salicylate showed the opposite pattern (Fig. 5). This indicates that variability in W+OS-elicited JA production and signaling accounted for few of the differences in VOC release.

W-induced JA levels showed a similar correlation to these three VOCs (Pearson's product-moment correlation; JA vs. *trans*-α-bergamotene, r = 0.910, P = 0.032; JA vs. unidentified sesquiterpene, r = 0.952, P = 0.013; JA vs. methyl salicylate, r = -0.924, P = 0.025). Control levels of JA were too low to detect any relationship to volatile emission after W+OS. Although there was no significant induction of SA by W or W+OS, control levels of SA were correlated only to methyl salicylate emission (r = 0.986, P = 0.002), perhaps reflecting an effect of induction on SA-JA crosstalk. Average JA-Ile and ABA levels in W+OS-treated plants were not correlated to any of the VOCs which were differentially emitted among accessions after W+OS treatment.

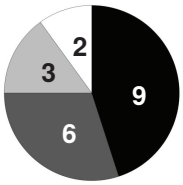
3.4.5 Total detectable VOC emission after MJ treatment

To determine whether differences in OS-elicited volatile emission are explained by differences in endogenous jasmonate signaling, we conducted a separate experiment to measure MJ-elicited VOC emissions. MJ treatment infuses plants with jasmonates and thereby short-circuits the transduction of OS into endogenous JA production. Because we did not yet know how great the variance among individuals within an accession might be, we used the same plants, but allowed them to recover for one week from the previous OS elicitation, at which time plants were elongated but not yet flowering. In a previous experiment using the same elicitation and trapping methods we found that for the UT line, one week is sufficient to eliminate any effect of OS elicitation on MJ-elicited VOC emissions (Supplemental Table S4), and the VOCs which are emitted by elongated plants after MJ treatment often differ quantitatively, but generally not qualitatively from those emitted by rosette-stage plants (Supplemental Table S5). A total of 43 compounds differed among MJ-elicited accessions, but only 20 differed after W+OS-elicitation (Supplemental Tables S1, S6, S7, Figs. 2 & 6), showing that MJ treatment did not produce comparable emissions among accessions but rather may have increased differences. A Pearson's correlation matrix of the four most abundant VOCs showed a significant positive correlation between *trans*-β-ocimene and *trans*-α-bergamotene, and a significant negative correlation between *trans*-β-ocimene and α-duprezianene emissions (Supplemental Table S8).

3.5 Discussion

We tested the hypothesis that co-occurring accessions (1-4) of the wild tobacco *N. attenuata* would emit similar VOC profiles after herbivore elicitation, and if not, that differences could be related to differences in endogenous jasmonate signaling. VOC blends emitted after simulated herbivory

(a)



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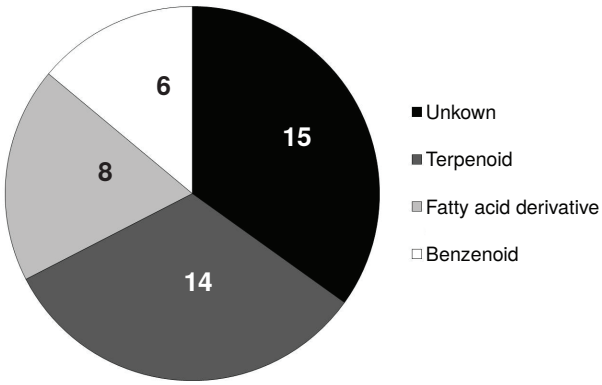

(c)

Origin	VOC	RT 1D (s)	RT 2D (s)	RI	Treatment	$F_{4,33}$	*P*	Accessions				
								UT	1	2	3	4
Terpenoid	*cis*-Ocimene	630	2.39	1047	W+OS	3.20	0.025	-	0.3 ± 0.2	0.6 ± 0.4	1.5 ± 0.5	0.5 ± 0.2
					MJ	5.58	0.002	-	0.3 ± 0.2	1.7 ± 0.7	4.1 ± 1.3	1.8 ± 0.5
Terpenoid	*trans*-β-Ocimene	648	2.42	1056	W+OS	5.23	0.0023	-	26.4 ± 7.2	60.5 ± 21.2	82.2 ± 19.1	38.4 ± 5.0
					MJ	8.30	< 0.0001	-	22.0 ± 9.6	84.9 ± 26.3	183.1 ± 43.1	88.5 ± 23.8
Terpenoid	*trans*-α-Bergamotene	1302	2.39	1435	W+OS	5.88	0.0001	5.2 ± 1.2	0.0 ± 0.0	0.7 ± 0.4	6.4 ± 1.7	1.6 ± 0.8
					MJ	4.16	0.008	15.8 ± 5.5	0.6 ± 0.3	8.4 ± 3.0	38.6 ± 11.3	17.8 ± 9.2

Figure 6 – Methyl jasmonate (MJ) treatment does not result in fewer differences in volatile emission among accessions. (a) & (b) Charts are scaled to represent total amounts of VOCs found to be differently emitted across accessions 24-32 h after (a) W+OS treatment or (b) MJ treatment of the same plants one week later, and the total number of VOCs in each category [see legend in (b)] is given in each segment. (c) Example of three terpenoid VOCs known to be emitted in similar levels over the developmental time scale of these two experiments. Differences in the emission of two isomers of ocimene are enhanced after MJ as compared to W+OS treatment (smaller *P*-values), whereas differences in the emission of *trans*-α-bergamotene become smaller after MJ treatment (larger *P*-value). Differences among accessions were determined by separate one-way ANOVAs ($P<0.05$) across accessions for each VOC (see also Table S7).

(W+OS) or jasmonate supplementation (MJ) were characterized in two separate experiments on the same plants, using both targeted (most abundant components) and untargeted approaches (Figs. 1 & 2), for a period approximating the time at which predators begin to respond to indirect defense signals (24-32 h after treatment; see for example Skibbe *et al.*, 2008). Accessions displayed striking differences in VOC emissions and elicited signaling traits (Figs. 1-4), but phenotypes were stable within accessions (Levene's tests of variance for individual traits).

Targeted analysis of the most abundant VOCs emitted by accessions after W+OS elicitation (Fig. 1, Table S7) revealed substantial variation which motivated us to investigate the total volatile blends trapped from the leaves of each accession by GCxGC-ToF analysis. This sensitive detection technique has been shown to yield high-quality mass spectra obtained from the enhanced chromatographic resolution (Shellie *et al.*, 2001) which accounts for its rapid adoption for non-targeted studies (Gaquerel *et al.*, 2009; Shellie *et al.,* 2005; Kusano *et al.,* 2007; Tu *et al.,* 2007). Untargeted analyses revealed differential emission among accessions for 20 VOCs after W+OS treatment (Figs. 2 & 6, Tables S1, S7) and for 43 after subsequent MJ treatment of the same plants (Fig. 6, Tables S6, S7). Because VOC emissions were found to be qualitatively if not quantitatively similar across the time frame of the two experiments, a comprehensive qualitative analysis of VOCs emitted by *N. attenuata* was possible (Tables S5 and S6), which revealed that differences among accessions were increased rather than obviated by MJ treatment, both in terms of the number of VOCs and the magnitude of some differences (Fig. 6).

Endogenous differences in levels of jasmonates (JA and JA-Ile) correlated well with differences in induced transcript accumulation among accessions (Fig. 4a,b). This pattern is consistent with the model of jasmonate-mediated, W+OS-elicited defense responses deduced from extensive work with transformed lines of *N. attenuata* silenced in various genes in these signaling pathways (Fig. 4d). Correlations among phytohormones demonstrated cross-talk between JA and SA signaling in all accessions, as has been shown by Spoel *et al.* (2003), Traw *et al.* (2003) and van Leeuwen *et al.* (2007) to occur variably in accessions of *Arabidopsis thaliana* . Interestingly, a positive correlation between JA-Ile and ABA was also discovered (Fig. 3c,d). ABA is thought to be involved both in synergistic and antagonistic modulation of JA signaling in defense, but its role, especially in herbivore defense, is unclear (Asselbergh *et al.*, 2008; Bodenhausen & Reymond, 2007). The induction of JA-Ile and ABA relative to control levels, but not of JA or SA, varied significantly among accessions after W and W+OS treatment (Table S2). Thus patterns of herbivore-elicited accumulation may be more conserved for JA and SA than for JA-Ile and ABA among individual *N. attenuata* plants. This is interesting given that only differences in JA levels corresponded to different transcript levels of signaling genes in W+OS-treated leaves (Fig. 4c). Also, only differences in JA and SA accumulation were correlated with differences in VOC emission after W+OS; this was true for only three of twenty VOCs (Figs. 2 and 5). Because elicitation of the same plants with MJ resulted in more rather than fewer differences (Fig. 6), these combined results suggest that most of the significant qualitative differences in VOC profiles after W+OS are explained by differences independent from, or downstream of OS-elicited jasmonate production.

Perhaps the most interesting result from the correlation of phytohormones with other responses is that endogenous peak JA levels are more indicative of transcript regulation and jasmonate-mediated VOC production than are peak JA-Ile levels, although JA-Ile and not JA has been shown to play a direct role in jasmonate signal perception (Chini *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008). Van Poecke and Dicke (2003) found that indirect defense ability was not affected in

JA-Ile-deficient *Arabidopsis* plants. Wang *et al.* (2007b) showed that many jasmonate-mediated responses in *N. attenuata* are dependent on jasmonates other than JA-Ile; JA-Ile also seems not to play a role in the indirect defense of *N. attenuata* (E. Gaquerel, N. Heinzel, M. Schuman and S. Meldau, unpublished data). Yet *N. attenuata* transformants with significantly impaired jasmonate biosynthesis and signaling showed reduced indirect defense ability in the same environment from which these accessions originated (Skibbe *et al.*, 2008; Halitschke *et al.*, 2007). Thus, although most differences in volatile profiles do not correlate well to differences in jasmonate signaling, it is likely that those which do are important for indirect defense, and the mechanism underlying this relationship is still poorly understood. Comparisons of two *N. attenuata* inbred lines originating from different accessions, the UT line and a line from Arizona (AZ), revealed that AZ is not impaired in indirect defense when compared to UT in the UT native habitat, despite having ca. 50% less JA and producing no *trans*-α-bergamotene (Glawe *et al.*, 2003; Steppuhn *et al.*, 2008; Wu *et al.*, 2008) similarly to accession 1. Many VOCs may be able to serve as effective indirect defense signals (Dicke *et al.*, 2003), and there may be species-specific threshold levels of JA which are important for their elicitation (Stork *et al.*, 2009).

For *trans*-α-bergamotene, which has been shown to function as an indirect defense in nature (Kessler & Baldwin, 2001; Halitschke *et al.*, 2008), differences in emission after W+OS treatment were significantly and positively correlated with average endogenous JA levels in each accession 1 h after W+OS treatment, and negatively correlated to endogenous SA levels after W+OS (Fig. 4). The same was true of only two other components found in total detectable leaf VOC profiles: an unidentified sesquiterpene which is thought to be co-regulated with *trans*-α-bergamotene (Gaquerel *et al.*, 2009), and methyl salicylate, for which emission levels corresponded well to the evident cross-talk between JA and SA in phytohormone analyses of individual leaves (Fig. 3). Supplementing plants with MJ to eliminate differences among accessions in OS-elicited jasmonate production did reduce differences in the emission of *trans*-α-bergamotene, which we may directly compare between the separate W+OS and MJ elicitations, because it is emitted in comparable levels across the plant growth stages used in these experiments (Table S5). Thus jasmonate deficiency may account for reduced *trans* -α-bergamotene production in some, but not all accessions: accession 1 emitted little or no *trans*-α-bergamotene after either treatment, and is likely mutated in the biosynthesis in this sesquiterpene rather than its signaling.

Because we conducted our experiments in a controlled environment, we assume that the differences we detected have a genetic basis. Delphia *et al.* (2009) found that variation in VOC emissions in co-occurring genotypes of horse nettle was heritable, and that inbreeding reduced variety, which is interesting given that we found several VOCs present in wild accessions which were not detected in UT (Fig. 2). Accessions of *N. attenuata* are likely polymorphic for one or more signaling genes as well as one or more genes directly controlling volatile biosynthesis (accession 1 produces almost no *trans*-α-bergamotene, and UT does not produce the monoterpene *trans*-β-ocimene: Fig. 1, Table S6). Wu *et al.* (2008) and Steppuhn *et al.* (2008) found many of the same differences between the UT and AZ accessions, and Wu *et al.* (2008) showed that these differences could be accounted for in part by expression level polymorphisms (ELPs) in known signaling genes such as *Nawipk* (see Fig. 4); ELPs may be particularly important in explaining intraspecific variation (Carroll 2008). Polymorphism in a single biosynthetic gene can also have a significant impact on defense signaling: a study by Pajerowska-Mukhtar *et al.* (2008) showed that different alleles of the *Solanum tuberosum* gene for allene oxide synthase (AOS, an enzyme necessary for jasmonate biosynthesis, Fig. 4d) resulted in different levels of resistance against pathogens when

expressed in *Arabidopsis thaliana* AOS null transformants. On the other hand, mechanisms underlying variation may be multiple and complex. Kliebenstein *et al.* (2002) found that for most glucosinolates produced by *A. thaliana*, differences among accessions are influenced by multiple quantitative trait loci (QTL), which also determine the plasticity of glucosinolate production in response to MJ and SA induction. Furthermore, epigenetic mechanisms are emerging as important players in polymorphism of herbivore induced responses (Pandey *et al.*, 2007).

Little is known about the mechanisms of functional genetic diversity in *N. attenuata* or its role in the fitness of individual plants. Polymorphism in the signaling response to herbivore attack may be widespread within, as well as between populations, as is thought to be the case for neutral genetic variation in this species due to its long and variable seed dormancy and recent spread (Bahulikar *et al.*, 2004). We predict that herbivores of *N. attenuata* and their predators must cope with a great variety of signaling and defense responses, even on a spatial scale relevant for a single insect. Perhaps the compounds which are most important for this interaction are limited to those which do not vary after elicitation, though this seems unlikely given the importance and variability of *trans*-α-bergamotene (Figs. 1 & 2, Tables S1 & S6) and of other volatiles including linalool which are emitted by only a few genotypes of *N. attenuata* (Kessler & Baldwin, 2001; Halitschke *et al.*, 2000; I.T. Baldwin, personal communication). Even if this is the case, the question remains: why should total VOC emission profiles vary so dramatically when plants are elicited? Is this a result of competing evolutionary pressures to be more or less apparent, or employ a more effective indirect defense than a neighboring plant - which in populations of *N. attenuata* is unlikely to come from the same parent? Is it just a byproduct of variation in biosynthetic pathways?

Results of studies like this are prerequisite to understanding how insects learn to respond to varying volatile signals in a polymorphic plant population, and how these responses may affect plant evolutionary fitness.

Acknowledgements

We thank Danny Kessler for collecting seeds from the field, Brigham Young University for the use of their awesome field station, the Lytle Ranch Preserve, Dr. Stephan von Reuss for providing standards of *cis*- and *trans*-α-bergamotene, Dr. A. F. Barrero for providing α-duprezianene, Firmenich (Switzerland) for providing a standard mixture of *cis*- and *trans*-β-ocimene, Emily Wheeler for editorial assistance, and the Max Planck Society for financial support.

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Supplemental

Supplemental data for the identification of α-duprezianene

EI MS of α-duprezianene and its hydrogenation product

peak 1 at 11.45 min: 204($M^{+\cdot}$, 43%), 189($M^{+\cdot}$-$CH_3^{\cdot}$, 15), 161 (100), 148($M^{+\cdot}$-C_4H_8, 35), 147(15), 137(20), 136(15), 135(25), 134(15), 133(30), 123(23), 122(23), 121(22), 120(40), 119(90), 109(35), 108(10), 107(39), 106(15), 105(60), 95(20), 94(25), 93(90), 92(15), 91(55), 82(18), 81(43), 79(55), 77(35), 69(80), 67(25), 55(38), 53(10).

Hydrogenation of 11.45 peak: peak at 11.50 min, 34%: 206($M^{+\cdot}$, 65%), 191($M^{+\cdot}$-$CH_3^{\cdot}$,85), 163(77), 149(8), 135(31), 124(8), 123(43), 122(60), 121(62), 109(30), 108(39), 107(65), 95(100), 93(76), 91(15), 82(88), 81(69), 79(38), 69(69), 67(23), 55(85), 43(42), 41(77).

peak at 12.08 min, 66%: 206($M^{+\cdot}$, 40%), 191($M^{+\cdot}$-$CH_3^{\cdot}$, 55), 178($M^{+\cdot}$-C_2H_4, 65), 163(100), 150($M^{+\cdot}$-C_4H_8, 20), 149(10), 135(35), 124(8), 123(43), 122(38), 121(48), 109(15), 107(40), 95(60), 93(43), 91(15), 82(50), 81(38), 79(15), 69(15), 67(23), 55(12).

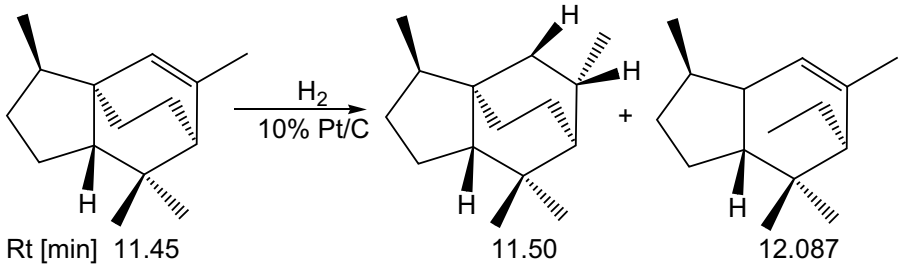

Figure S1. Hydrogenation products

Note: This structure is identified as α-duprezianene in accordance with Barrero *et al.* (1996, 2000). The NIST mass spectral database incorretly lists this spectrum as belonging to β-duprezianene.

Table S1 – Leaf volatile organic compounds (VOCs) differentially emitted ($P < 0.05$, one-way ANOVAs) among five *N. attenuata* accessions 24 to 32 h after wounding and application of *M. sexta* oral secretions (W+OS) are listed with their chemical class, their retention times (RT) in seconds (s) on the first (1D) and second dimensions (2D), their retention indices (RI), their F and *P* values and their average intensity for each accession (in % of internal standard ± SEM).

VOC	Class	RT 1D (s)	RT 2D (s)	RI	W+OS elicitation											
							Accessions									
					$F_{4.33}$	*P*	UT		1		2		3		4	
3-Methylbut-2-enal	Terpenoid	162	2.17	ND	11.68	< 0.0001	0.2 ± 0.1		0.3 ± 0.1		0.4 ± 0.2		1.4 ± 0.2		0.6 ± 0.2	
Unknown	Unknown	234	2.23	851	4.73	0.004	4.6 ± 1.1		1.1 ± 0.3		3.1 ± 0.8		6.2 ± 1.4		3.2 ± 0.5	
cis-3-Hexenol	Fatty acid derivative	246	2.65	859	8.84	< 0.0001	34.9 ± 10.0		25.0 ± 5.6		81.6 ± 21.2		131.9 ± 18.9		36.7 ± 12.2	
Unknown	Unknown	360	4.18	922	3.10	0.028	0.5 ± 0.1		0.6 ± 0.1		0.7 ± 0.2		1.2 ± 0.3		1.1 ± 0.2	
cis-Ocimene	Terpenoid	630	2.39	1047	3.20	0.025	-		0.3 ± 0.2		0.6 ± 0.4		1.5 ± 0.5		0.5 ± 0.2	
trans-β-Ocimene	Terpenoid	648	2.42	1056	5.23	0.0023	-		26.4 ± 7.2		60.5 ± 21.2		82.2 ± 19.1		38.4 ± 5.0	
Unknown	Unknown	762	3.60	1112	2.83	0.0402	-		0.1 ± 0.0		0.1 ± 0.0		0.2 ± 0.1		0.2 ± 0.1	
Benzyl cyanide	Benzenoid	822	5.64	1147	9.04	< 0.0001	0.1 ± 0.1		0.4 ± 0.1		-		0.6 ± 0.1		1.3 ± 0.3	
Unknown	Unknown	828	2.97	1149	5.30	0.0021	-		0.7 ± 0.2		1.2 ± 0.3		1.8 ± 0.5		1.2 ± 0.2	
Methyl salicylate	Benzenoid	912	4.03	1192	5.19	0.0023	0.7 ± 0.3		4.9 ± 1.6		8.8 ± 2.1		1.3 ± 0.5		3.4 ± 1.4	
Unknown	Unknown	912	2.71	1192	5.09	0.0026	0.8 ± 0.4		1.7 ± 0.3		6.5 ± 2.3		12.8 ± 4.4		2.2 ± 0.9	
Unknown	Unknown	948	4.47	1213	10.59	< 0.0001	2.7 ± 0.6		0.4 ± 0.1		0.5 ± 0.1		2.1 ± 0.5		2.7 ± 0.4	
Unknown	Unknown	996	3.05	1242	4.99	0.0029	-		-		-		0.2 ± 0.1		-	
Unknown	Unknown	1092	4.27	1298	9.15	< 0.0001	1.2 ± 0.4		0.1 ± 0.1		0.2 ± 0.1		0.1 ± 0.1		-	
cis-3-hexenyl tigilate	Fatty acid derivative	1140	2.98	1329	4.47	0.0059	-		-		0.6 ± 0.3		1.0 ± 0.2		0.3 ± 0.2	
cis-Hexenyl hexanoate	Fatty acid derivative	1164	2.59	1345	9.81	< 0.0001	-		-		-		0.5 ± 0.2		0.1 ± 0.1	
Unidentified sesquiterpene	Terpenoid	1176	3.39	1353	6.72	0.0005	2.7 ± 0.9		0.1 ± 0.0		0.4 ± 0.2		2.2 ± 0.6		0.5 ± 0.3	
trans-α-Bergamotene	Terpenoid	1302	2.39	1435	5.88	0.0001	5.2 ± 1.2		-		0.7 ± 0.4		6.4 ± 1.7		1.6 ± 0.8	
α-Farnesene	Terpenoid	1410	2.55	1510	3.15	0.0009	-		-		0.1 ± 0.0		1.5 ± 0.6		-	
cis-Hexenyl benzoate	Fatty acid derivative	1494	3.76	1572	2.68	0.049	-		0.2 ± 0.2		1.0 ± 0.5		1.0 ± 0.4		0.2 ± 0.2	

Table S2 – Control, wounding plus water- (W, control for W+OS), and W+OS-induced levels of phytohormones in treated leaves of *N. attenuata* at 1 h post-induction and results of 2-way ANOVAs for each phytohormone with treatment and accession as factors; significant *P* -values are in **bold**.

Hor-mone	Accession	Treatment	Average ± SEM	N	Accession effect	Treatment effect	Interaction
JA	UT	Control	162.6 ± 77.85	5	$F_{4,59} = 9.49$	$F_{2,59} = 273$	$F_{8,59} = 1.40$
		W	1438 ± 219.5	5	***P* < 0.0001**	***P* < 0.0001**	$P = 0.216$
		W+OS	5372 ± 591.2	5			
	1	Control	159.3 ± 46.07	5			
		W	733.1 ± 67.41	4			
		W+OS	2927 ± 193.3	5			
	2	Control	101.0 ± 13.50	5			
		W	482.8 ± 93.67	5			
		W+OS	1413 ± 195.6	5			
	3	Control	224.3 ± 90.90	5			
		W	1267 ± 58.86	5			
		W+OS	5520 ± 480.2	5			
	4	Control	256.1 ± 126.7	5			
		W	777.0 ± 93.52	5			
		W+OS	3630 ± 471.8	5			
JA-Ile	UT	Control	5.047 ± 0.9411	5	$F_{4,59} = 16.2$	$F_{2,59} = 369$	$F_{8,59} = 7.09$
		W	40.55 ± 3.058	5	***P* < 0.0001**	***P* < 0.0001**	***P* < 0.0001**
		W+OS	112.0 ± 7.984	5			
	1	Control	5.946 ± 1.072	5			
		W	11.48 ± 0.6502	4			
		W+OS	25.98 ± 2.237	5			
	2	Control	4.698 ± 0.6017	5			
		W	17.70 ± 2.371	5			
		W+OS	51.58 ± 5.382	5			
	3	Control	5.503 ± 0.3859	5			
		W	15.19 ± 1.413	5			
		W+OS	38.21 ± 2.282	5			
	4	Control	6.452 ± 2.379	5			
		W	18.11 ± 3.697	5			
		W+OS	84.34 ± 9.484	5			
SA	UT	Control	101.3 ± 36.30	5	$F_{4,59} = 2.58$	$F_{2,59} = 1.62$	$F_{8,59} = 1.09$
		W	243.6 ± 87.18	5	***P* = 0.047**	$P = 0.207$	$P = 0.386$
		W+OS	165.2 ± 31.55	5			
	1	Control	329.7 ± 110.6	5			
		W	420.0 ± 150.8	4			
		W+OS	461.5 ± 104.2	5			
	2	Control	599.4 ± 195.5	5			
		W	306.9 ± 34.93	5			
		W+OS	583.3 ± 97.80	5			
	3	ControlW	191.2 ± 42.00281.5 ± 65.87	55			
		W+OS	194.0 ± 19.72	5			
	4	Control	303.0 ± 130.1	5			
		W	268.7 ± 59.37	5			
		W+OS	473.3 ± 221.2	5			
ABA	UT	Control	143.1 ± 16.92	5	$F_{4,59} = 21.7$	$F_{2,59} = 9.49$	$F_{8,59} = 3.35$
		W	148.6 ± 12.58	5	***P* < 0.0001**	***P* = 0.0003**	***P* = 0.0032**
		W+OS	145.8 ± 21.77	5			
	1	Control	73.58 ± 12.18	5			
		W	72.38 ± 5.074	4			
		W+OS	89.52 ± 6.299	5			
	2	Control	62.12 ± 6.41	5			
		W	100.0 ± 10.15	5			
		W+OS	83.76 ± 7.752	5			
	3	Control	55.01 ± 9.128	5			
		W	128.7 ± 17.70	5			
		W+OS	110.6 ± 16.20	5			
	4	Control	140.7 ± 12.51	5			
		W	127.7 ± 8.230	5			
		W+OS	189.0 ± 12.70	5			

Table S3 – Control, W-, and W+OS-induced transcript levels for genes involved in JA-mediated signaling in treated leaves of *N. attenuata* at 1 h post-induction, and results of 2-way ANOVAs with accession and treatment as factors; significant *P*-values are in **bold** .

Gene	Acces-sion	Treatment	Average ± SEM	N	Accession effect	Treatment effect	Interaction
Nawipk	UT	Control	0.0034 ± 0.0005	5	$F_{4,58} = 1.81$	$F_{2,58} = 651$	$F_{8,58} = 2.31$
		W	0.0321 ± 0.0038	5	$P = 0.139$	$\mathbf{P < 0.0001}$	$\mathbf{P = 0.032}$
		W+OS	0.2118 ± 0.0178	5			
	1	Control	0.0048 ± 0.0009	4			
		W	0.0252 ± 0.0035	5			
		W+OS	0.1718 ± 0.0127	5			
	2	Control	0.0087 ± 0.0024	5			
		W	0.0363 ± 0.0062	5			
		W+OS	0.1551 ± 0.0161	5			
	3	Control	0.0045 ± 0.0005	4			
		W	0.0497 ± 0.0108	5			
		W+OS	0.2312 ± 0.0249	5			
	4	Control	0.0043 ± 0.0005	4			
		W	0.0344 ± 0.0044	5			
		W+OS	0.2107 ± 0.0174	5			
Nalox3	UT	Control	0.0746 ± 0.0109	5	$F_{4,58} = 2.85$	$F_{2,58} = 201$	$F_{8,58} = 0.764$
		W	0.2024 ± 0.0195	5	$\mathbf{P = 0.032}$	$\mathbf{P < 0.0001}$	$P = 0.635$
		W+OS	0.3511 ± 0.0272	5			
	1	Control	0.0693 ± 0.0077	5			
		W	0.1227 ± 0.0078	5			
		W+OS	0.2677 ± 0.0161	5			
	2	Control	0.0691 ± 0.0050	5			
		W	0.1635 ± 0.0109	5			
		W+OS	0.2654 ± 0.0439	5			
	3	Control	0.0734 ± 0.0119	5			
		W	0.1914 ± 0.0252	5			
		W+OS	0.3409 ± 0.0402	5			
	4	Control	0.0698 ± 0.0093	5			
		W	0.1506 ± 0.0137	4			
		W+OS	0.2858 ± 0.0229	4			
Najaz1	UT	Control	0.0016 ± 0.0006	5	$F_{4,55} = 3.91$	$F_{2,55} = 534$	$F_{8,55} = 0.660$
		W	0.0801 ± 0.0151	5	$\mathbf{P = 0.007}$	$\mathbf{P < 0.0001}$	$P = 0.724$
		W+OS	0.2347 ± 0.0326	5			
	1	Control	0.0009 ± 0.0007	4			
		W	0.0402 ± 0.0054	5			
		W+OS	0.1482 ± 0.0263	5			
	2	Control	0.0008 ± 0.0003	4			
		W	0.0538 ± 0.0101	5			
	3	W+OS	0.1215 ± 0.0171	5			
	3	Control	0.0010 ± 0.0004	4			
		W	0.0789 ± 0.0218	4			
		W+OS	0.1959 ± 0.0279	5			
	4	Control	0.0005 ± 0.0002	5			
		W	0.0501 ± 0.0023	4			
		W+OS	0.1542 ± 0.0187	5			

Table S4 – Results of individual t-tests comparing intensities measured for the emission of 60 common plant volatiles 24-32 h after methyl jasmonate (MJ) elicitation from UT plants (N = 6) which had either been treated by wounding and applying *Manduca sexta* oral secretions (W+OS) 1 week prior to MJ elicitation (W+OS T1 → MJ T2) or not treated (CRTL T1 → MJ T2)

RT (1D, 2D) (s)	Plant volatiles	W+OS T1→ MJ T2 Mean		CTRL T1 → MJ T2 Mean		P value	S:significant (P < 0.05) NS: non significant
		Average	Stdev	Average	Stdev		
174 , 1.830	hexanal	1.10	0.94	1.79	0.70	0.1819	NS
174 , 1.985	cis-3-hexenal	-	-	-	-	-	-
240 , 2.535	trans-3-hexen-1-ol	1.81	4.43	2.39	5.86	0.8506	NS
246 , 2.630	cis-3-hexen-1-ol	15.87	17.57	22.87	23.00	0.5683	NS
246 , 2.895	trans-2-hexen-1-al	-	-	-	-	-	-
270 , 2.770	trans-2-hexen-1-ol	0.94	2.30	0.37	0.58	0.5802	NS
276 , 2.600	1-hexanol	4.00	2.43	5.27	2.82	0.4263	NS
276 , 2.840	cis-2-hexen-1-ol	-	-	-	-	-	-
294 , 3.210	cis-4-hexen-1-ol	-	-	-	-	-	-
384 , 2.985	cis-3-hexenyl formate	-	-	-	-	-	-
396 , 2.055	alpha-pinene	13.58	3.64	7.79	8.16	0.1636	NS
462 , 4.740	benzaldehyde	29.97	23.08	28.52	20.81	0.9113	NS
486 , 2.425	β-pinene	-	-	-	-	-	-
528 , 2.240	β-myrcene	2.98	1.12	2.59	1.02	0.5462	NS
546 , 2.445	α-phellandrene	-	-	-	-	-	
570 , 2.950	cis-3-hexenyl acetate	1.45	0.85	1.39	0.81	0.9096	NS
576 , 2.475	1,4-cineole	-	-	-	-	-	-
576 , 3.610	unknown	-	-	-	-	-	-
588 , 2.620	hexylacetate	-	-	-	-	-	-
594 , 2.945	trans-2-hexenyl acetate	-	-	-	-	-	-
600 , 2.350	D-limonene	6.80	2.06	7.44	2.70	0.6569	NS
606 , 2.650	1,8-cineole	-	-	-	-	-	
624 , 3.820	unknown	-	-	-	-	-	-
624 , 4.475	benzylalcohol	35.49	22.75	37.73	22.95	0.8686	NS
630 , 2.370	cis-β-ocimene	-	-	-	-	-	-
648 , 2.370	trans-β-ocimene	-	-	-	-	-	-
684 , 3.760	4-ethyl-cyclohexanonen	-	-	-	-	-	-
738 , 4.080	methyl benzoate	0.11	0.18	0.32	0.52	0.3755	NS
750 , 2.660	linalool	0.09	0.14	0.23	0.29	0.3286	NS
750 , 2.965	α-thujone	-	-	-	-	-	-
756 , 2.780	cis-3-hexenyl propionate	-	-	-	-	-	
768 , 2.530	hexyl propionate	-	-	-	-	-	-
774 , 2.820	trans-2-hexenyl propionate	-	-	-	-	-	-
834 , 2.610	cis-3-hexenyl isobutyrate	0.38	0.55	0.36	0.30	0.9342	NS
840 , 2.410	hexyl isobutyrate	-	-	-	-	-	-
876 , 3.860	ethyl benzoate	1.66	1.33	1.60	1.05	0.9321	NS
906 , 3.090	alpha-terpineol	4.40	2.13	3.57	1.51	0.4554	NS
912 , 2.695	cis-3-hexenyl butyrate	1.31	2.31	0.60	1.23	0.5289	NS
918 , 2.510	hexylbutyrate	-	-	-	-	-	-
924 , 2.710	trans-2-hexenyl butyrate	-	-	-	-	-	-
984 , 2.555	cis-3-hexenyl-2-methylbutanoate	0.23	0.46	0.19	0.33	0.8789	NS
996 , 2.555	cis-3-hexenyl-3-methylbutanoate	-	-	-	-	-	-
1002 , 4.455	benzylacetone	-	-	-	-	-	
1026 , 2.955	geraniol	-	-	-	-	-	-
1044 , 3.645	ethyl salicylate	-	-	-	-	-	-
1140 , 2.980	cis-3-hexenyl tiglate	0.23	0.55	0.20	0.31	0.9153	NS
1230 , 2.625	cis-3-hexenyl hexanoate	-	-	-	-	-	-
1236 , 2.435	hexyl hexanoate	-	-	-	-	-	-
1242 , 2.660	trans-2-hexenyl caproate	-	-	-	-	-	-
1248 , 2.605	longifolene	-	-	-	-	-	-
1248 , 4.020	cis-jasmone	-	-	-	-	-	
1260 , 2.445	alpha-gurjunene	-	-	-	-	-	-
1260 , 2.515	alpha-cedrene	0.03	0.07	0.03	0.08	0.9764	NS
1272 , 2.565	trans-β-caryophyllene	-	-	-	-	-	
1302 , 2.395	trans-α-bergamotene	3.88	5.16	1.41	0.99	0.3014	NS
1320 , 2.535	citronellylpropionate	-	-	-	-	-	-
1338 , 2.355	β-farnesene	-	-	-	-	-	-
1494 , 3.750	cis-3-hexenyl benzoate	-	-	-	-	-	-
1602 , 4.080	methyl jasmonate	-	-	-	-	-	-
1698 , 3.035	trans-trans-farnesol	-	-	-	-	-	-

Table S5 – Results of individual t-tests comparing intensities measured for the emission of 60 common plant volatiles 24-32 h after methyl jasmonate (MJ) elicitation from UT plants (N = 6) at rosette stage or elongation stage. Rosette plants were elicited and volatiles were trapped 1 week prior to elicitation and trapping of elongated plants under the same conditions. Compounds highlighted in blue appear in elongated but not in rosette-stage plants.

RT (1D, 2D) (s)	Plant volatiles	P value	S:significant ($P < 0.05$) NS: non significant
174 , 1.830	hexanal	0.044101544	S
174 , 1.985	cis-3-hexenal	-	-
240 , 2.535	trans-3-hexen-1-ol	0.5034848	NS
246 , 2.630	cis-3-hexen-1-ol	0.009200811	S
246 , 2.895	trans-2-hexen-1-al	-	-
270 , 2.770	trans-2-hexen-1-ol	0.008814719	S
276 , 2.600	1-hexanol	0.0915154	NS
276 , 2.840	cis-2-hexen-1-ol	-	-
294 , 3.210	cis-4-hexen-1-ol	-	-
384 , 2.985	cis-3-hexenyl formate	0.08933105	NS
396 , 2.055	alpha-pinene	0.01960987	S
462 , 4.740	benzaldehyde	0.09791989	NS
486 , 2.425	β-pinene	-	-
528 , 2.240	β-myrcene	0.048949633	S
546 , 2.445	α-phellandrene	0.6266898	NS
570 , 2.950	cis-3-hexenyl acetate	0.014109192	S
576 , 2.475	1,4-cineole	-	-
576 , 3.610	unknown	-	-
588 , 2.620	hexylacetate	-	-
594 , 2.945	trans-2-hexenyl acetate	-	-
600 , 2.350	D-limonene	0.060221992	NS
606 , 2.650	1,8-cineole	0.030538024	S
624 , 3.820	unknown	-	-
624 , 4.475	benzylalcohol	0.11708719	NS
630 , 2.370	cis-β-ocimene	-	-
648 , 2.370	trans-β-ocimene	0.09613336	NS
684 , 3.760	4-ethyl-cyclohexanonen	-	-
738 , 4.080	methyl benzoate	0.57467824	NS
750 , 2.660	linalool	0.03297097	S
750 , 2.965	α-thujone	-	-
756 , 2.780	cis-3-hexenyl propionate	0.008838891	S
768 , 2.530	hexyl propionate	0.019406999	S
774 , 2.820	trans-2-hexenyl propionate	-	-
834 , 2.610	cis-3-hexenyl isobutyrate	0.017076833	S
840 , 2.410	hexyl isobutyrate	-	-
876 , 3.860	ethyl benzoate	0.013269375	S
906 , 3.090	alpha-terpineol	0.014271427	S
912 , 2.695	cis-3-hexenyl butyrate	0.0204527	S
918 , 2.510	hexylbutyrate	-	-
924 , 2.710	trans-2-hexenyl butyrate	0.37390098	NS
984 , 2.555	cis-3-hexenyl-2-methylbutanoate	0.016881254	S
996 , 2.555	cis-3-hexenyl-3-methylbutanoate	-	-
1002 , 4.455	benzylacetone	0.13557152	NS
1026 , 2.955	geraniol	-	-
1044 , 3.645	ethyl salicylate	-	-
1140 , 2.980	cis-3-hexenyl tiglate	0.017050792	S
1230 , 2.625	cis-3-hexenyl hexanoate	-	-
1236 , 2.435	hexyl hexanoate	-	-
1242 , 2.660	trans-2-hexenyl caproate	-	-
1248 , 2.605	longifolene	-	-
1248 , 4.020	cis-jasmone	0.36321747	NS
1260 , 2.445	alpha-gurjunene	-	-
1260 , 2.515	alpha-cedrene	0.36321747	NS
1272 , 2.565	trans-β-caryophyllene	0.5819179	NS
1302 , 2.395	trans-α-bergamotene	0.22573934	NS
1320 , 2.535	citronellylpropionate	-	-
1338 , 2.355	β-farnesene	-	-
1494 , 3.750	cis-3-hexenyl benzoate	0.3632175	NS
1602 , 4.080	methyl jasmonate	-	-
1698 , 3.035	trans-trans-farnesol	-	-

Table S6 – Leaf volatile organic compounds (VOCs) differentially emitted ($P < 0.05$, one-way ANOVAs) among five *N. attenuata* accessions 24 to 32 h after application of methyl jasmonate (MJ) in lanolin are listed with their chemical class, their retention times (RT) in seconds (s) on the first (1D) and second dimensions (2D), their retention indices (RI), their F and *P* values and their average intensity for each accession (in % of internal standard ± SEM).

VOC	Class	RT 1D (s)	RT 2D (s)	RI	MJ supplementation									
							Accessions							
					$F_{4,33}$	*P*	UT	1	2	3	4
3-Methylbut-2-enal	Terpenoid	162	2.17	ND	5.92	0.001	2.3 ± 0.4	2.7 ± 0.5	2.4 ± 0.3	6.8 ± 1.5	2.9 ± 0.7
cis-Hexenal	Fatty acid derivative	180	2.09	811	3.80	0.012	-	1.3 ± 0.9	2.4 ± 0.8	8.9 ± 3.7	4.2 ± 1.3
cis-3-Hexenol	Fatty acid derivative	246	2.65	859	4.16	0.008	377.6 ± 41.8	252.6 ± 38.6	190.8 ± 41.9	295.1 ± 36.7	173.5 ± 33.9
Unknown	Unknown	258	5.33	868	4.44	0.006	3.3 ± 1.0	0.1 ± 0.1	1.0 ± 0.6	0.3 ± 0.2	1.1 ± 0.6
Unknown	Unknown	294	2.69	886	4.29	0.007	2.0 ± 0.9	7.5 ± 2.2	0.8 ± 0.8	6.3 ± 1.5	5.0 ± 1.5
α-Terpineol	Terpenoid	390	3.13	936	3.18	0.026	13.5 ± 2.5	7.2 ± 1.2	7.8 ± 1.3	20.3 ± 5.5	9.5 ± 2.4
Unknown	Unknown	444	3.50	961	3.65	0.015	-	0.1 ± 0.1	0.4 ± 0.1	0.8 ± 0.4	0.1 ± 0.1
Unknown	Unknown	480	4.91	977	3.73	0.013	1.0 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
Unknown	Unknown	504	4.52	986	7.33	0.0003	0.1 ± 0.1	1.0 ± 0.4	2.5 ± 0.5	3.0 ± 0.6	1.7 ± 0.5
β-Myrcene	Terpenoid	528	2.31	994	3.24	0.024	2.2 ± 0.3	1.8 ± 0.3	2.7 ± 0.6	4.5 ± 0.9	2.1 ± 0.7
6-Methylhept-5-en-2-one	Terpenoid	528	3.27	994	12.22	< 0.0001	1.4 ± 0.2	1.4 ± 0.3	1.8 ± 0.3	10.4 ± 2.4	3.5 ± 0.8
Unknown	Unknown	540	2.98	998	10.56	< 0.0001	-	0.1 ± 0.1	0.9 ± 0.4	2.7 ± 0.6	0.9 ± 0.3
cis-3-Hexenyl acetate	Fatty acid derivative	570	2.97	1015	3.89	0.011	0.1 ± 0.1	0.5 ± 0.2	-	0.3 ± 0.1	0.1 ± 0.1
Benzyl alcohol	Benzenoid	618	4.66	1042	3.95	0.010	43.5 ± 10.3	37.5 ± 12.1	123.0 ± 31.0	80.2 ± 15.6	35.5 ± 15.4
cis-Ocimene	Terpenoid	630	2.39	1047	5.58	0.002	-	0.3 ± 0.2	1.7 ± 0.7	4.1 ± 1.3	1.8 ± 0.5
trans-β-Ocimene	Terpenoid	648	2.42	1056	8.30	< 0.0001	-	22.0 ± 9.6	84.9 ± 26	183.1 ± 43.1	88.5 ± 23.8
Unidentified monoterpene	Terpenoid	678	2.73	1070	2.87	0.039	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	-	0.2 ± 0.0
Methyl benzoate	Benzenoid	738	4.19	1098	4.55	0.005	0.4 ± 0.2	0.5 ± 0.1	3.2 ± 1.0	1.1 ± 0.4	0.9 ± 0.2
Unknown	Unknown	762	3.21	1112	10.79	< 0.0001	-	0.5 ± 0.3	1.3 ± 0.3	3.9 ± 0.8	1.5 ± 0.5
Unknown	Unknown	762	3.60	1112	8.95	< 0.0001	-	0.2 ± 0.1	0.6 ± 0.1	1.5 ± 0.4	0.6 ± 0.1
Unknown	Unknown	780	3.94	1122	2.71	0.048	0.3 ± 0.1	0.4 ± 0.2	0.6 ± 0.2	0.9 ± 0.2	0.6 ± 0.0
Unidentified monoterpene oxide	Terpenoid	804	3.12	1136	4.24	0.007	0.4 ± 0.1	-	0.1 ± 0.1	-	0.1 ± 0.1
Unknown	Unknown	816	3.08	1142	7.30	0.0003	-	0.4 ± 0.3	0.9 ± 0.4	3.0 ± 0.7	1.3 ± 0.5
Benzyl cyanide	Benzenoid	822	5.64	1147	10.73	< 0.0001	0.4 ± 0.1	2.0 ± 0.5	0.1 ± 0.1	1.6 ± 0.3	2.3 ± 0.4
Unknown	Unknown	828	2.97	1148.64	8.39	< 0.0001	-	1.0 ± 0.5	2.5 ± 0.6	7.6 ± 2.1	2.7 ± 0.7
cis-3-Hexenyl isobutyrate	Fatty acid derivative	834	2.63	1152	3.54	0.017	7.2 ± 1.0	4.9 ± 0.9	4.0 ± 0.8	7.5 ± 0.9	4.2 ± 0.5
Acetic acid benzyl ester	Benzenoid	870	4.30	1171	4.62	0.005	-	0.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Ethyl benzoate	Benzenoid	876	3.93	1174	4.36	0.006	2.8 ± 0.2	2.9 ± 0.6	5.6 ± 1.0	3.0 ± 0.5	2.3 ± 0.4
cis-3-Hexenyl ester	Fatty acid derivative	882	2.84	1177	3.89	0.011	0.7 ± 0.2	0.8 ± 0.2	0.4 ± 0.1	1.3 ± 0.2	0.8 ± 0.1
Unidentified monoterpene	Terpenoid	888	3.43	1180	4.13	0.008	-	0.1 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.1
Carenal	Terpenoid	978	3.71	1232	7.64	0.0002	1.5 ± 0.5	-	0.1 ± 0.1	-	-
Unknown	Unknown	1092	3.81	1297.33	7.55	0.0002	0.4 ± 0.1	0.3 ± 0.1	1.6 ± 0.3	0.8 ± 0.2	0.3 ± 0.1
Unknown	Unknown	1098	5.33	1302.49	2.84	0.040	0.1 ± 0.0	0.4 ± 0.2	-	0.1 ± 0.1	0.4 ± 0.1
cis-Hexenyl ester	Fatty acid derivative	1236	2.85	1389.8	4.62	0.005	1.1 ± 0.3	0.8 ± 0.3	1.1 ± 0.4	3.1 ± 0.7	1.1 ± 0.3
Unidentified sesquiterpene	Terpenoid	1236	2.44	1389.55	3.45	0.019	1.6 ± 0.6	-	0.5 ± 0.5	-	-
Methyl salicylate	Benzenoid	912	4.03	1192.39	10.53	< 0.0001	0.1 ± 0.1	1.2 ± 0.3	3.4 ± 0.8	0.2 ± 0.1	0.8 ± 0.3
Unknown	Unknown	1092	4.27	1298.12	9.64	< 0.0001	5.3 ± 1.3	0.1 ± 0.0	1.3 ± 0.6	-	0.8 ± 0.5
cis-Hexenyl hexanoate	Fatty acid derivative	1164	2.59	1344.51	8.57	< 0.0001	0.9 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	2.1 ± 0.6	0.6 ± 0.2
Unidentified sesquiterpene	Terpenoid	1176	3.39	1352.73	8.49	< 0.0001	4.1 ± 1.1	0.4 ± 0.3	3.6 ± 0.8	11.8 ± 2.2	6.1 ± 2.0
trans-α-Bergamotene	Terpenoid	1302	2.39	1435.02	4.16	0.008	15.8 ± 5.5	0.6 ± 0.3	8.4 ± 3.0	38.6 ± 11.3	17.8 ± 9.2
α-Farnesene	Terpenoid	1410	2.55	1509.68	8.72	< 0.0001	0.1 ± 0.1	-	0.4 ± 0.2	13.4 ± 4.4	2.4 ± 0.9
Unknown	Unknown	1476	3.35	1558.81	3.46	0.019	2.6 ± 0.3	2.0 ± 0.5	1.4 ± 0.5	1.2 ± 0.3	0.9 ± 0.1
cis-Hexenyl benzoate	Fatty acid derivative	1494	3.76	1571.96	3.03	< 0.0001	1.1 ± 0.4	0.6 ± 0.2	5.3 ± 2.1	3.3 ± 1.0	1.5 ± 0.4

Table S7 – Results of Wilks' Lambda Test in MANOVA analyses of VOCs differentially emitted (*P* values < 0.05, one-way ANOVAs) among five *N. attenuata* accessions 24 to 32 h after W+OS or MJ elicitation using data from GCxGC-TOF analysis of samples.

Analysis[a]	**Num DF**	**Den DF**	**F**	**P**
Abundant VOCs (Fig. 1) after W+OS	12	82	9.615	< 0.0001
Abundant VOCs (Fig. 1) after MJ	12	82	45.762	< 0.0001
20 VOCs different after W+OS	80	58	3.29	< 0.0001
14 VOCs different after W+OS and MJ, MJ levels	56	80	4.164	< 0.0001
29 VOCs different after MJ	116	22	2.377	0.0094

Table S8 – Pearson's correlation matrix of abundant VOCs emitted from five *N. attenuata* accessions 24 to 32 h after W+OS or MJ elicitation; significant correlations (Fisher's r to Z) are in **bold**.

W+OS	α-duprezianene	*cis*-3-hexenol	*trans*-β-ocimene	*trans*-α-bergamotene				
	r	*P*	r	*P*	r	*P*	r	*P*
β-duprezianene	1	-	-0.052	0.769	0.016	0.930	0.183	0.295
cis-3-hexenol	-0.052	0.769	1	-	0.323	0.058	0.202	0.247
trans-β-ocimene	0.016	0.930	0.323	0.058	1	-	0.251	0.146
trans-α-bergamotene	0.183	0.295	0.202	0.247	0.251	0.146	1	-
MJ	α-duprezianene	*cis*-3-hexenol	*trans*-β-ocimene	*trans*-α-bergamotene				
	r	*P*	r	*P*	r	*P*	r	*P*
β-duprezianene	1	-	0.142	0.405	**-0.439**	**0.006**	-0.246	0.142
cis-3-hexenol	0.142	0.405	1	-	0.310	0.062	0.090	0.598
trans-β-ocimene	**-0.439**	**0.006**	0.310	0.062	1	-	**0.599**	**<0.001**
trans-α-bergamotene	-0.246	0.142	0.090	0.598	**0.599**	**<0.001**	1	-

CHAPTER 4

Jasmonate and ppHsystemin regulate key malonylation steps of 17-hydroxygeranyllinalool diterpene glycosides, the most abundant and effective direct defense against herbivores in *Nicotiana attenuata*

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Published in *The Plant Cell*, doi:10.1105/tpc.109.071449 (2010)

4.1 Abstract

We identified eleven 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) that occur in concentrations equivalent to starch (mg/g FM) in aboveground tissues of coyote tobacco (*Nicotiana attenuata*) and differ in their sugar moieties and malonyl sugar esters (0-2). Concentrations of HGL-DTGs, particularly malonylated compounds, are highest in young and reproductive tissues. Within a tissue, herbivore elicitation changes concentrations and biosynthetic kinetics of individual compounds. Using stably transformed *N. attenuata* plants silenced in jasmonate production (IR*lox3*) and perception (IR*coi1*), or production of *N. attenuata* hydroxyproline-rich glycopeptide systemin precursor (NappHS, IR*sys*) by RNAi (IR), we identified malonylation as

the key biosynthetic step regulated by herbivory and jasmonate signaling. We stably silenced *N. attenuata* geranylgeranyl diphosphate synthase (*Naggpps*, IR*ggpps*) to reduce precursors for the HGL-DTG skeleton, resulting in reduced total HGL-DTGs and greater vulnerability to native herbivores in the field. Larvae of the specialist tobacco hornworm (*Manduca sexta*) grew up to ten times as large on IR*ggpps*, and IR*ggpps* suffered significantly more damage from herbivores in *N. attenuata*'s native habitat than wild-type (WT) plants. We propose that high concentrations of HGL-DTGs effectively defend valuable tissues against herbivores, and that malonylation may play an important role in regulating the distribution and storage of HGL-DTGs in plants.

4.2 Introduction

Diterpene glycosides (DTGs) are a diverse group of terpenoid metabolites known for their elaborate structures and potential medical and dietary applications in humans (see for example Schiffman and Gatlin, 1993; Kim and Kinghorn, 2001; Gregersen *et al.*, 2004; DeMarino *et al.*, 2006). DTGs consist of a cyclic or acyclic diterpene (C_{20}) skeleton and attached sugar moieties which may bear additional functional groups. Stevioside, a cyclic DTG produced by stevia (*Stevia rebaudiana* Bertoni) and used as a sugar substitute, is 300 times as sweet as sugar (Hanson and Deolivera, 1993) and inhibits monosaccharide metabolism in the rat liver (Ishii *et al.*, 1987); Capsianoside, a linear DTG with a hydroxygeranyllinalool skeleton from pepper (*Capsicum annuum*), may alter the permeability of intestinal tight junctions in human cell lines (Hashimoto *et al.*, 1997). The term "terpenoid" encompasses all molecules derived from the condensation of the C5 precursor isopentenyl pyrophosphate (IPP; C_5) and its allylic isomer dimethylallyl pyrophosphate (DMAPP; C_5), which in higher plants are synthesized from one of two independent pathways: the mevalonic acid (MVA) pathway in the cytosol or the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway) in plastids (Newman & Chappell 1999, Lichtenthaler 1999). Plastidial geranylgeranyl pyrophosphate synthases (GGPPSs) catalyze the condensation of three molecules of IPP with DMAPP to produce the diterpenoid precursor geranylgeranyl pyrophosphate (GGPP, C_{20}) (Ohnuma *et al.* 1998; Dewick 2002).

Hydroxygeranyllinalool (HGL)-DTGs consist of a acyclic C_{20} 17-hydroxygeranyllinalool skeleton conjugated to sugar groups (glucose and rhamnose) via bonds at C-3 and C-17 hydroxylated carbons; these sugars are conjugated to the C'-2, C'-4 or C'-6 hydroxyl groups and malonyl groups which are typically connected to the C'-6-hydroxyl group of the glucose(s) (Taguchi et al., 2005; Xiao-Hong Yu et al., 2008). HGL-DTGs have been isolated from many members of the *Solanaceae* including tobacco (*Nicotiana spp.*) (Shinozaki, 1996; Snook, 1997; Jassbi, 2006), *C. annuum* (Izumitani, 1990; Hashimoto, 1997; Lee, 2006, 2007, 2008; De Marino, 2006) and wolfberry (*Lycium chinense*) (Terauchi, 1995, 1998). Snook and colleagues (1997) found HGL-DTGs to be highly abundant (>2.5% dry mass) in at least 26 *Nicotiana* species. Several studies have found negative correlations between total HGL-DTG content and the mass gained by lepidopteran larvae feeding on different species of *Nicotiana* (Lou and Baldwin, 2003), different cultivars of tobacco (*N. tabacum*) (Snook et al., 1997), or different transformed lines of the wild tobacco *N. attenuata* (Mitra *et al.*, 2008). No-choice assays in which isolated plant HGL-DTG fractions have been mixed into artificial diet indicate that these compounds have an antifeedant effect on lepidopteran larvae: the generalist tobacco budworm (*Heliothis virescens*) (Snook et al., 1997) and the solanaceous specialist tobacco hornworm *Manduca sexta* (Jassbi et al. , 2006).

Little is known about the dynamics and regulation of HGL-DTGs in *N. attenuata* and other plants. HGL-DTGs are not found on the surface or in the trichomes of *N. attenuata* leaves (Roda et al., 2003), although other diterpenoids have been found in trichomes of *N. tabacum* (Lin and Wagner, 1994; Guo and Wagner, 1995). Concentrations of particular HGL-DTGs vary seasonally in flowers of *L. chinense* (Terauchi *et al.*, 1997b). HGL-DTG biosynthesis depends on the production of GGPP in the plastid and sufficient free sugars and malonic acid and is thus likely correlated with photosynthesis. In *N. attenuata*, total HGL-DTG concentrations in leaves increase after treatment with the plant stress hormone methyl jasmonic acid (MJ) or feeding by *M. sexta* larvae (Keinänen *et al.*, 2001; Jassbi *et al.*, 2008).

A GGPPS characterized in *N. attenuata* (NaGGPPS) provides GGPP for HGL-DTG biosynthesis and plays a minimal role in primary metabolism (Jassbi et al., 2008). Jassbi and colleagues (2008) used transient virus induced gene silencing (VIGS) of *Naggpps* to reduce total HGL-DTG levels in *N. attenuata* plants, and found that *M. sexta* larvae grew three times as large on VIGS-*Naggpps* plants as on VIGS empty vector controls, and significantly larger than larvae on VIGS-*Napmt* (reduced nicotine) or VIGS-*Natpi* plants (reduced trypsin protease inhibitors: TPIs). Both nicotine and TPIs have a significant negative effect on *M. sexta* larval growth (Steppuhn *et al.*, 2004; Zavala et al., 2004). HGL-DTGs are thus a potent defense against lepidopteran herbivores, but their regulation and mechanism of action *in planta*, as well as their role in defense against plants' native herbivores have not been examined.

Most known herbivore-elicited defense metabolites are regulated by the jasmonate signaling pathway, which also mediates plant responses to abiotic stresses such as ultraviolet radiation and ozone, and developmental processes including tuberization, senescence and reproductive development (Schilmiller and Howe, 2005; Creelman and Mullet 1997; Lorenzo and Solano 2005). The first committed step in jasmonate biosynthesis is the oxygenation of -linolenic acid by a 13-lipoxygenase (NaLOX3 in *N. attenuata*) to the intermediate 13-HPOT (Vick and Zimmermann, 1984). Inverted repeat RNAi lines deficient in *Nalox3* (IR*lox3*) have severely reduced levels of all jasmonate hormones and are useful in revealing the role of jasmonates in coordinating induced defense responses (shown for antisense AS*lox3* lines in Halitschke and Baldwin, 2003). Some or all jasmonates effect signaling via interaction with the F-box protein COI1, a subunit of the SCF^{COI1} E3 ubiquitin ligase complex, which triggers the degradation of JAZ repressors of jasmonate signaling and permits the transcription of jasmonate-elicited genes (Xie *et al.*, 1998; Chini *et al.*, 2007; Thines *et al.*, 2007; Chung *et al.*, 2008; Ribot *et al.*, 2008). Inverted repeat RNAi lines deficient in *Nacoi1* (IR*coi1*) are impaired in both jasmonate biosynthesis and perception because jasmonates stimulate their own biosynthesis (Paschold *et al.*, 2007, 2008). Responses dependent on COI1-mediated jasmonate perception can be identified by supplementing IR*lox3* and IR*coi1* plants with jasmonates and determining which responses are restored in IR*lox3* , but not in IR*coi1*. In general, plants deficient in LOX3 or COI1 are highly susceptible to attack from herbivores and pathogens (Reymond *et al.*, 2000; Ellis and Turner 2002; Kessler *et al.*, 2004; Li et al., 2004; Mewis *et al.*, 2005; Chung *et al.*, 2008).

The hydroxyproline-rich systemin glycopeptides, encoded by a 146 aa precursor in tomato (*Lycopersicon esculentum*) (Pearce and Ryan, 2003) are wound-induced signals which trigger the induction of JA-mediated defenses. Two hydroxyproline-rich systemin glycopeptides derived from a 165 aa systemin-like glycopeptide precursor have been reported in *N. tabacum* (Pearce et al.,

2001; Ryan and Pearce, 2003), and a homologue of the *N. tabacum* precursor is known in *N. attenuata* (NappHS; Berger and Baldwin, 2007). Unlike the systemin-like glycopeptides in tomato (Pearce *et al*., 2008) and *N. tabacum* (Ren & Lu, 2006), NappHS does not play a central role in the induction of JA-mediated defense in vegetative tissues: inverted repeat NappHS lines (IR*sys*) are not more susceptible to native herbivores than are WT plants and have normal herbivore-elicited oxylipin dynamics (Berger and Baldwin, 2007). However, NappHS may influence JA signaling in flowers (Berger and Baldwin, 2009). Recently, Pearce and colleagues (2009) isolated a homologue of the tobacco and tomato hydroxyproline-rich systemin glycopeptides from black nightshade (*Solanum nigrum*) and showed that this peptide may be involved in early jasmonate signaling.

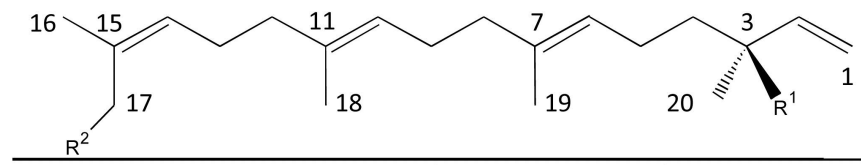
We identified several HGL-DTGs previously unknown in *N. attenuata*, bringing the total number to 11 in this species. We characterized the accumulation of these compounds in vegetative and reproductive plant tissues and followed their dynamics in plants which were left unelicited or elicited with wounding and *M. sexta* oral secretions (W+OS). Using IR*sys*, IR*lox3*, and IR*coi1* plants, we determined the roles of NappHS and jasmonate signaling in the herbivore-induced dynamics of individual HGL-DTGs. Lines stably silenced in *Naggpps* (IR *ggpps*) allowed us to assess the effect of total HGL-DTGs on the specialist *M. sexta* and generalist herbivores in *N. attenuata*'s native habitat. Given that IR*sys* plants, which we show to be impaired in the jasmonate-mediated accumulation of malonylated HGL-DTGs, are not more susceptible to native herbivores (Berger and Baldwin, 2007), we discuss the importance of total HGL-DTGs as a defense, and the possible roles of malonylation in the metabolism of HGL-DTGs.

4.3 Results

4.3.1 Identification, analysis and structure of HGL-DTGs in *N. attenuata*

We identified eleven HGL-DTGs which are highly abundant in aboveground tissues of *N. attenuata* (Figure 1; Supplemental Tables 1A-1D; Supplemental mass spectral data). HGL-DTGs differ in their sugar moieties and number of malonyl sugar esters (0-2) and can be divided into three classes: the precursor lyciumoside I, core molecules which are fully glycosylated but have no malonyl groups (lyciumoside IV, nicotianoside III, attenoside), singly malonylated compounds (nicotianosides I, IV, VI), and dimalonylated compounds (nicotianosides II, V, VII) (Figure 1). Malonylated HGL-DTGs are poorly described (Terauchi *et al*., 1998). We optimized the buffer composition (Supplemental Figure 1) and dilution of our extraction to maximize the yield of each individual compound; 40% methanol at a slightly acidic pH was the best compromise that maximized the yield of more water-soluble (precursor and core) and less water-soluble (malonylated) compounds and their ionization in liquid chromatography-mass spectrometry (LC-MS) measurements. Samples were diluted 50 to ensure that all compounds were measured in the linear range. From standard addition curves, we calculated that the most abundant HGL-DTGs are present at a concentration of 1-5 mg/g FM in WT leaf tissue (Supplemental Figure 2). We tested the durability and robustness of signal mass detection and the reproducibility of extraction and measurement conditions by analyzing injection replicates of an single leaf extract (N = 20). Reproducibility of extraction and measurement conditions was confirmed (relative standard deviation [RSD] lower than 10 %) for all but one HGL-DTG and for two internal standards (glycyrrhizinic acid and ^{2}D jasmonic acid): attenoside had a higher error (RSD 21%).

A



	Group*	R1 Sugar 1	R1 Sugar 2	R2 Sugar 1	R2 Sugar 2	Malonyl-groups
Lyciumoside I	1	Glc		Glc		
Lyciumoside II	1	Glc		Glc	Glc	
Lyciumoside IV	2	Glc	Rh	Glc		
Nicotianoside I	3	Glc	Rh	Glc		**1**
Nicotianoside II	4	Glc	Rh	Glc		**2**
Nicotianoside III	2	Glc	Rh	Glc	Rh	
Nicotianoside IV	3	Glc	Rh	Glc	Rh	**1**
Nicotianoside V	4	Glc	Rh	Glc	Rh	**2**
Attenoside	2	Glc	Rh	Glc	Glc	
Nicotianoside VI	3	Glc	Rh	Glc	Glc	**1**
Nicotianoside VII	4	Glc	Rh	Glc	Glc	**2**

* 1 Precursor 2 Core 3 Malonylated 4 Dimalonylated

B

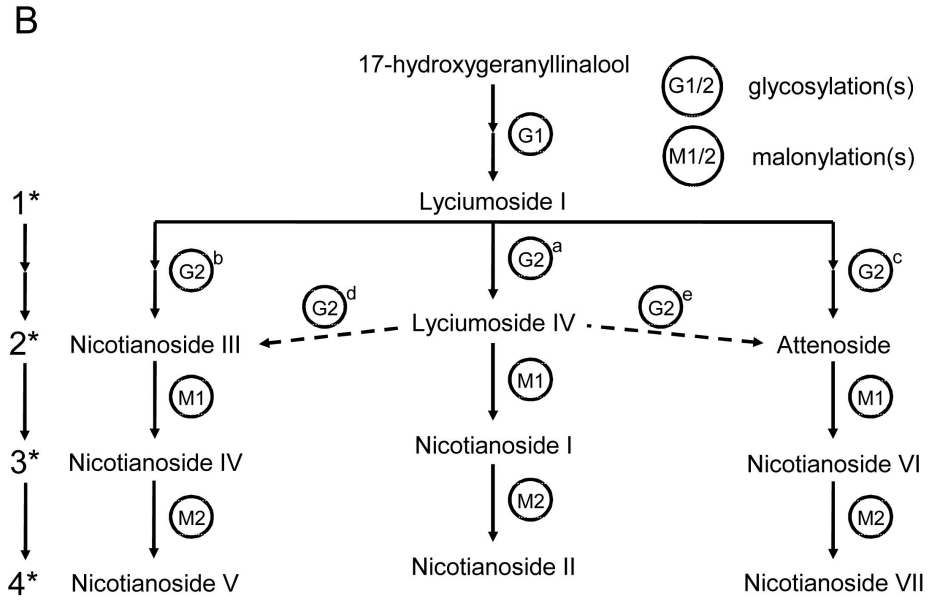


Figure 1 – Identity and Biosynthesis of 17-Hydroxygeranyllinalool Diterpene Glycosides (HGL-DTGs) (A) Newly identified and previously described compounds from leaves of *Nicotiana attenuata* differing in their sugar and malonyl groups are organized into the following biosynthetic groups: (1) precursor, (2) core, (3) singly malonylated, and (4) dimalonylated compounds. (B) Biosynthetic pathway: 17-hydroxygeranyllinalool is glycosylated at the C-3 and C-17 hydroxyl groups to lyciumoside I, the precursor for all HGL-DTGs. Additional glycosylations of lyciumoside I (G2a, b, c) or lyciumoside IV (G2d, e) produce larger " core" HGL-DTGs which may be malonylated once (M1) or twice (M2).

Table 1 – Normalized Tissue-Specific Pools of HGL-DTGs in Aboveground Tissues of Young Flowering (54 d old) Plants.

Tissue	Normalized tissue-specific pool (% total HGL-DTGs/% total FM)
senescent rosette leaves	31
senescing rosette leaves	99
young rosette leaves	170
elicited leaf position (S1)*	223
large stem leaves	265
small stem leaves	277
calyxes	215
flowers	296
buds	430

*First stem leaf. See tissues in Figure 2B

4.3.2 Ontogeny of HGL-DTG accumulation

We analyzed the distribution of HGL-DTG accumulation in aboveground tissues of *N. attenuata* plants at different growth stages (rosette, elongated, younger and older flowering plants) that had been left unelicited (control), or elicited with W+OS to mimic herbivory with a precisely defined kinetic. In a previous experiment, no HGL-DTGs were found in roots (not shown).

Plants' total pools of HGL-DTGs increased during growth until plants began to flower (Figure 2A): elongated plants produced more total HGL-DTGs than did rosette plants (multiple t-tests with Bonferroni-corrected P's; $P \leq 0.001$), and young flowering plants produced more than elongated plants ($P \leq 0.001$) regardless of treatment. Old flowering plants had 6-8 times the total HGL-DTG content as rosette-stage plants, but did not contain more total HGL-DTGs than young flowering plants ($P > 0.075$). Because young flowering plants contained the most vegetative and reproductive tissue types (only the ripe seed capsules were missing from this stage of growth) and still responded dynamically to W+OS elicitation, we present the allometrically corrected distribution of HGL-DTGs in this growth stage (Figure 2B; allometric correction explained in Baldwin, 1996).

The distribution of HGL-DTGs varied greatly among tissue types, ranging from nearly undetectable levels in stems to the highest concentrations in young leaves and reproductive organs. The ratio of mono- to di-malonylated DTGs was markedly different in reproductive tissues versus leaves. While dimalonylated HGL-DTGs were more abundant in leaves, singly malonylated compounds dominated the pools of buds, calyxes and flowers. For young flowering plants, the percentage of total HGL-DTGs in each tissue type was calculated and compared to the percentage of mass that different tissues represent in the plant to derive the normalized tissue-specific pools. This presentation reveals that HGL-DTGs are preferentially allocated to young and reproductive tissues (Table 1).

A single W+OS elicitation changed the allocation of HGL-DTGs in individual tissues but did not significantly change the whole-plant pool for any growth stage. Concentrations of singly malonylated HGL-DTGs increased in the elicited leaf position of elongated and young flowering plants

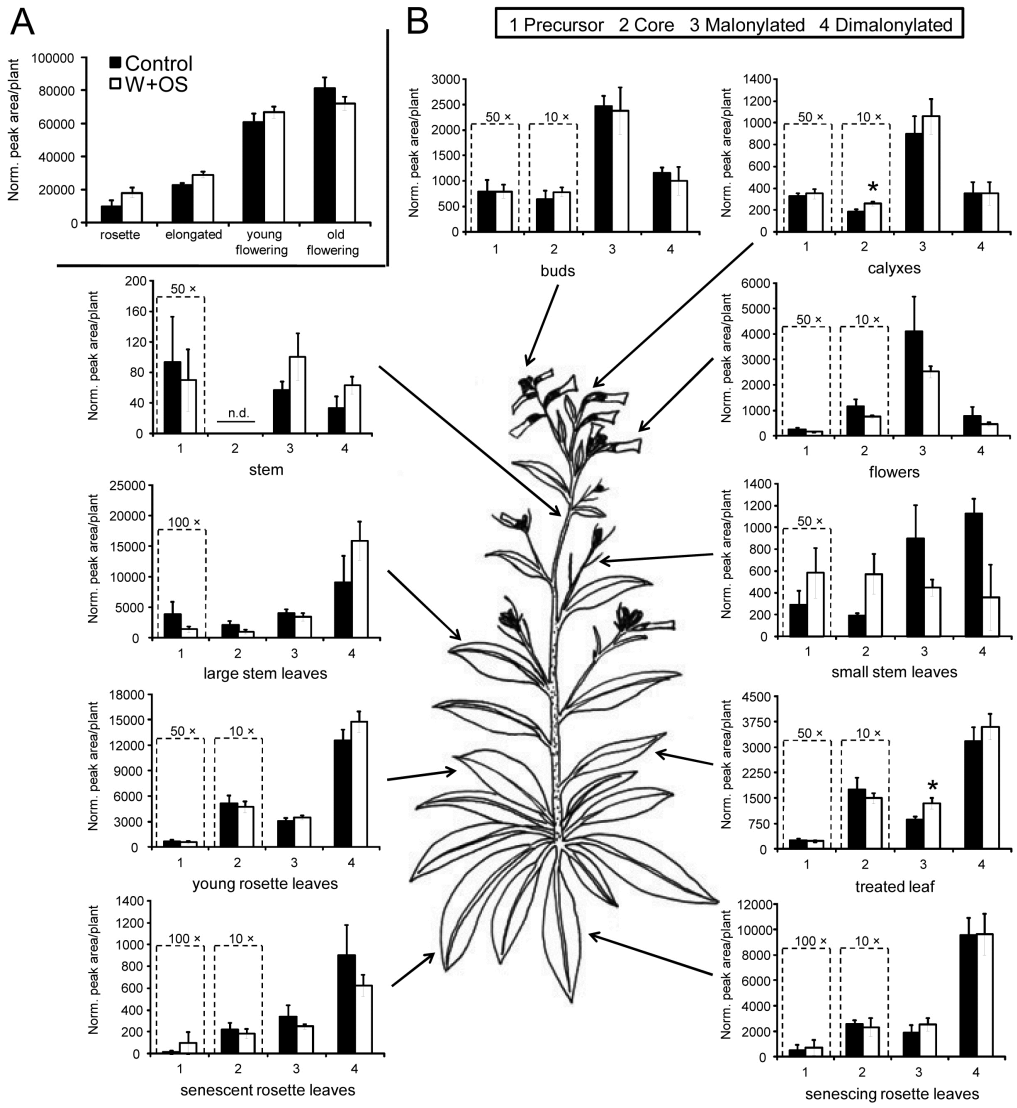


Figure 2 – *N. attenuata* Accumulates HGL-DTGs During Growth and Differentially Accumulates Them to Young and Reproductive Tissues. Plants were left unelicited (Control), or one leaf per plant was elicited with wounding and *Manduca sexta* oral secretions (W+OS). Aboveground tissues were harvested 3 d after treatment and pooled by tissue type. In a previous experiment, no HGL-DTGs were found in roots (not shown). (A) HGL-DTGs (average +SEM) accumulate as plants grow from the rosette stage (34 d old) and elongate (44 d old) through the initiation of flowering; total HGL-DTG pools are no different in young (54 d old) versus old (68 d) flowering plants. (B) Accumulation of HGL-DTGs in a young flowering plant. Singly malonylated compounds accumulate in reproductive tissues, whereas doubly malonylated compounds accumulate in leaves. The greatest amounts of HGL-DTGs are found in young and reproductive tissues. W+OS elicitation of a leaf changes the composition of HGL-DTGs in that leaf, likely by reallocation from the rest of the plant, because a single treatment has no significant effect on total HGL-DTG pools in (A). Note that some compounds have been multiplied by constants to fit the scale of the graph, and that scales change between graphs. *$P < 0.05$ in a Student's t-test.

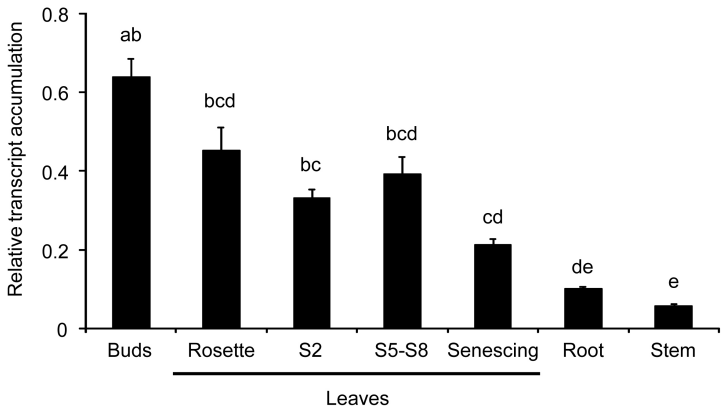


Figure 3 – *Naggpps* Transcript Accumulation in Tissues of Young Flowering Plants (54 d old) Relative to *N. attenuata actin* (Average +SEM). Different letters indicate significant differences ($P < 0.05$) in Bonferroni-corrected post-hoc tests following an ANOVA.

(individual t-tests; elongated, P = 0.010; young flowering, P = 0.031), whereas levels of core compounds increased in calyxes of W+OS-elicited young flowering plants (P = 0.044). There was no significant increase in the other HGL-DTG classes in elicited leaves, and no significant changes in any HGL-DTGs in elicited leaves of rosette-stage or old flowering plants.

Naggpps transcript accumulation in young flowering plants was found to be highest in buds, followed by green leaves (rosette and stem leaves had similar levels; Figure 3). Senescing leaves accumulated fewer *Naggpps* transcripts, likely due to their loss of active chloroplasts, and roots and stems had only very low levels (ANOVA, $F_{6,20} = 28.30$, $P < 0.0001$). Thus the expression of *Naggpps* correlates well with the relative concentrations of HGL-DTGs in different tissues.

4.3.3 Kinetics of HGL-DTG biosynthesis and elicitation

We analyzed the kinetics of HGL-DTG accumulation over one week in five green leaves of rosette-stage control and W+OS-elicited plants. Each biosynthetic group showed a distinct pattern of constitutive and W+OS-elicited dynamics which can be described as follows: concentrations of the precursor lyciumoside I always increased first and then fell as concentrations of core compounds increased; core compounds then stabilized as concentrations of malonylated compounds increased; and W+OS elicitation significantly increased the rate of biosynthesis of core and malonylated compounds (Student's t-tests, Figure 4, Supplemental Table 2). By the end of the experiment, concentrations of all core and malonylated HGL-DTGs except attenoside and nicotianoside II had increased significantly, whereas the precursor lyciumoside I had returned to initial levels (unpaired *t*-tests between the first and the last time point in control plants: nicotianoside II: P = 0.108; attenoside: P = 0.067; lyciumoside I: P = 0.137; all others, $P < 0.05$).

In W+OS-elicited leaves, accelerated biosynthesis of HGL-DTGs apparently resulted in a greater accumulation of the precursor lyciumoside I, leading to sustained increases in malonylated compounds above control levels. Malonylated and dimalonylated HGL-DTGs first accumulated in

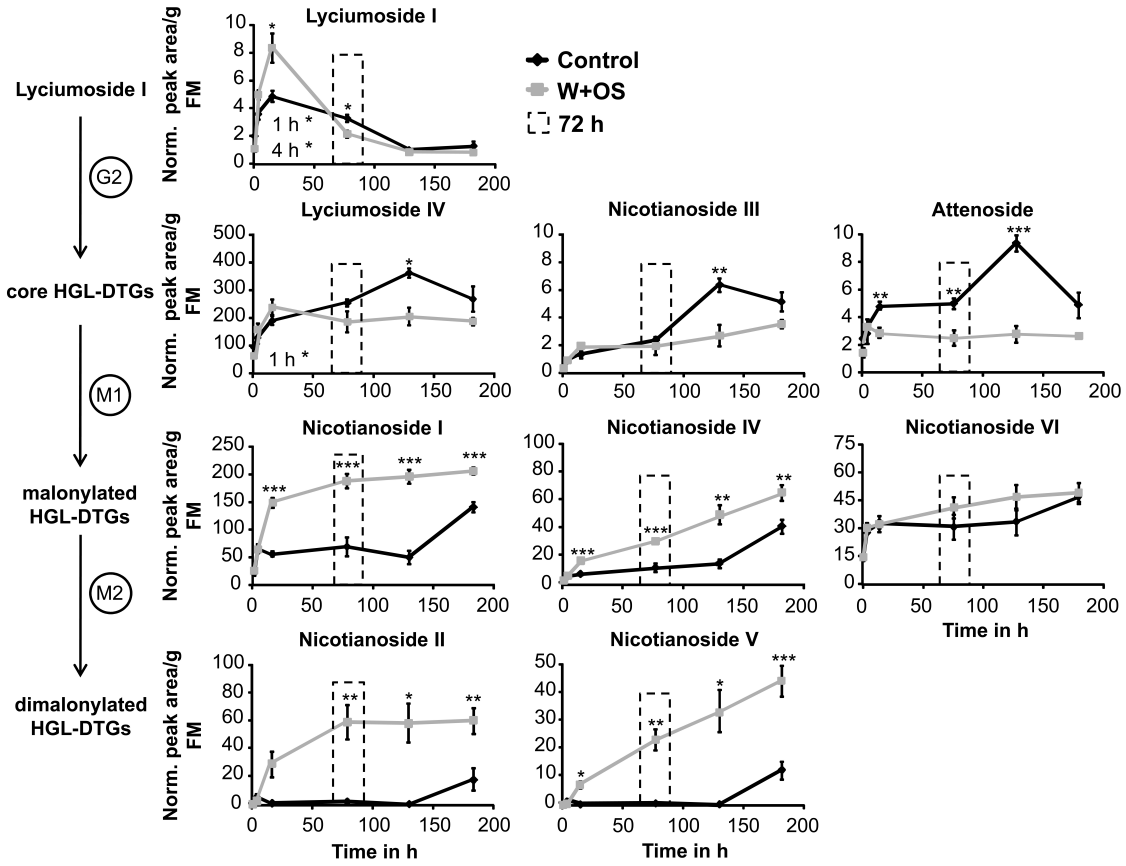


Figure 4 – The Different Biosynthetic Groups of HGL-DTGs Display Characteristic Dynamic Patterns Over One Week of Growth in Rosette-Stage Plants. Plants were left unelicited (Control), or five leaves per plant were elicited with W+OS. Elicited leaf positions were harvested and pooled from each plant (1 pooled sample per plant, N = 5) at one of five time points: 1 h, 4h, 14 h (night), and 3 d, 5 d and 7 d. The precursor lyciumoside I (average + SEM) attains maximum concentration during the night at 14 h and then decreases as other compounds accumulate. The peak value is significantly increased by W+OS elicitation, as is the slope of the decrease following the peak. The kinetics of core compound accumulation depend on the rate at which precursor levels decline: in unelicited leaves the core compounds peak at 120 h, after the precursor has declined from its uninduced peak, and then sharply decrease as malonylated compounds begin to accumulate. In W+OS-elicited leaves, the sharp decline in precursor levels following their 14 h peak likely supply substrates for the subsequent accumulation of core compounds, which do not peak, but are maintained at a constant level as they provide substrate for the synthesis of malonylated compounds. Singly malonylated compounds increase first, followed by dimalonylated compounds. The elevation in malonylated compounds begins concurrently with the elevation in core compounds and precursor levels in W+OS-elicited leaves, but at 7 d in unelicited leaves. All compounds reach stable elicited levels beginning at 3 d after W+OS elicitation (highlighted) with the exception of malonylated compounds derived from nicotianoside III, which continue to increase. Note that scales change among graphs. Asterisks indicate significant differences between W+OS-treated and control samples in Student's t-tests within each time point (*P ≤ 0.05, ** P < 0.01, *** P < 0.001).

W+OS-elicited leaves by 14 h: more than 6 d earlier than in control leaves. Concentrations of lyciumoside I were lower in W+OS-elicited than in control leaves at 1 h, but increased to concentrations much greater than in control leaves by 14 h before returning to control levels. Interestingly, lyciumoside I concentrations also peaked at 14 h in control leaves: as this was the only night time harvest, it suggests that precursors increase in concentrations during the night.

The core compounds lyciumoside IV and nicotianoside III accumulated similarly in elicited and control leaves until 3 d (although levels of lyciumoside IV were somewhat higher in control leaves 1 h after treatment), but then stabilized, whereas concentrations in control leaves continued to increase until d 5 and then fell concurrent with the rise in control levels of malonylated compounds at d 7. Among the core compounds, attenoside was the exception: concentrations were lower in elicited leaves beginning at 14 h. The "missing" attenoside could have been routed to synthesis of nicotianoside VII, its dimalonylated adduct, which was not determined in this experiment. The singly malonylated adduct of attenoside, nicotianoside VI was the only malonylated compound which accumulated no differently in W+OS-elicited leaves.

4.3.4 Jasmonate and NappHS signaling are required for HGL-DTG biosynthesis

WT plants and lines silenced in jasmonate (IR*lox3* and IR *coi1*) and NaHPPS signaling (IR*sys*) were elicitated with W+OS or jasmonate supplementation with methyl jasmonate in lanolin paste (lan+MJ). Concentrations of malonylated HGL-DTGs increased after W+OS or Lan+MJ treatment in WT leaves compared to control or lanolin (lan)-treated plants; precursor and core compounds decreased or remained constant (Supplemental Figure 3; ANOVA and Bonferroni post-hoc test results are in Supplemental Tables 5 and 6). The malonylated adducts of attenoside behaved differently than the other malonylated DTGs in that they increased more after W+OS than after Lan+MJ treatment, suggesting that the metabolism of attenoside is not entirely jasmonate-dependent or that nicotianosides VI and VII become substrates for unknown jasmonate-inducible product(s). For all genotypes, all differences which were significant between Lan and Lan+MJ-treated plants were also significant between control and Lan+MJ-treated plants except for nicotianoside IV and nicotianoside VI in WT, and Lan treatment did not affect control levels of HGL-DTGs (Supplemental Table 6).

Jasmonate and NaHPPS-silenced lines did not display WT accumulation of HGL-DTGs in leaves at 3 d after elicitation (Figure 5), when most induced changes in HGL-DTG metabolism are stable (Figure 4). The signaling mutants IR*lox3* , IR*coi1*, and IR*sys* all had reduced levels of malonylated HGL-DTGs, but this phenotype was far stronger in the jasmonate-silenced lines IR*lox3* and IR*coi1* (Figure 5; Supplemental Figure 3; ANOVA and Bonferroni post-hoc test results are in Supplemental Tables 3, 4 and 5). In IR*lox3*, reduced levels of malonylated compounds after W+OS elicitation could be fully recovered by Lan+MJ treatment for singly malonylated and partially recovered for dimalonylated HGL-DTGs. Lan+MJ treatment could not recover singly or dimalonylated HGL-DTGs in IR*coi1* plants, which had the greatest reduction in malonylated compounds and nearly undetectable levels of dimalonylated compounds. Thus malonylation is strongly dependent on jasmonate biosynthesis and perception, and higher levels of perceived jasmonates are required to add the second malonyl moiety; NappHS also influences the second malonylation step.

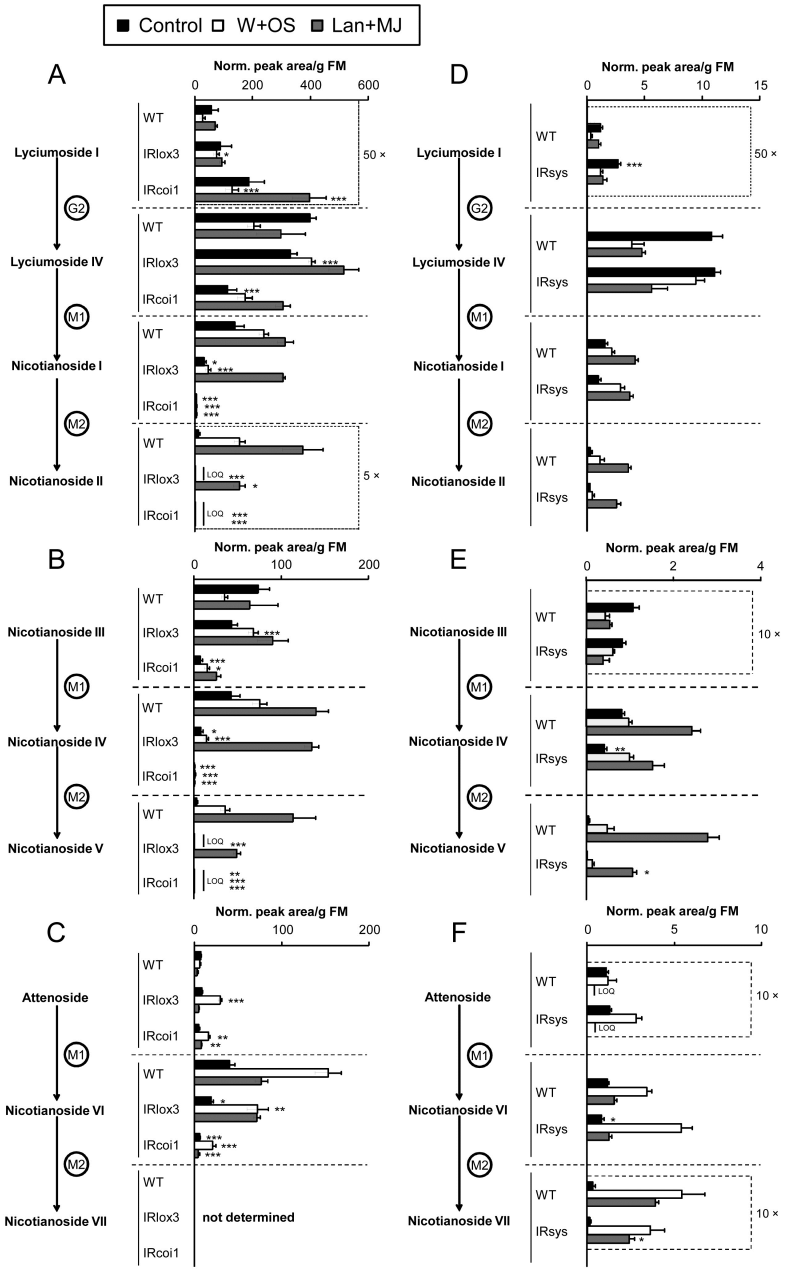


Figure 5 – Malonylation of HGL-DTGs is Regulated by Jasmonate Signaling (NaLOX3, NaCOI1) and Tuned by NappHS Signaling. Elongated *N. attenuata* plants were left unelicited (Control), or one leaf per plant was elicited with W+OS to mimic herbivory, with 150 µg methyl jasmonate (MJ) in 20 µL of lanolin paste to supplement jasmonate production, or with 20 µL of lanolin paste (lan) as a control for the Lan+MJ treatment. Elicited leaves were harvested at 3 d. Plants impaired in jasmonate biosynthesis (IR*lox3*) and perception (IR*coi1*) or in systemin-mediated signaling (IR*sys*) were compared to WT plants in two separate experiments using identical methods. In this figure we compare levels of each HGL-DTG among silenced lines and WT which have received the same treatment. The effects of treatments within genotypes are shown in Supplemental Figure 3 and Supplemental Tables 5 and 6. (continued on p. 112)

Continued Figure 5 – (A)-(C) Experiment 1: WT, IR*lox3*, IR*coi1*. (D)-(F) Experiment 2: IR*sys*, WT. Asterisks indicate significant differences from WT in one-way ANOVAs (WT, IR*lox3*, IR*coi1*, A-C) or Student's t-tests (WT, IR*sys*, D-F) within each treatment: *P ≤ 0.05, ** P < 0.01, *** P < 0.001 in Bonferroni-corrected tests. (Student's t-tests were also subjected to the Bonferroni comparison as the same data was used to analyze the effect of treatment on HGL-DTG accumulation within genotypes in Supplemental Figure 3 and Supplemental Tables 5 and 6.) Each graph represents the full biosynthetic pathway from glycosylation through malonylation for one set of HGL-DTGs. For example in (A), IR*coi1* plants accumulate the precursor lyciumoside I (average +SEM) and are impaired in their ability to convert it to the core compound lyciumoside IV at a WT rate, especially after Lan+MJ treatment; IR*lox3* plants accumulate lyciumoside IV, especially after W+OS elicitation. Both IR*coi1* and IR*lox3* are unable to malonylate lyciumoside IV in control and W+OS-elicited leaves. Malonylation is recovered in IR*lox3* and not in IR*coi1* by Lan+MJ. In contrast, (D) IR*sys* plants are able to synthesize WT levels of lyciumosides IV and nicotianosides I and II but like IR*coi1*, accumulate lyciumoside I in unelicited tissue; however, (E) and (F) IR*sys* plants are unable to accumulate WT levels of other dimalonylated HGL-DTGs (nicotianosides V and VII) after Lan+MJ treatment. Note that some compounds have been multiplied by constants to fit the scale of the graph, and that scales change between graphs. LOQ = below limit of quantification.

Whereas both NaCOI1 and NaLOX3 are required for malonylation, NaCOI1, but not NaLOX3 is involved in glycosylation of lyciumoside I to generate core compounds. Core compounds decreased in WT leaves after treatment with W+OS and Lan+MJ (except for attenoside after W+OS and nicotianoside III after Lan+MJ) but accumulated in IR*lox3* plants without Lan+MJ treatment (Supplemental Tables 3 and 6, Figure 5). IR*coi1* had reduced levels of core compounds other than attenoside in control leaves, but near-WT levels after W+OS elicitation. Lan+MJ treatment recovered concentrations of core molecules to WT levels in IR*coi1* leaves, likely due to an increase in the precursor pool rather than an increase in precursor glycosylation. Interestingly, concentrations of attenoside were not reduced in IR*coi1* leaves and were greater than WT concentrations after W+OS and Lan+MJ. IR*lox3* plants had WT levels of lyciumoside I, but IR*coi1* plants had concentrations 3.75 greater than WT after W+OS and 4.5 greater after Lan+MJ. In WT leaves the precursor lyciumoside I decreased after treatment with W+OS but did not change after treatment with Lan+MJ (Supplemental Table 6, Figures 5A and 5D). Control leaves of IR*sys* plants also had higher concentrations of lyciumoside I (Supplemental Table 4, Figure 5D).

Only the combination of deficient jasmonate biosynthesis and perception in IR *coi1* strongly influenced total levels of HGL-DTGs (Figure 6): both IR *lox3* plants and IR*coi1* plants had lower levels of HGL-DTGs, but the difference between IR*lox3* and WT was no longer significant following W+OS elicitation (Figure 6A) due to accumulation of core molecules by IR*lox3* (Figure 5A-5C). Accumulation of precursors in IR *coi1* plants did not compensate for their reduced production of most core and all malonylated compounds (Figures 5A-5C, Figure 6A; $P < 0.001$). After Lan+MJ treatment, total HGL-DTG levels were identical in WT and IR *lox3*, but still strongly reduced in IR*coi1* plants ($P < 0.001$). IR*sys* plants were not impaired in total HGL-DTG accumulation (Figure 6B).

4.3.5 Stably silencing *Naggpps* reduces total HGL-DTGs in *N. attenuata* with minimal non-target effects

Although silencing *Naggpps* may have unwanted effects on other GGPP-dependent metabolites, such as carotenoids, we concluded that it is the best gene target for knocking down total HGL-

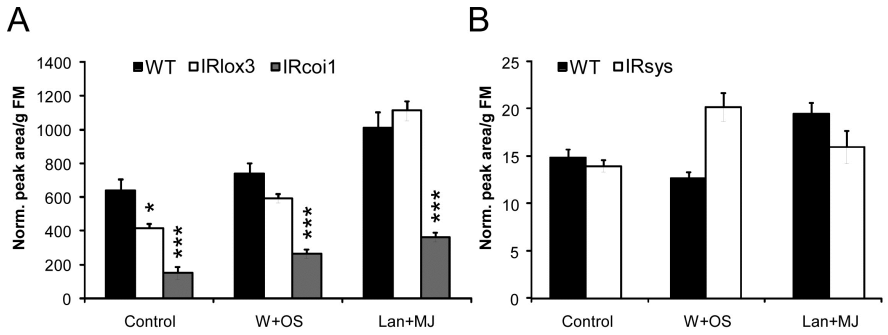


Figure 6 – Total HGL-DTG Accumulation (Average + SEM) Depends on Jasmonate Perception as Mediated by COI1. Total HGL-DTGs were measured after no treatment (control) or treatment with W+OS or Lan+MJ in WT, IR*lox3*, and IR*coi1* leaves in one experiment, and in WT versus IR*sys* leaves in a replicate experiment conducted under the same conditions. (A) Experiment 1: IR*lox3* accumulates nearly WT levels of HGL-DTGs after W+OS elicitation due to buildup of core compounds (Figure 5), and is completely restored to WT levels by Lan+MJ treatment; neither W+OS nor Lan+MJ treatment restores total HGL-DTG levels in IR*coi1*. (B) Experiment 2: IR*sys* plants are not impaired in the accumulation of total HGL-DTGs. Asterisks indicate significant differences from WT in a one-way ANOVA followed by Bonferroni-corrected tests (WT, IR*lox3*, IR*coi1*, A) or Student's t-tests (WT, IR*sys*, B) within each treatment: *P ≤ 0.05, ** P < 0.01, *** P < 0.001.

DTG production in *N. attenuata*. Other potential targets for reducing levels of total HGL-DTGs include enzymes which convert GGPPS to the 17-hydroxygeranyllinalool DTG skeleton: geranyllinalool synthase (GLS) and a cytochrome p450 which hydroxylates geranyllinalool. Silencing GLS could produce a buildup of GGPP, which might result in the formation of other diterpene toxins or antifeedants, and worse, silencing the Cyp450 would likely produce elevated levels of geranyllinalool, which can be toxic to insects (Lemaire *et al.*, 1990).

In our experiments, we were careful to monitor plant growth and levels of non-target defensive metabolites during all bioassays. As demonstrated in pictures, growth and defensive metabolite data from field-grown plants (Figure 8, Supplemental Figures 6 and 7), the main difference between IR*ggpps* and WT plants during the time over which we conducted bioassays was the level of HGL-DTGs. Data from a competition assay in the glasshouse (Supplemental Figure 5) confirm that IR*ggpps* plants grow normally and do not suffer a reduction in seed production under glasshouse conditions, implying that there is no strong effect of silencing *Naggpps* on primary metabolism. To the extent that IR*ggpps* plants accumulate less of any primary metabolites, the comparison of herbivore damage and growth on IR*ggpps* versus WT plants provides a conservative estimation of the defensive effect of HGL-DTGs. Thus, in the worst case, silencing *Naggpps* underestimates rather than inflates our estimation of the importance of HGL-DTGs in plant defense.

4.3.6 HGL-DTGs dramatically increase resistance against a specialist herbivore

M. sexta larvae were fed on WT plants or one of two independently silenced lines of IR*ggpps* for 13 d (silencing efficiency Line 1: 69.7% ± 4.5%, Line 2: 94.2% ± 1.0%; Supplemental Figure 4A). Mortality of *M. sexta* larva on IR*ggpps* lines was one quarter to one half of that on WT plants

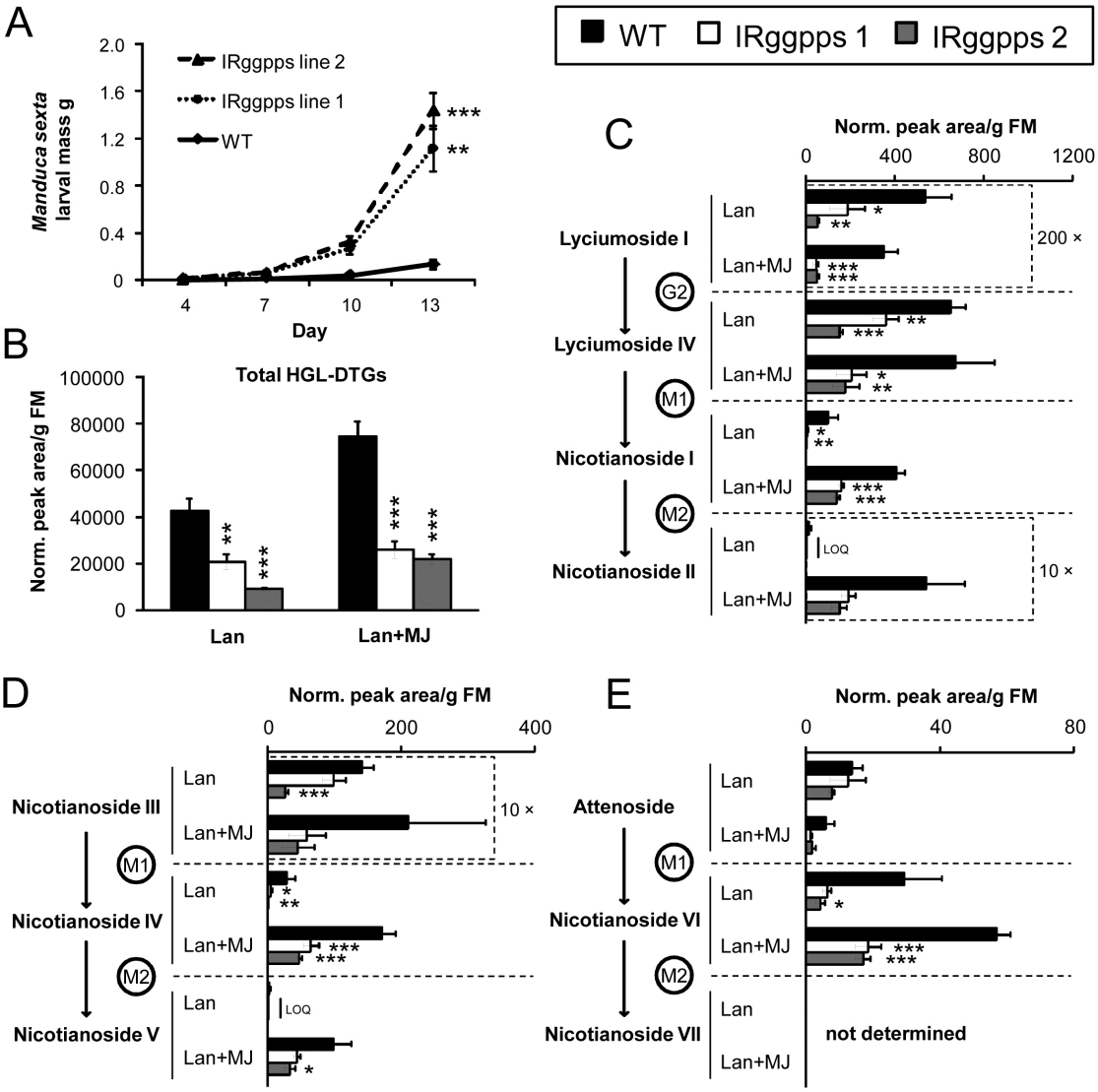


Figure 7 – Total HGL-DTGs Have a Significant Effect on the Growth of the Specialist Herbivore *M. sexta*. (A) Mass of *M. sexta* larvae feeding on two independently silenced IR *ggpps* lines versus WT (average ± SEM). Larvae grow significantly larger on both lines of IR*ggpps*: growth on IR*ggpps* line 1 was significantly faster by day 4 ($P = 0.018$) and on both IR *ggpps* lines by day 7 (Line 1, $P = 0.011$; Line 2, $P = 0.016$) as determined by a repeat-measures ANOVA followed by Bonferroni-corrected tests for each day. For clarity, significance is shown only for day 13: ** $P < 0.01$, *** $P < 0.001$. (B) Total HGL-DTGs and (C)-(E) concentrations of individual compounds in mature young rosette leaves of WT and both lines of IR*ggpps* 3 d after treatment with Lan or Lan+MJ (average + SEM).Note that some compounds have been multiplied by constants to fit the scale of the graph, and that scales change between graphs. Asterisks indicate significantly lower levels of HGL-DTGs in IR*ggpps* in one-way ANOVAs within treatment (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in Bonferroni-corrected tests). LOQ = below limit of quantification.

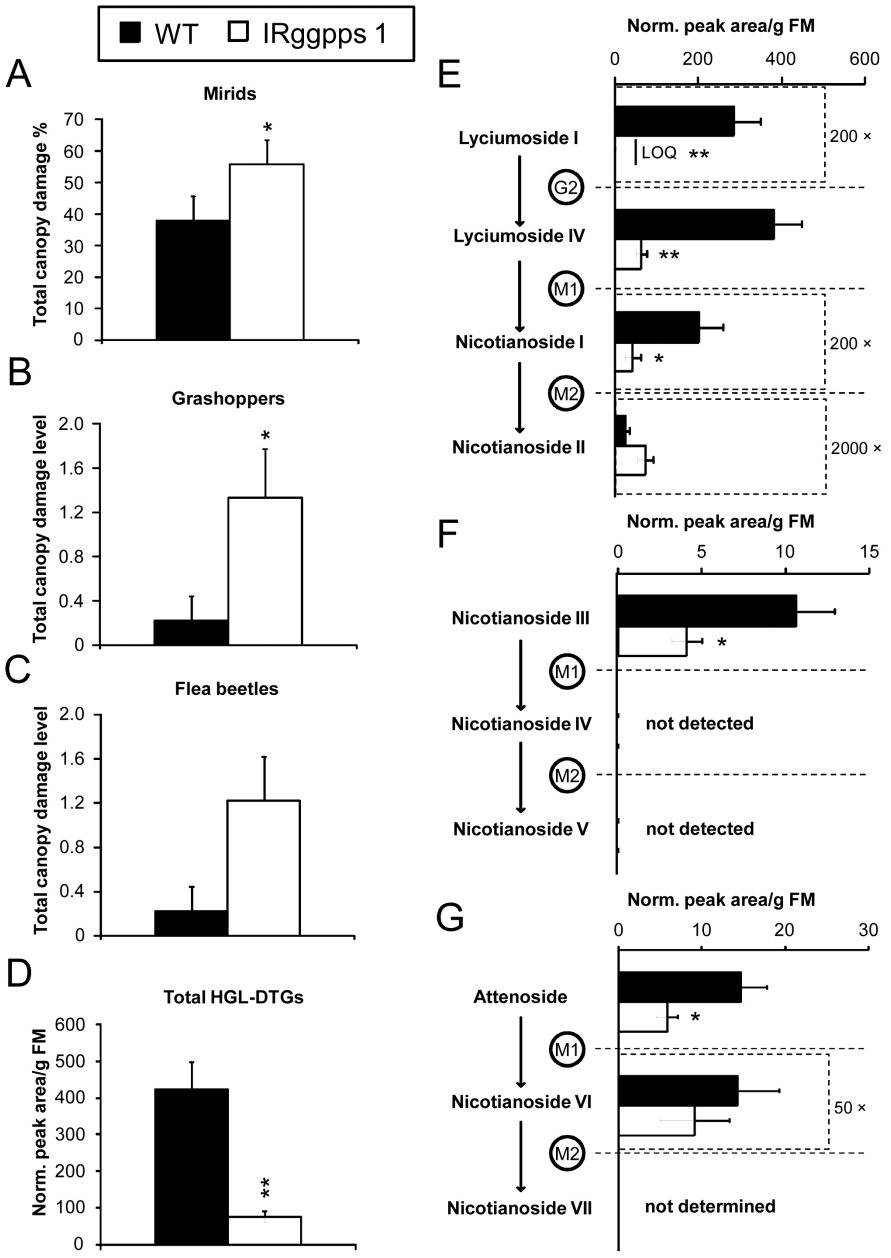


Figure 8 – HGL-DTGs Reduce Damage from Generalist Herbivores in Nature. (A) The most abundant herbivore in the 2008 field season, mirids caused significantly more damage on IR*ggpps* line 1 than on WT plants in the field by May 16th (average +SEM, *P $<$ 0.05 in paired t-tests). (B) Grasshoppers caused significantly more damage on IR*ggpps* line 1 by May 16th (average +SEM , *P $<$ 0.05 in Wilcoxon's sign-rank test). (C) Flea beetles tended to cause more damage on IR*ggpps* line 1 at the beginning of damage measurements on May 8th; differences are not statistically significant (average +SEM, Wilcoxon's sign-rank test). Scale for less abundant herbivores in (B) and (C): Damage Level 0 = 0% total canopy damage; Level 1, <1%; Level 2, $<$ 5%; Level 3, 5-10%. (D) Total HGL-DTGs and (E)-(F) concentrations of individual compounds (average +SEM) in undamaged systemic leaves harvested from pairs of WT and IR *ggpps* Line 1 on May 15th. Note that some compounds have been multiplied by constants to fit the scale of the graph, and that scales change. *P $<$ 0.05, ** P $<$ 0.01 in paired t-tests. LOQ = below limit of quantification.

(26% on IR*ggpps* Line 1, 13% on IR *ggpps* Line 2, 52% on WT). Larvae on either IR*ggpps* line gained up to ten times as much mass as those which survived on WT; there was no difference in the average mass of larvae feeding on IR*ggpps* Line 1 or Line 2 (Figure 7A; repeated-measures ANOVA followed by Bonferroni post-hoc tests, $F_{6,32}$= 10.76, P $<$ 0.0001).

HGL-DTGs are likely responsible for the dramatic difference in larval mass on IR *ggpps* versus WT plants as all other secondary metabolites known to influence *M. sexta* growth were not altered in IR *ggpps* plants. Levels of all types of HGL-DTGs in Lan (control) or lan+MJ-treated leaves were consistently lower for both lines of IR *ggpps* than for WT, although the difference in levels of dimalonylated compounds, which were present in low concentrations and were highly variable in WT leaves, was not always statistically significant (Figures 5B and 5C, ANOVAs within treatment followed by Bonferroni post-hoc tests; total HGL-DTGs, Lan: $F_{2,11}$= 17.9, P $<$ 0.001, Lan+MJ: $F_{2,11}$= 51.4, P $<$ 0.001; for individual compounds see Supplemental Table 7 and Figures 7C-7D). Other defense-related metabolites were similar to WT levels in IR*ggpps* plants, although nicotine was slightly higher and chlorogenic acid slightly lower, and IR*ggpps* plants grew similarly to WT plants in both the field as well as in the glasshouse (Supplemental Figures 4B-4E and 5, results of paired t-tests for growth and fitness are in Supplemental Table 8; ANOVAs within each treatment for secondary metabolites: trypsin protease inhibitors [TPI], lan: $F_{2,10}$=3.50, P = 0.066, Lan+MJ: $F_{2,10}$=3.45, P = 0.073; nicotine, Lan: $F_{2,11}$=5.90, P = 0.018, Lan+MJ: $F_{2,10}$=12.5, P = 0.002; rutin, Lan: $F_{2,11}$=19.1, P $<$ 0.001, Lan+MJ: $F_{2,10}$ =8.67, P = 0.007; chlorogenic acid, Lan: $F_{2,11}$=2.57, P = 0.121, Lan+MJ: $F_{2,10}$=4.20, P = 0.047).

4.3.7 Plants deficient in HGL-DTGs are more susceptible to herbivores in nature

IR*ggpps* Line 1 plants were grown in a paired design with WT plants in a field plot experiment in *N. attenuata*'s native habitat and damage by native herbivores was observed for 11 days; at the end of this period, tissue samples were taken for analysis of secondary metabolites. Field-grown IR*ggpps* plants produced significantly lower concentrations of HGL-DTGs, and received significantly greater damage from native herbivores, which could indicate decreased resistance or increased apparancy to herbivores. However, there was no observed difference in herbivore presence on IR*ggpps* versus WT plants, indicating that plants were equally apparent to herbivores and that increased damage on IR*ggpps* was due to its increased palatability. When herbivore attack was at its highest (May 16^{th}), IR*ggpps* Line 1 received significantly more damage from both abundant mirids (*Tupiocoris notatus*) and less abundant grasshoppers (*Trimeropteris spp.*) than did WT plants (Figures 8A and 8B; mirids; paired *t*-test, P = 0.029; grasshoppers; Wilcoxon sign-rank test, P = 0.043). IR*ggpps* also tended to receive more damage from flea beetles (*Epitrix spp.*), which damaged IR *ggpps* plants before beginning to damage their WT neighbors. The difference in flea beetle damage between WT and IR*ggpps* was at a maximum, but was not statistically significant on May 8^{th} (Figure 8C).

Levels of all types of HGL-DTGs were consistently lower in IR*ggpps* Line 1 than in WT, although the difference in levels of malonylated compounds, which were present in lower concentrations and were highly variable in WT leaves, was not always statistically significant (Figures 8D-8G; paired *t*-tests; total HGL-DTGs: P = 0.003; nicotianoside II, P = 0.057; nicotianosides IV and V, below the limit of quantification; nicotianoside VI, P = 0.364; all others, P $<$ 0.05). There was no

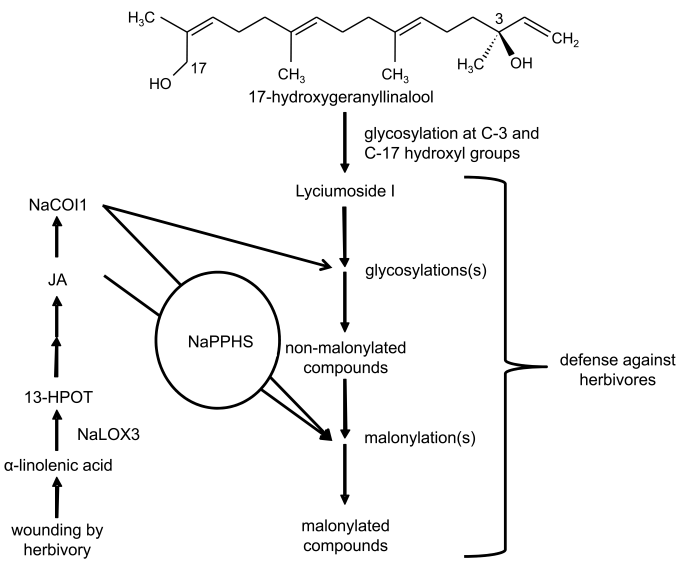


Figure 9 – Proposed Model for the Regulation of HGL-DTG Biosynthesis and Effect on Herbivores in *N. attenuata*. A simplified biosynthetic pathway is shown for HGL-DTGs from the 17-hydroxygeranylinalool skeleton to the higher molecular weight HGL-DTGs and their mono- and dimalonylated forms. Jasmonate biosynthesis (Na*lox3*) and perception (Na*coi1*) strongly regulate malonylation of HGL-DTGs, and jasmonate perception accelerates glycosylation. NaPPHS may fine-tune jasmonate-mediated malonylation by altering either the perception of jasmonates or the positive feedback of jasmonates on their own biosynthesis.

significant difference between IR *ggpps* Line 1 and WT in any other defense-related metabolite on May 15 th (Supplemental Figure 6). Plants in a pair were of similar size, but IR *ggpps* plants grew slightly faster than WT and were taller by the end of the season, when herbivore damage was no longer monitored (Supplemental Figure 7; paired *t*-tests: stalk length May 22, P = 0.003; maximum rosette diameter May 6, P = 0.005, May 7, P = 0.040).

4.4 Discussion

In this study, we have described the dynamic biosynthesis of HGL-DTGs abundant in the wild tobacco *N. attenuata* and highlighted glycosylation and malonylation as the key biosynthetic steps producing the diversity of compounds observed (Figure 1). Optimal Defense Theory (ODT) predicts that plant parts with the highest fitness value, such as reproductive organs, should be the best-defended (Rhoades, 1979; McKey, 1979), and we suggest that the distribution of HGL-DTGs in *N. attenuata* corresponds to the predictions of ODT in that HGL-DTGs accumulate most in young and reproductive tissues (Figures 2B and 3). Field and glasshouse bioassays with IR *ggpps* plants showed that the reduction of total HGL-DTGs has a highly significant impact on the growth of, and damage inflicted by multiple native herbivores of *N. attenuata* (Figures 5 and 6). Here we discuss the roles of jasmonate and NappHS signaling in the control of HGL-DTG biosynthesis, particularly their malonylation, and the likely contributions of total versus malonylated compounds to defense (Figure 9). We propose that malonylation is primarily a mechanism for plants to regulate HGL-DTG accumulation and distribution, which according to ODT is vital to the defensive value of these compounds.

4.4.1 Dynamics of HGL-DTG biosynthesis

Plants accumulate HGL-DTGs as they grow (Figure 2A) and hence their pool sizes need to be allometrically analyzed and concentrations can only be compared between plants at the same stage of growth with the same distribution of plant parts (see for example Baldwin and Schmelz, 1996 for an allometric analysis of nicotine production). The accumulation of HGL-DTGs in whole plants differs for each biosynthetic class (Figures 1 and 2): malonylated compounds generally accumulate more than core or precursor molecules; singly malonylated compounds accumulate to higher levels in reproductive tissues, and dimalonylated compounds accumulate in leaves. The biosynthesis of malonylated compounds takes between 6 and 7 d in unelicited leaves, but begins only 14 h following W+OS elicitation. Three days after W+OS elicitation, the elicited leaves of rosette-stage (Figure 4), elongated (Figure 5, WT) and young flowering plants (Figure 2B) contain significantly higher levels of malonylated HGL-DTGs. This may result from transport from neighboring tissues or local biosynthesis: W+OS elicitation to multiple leaves significantly elevated precursor levels in those leaves (Figure 4).

Wild *Nicotiana* plants also accumulate nicotine in a similar pattern to HGL-DTGs: total pools increase as they grow, preferentially in young and reproductive tissues, and damage or jasmonate application increases the rate of accumulation (Baldwin and Schmelz, 1996; Baldwin, 1999; Ohnmeiss and Baldwin, 2000). HGL-DTGs display a similar kinetic to nicotine, which reaches maximum levels 5 d following wounding or jasmonate elicitation (Baldwin, 1999). Because nicotine is synthesized in roots, the eliciting jasmonate signal must be transported from leaves to roots and the synthesized nicotine transported from roots back to leaves; HGL-DTGs may have a similar mechanism of elicitation but respond with a faster kinetic, where induced changes are stable within 3 d, because they are synthesized in leaf tissues (Figure 3).

4.4.2 Jasmonates regulate HGL-DTG biosynthesis in *N. attenuata*

Comparison of the accumulation of each HGL-DTG in WT plants and plants deficient either in jasmonate biosynthesis (IR*lox3*) or both biosynthesis and perception (IR*coi1*) revealed separate but overlapping roles for *Nalox3* and *Nacoi1* in the regulation of HGL-DTG biosynthesis (Figure 5). Neither jasmonate mutant was able to induce WT levels of malonylated compounds in W+OS-elicited leaves, but this phenotype was more severe in IR*coi1* than in IR*lox3*. IR*lox3* plants accumulated core compounds, whereas IR*coi1* plants accumulated the precursor lyciumoside I (and attenoside, Figure 5C). Jasmonate supplementation with Lan+MJ recovered most compounds to WT levels in *lox3*, but not in IR*coi1*. Dimalonylated compounds could not be fully recovered in IR*lox3*, indicating that their induced synthesis depends on a sustained or amplified jasmonate signal resulting from the positive feedback of jasmonates on their own biosynthesis which occurs in WT, but not in IR*lox3* plants. Because IR*coi1* plants were able to accumulate similar levels of core compounds as WT plants, albeit from a larger precursor pool, we conclude that jasmonate biosynthesis and perception strongly regulate malonylation, and jasmonate perception accelerates glycosylation (Figure 9). IR*lox3* plants accumulate core compounds either because their residual pool of jasmonates is sufficient to allow glycosylation but not malonylation to proceed at WT rates, or because non-jasmonate signaling molecules can interact with NaCOI1 to influence glycosylation.

In WT plants, W+OS elicitation reduces concentrations of precursors by 3 d as compared to control plants, but Lan+MJ treatment does not (Figure 5A); and IR*coi1* plants are unable to produce WT levels of total HGL-DTGs (Figure 6A). Thus jasmonates also promote the initiation of HGL-DTG biosynthesis, and the precursor pool is maintained at a level corresponding to the magnitude or duration of jasmonate elicitation - presumably to permit the more rapid biosynthesis of malonylated compounds. Again we see a parallel to nicotine accumulation: increasing concentrations of jasmonates correspond to a higher biosynthetic " set point" for the period post-elicitation (Baldwin and Schmelz, 1996; Baldwin, 1996, 1999).

4.4.3 Jasmonate regulation of HGL-DTG biosynthesis is tuned by NappHS

IR*sys* plants tend to accumulate lower levels of malonylated compounds than WT plants, and this tendency is significant after Lan+MJ treatment for two of three dimalonylated compounds (Figures 4D-4F). Like IR *coi1*, unelicited IR*sys* plants also accumulate significantly higher concentrations of the precursor lyciumoside I than do WT plants, although of the malonylated compounds only nicotianoside VI is significantly lower in unelicited IR*sys* plants (Figures 4D and 4E). Unlike systemin precursor proteins in *L. esculentum*, NappHS overexpression does not enhance herbivore-induced defense responses of *N. attenuata* (Berger and Baldwin, 2007), consistent with the hypothesis that NappHS is involved in tuning, rather than initiating the HGL-DTG biosynthetic cascade. IR*sys* plants do have lower levels of the jasmonoyl isoleucine conjugate (JA-Ile) in flowers and an altered kinetic of jasmonate accumulation in the leaves: peak levels of jasmonic acid (JA) are no different from WT plants, but the increase after herbivore elicitation is not as rapid (Berger and Baldwin 2007, 2009). This may indicate a role for NappHS in modifying sensitivity to jasmonates as has been proposed for systemin in tomato (Ryan and Pearce, 2003). It is tempting to speculate that NappHS may modify jasmonate-mediated malonylation by altering the perception of jasmonates or the positive feedback of jasmonates on their own biosynthesis (Figure 9).

4.4.4 Role of malonylation in HGL-DTG regulation and activity

IR*sys* plants are impaired in the accumulation of dimalonylated compounds (Figure 5), which accumulate to the highest levels in WT leaves (Figure 2B), but their foliage is not more susceptible to herbivores (Berger and Baldwin, 2007), which indicates that malonylation is not critical for any toxic or deterrent function of HGL-DTGs. Furthermore, we discovered during optimization of the extraction protocol that the malonyl group is easily lost to non-enzymatic cleavage in basic solutions (not shown) and thus it is unlikely that malonylated compounds would survive in the very high pH of the lepidopteran midgut (Dow, 1992). It is unlikely that the loss of the malonyl group would result in a more toxic compound, but rather that cleavage of the sugar moieties and exposure of the geranyllinalool skeleton could be toxic (LeMaire *et al.*, 1990). We propose that malonyl moieties are more likely involved in regulating within-plant transport and storage of HGL-DTGs.

Malonylation of secondary metabolites such as flavonoids, isoflavonoids and anthocyanins is a common phenomenon in many plant species, among them *Medicago trunculata*, lupins (*Lupinus* spp.) and *N. tabacum*, and may alter molecular properties in several ways: by stabilizing labile

structures (Mater *et al*., 1983b; Suzuki et al., 2002), enhancing their solubility in water (Heller and Forkman, 1994), detoxifying xenobiotics (Sandermann, 1991 and 1994) and biogenic compounds (Taguchi *et al*., 2005), preventing the enzymatic degradation of glycoconjugates (Yu *et al*., 2005), or sequestering compounds to different cellular compartments including vacuoles and the cell wall (Harborne, 2000; Markham, 2000). Malonylation might also activate HGL-DTGs prior to dimer formation: dimeric HGL-DTGs in *C. annuum* can modulate the reorganization of actin filaments and thus change the structure and permeability of tight junctions in human intestinal cells (Hashimoto *et al*., 1997).

Unlike some phenolic glucosides, malonylated HGL-DTGs are less soluble in water than are core compounds (Supplemental Figure 1; Taguchi *et al*., 2005), which may permit them to more easily cross membranes, but should impede phloem transport. Morris and Larcombe (1995) used ^{14}C labeling to show that the water-soluble ethylene precursor [2,3-^{14} C]1-aminocyclopropane-1-carboxylic acid ([^{14}C]ACC) was freely transported in the phloem of cotton plants (*Gossypium hirsutum* L.) and quickly converted to the less-soluble malonyl-[^{14}C]ACC ([^{14}C]MACC) in destination tissues; [^{14}C]MACC did not re-enter the phloem, and MACC was not found in phloem exudates. Bouzayen and colleagues (1989) demonstrated ATP-dependent active transport of MACC into vacuoles isolated from the Madagascar periwinkle (*Catharanthus roseus*) and proposed this as a mechanism for recovering stress-induced amino acid conjugates from the phloem. In senescing leaves, chlorophyll is broken down into fragments including the linear diterpene alcohol phytol, which has a similar structure to geranyllinalool, and these cleavage products are often found in the vacuole as glycosides or malonyl conjugates (Martinoia *et al*., 2000). Thus malonylation likely traps HGL-DTGs in target tissues, perhaps by marking them for transport into vacuoles (Matern *et al*., 1983).

W+OS-elicited young flowering plants accumulated core rather than malonylated HGL-DTGs in their calyxes (Figure 2B), which could be due to the arrival of core compounds at the calyxes via phloem transport. In fact, although treatment reliably elevated concentrations of malonylated HGL-DTGs across our experiments, relative concentrations of core versus malonylated compounds were highly variable. This variability may be a snapshot of the current status of HGL-DTG import versus export, or biosynthesis versus fixation in the tissue sampled. Alternatively, malonylated HGL-DTGs could become substrates for HGL-DTG dimerization (Hashimoto *et al*., 1997), which would explain the unexpected decrease in concentrations of malonylated attenoside products following Lan+MJ elicitation (Figure 5). Further research must determine the role of malonylation in the metabolism and allocation of HGL-DTGs, and the extent to which it is important for the function of HGL-DTGs in defending different plant tissues from their attackers.

4.4.5 Ecological significance and metabolic role of HGL-DTGs

HGL-DTGs are biosynthetically classified as secondary metabolites but are present in WT leaves in amounts similar to starch (mg/g FM; Supplemental Figure 2) and their regulation is correlated with patterns in primary metabolism: concentrations of all except malonylated HGL-DTGs increased in unelicited plants during the night (14 h, Figure 4), suggesting that HGL-DTG biosynthesis may occur in the dark following daytime photosynthetic fixation, parallel to starch degradation in the plastid and transport of monomeric sugar to the cytosol (Lu *et al*., 2005). Although the

production of sugar and malonyl moieties and the plastid-produced diterpene skeleton of HGL-DTGs are all directly linked to photosynthesis, nothing is known about the role of diurnal rhythms or light in regulating concentrations of individual and total HGL-DTGs. There are seasonal fluctuations in levels of lyciumosides I, II and III in *L. chinense* (Terauchi *et al*., 1997a and 1997b), which could be due in part to changing light levels. Further studies must address the separate influences of light, diurnal rhythms, and photosynthetic rates on HGL-DTG biosynthesis, as well as the potential role of HGL-DTGs as a sugar storage form which the plant, but not its enemies, may later draw on for nutrition.

A growing number of metabolites are known to function both in primary metabolism as well as in defense signaling or altering the nutritive value of plant tissue (Schwachtje and Baldwin, 2008). Some secondary metabolites are thought to be remobilized and degraded for use of the component nutrients in primary metabolism. For example, ammonia or hydrogen cyanide from the breakdown of cyanogenic glycosides in barley (*Hordeum vulgare*), cassava (*Manihot esculenta*), rubber tree (*Hevea brasiliensis*) and sorghum (*Sorghum bicolor*) may provide an important source of nitrogen for germinating seeds and mature plants (Selmar *et al*., 1988; Forslund and Jonsson, 1997; Siritunga and Sayre, 2004; Jenrich *et al*., 2007). Moreover, cleavage of cyanogenic glycosides to release benzaldehyde may also act as an indirect defense during aphid feeding (Han and Chen, 2002; Hatano *et al*., 2008).

Jasmonates are known to regulate primary metabolism as well as the biosynthesis of defense compounds, and are linked to primary metabolism via sugar signaling and JA-Ile formation (Creelman and Mullet, 1997; Schwachtje and Baldwin, 2008). Likewise, systemin and systemin-like precursor proteins regulate growth and development as well as jasmonate-mediated defense (Berger and Baldwin, 2009; Schmidt and Baldwin, 2009). Thus the regulation of malonylation by jasmonates and NappHS could reflect the original importance of malonylated HGL-DTGs in primary or secondary metabolism, or both.

A 50-75% reduction of total HGL-DTGs in IR*ggpps* lines corresponded to a tremendous increase in *M. sexta* growth and to increased damage by native generalist herbivores in a natural habitat (Figures 5 and 6). Although silencing a GGPPS gene has the potential to reduce levels of other diterpenoid derivatives, growth and defensive metabolite data from field-grown plants (Figure 8, Supplemental Figures 6 and 7) show that the main difference between IR*ggpps* and WT plants during the time over which we conducted bioassays was the level of HGL-DTGs. Data from a competition assay in the glasshouse (Supplemental Figure 5) confirm that IR*ggpps* plants grow normally and do not suffer a reduction in seed production under glasshouse conditions, implying that there is no strong effect of silencing *Naggpps* on primary metabolism. This is consistent with results from Jassbi et al. (2008) which showed that there was no effect on carotenoid content from the transient silencing of *Naggpps* using virus-induced gene silencing (VIGS), in contrast to the complete photobleaching that is observed after VIGS of *N. attenuata* phytoene desaturase (Saedler and Baldwin, 2004). They concluded that *Naggpps* is one of multiple GGPPS enzymes in *N. attenuata* and that it provides substrate mainly for HGL-DTGs. Furthermore, silencing *Naggpps* does not lead to the accumulation of any toxic compounds, and although it could increase flux to other areas of terpenoid metabolism, we have not seen evidence that this occurs (e.g., levels of terpenoid volatiles were not elevated in IR*ggpps* plants grown under normal glasshouse conditions). Finally, Jassbi *et al.* (2008) found no evidence of other diterpenoid secondary metabolites which might be formed from GGPP, including the cembrane- and labdane-type diterpenoids found in

N. tabacum. Thus *Naggpps* silencing is an effective tool for investigating the role of HGL-DTGs in *N. attenuata*.

Differences in naturally-occurring levels of HGL-DTGs are known to be directly correlated with differences in resistance against herbivores for *N. attenuata* and *N. tabacum* (Snook *et al.*, 1997; Mitra *et al.*, 2008) and transient silencing of *Naggpps* had a greater positive effect on *M. sexta* larval growth than did reducing nicotine or TPIs and did not influence the levels of non-target diterpenes such as chlorophyll (Jassbi et al., 2008). It is clear that HGL-DTGs contribute substantially to defense in *Nicotiana spp.* In contrast, it is entirely unclear what role, if any, malonylation plays in the defensive function of HGL-DTGs, and why it is apparently the most regulated step in HGL-DTG biosynthesis. Determining the effect of malonylation on the properties and distribution of HGL-DTGs will be critical to understanding their function in plants.

4.5 Methods

4.5.1 Plant material and growth conditions

Seed germination, glasshouse growth conditions, and the *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure are described in Krügel *et al.* (2002). Seeds of the 30^{th} generation of an inbred line of *Nicotiana attenuata* Torr. Ex Watts were used as the wild type (WT) plant in all experiments. For the field experiment, seedlings were transferred to 50-mm peat pellets (Jiffy) 15 days after germination and gradually hardened to the environmental conditions of high sun exposure and low relative humidity over 2 weeks. Small, adapted, rosette-stage plants of equal size were transplanted into a field plot in a native habitat in Utah and watered daily for two weeks until roots were established.

4.5.2 Purification of HGL-DTGs

Leaf material was ground in liquid nitrogen, and was split into 200 mg aliquots. Each aliquot was extracted in 1 ml extracting solution (40% MeOH and 0.05% formic acid) with 900 mg lysing matrix (BIO 101, Qbiogen, Vista, CA, USA) by shaking twice at 6.5 $msec^{-1}$ for 20s (FastPrep FP 120 Thermo Savant). Homogenized samples were centrifuged at 16,000 g for 20 min at 4 °C. Supernatants were pooled in a round flask and evaporated in a rotary evaporator to 1/4 of the original volume. The supernatant was centrifuged again at 16,000 g for 3 min at 4 °C. The concentrated solution was fractionated on a reverse-phase HPLC (Luna C-18 (2), 250 10 mm Phenomenex, Aschaffenburg, Germany) at a 2 ml/min flow rate. Mixtures of two solvents were used to elute analytes from the column: A (Millipore water, 0.1% acetonitrile, 0.05% formic acid) and B (acetonitrile, 0.05% formic acid). HPLC-grade acetonitrile was purchased from Malinckrodt Baker BV (Deventer, Holland), formic acid was purchased from Fluka (Taufkirchen, Germany) and ultrapure water was obtained using a Millipore (Billerica, MA, USA) model Milli-Q Advantage A10. Sample elution steps were as follows: 0-2 min, 0 % of B, 2-25 min, 0-70 % of B, 25-27 min, 70 % of B, 27-35 min, 70-98 % of B, 35-37 min, 98 % of B, 37-40 min, 98-0 % of B. Nearly pure nicotianosides I, II, III and lyciumoside I were collected from fractions 40 to 48. Fractions were passed through an Oasis (Waters, Milford, Massachusetts, USA) C-18 cartridge to remove formic acid. Extracts

were then evaporated completely in a vacuum concentrator (3.7 mbar; Concentrator 5301, Eppendorf).

4.5.3 Structural determination by nuclear magnetic resonance spectroscopy (NMR)

1H and 13C NMR Spectra were determined on a Bruker Avance AV-500 NMR Spectrometer at 500 and 125 MHz.

4.5.4 HGL-DTG quantification in plant tissues

Buffer A consisted of 60% solution 1 (2.3 ml/l of acetic acid, 3.41 g/l ammonium acetate adjusted to pH 4.8 with 1 M NH_4OH) and 40% MeOH. Buffer B consisted of Buffer A (10%) and 40% MeOH (90%). HGL-DTGs were extracted from plant material in 1 ml Buffer A spiked with the following internal standards (Buffer A + ISTDs): 600 ng/ml reserpine (Spectrum Laboratory, New Brunswick, NJ, USA), 10000 ng/ml 9, 10-dihydro-dideuterio-jasmonic acid (^{2}D-JA), 200 ng/ml atropine (Fluka) and 1000 ng/ml glycyrrhizinic acid (Sigma-Aldrich, Seelze, Germany).

Plant tissue samples were flash-frozen in liquid nitrogen and stored at -80C until extraction. Samples were ground in liquid nitrogen and 100 mg aliquots (mass was recorded and later used for normalization) were extracted in 1 mL Buffer A + ISTDs with 900 mg lysing matrix by homogenization twice at 6.5 m sec $^{-1}$for 45 s (FastPrep FP 120 Thermo Savant). Homogenized samples were centrifuged at 16,000 g for 20 min at 4 °C. Supernatants were removed and again centrifuged at 16,000 g for 20 min at 4 °C. Particle-free supernatants were diluted 1:50 with Buffer B for HPLC-MS/MS analysis.

Ten µl of undiluted, particle-free extract was analyzed by reverse-phase HPLC coupled to a Varian (Hansen Way, Palo Alto, CA, USA) 1200 L triple-quad mass spectrometry (MS/MS/MS) system. HPLC separation was achieved using a ProntoSIL UHC-520 (50 2.0 mm) (BISCHOFF Analysentechnik und -geräte GmbH, Leonberg, Germany), with a mobile phase flow rate of 0.2 ml/min. The mobile phase was a gradient of A (water, 0.1% acetonitrile, 0.05% formic acid) and B (acetonitrile, 0.05% formic acid) starting at 20% B and rising linearly to 70% over 3.5 min. The gradient was held at 70% B for 7.5 min, followed by re-equilibration for 3.5 min. Multiple reaction monitoring (MRM) was conducted on parent-ion/daughter-ion selections after negative ionization: 213/59 (D2-JA), 629.4/467.4 (lyciumoside I), 775.4/629.4 (lyciumoside IV), 791.4/629.4 (lyciumoside II), 821.4/351.4 (glycyrrhizinic acid), 861.4/758.4 (nicotianoside I), 921.4/775.4 (nicotianoside III), 937.4/791.4 (attenoside),947.4/859.4 (nicotianoside II), 1007.5/963.5 (nicotianoside IV), 1023.47/979.46 (nicotianoside VI), 1093.5/1005.5 (nicotianoside V), 1109.5/979.46 (nicotianoside VII). The area beneath the MRM product ion peak was recorded for detected analytes and ISTDs using the operating Software from Varian© (MS Workstation).

Concentrations of individual compounds were calculated in normalized peak area (PA) per g FM as $(PA_{compound})/(PA_{glycyrrhizinicacid}\ FM_{extracted})$. Classes of HGL-DTGs per tissue type (e.g. total malonylated compounds) were calculated as $(PA_{compound}/PA_{glycyrrhizinicacid})/FM_{extracted}$. These concentrations were multiplied by the average FM of each tissue type to calculate total HGL-DTGs per tissue.

4.5.5 High performance liquid chromatography/time of flight mass spectrometry (HPLC-TOF/MS)

Samples were extracted as above (*HGL-DTG quantification*) and the same solvents (A and B) were used for separation. HPLC was performed using an Agilent HPLC 1100 Series system (Palo Alto, CA, USA), combined with an Phenomenex Gemini NX 3u (150 2.0 mm) column. Sample elution steps were as follows: 0-2 min isocratic at 5% B, 2-30 min linear gradient up to 80% of B, 30-35 min isocratic at 80% of B followed by a return to starting conditions and column equilibration steps. The injection volume was 2 µl and the flow rate 0.2 ml/min.

MS was performed using a Bruker microTOF (Bruker Daltonics, Bremen, Germany) time of flight mass spectrometer with an electrospray ionization (ESI) source operating both in positive and in negative ion mode. ESI conditions: TOF 2100 V, capillary voltage 4500 V, capillary exit 130 V, dry temperature 200 °C, dry gas flow of 8 l/min. Mass calibration was performed using sodium formiate (50 ml isopropanol, 200 µl formic acid, 1 ml 1M NaOH in water).

Compounds and types of compounds in samples were quantified as described in *HGL-DTG quantification*.

4.5.6 Ontogeny of HGL-DTG accumulation

To determine the within-plant dynamics of HGL-DTGs in plants of different ages, one mature, green leaf per plant on 34-day-old rosette, 44-day-old elongated, 54-day-old flowering and 68-day-old flowering-stage plants was wounded with a pattern wheel to produce 3 rows of holes on each side of the midvein, and 50 µL of *Manduca sexta* oral secretions diluted 1:5 with distilled water (OS) was applied to the wounds by gently rubbing the pipetted liquid across the leaf surface using a gloved finger (W+OS) (N = 4). All tissue types were harvested from elicited and control plants (N = 4) 3 days. Depending on the developmental stage, harvested tissues from the same plant were pooled into the following groups: ripe seed capsules, unripe seed capsules, flowers, calyxes, buds, small stem leaves (on side stems), large stem leaves (on the main stem) young rosette leaves (still green), senescing rosette leaves and senescent rosette leaves (completely yellow). The stem, and the elicited leaf or the equivalent leaf position on control plants were harvested separately. During the harvest, control plants were alternated with W+OS-elicited plants, and growth stages were alternated, so that the harvesting of all treatments and growth stages was distributed throughout the entire harvesting period. Samples were flash-frozen in liquid nitrogen, then stored at −80C until use.

HGL-DTGs were extracted from each tissue type of each growth stage as described in *HGL-DTG quantification in plant tissues*. We harvested and weighed the total tissues of each type (FM) from separate, unelicited plants of the same four growth stages (N = 5), and the total mass of each tissue was used to calculate the allometric distribution of HGL-DTGs (total amount in each tissue type) based concentrations measured in each tissue. We built a mixed linear model which used the total HGL-DTGs in each tissue sample from a particular growth stage to estimate the average total HGL-DTG contents of the whole plant at each growth stage with a calculated standard error using R package lme4 (*R-Project,* http://www.r-project.org).

RNA from separate, unelicited 54-d-old WT plants was used to analyze *Naggpps* transcripts in different tissues. Synthesis of cDNA from 0.5 g of RNA per sample and TaqMan ® (Applied Biosystems, Foster City, CA, USA) quantitative polymerase chain reaction (qPCR) were conducted as described in Wu *et al.* (2007) with primers and a probe designed from positions 966-1019 of the *Naggpps* sequence EF382626 using Primer Express v1.5 from Applied Biosystems. Expression was quantified relative to *N. attenuata actin* (GenBank EU273278).

4.5.7 Dynamics of HGL-DTGs after simulated herbivory

In order to analyze the dynamics of different HGL-DTGs after herbivore attack, five leaves per plant on 30-day-old rosette-stage plants were wounded with a pattern wheel to produce 3 rows of holes on each side of the midvein, and 20 µL of OS was applied to the wounds (W+OS) as described in the previous section. Leaves at the elicited positions were harvested from elicited and unelicited (control) plants at 1, 4, 14, and 72 h, 5, and 7 d after elicitation (N = 5 plants/treatment/time point). All five elicited leaves, or the equivalent leaves on control plants, were pooled together for each individual plant prior to extraction, but samples were not pooled among plants. Samples were flash-frozen in liquid nitrogen, then stored at -80C until use. HGL-DTGs were extracted and quantified.

4.5.8 Jasmonate and systemin signaling in HGL-DTG biosynthesis

To determine the role of jasmonate biosynthesis and perception in the elicitation of different HGL-DTGs in *N. attenuata*, the oldest stem leaves (S1) of 44-day-old elongated IR*lox3*, IR*coi1*, and WT plants received one of three treatments. To simulate herbivory, leaves on 5 plants/line were elicited with W+OS using 20 µL of OS, and to supplement jasmonates, another 5 plants/line were elicited with either with 20 µL of lanolin paste containing 150 µg Lan+MJ (Lan+MJ), or with 20 µL of lanolin (Lan). Elicited leaves and S1 leaves from unelicited (control) plants were harvested after 3 d and flash-frozen in liquid nitrogen, then stored at -80C until use. Plants impaired in systemin-mediated signaling (IR*sys*) were compared to WT plants in a second experiment using identical methods. HGL-DTGs were extracted and quantified.

4.5.9 Generation and characterization of stable transformants

The *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure is described in Krügel *et al.* (2002). To determine the role of jasmonate and systemin signaling in HGL-DTG elicitation, we used the inverted repeat RNAi transformants IR*coi1* (Paschold *et al.*, 2007), IR*lox3* (inverted repeat construct created with a 467 bp segment of the *Nalox3* fragment used in Krügel *et al.*, 2002 to create the antisense as-*lox* plants characterized in Halitschke and Baldwin, 2003) and IR*sys* (Berger and Baldwin, 2007). To determine the role of total NaGGPPS products in defense against herbivores, we used two independently transformed RNAi lines (IR*ggpps* Lines 1 and 2) containing an inverted repeat construct generated from a unique fragment comprising positions 506-823 of the *Naggpps* sequence (GenBank EF382626, http://www.ncbi.nlm.nih.gov).

Vector construction and the pRESC5 plasmid are described in Steppuhn et al. (2004). Homozygosity of T2 plants was determined by screening for resistance to hygromycin (hygromycin phosphotransferase II gene from pCAMBIA-1301, GenBank AF234297, contained in the pRESC5 vector).

Two independently transformed lines were chosen for further characterization based on high constitutive silencing efficiency of *Naggpps* and similar metabolite production, growth and morphology to WT plants (Supplemental Figures 4 and 5). Transcript accumulation was determined by TaqMan ® qPCR as described in *Ontogeny of HGL-DTG accumulation*. Flow cytometric analysis as described in Bubner et al. (2006) confirmed that both lines were diploid. Growth and fitness were measured in a competition experiment where one IRggpps Line 1 plant was paired with one empty vector (EV) plant in a 2 L pot (pRESC EV characterized in Schwachtje *et al.*, 2008); beginning on day 33 after germination, the two plants in a pot were elicited 1/wk either with Lan or Lan+MJ (N = 10) to the leaf one older than the source/sink transition leaf (+1) on rosette-stage and bolting plants or to the base of the stem on elongated plants. The +1 leaf on 10 35-day-old rosette plants/line was elicited either with Lan or Lan+MJ (N = 5) as described in *Jasmonate and systemin signaling in HGL-DTG biosynthesis* and harvested after 3 d for analysis of HGL-DTGs and other metabolites involved in defense against herbivores (nicotine, trypsin protease inhibitors [TPI]) and UV tolerance (rutin, chlorogenic acid). Nicotine, chlorogenic acid and rutin were analyzed on an Agilent high performance liquid chromatography (HPLC) system according to Keinänen *et al.* (2001); TPI activity levels were determined by radial diffusion assay as described in van Dam *et al.* (2001); determination of HGL-DTG concentrations is described under *HGL-DTG quantification*.

Growth of Line 1 was also compared to WT in the field: maximum rosette diameter and stalk elongation were measured on 6, 8, 15, and 21 May from before the rosette was fully developed through the onset of flowering. Growth was always measured after arthropod presence and herbivore damage had been recorded. One undamaged or minimally damaged green leaf per plant was collected from field-grown IR*ggpps* Line 1 and WT on 16 May (when herbivore attack was at its highest), extracted and analyzed for levels of HGL-DTGs and other secondary metabolites as described above; harvested leaf positions were similar within pairs.

4.5.10 Effect of HGL-DTGs on the specialist

M. sexta WT plants and plants of both lines of IR*ggpps* were grown in the glasshouse in Jena as described in *Plant material and growth conditions*. *M. sexta* eggs were taken from our own colony. One *M. sexta* neonate was placed on the S1 leaf of each 39-day-old bolting plants (N = 23) of both lines of IR*ggpps* and WT. Larval mass was determined on days 4, 7, 10 and 13 by which time larvae were in the 3^{rd} to 4^{th} instar. Due to mortality and to larval movement off the plants, replicate number decreased during the assay: by day 13, 7 larvae remained on WT, 15 on IR*ggpps* line 1, and 13 on IR*ggpps* line 2. Plants were randomized spatially on the same table in the greenhouse, and placement and weighing of larvae was also randomized among genotypes.

4.5.11 Field bioassays

Ten replicates consisting of pairs of WT and IR*ggpps* Line 1 plants were transplanted on 21st April 2008 into a field plot within a natural *N. attenuata* habitat on Lytle Ranch near Santa Clara, Utah, under Animal and Plant Health Inspection Service (APHIS) notification number 06-242-3r, as described in *Plant material and growth conditions*.

Presence of arthropod herbivores and predators, and leaf area damage as percentage of canopy damaged by herbivores were monitored on 6, 8, 12, and 16 May. Small amounts of damage were estimated using categories: 0%, <1%, <5%, 5%-10%; larger amounts of damage were estimated to the nearest 5% of total canopy area. Pairwise comparisons allowed even small differences in canopy damage to be accurately assessed within pairs, although the absolute percentage of canopy damage was not measured with the same accuracy. Characteristic damage caused by Noctuidae larvae (*Spodoptera* spp.), flea beetles (*Epitrix hirtipennis* Melsheimer and *Epitrix subcrinita* Le Conte), grasshoppers (*Trimerotropis* spp.), mirids (*Tupiocoris notatus* Distant), leaf miners or due to mechanical damage was recorded separately.

Field data cited for IR*sys* lines comes from 14 pairs of IR *sys* and WT planted into the same field plot in 2006 (Berger and Baldwin, 2007).

4.5.12 Statistical analysis

Data were analyzed with Excel (Microsoft Corporation, Redmond, Washington, USA), SPSS 17.0 (*SPSS Inc., Chicago, IL, USA*), StatView 5.0 (SAS Institute Inc., Cary, NC, USA) or R (R-Project, *http://www.r-project.org*). When necessary, data were log-transformed to meet requirements for homogeneity of variance: raw values were augmented by 1 to include any zero values and the resulting values were log2 transformed. Unless otherwise stated, parametric data were compared using ANOVAs followed by Bonferroni post-hoc tests or Student's t -tests with Ps altered as necessary for multiple comparisons using the Bonferroni correction. Larval mass in the glasshouse bioassay was analyzed by a repeat-measures ANOVA followed by Bonferroni post-hoc tests on individual days. Paired t-tests were used for field data. If variance was not homogeneous following transformation, data were compared via Kruskal-Wallis (multiple comparisons), or either Wilcoxon signed-rank or Welch (t-test) tests.

4.5.13 Accession numbers

Sequence data from this article can be found in the GenBank data library under the following accession numbers: hygromycin phosphotransferase II from pCAMBIA-1301 complete gene sequence (used in the pRESC5 vector): AF234297, *Naggpps* complete cDNA: EF382626, *N. attenuata actin* (used as an internal standard in TaqMan [Applied Biosystems] quantitative PCR analysis): EU273278.

Acknowledgments

We thank the gardening staff at the Max Planck Institute for Chemical Ecology and the 2008 field crew, in particular Celia Diezel and Danny Kessler, for plant growth and care, and Christiane Schubert, Tim Sehrt, and Richard Golnik for help with harvesting and processing samples. We also thank Dr. Jens Schumacher, Dr. Nicolas Heinzel and Dr. Emmanuel Gaquerel for analytical and statistical advice. Thanks go as well to Brigham Young University for the use of their awesome field station, APHIS for careful regulatory oversight, and the Max Planck Society and the International Max Planck Research School on the Exploration of Ecological Interactions with Chemical and Molecular Techniques for financial support.

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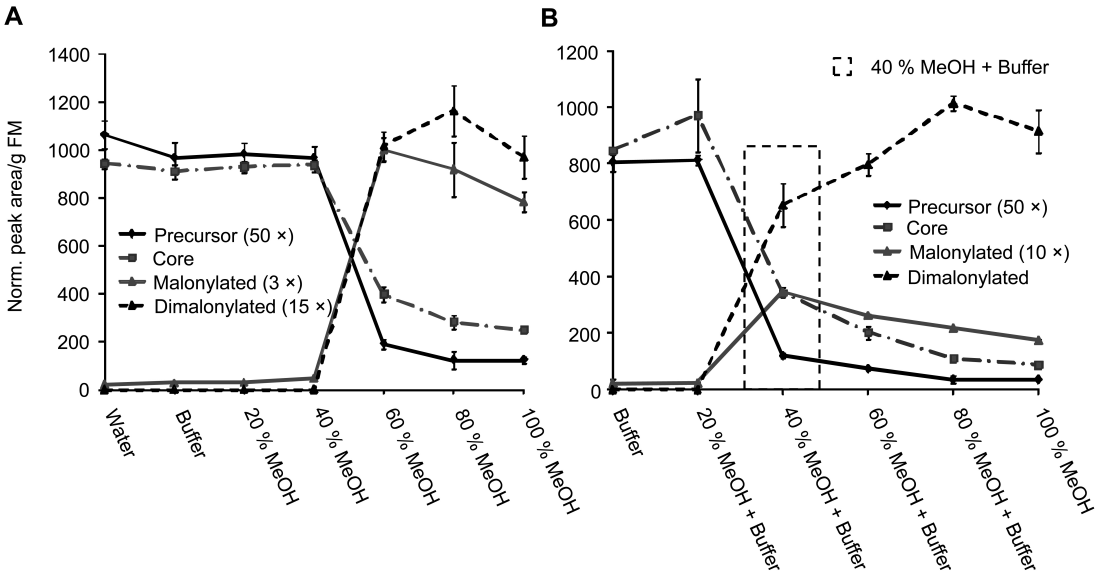

Figure S1 – Optimization of Buffer Composition for Extraction of HGL-DTGs from *N. attenuata* leaves. Leaf material was harvested from 54 d old flowering *N. attenuata* plants, pooled and extracted using different ratios of (A) methanol to distilled water or (B) methanol to 0.341% (w/v) ammonium actate buffer, pH 4.8

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Supplemental

Supplemental mass spectral data for All HGL-DTGs Measured in *Nicotiana attenuata*.

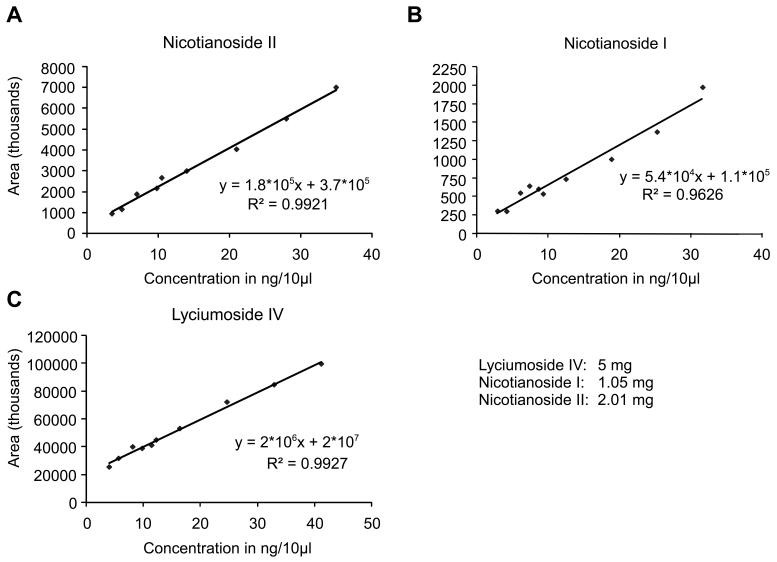

Figure S2 – Standard Addition Curve for Mass Spectrometry (MS) Measurements and Calculated Quantities of HGL-DTGs in Leaf Tissue. Leaf material was harvested from 54 d old flowering *N. attenuata* plants, spiked with a known amount of pure compound, pooled and extracted. Quantities were calculated by regression for the most abundant compounds in these extracts, (A) nicotianoside II, (B) nicotianoside I and (C) lyciumoside IV.

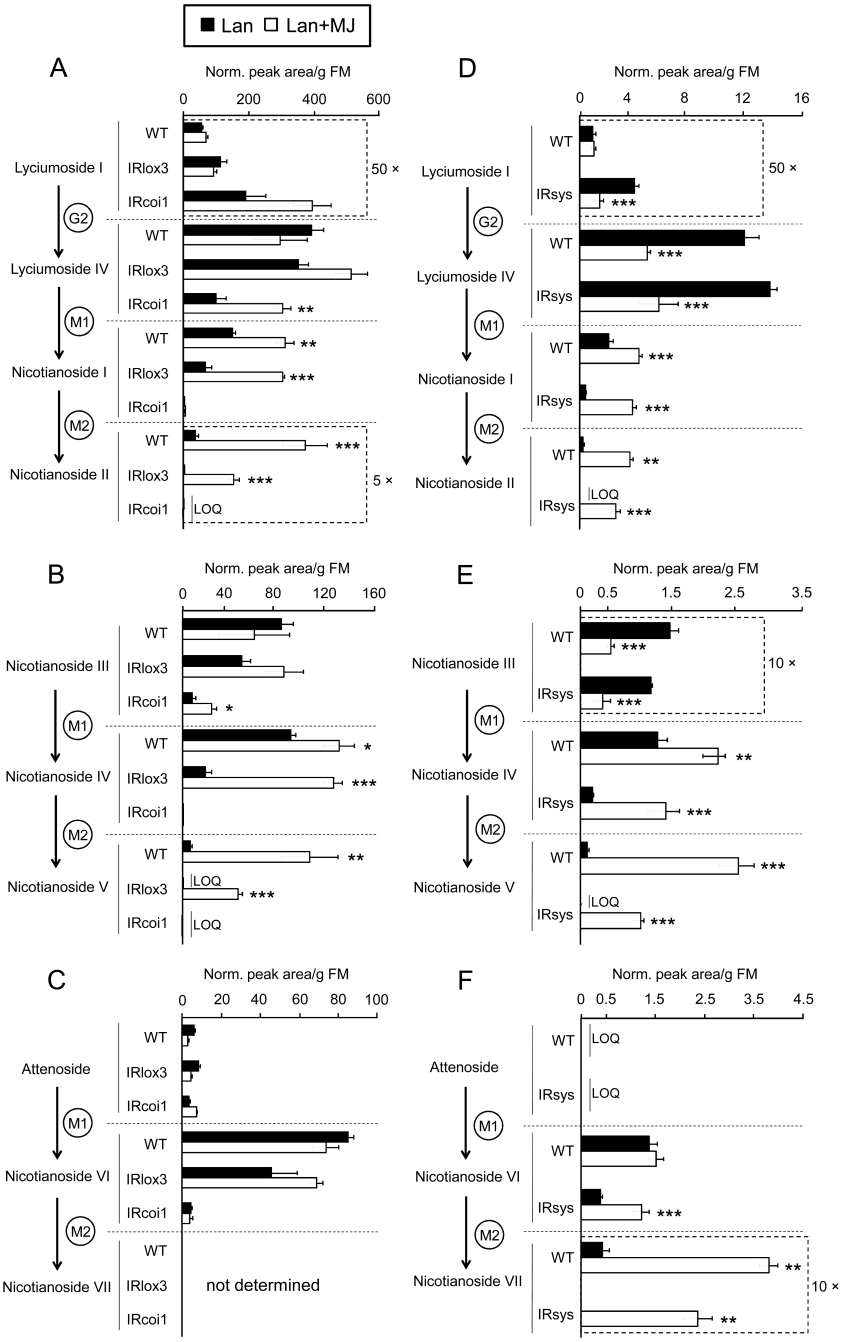

Figure S3 – Effects of Methyl Jasmonate Application on HGL-DTG Biosynthesis in WT Plants and Plants Impaired in Jasmonate Biosynthesis (IR*lox3*) and Perception (IR*coi1*) or NappHS Signaling (IR*sys*). (A)-(F) In two experiments using identical methods, HGL-DTGs were measured after 3 d in control S1 leaves of elongated plants or S1 leaves treated on d 41 with W+OS, Lan+MJ, or Lan, N = 5. All differences which are significant between Lan- and Lan+MJ-treated leaves (Supplemental Table 5) are also significant between Control (untreated) and Lan+MJ-treated leaves. Asterisks indicate significant differences between Lan and Lan+MJ within a genotype in t-tests following Bonferroni's correction for multiple testing. *P ≤ 0.05, **P < 0.01, ***P < 0.001.

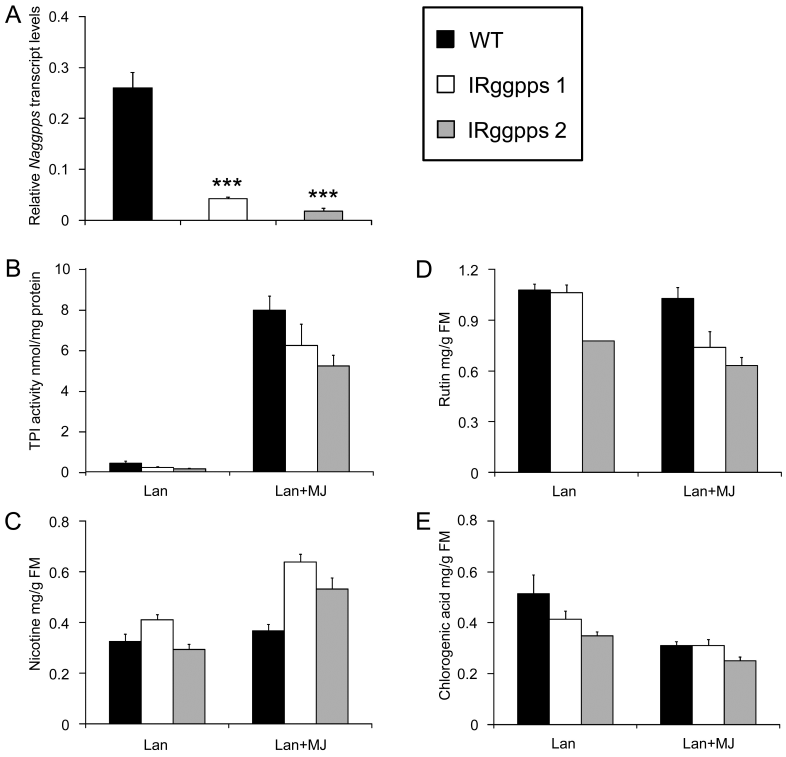

Figure S4 – Silencing Efficiency and Secondary Metabolites in IR*ggpps* Lines Versus WT. (A) Transcript levels (average + SEM) of *Naggpps* relative to *N. attenuata actin* in young rosette leaves of WT plants and both lines of IR *ggpps*. Transcripts are approximately 25% of WT levels in Line 1 and 10% in Line 2. Asterisks indicate significant differences from WT in Bonferroni-corrected post-hoc tests following an ANOVA: *P ≤ 0.05, **P < 0.01, ***P < 0.001. (B)-(E) Secondary metabolites (average + SEM): (B) Trypsin protease inhibitor (TPI) activity, (C) nicotine, (D) rutin, and (E) chlorogenic acid (average +SEM) are similar in WT and both lines of IR*ggpps* 3 d after Lan or Lan+MJ treatment of the youngest mature rosette leaf (position +1).

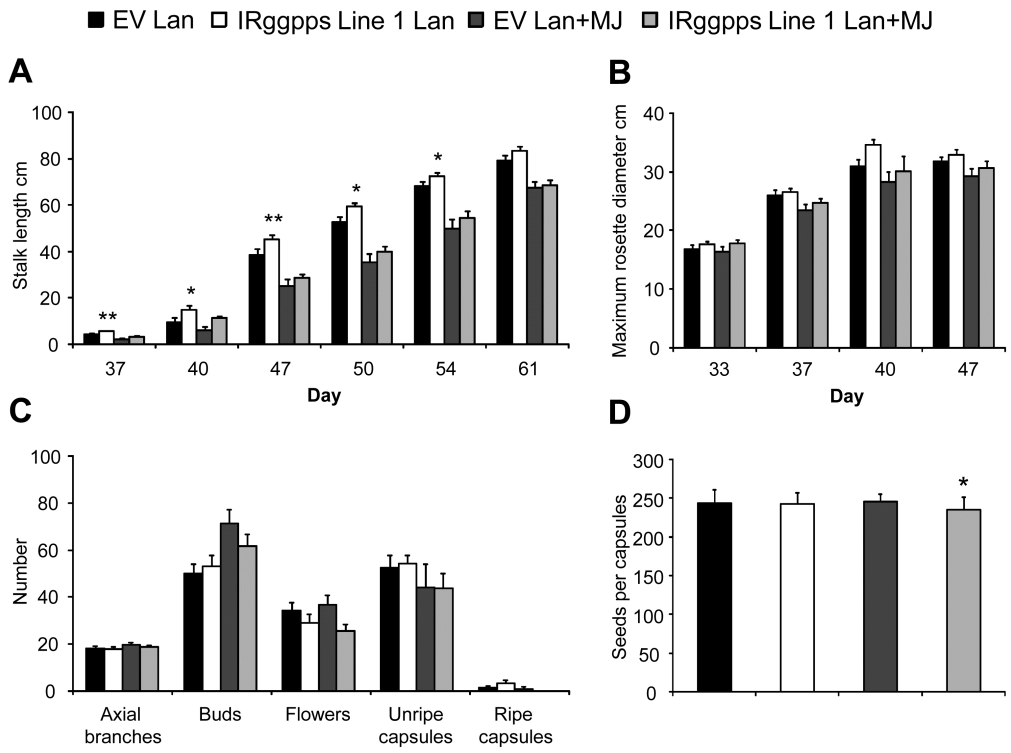

Figure S5 – Growth and Fitness of Empty Vector (EV) and IR *ggpps* Line 1 Plants in a Competition Experiment in the Glasshouse. IR*ggpps* Line 1 and EV plants were planted in pairs into 2 L pots and grown in the glasshouse. Beginning at 33 d post-germination, pairs were elicited 1/wk with Lan or Lan+MJ (N = 10) on the +1 leaf (rosette and bolting plants) or the base of the stem (elongated plants). Growth was measured regularly as (A) stalk length and (B) maximum rosette diameter (average +SEM). Darwinian fitness was estimated as (C) number of reproductive organs on flowering plants and (D) number of seeds per ripe seed capsule. Fitness units were counted at the same time for plants in a pair, and number of seeds/capsules was counted from capsules from ripe capsules at the branching point of the main stem collected on the same day from all plants. *$P < 0.05$, ** $P < 0.01$ when compared to the EV plant receiving the same treatment in paired t-tests.

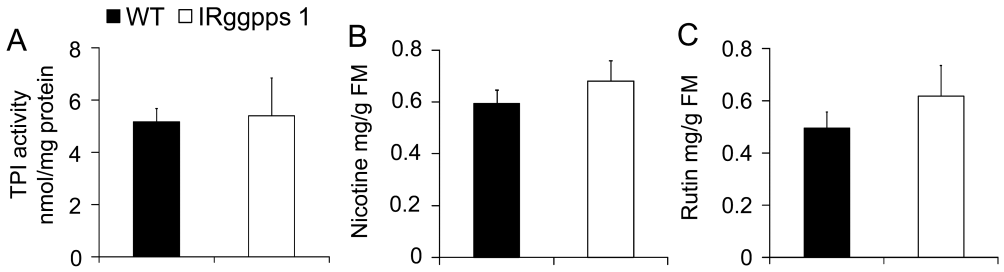

Figure S6 – Secondary Metabolites in WT and IR *ggpps* Line 1 Plants Grown in a Paired Field Plot Experiment in *N. attenuata*'s Native Habitat. (A) TPI activity, (B) nicotine and (C) rutin levels (average +SEM) are similar in WT and IR*ggpps* line 1 plants. Compounds were measured in systemic leaf tissue harvested when herbivore attack was at its highest (May 15th). A similar green leaf was harvested from both plants in a pair.

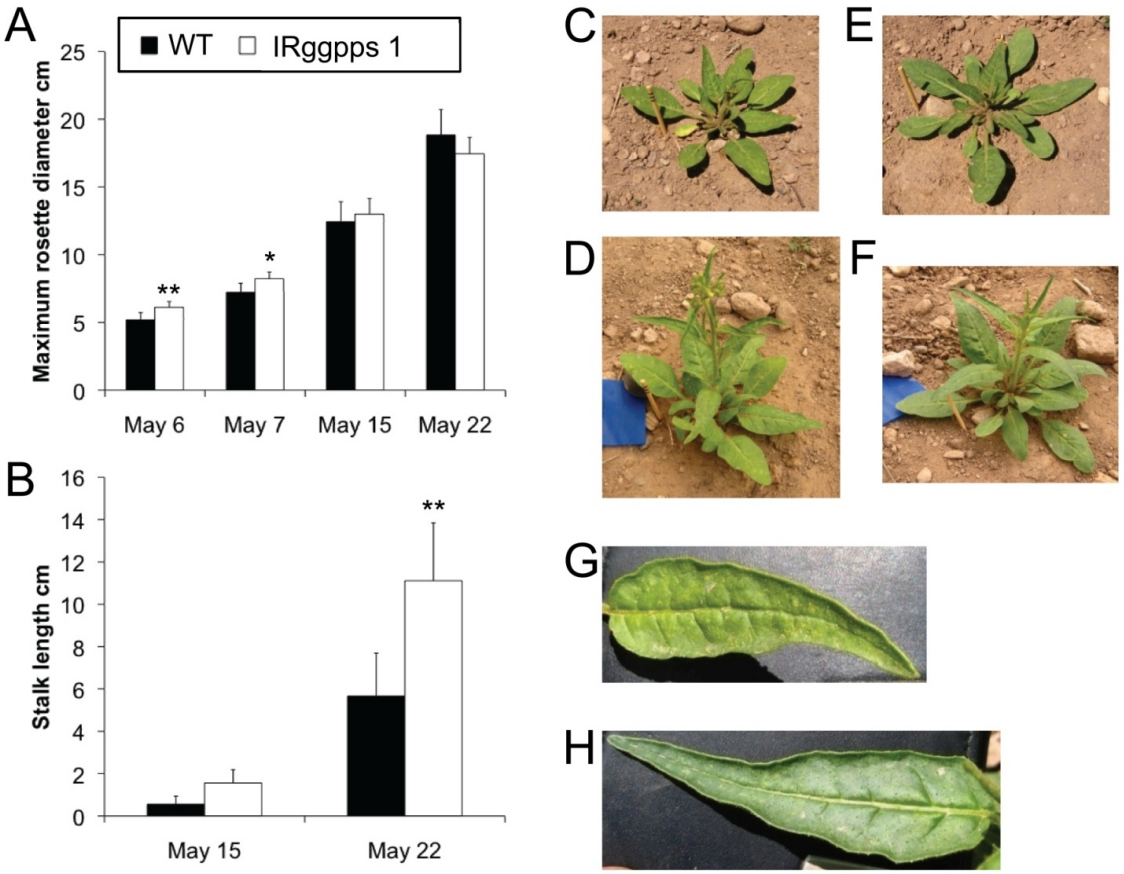

Figure S7 – Growth and Morphology of WT and IR *ggpps* Line 1 Plants Grown in a Paired Field Plot Experiment in *N. attenuata*'s Native Habitat. (A) Maximum rosette diameter and (B) stalk length (average +SEM). *P $<$ 0.05, ** P $<$ 0.01 in paired t-tests. (C) IR*ggpps* and (D) WT on May 15th and (E) IR*ggpps* and (F) WT on May 21st. Similar levels of mirid damage are pictured on leaves of (G) IR*ggpps* and (H) WT.

Supplemental table 1A: ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz from HMBC and HSQC), NMR in DMSO-d6 and MeOD

	Nicotianoside II		**Nicotianoside Ia**		**Nicotianoside Ib**	
position	δ_H[a]	δ_C	δ_H[a]	δ_C	δ_H[a]	δ_C
	Backbone		Backbone		Backbone	
1	5.20, dd (J=17.6, 1.0)	114.4	5.24, dd (J=1.3, 16.8)	114.3	5.19, dd (J=11.1, 1.3)	114.3
	5.12, dd (J=10.9, 1.0)		5.19, dd (J=1.3, 11.0)		5.22, dd (J=1.3, 17.4)	
2	5.83, dd (J=17.6, 10.9)	142.9	5.9, dd (J=11.0, 16.8)	144.7	5.93, dd (J=17.4, 11.1)	144.9
3		78.9		81.6		81.5
4	1.48, t (J=8.4)	40.5	1.59, t (J=8.5)	40.9	1.59, t (J=8.5)	40.9
5	1.94[b], m	21.7	2.04, dt (J=8.5, 6.9)	23.3	2.04, dt (J=8.5, 6.9)	23.3
6	5.06, br t (J=7.1)	124.1	5.12, t (J=6.9)	125.6	5.12, t (J=6.9)	125.6
7		134.1		135.4		135.4
8	1.92[b], m	38.9	1.96, t (J=8.0)	39.5	1.96, t (J=8.0)	39.5
9	2.00, m	25.8	2.08, dt (J=8.0, 7.0)	26.1	2.08, dt (J=8.0,7.0)	26.1
10	5.06, br t (J=7.1)	124.1	5.12, t (J=7.0)	125.6	5.12, t (J=7.0)	125.6
11		133.8		135.4		135.4
12	1.92[b], m	38.9	1.97, t (J=7.0)	39.5	1.97, t (J=7.5)	39.5
13	2.08, m	25.2	2.17, dt (J=7.0, 8.0)	27.2	2.17, dt (J=7.5, 8.0)	27.2
14	5.30, br t (J=7.1)	128.9	5.38, t (J=8.0)	131.7	5.38, t (J=8.0)	131.7
15		130.9		132.4		132.4
16	3.97, d (J=11.8)	65.3	4.19, d (J=11.3)	67.9	4.19, d (J=11.3)	67.9
	4.17, d (J=11.8)		4.33, d (J=11.3)		4.33, d (J=11.3)	
17	1.22, s	22.6	1.35	23.11	1.37	23.08
18	1.53*, s	15.5	1.60	16.2	1.60	16.2
19	1.54*, s	15.5	1.60	16.2	1.60	16.2
20	1.68, s	21.1	1.78	22.0	1.78	22.0
	Glu I		Glu I		Glu I	
1	4.20, d (J=8.0)	97.5	4.36, d (J=7.27)	98.1	4.38, d (J=7.27)	99.5
2	3.00, dd (J=8.0, 8.4)	73.3	3.20	73.4	3.21	78.4
3	3.24	74.9	3.42	73.2	3.31	76.8
4	3.25	77.9	3.51	78.8	3.44	78.6
5	3.26	73.3	3.24, m	74.9	3.50	79.7
6	4.29; 4.09	63.6	3.75; 3.63, dd (J=4.0, 11.3, 1.62)	60.4	4.33; 4.29	67.8
	Glu II		Glu II		Glu II	
1	4.04, d (J=8.0)	100.4	4.22, d (J=8.1)	102.8	4.21, d (J=8.0)	102.2
2	2.97, dd (J=8.0, 8.4)	72.9	3.19	74.8	3.19	74.8
3	3.12, dd (J=8.7, 8.7)	76.2	3.31	70.6	3.31	71.9
4	3.38	70.3	3.51	79.3	3.43	75.4
5	3.06, dd (J=9.4, 9.4)	69.8	3.42, m	74.7	3.21	74.6
6	4.17, 4.09	64.2	4.45; 4.28, dd (J=12.1, 6.5, 2.4)	65.5	3.85; 3.69	62.5
	Rha I		Rha I		Rha I	
1	4.63, br s	100.6	4.85, d (J=1.62)	101.3	4.8, d (J=2.9)	101.8
2	3.60, br s	70.4	3.82, dd (J=4.0, 1.62)	72.8	3.85, dd (J=4.0, 2.9)	73.1
3	3.38[u], dd	71.7	3.62, dd (J=8.9, 4.0)	72.6	3.62, dd (J=8.9, 4.0)	72.6
4	3.18, dd (J=9.4, 9.4)	71.5	3.39, dd (J=9.3, 8.9)	73.4	3.4, dd (J=9.3, 8.9)	73.4
5	3.75, dq (J=9.4, 6.2)	68.8	3.95, dq (J=9.3, 5.7)	69.1	3.9, dq (J=5.7, 9.3)	69.7
6	1.09, d (J=6.2)	17.5	1.26, d (J=5.7)	18.0	1.26, d (J=5.7)	18.0
	Malonic acid I				Malonic acid I	
1		166.6				169.1
2	3.31[u], HDO	41.5				
3		167.7				169.1
	Malonic acid II		Malonic acid II			
1		166.6		169.1		
2	3.31[u], HDO	41.5				
3		167.7		169.1		

[a] coupling constants J (in Hz) are given in parentheses, [b] overlapped signals

Table S1a – Mass Spectral (MS) Assignments and Nuclear Magnetic Resonance (NMR) Spectra of 17-Hydroxygernalyllinalool Diterpene Glycosides (HGL-DTGs) in *Nicotiana attenuata*.

Supplemental table 1B: ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz from HMBC and HSQC), NMR in MeOD

	Nicotianoside III		**Lyciumoside I**	
position	δ_H[a]	δ_C	δ_H[a]	δ_C
1	5.19, dd (J=10.5, 1.0)	114.3	5.21, dd (J=11.2, 1.0)	115.8
	5.23, dd (J=17.7, 1.0)		5.25, dd (J=17.9, 1.0)	
2	5.93, dd (J=10.5, 17.7)	143	5.93, dd (J=11.2, 17.9)	144.7
3		80.0		81.6
4	1.6, t (J=8.5)	41.1	1.6, t (J=8.2)	42.8
5	2.05, m (J=8.5, 7.0)	22.0	2.04, m (J=8.2, 7.5)	23.8
6	5.11, t (J=7.0)	124.3	5.12, t (J=7.5)	125.9
7		134.1		136.0
8	1.97, m	39.5	1.99, m	41.2
9	2.08, m (J=6.8)	26.1	2.08, m (J=7.5)	27.8
10	5.11, t (J=6.8)	124.3	5.12, t (J=7.5)	125.9
11		134.1		126
12	1.99, m	39.5	1.99, m (J=8.4)	40.9
13	2.17, m (J=7.3)	25.9	2.17, m (J=8.4, 7.2)	27.1
14	5.39, t (J=7.3)	129.7	5.38, t (J=7.2)	131.5
15		131.1		132.8
16	4.17, d (J=11.3)	66.4	4.19, d (J=11.8)	68.1
	4.33, d (J=11.3)		4.33, d (J=11.8)	
17	1.37		1.37	22.1
18	1.60	14.7	1.59	16.2
19	1.60	14.7	1.59	16.2
20	1.77	20.5	1.77	22.1
	Glu I		Glu I	
1	4.37, d (J=8.9)	98.0	4.35, d (J=8.5)	99.6
2	3.21, dd (J=8.5, 8.9)	73.8	3.15	75.1
3	3.41, dd	75.6	3.28	71.8
4	3.52	78.3	3.40	74.0
5	3.23	75.2	3.17	77.8
6	3.75; 3.63	60.7	3.80; 3.63, dd (J=14.0, 6.6)	63.0
	Glu II		Glu II	
1	4.23, d (J=8.9)	100.7	4.22, d (J=9.6)	102.5
2	3.23, dd (J=8.9, 8.5)	73.7	3.18	75.2
3	3.44, dd	75.6	3.28	71.6
4	3.56	78.3	3.33	71.8
5	3.27	75.4	3.21	77.9
6	3.81; 3.68	60.5	3.85; 3.69, dd (J=12.1, 6.8)	62.9
	Rha I			
1	4.86, d	101.4		
2	3.84	71.1		
3	3.64	70.9		
4	3.4, dd (J=10.1, 11.0)	72.4		
5	3.97, dq (J=5.7, 10.1)	69.1		
6	1.26, d (J=5.7)	16.2		
	Rha II			
1	4.86, d	101.4		
2	3.83	71.1		
3	3.63	70.9		
4	3.39, dd (J=10.1, 11.0)	72.4		
5	3.97, dq (J=5.7, 10.1)	69.1		
6	1.26, d (J=5.7)	16.2		

[a] coupling constants J (in Hz) are given in parentheses, [b] overlapped signals

Table S1b – Mass Spectral (MS) Assignments and Nuclear Magnetic Resonance (NMR) Spectra of 17-Hydroxygernalyllinalool Diterpene Glycosides (HGL-DTGs) in *Nicotiana attenuata*.

Supplemental table 1C: Assignment by mass spectroscopy of novel and known HGL-DTGs

RT (min)		Fragments	Mol Form	Adduct	Theo. Mass	*m/z*	Mean (ppm)	Putative ID	Reference
Av	StDev								
9.017	0.033	451, 433, 289, 271	$C_{32}H_{54}O_{12}$	$M+NH_4^+$	648.3954	648.3942	1.8	Lyciumoside I	Yahara et al. (1993)
8.518	0.042	613, 451, 433, 289, 271	$C_{38}H_{64}O_{17}$	$M+H^+$	793.4216	793.4160	7.1	Lyciumoside II	Yahara et al. (1993)
8.868	0.013	759, 597, 579, 561, 543, 451, 435, 417, 399, 381, 289, 271	$C_{38}H_{64}O_{16}$	$M+NH_4^+$	794.4533	794.4526	-0.5	Lyciumoside IV	Terauchi et al. (1998)
8.474	0.040	921, 759, 741, 613, 597, 579, 451, 433, 417, 399, 289, 271	$C_{44}H_{74}O_{21}$	$M+NH_4^+$	956.5061	956.5066	4.6	Attenoside	Jassbi et al. (2006)
9.150	0.011	845, 683, 665, 647, 629, 579, 537, 519, 501, 483, 435, 417, 399, 381, 289, 271	$C_{41}H_{66}O_{19}$	$M+NH_4^+$	880.4537	880.4513	2.7	Nicotianoside I	
9.424	0.010	931, 683, 665, 647, 537, 519, 501, 417, 399, 289, 271	$C_{44}H_{68}O_{22}$	$M+NH_4^+$	966.4541	966.4504	3.8	Nicotianoside II	
8.788	0.038	905, 743, 597, 581, 563, 435, 417, 399, 289, 271	$C_{44}H_{74}O_{20}$	$M+NH_4^+$	940.5112	940.5105	0.7	Nicotianoside III	
9.020	0.011	991, 829, 811, 743, 683, 665, 563, 537, 519, 433, 417, 399, 289, 271	$C_{47}H_{76}O_{23}$	$M+NH_4^+$	1026.5116	1026.5029	8.4	Nicotianoside IV	
9.288	0.014	829, 683, 665, 417, 399, 289, 271	$C_{50}H_{78}O_{26}$	$M+NH_4^+$	1112.5120	1112.5101	1.7	Nicotianoside V	
8.730	0.014	1007, 845, 827, 699, 683, 665, 647, 537, 519, 417, 399, 289, 271	$C_{47}H_{76}O_{24}$	$M+NH_4^+$	1042.5065	1042.5041	2.3	Nicotianoside VI	
8.974	0.013	1093, 931, 913, 845, 827, 699, 683, 665, 537, 519, 417, 399, 289, 271	$C_{50}H_{78}O_{27}$	$M+NH_4^+$	1128.5069	1128.4976	8.2	Nicotianoside VII	

RT, Retention time on Varian 1200 System, in minutes; Av, average; StDev, standard deviation; *m/z*, average mass signal; Mol Form, molecular formula of the metabolite; Adduct, Adduct of the metabolite in positive mode; Theo. Mass, theoretical mass calculated for adduct; Mean delta (ppm), deviation between the average of found accurate mass and real accurate mass, in ppm; Putative ID, putative identification of metabolite

Table S1c – Mass Spectral (MS) Assignments and Nuclear Magnetic Resonance (NMR) Spectra of 17-Hydroxygernalyllinalool Diterpene Glycosides (HGL-DTGs) in *Nicotiana attenuata*.

Supplemental table 1D: Assignment by mass spectroscopy of novel and known HGL-DTGs

Ret (min) Av	Ret (min) StDev	Compound	CA index name	Reference
20.210	0.034	Lyciumoside I	β-D-Glucopyranoside, (2Z,6E,10E,14S)-14-ethenyl-2,6,10,14-tetramethyl-2,6,10-tetradecatriene-1,14-diyl bis- (9CI)	Yahara et al. (1993)
19.110		Lyciumoside II	β-D-Glucopyranoside, (2Z,6E,10E,14S)-14-(β-D-glucopyranosyloxy)-2,6,10,14-tetramethyl-2,6,10,15-hexadecatetraen-1-yl 2-O-β-D-glucopyranosyl-	Yahara et al. (1993)
20.403	0.030	Lyciumoside IV	β-D-Glucopyranoside, (1S,4E,8E,12Z)-14-(β-D-glucopyranosyloxy)-1-ethenyl-1,5,9,13-tetramethyl-4,8,12-tetradecatrien-1-yl 4-O-(6-deoxy-α-L-mannopyranosyl)-	Terauchi et al. (1998)
20.543	0.029	Attenoside	β-D-Glucopyranoside, (2Z,6E,10E,14S)-14-[[4-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6,10,14-tetramethyl-2,6,10,15-hexadecatetraen-1-yl 2-O-β-D-glucopyranosyl-	Jassbi et al. (2006)
20.777	0.030	Nicotianoside I	(1S,4E,8E,12Z)-14-{[6-O-(carboxyacetyl)-hexopyranosyl]oxy}-1,5,9,13-tetramethyl-1-vinyltetradeca-4,8,12-trien-1-yl 4-O-(6-deoxyhexopyranosyl)hexopyranoside	
20.957	0.030		(1S,4E,8E,12Z)-14-(hexopyranosyloxy)-1,5,9,13-tetramethyl-1-vinyltetradeca-4,8,12-trien-1-yl 6-O-(carboxyacetyl)-4-O-(6-deoxyhexopyranosyl)hexopyranoside	
21.458	0.030			
21.822	0.034	Nicotianoside II	(1S,4E,8E,12Z)-14-{[6-O-(carboxyacetyl)hexopyranosyl]oxy}-1,5,9,13-tetramethyl-1-vinyltetradeca-4,8,12-trien-1-yl 6-O-(carboxyacetyl)-4-O-(6-deoxyhexopyranosyl)hexopyranoside	
22.642	0.031			
19.662	0.029	Nicotianoside III	(2Z,6E,10E,14S)-14-{[4-O-(6-deoxyhexopyranosyl)hexopyranosyl]oxy}-2,6,10,14-tetramethylhexadeca-2,6,10,15-tetraen-1-yl 4-O-(6-deoxyhexopyranosyl)hexopyranoside	
20.543	0.029	Nicotianoside IV	malonylated Nicotianoside III	
21.516	0.031	Nicotianoside V	dimalonylated Nicotianoside III	
19.550	0.030	Nicotianoside VI	malonylated Attenoside	
20.403	0.030	Nicotianoside VII	dimalonylated Attenoside	

RT, Retention time on HPLC-TOF/MS, in minutes; Av, average; StDev, standard deviation, putative ID, putative identification of metabolite

Table S1d – Mass Spectral (MS) Assignments and Nuclear Magnetic Resonance (NMR) Spectra of 17-Hydroxygernalyllinalool Diterpene Glycosides (HGL-DTGs) in *Nicotiana attenuata*.

Table S2 – Mass spectral data for All HGL-DTGs measured in *Nicotiana attenuata*.

Attenoside. HPLC/ESI(+)-TOF/MS, t_r = 18.96 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 939.470 (6.5) [M+H^+] (calc. for $C_{44}H_{75}O_{21}^+$: 939.479), 961.455 (58.9) [M+Na^+] (calc. for $C_{44}H_{74}O_{21}Na^+$: 961.461), 956.507 (32.7) [M+NH_4^+] (calc. for $C_{44}H_{74}O_{21}NH_4^+$: 956.506). Fragmentation: *m/z* (rel.int. (%)) = 921.462 (6.2) (calc. for $C_{44}H_{73}O_{20}^+$: 921.468), 759.420 (18.8) (calc. for $C_{38}H_{63}O_{15}^+$: 759.416), 741.412 (4.0) (calc. for $C_{38}H_{61}O_{14}^+$: 741.405), 613.359 (5.1) (calc. for $C_{32}H_{53}O_{11}^+$: 613.358), 597.369 (10.8) (calc. for $C_{32}H_{53}O_{10}^+$: 597.363), 579.355 (3.8) (calc. for $C_{32}H_{49}O_9^+$: 579.352), 451.302 (8.3) (calc. for $C_{26}H_{43}O_6^+$: 451.305), 433.294 (5.7) (calc. for $C_{26}H_{41}O_5^+$: 433.294), 417.303 (19.3) (calc. for $C_{26}H_{41}O_4^+$: 417.299), 399.287 (3.6) (calc. for $C_{26}H_{39}O_3^+$: 399.289), 289.253 (12.9) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.242 (100) (calc. for $C_{20}H_{31}^+$: 271.242).

Lyciumoside I. HPLC/ESI(+)-TOF/MS, t_r = 20.35 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 653.346 (100) [M+Na^+] (calc. for $C_{32}H_{54}O_{12}Na^+$: 653.350), 648.392 (46.7) [M+NH_4^+] (calc. for $C_{32}H_{54}O_{12}NH_4^+$: 648.395). Fragmentation: *m/z* (rel.int. (%)) = 451.304 (24.3) (calc. for $C_{26}H_{43}O_6^+$: 451.305), 433.288 (5.9) (calc. for $C_{26}H_{41}O_5^+$: 433.294), 289.224 (12.4) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.240 (96.2) (calc. for $C_{20}H_{31}^+$: 271.242).

Lyciumoside II. HPLC/ESI(+)-TOF/MS, t_r = 19.11 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 815.397 (90.3) [M+Na^+] (calc. for $C_{38}H_{64}O_{17}Na^+$: 815.403), 810.446 (53.1) [M+NH_4^+] (calc. for $C_{38}H_{64}O_{17}NH_4^+$: 810.448). Fragmentation: *m/z* (rel.int. (%)) = 775.414 (4.7) (calc. for $C_{38}H_{63}O_{16}^+$: 775.411), 613.346 (11.7) (calc. for $C_{32}H_{53}O_6^+$: 613.358), 451.306 (10.2) (calc. for $C_{26}H_{43}O_6^+$: 451.305), 433.306 (7.2) (calc. for $C_{26}H_{41}O_5^+$: 433.294), 289.246 (19.6) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.242 (100) (calc. for $C_{20}H_{31}^+$: 271.242).

Lyciumoside IV. HPLC/ESI(+)-TOF/MS, t_r = 20.03 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 777.425 (4.8) [M+H^+] (calc. for $C_{38}H_{65}O_{16}^+$: 777.426), 799.406 (51.7) [M+Na^+] (calc. for $C_{38}H_{64}O_{16}Na^+$: 799.408), 794.451 (47.7) [M+NH_4^+] (calc. for $C_{38}H_{64}O_{16}NH_4^+$: 794.453). Fragmentation: *m/z* (rel.int. (%)) = 759.414 (12.3) (calc. for $C_{38}H_{63}O_{15}^+$: 759.416), 597.361 (31.0) (calc. for $C_{32}H_{53}O_{10}^+$: 597.363), 579.353 (7.3) (calc. for $C_{32}H_{49}O_9^+$: 579.352), 561.340 (3.1) (calc. for $C_{32}H_{49}O_8^+$: 561.342), 543.329 (1.4) (calc. for $C_{32}H_{47}O_7^+$: 543.331), 451.304 (13.7) (calc. for $C_{26}H_{43}O_6^+$: 451.305), 435.310 (11.2) (calc. for $C_{26}H_{43}O_5^+$: 435.310), 417.299 (36.5) (calc. for $C_{26}H_{41}O_4^+$: 417.299), 399.288 (9.1) (calc. for $C_{26}H_{39}O_3^+$: 399.289), 381.274 (3.9) (calc. for $C_{26}H_{37}O_2^+$: 381.278), 289.251 (23.8) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.241 (100) (calc. for $C_{20}H_{31}^+$: 271.242).

Nicotianoside I. HPLC/ESI(+)-TOF/MS, t_r = 20.89 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 863.422 (2.1) [M+H^+] (calc. for $C_{41}H_{67}O_{19}^+$: 863.427), 885.400 (100) [M+Na^+] (calc. for $C_{41}H_{66}O_{19}Na^+$: 885.409), 880.447 (47.7) [M+NH_4^+] (calc. for $C_{41}H_{66}O_{19}NH_4^+$: 880.453). Fragmentation: *m/z* (rel.int. (%)) = 845.407 (12.1) (calc. for $C_{41}H_{65}O_{18}^+$: 845.416), 683.361 (33.7) (calc. for $C_{35}H_{55}O_{13}^+$: 683.361), 665.346 (18.0) (calc. for $C_{35}H_{53}O_{12}^+$: 665.353), 647.340 (7.8) (calc. for $C_{35}H_{51}O_{11}^+$: 647.342), 629.328 (3.2) (calc. for $C_{35}H_{49}O_{10}^+$: 629.332), 579.344 (3.5) (calc. for $C_{32}H_{49}O_9^+$: 579.352), 537.303 (29.8) (calc. for $C_{29}H_{45}O_9^+$: 537.305), 519.293 (10.2) (calc. for $C_{29}H_{43}O_8^+$: 519.295), 501.281 (3.7) (calc. for $C_{29}H_{41}O_7^+$: 501.284), 483.273 (<1) (calc. for $C_{29}H_{35}O_6^+$: 483.274), 435.311 (3.9) (calc. for $C_{26}H_{43}O_5^+$: 435.310), 417.297 (45.1) (calc. for $C_{26}H_{41}O_4^+$: 417.299), 399.289 (11.5) (calc. for $C_{26}H_{39}O_3^+$: 399.289), 381.276 (4.2) (calc. for

$C_{26}H_{37}O_2^+$: 381.278), 289.249 (12.5) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.240 (91.6) (calc. for $C_{20}H_{31}^+$: 271.242).

Nicotianoside II. HPLC/ESI(+)-TOF/MS, t_r = 22.02 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 949.426 (6.8) [M+H $^+$] (calc. for $C_{44}H_{69}O_{22}^+$: 949.427), 971.405 (100) [M+Na^+] (calc. for $C_{44}H_{68}O_{22}Na^+$: 971.409), 966.449 (89.2) [M+NH_4^+] (calc. for $C_{44}H_{69}O_{22}NH_4^+$: 966.454). Fragmentation: *m/z* (rel.int. (%)) = 931.411 (4.2) (calc. for $C_{44}H_{67}O_{21}^+$: 931.416), 683.366 (28.6) (calc. for C $_{35}H_{55}O_{13}^+$: 683.361), 665.353 (20.5) (calc. for $C_{35}H_{53}O_{12}^+$: 665.353), 647.341 (7.6) (calc. for $C_{35}H_{51}O_{11}^+$: 647.342), 537.305 (38.3) (calc. for $C_{29}H_{45}O_9^+$: 537.305), 519.292 (15.5) (calc. for $C_{29}H_{43}O_8^+$: 519.295), 501.279 (5.4) (calc. for $C_{29}H_{41}O_7^+$: 501.284), 417.298 (38.7) (calc. for $C_{26}H_{41}O_4^+$: 417.299), 399.292 (10.6) (calc. for $C_{26}H_{39}O_3^+$: 399.289), 289.250 (15.4) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.240 (99.7) (calc. for $C_{20}H_{31}^+$: 271.242).

Nicotianoside III. HPLC/ESI(+)-TOF/MS, t_r = 19.73 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 923.480 (24.6) [M+H $^+$] (calc. for $C_{44}H_{75}O_{20}^+$: 923.480), 945.463 (74.6) [M+Na^+] (calc. for $C_{44}H_{74}O_{20}Na^+$: 945.466), 940.500 (62.7) [M+NH_4^+] (calc. for $C_{44}H_{74}O_{20}NH_4^+$: 940.511). Fragmentation: *m/z* (rel.int. (%)) = 905.469 (16.9) (calc. for $C_{44}H_{73}O_{19}^+$: 905.474), 743.418 (39.9) (calc. for C $_{38}H_{63}O_{14}^+$: 743.421), 597.357 (40.3) (calc. for $C_{32}H_{53}O_{10}^+$: 597.363), 581.350 (<1) (calc. for $C_{32}H_{53}O_9^+$: 581.368), 563.353 (24.0) (calc. for $C_{32}H_{51}O_8^+$: 563.357), 435.308 (26.5) (calc. for $C_{26}H_{43}O_5^+$: 435.310), 417.297 (64.7) (calc. for $C_{26}H_{41}O_4^+$: 417.299), 399.288 (13.4) (calc. for $C_{26}H_{39}O_3^+$: 399.289), 289.240 (36.3) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.244 (100) (calc. for $C_{20}H_{31}^+$: 271.242).

Nicotianoside IV. HPLC/ESI(+)-TOF/MS, t_r = 20.65 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 1009.480 (4.7) [M+H $^+$] (calc. for $C_{47}H_{77}O_{23}^+$: 1009.485), 1031.464 (30.1) [M+Na^+] (calc. for $C_{47}H_{76}O_{23}Na^+$: 1031.466), 1026.515 (49.5) [M+NH_4^+] (calc. for $C_{47}H_{76}O_{23}NH_4^+$: 1026.511). Fragmentation: *m/z* (rel.int. (%)) = 991.457 (<1) (calc. for $C_{47}H_{75}O_{22}^+$: 991.474), 829.411 (19.9) (calc. for $C_{41}H_{65}O_{17}^+$: 829.421), 811.396 (<1) (calc. for $C_{41}H_{63}O_{16}^+$: 811.411), 743.418 (<1) (calc. for $C_{38}H_{63}O_{14}^+$: 743.421), 683.370 (21.6) (calc. for $C_{35}H_{55}O_{13}^+$: 683.361), 665.356 (8.7) (calc. for $C_{35}H_{53}O_{12}^+$: 665.353), 563.366 (8.3) (calc. for $C_{32}H_{51}O_8^+$: 563.357), 537.291 (15.8) (calc. for $C_{29}H_{45}O_9^+$: 537.305), 519.294 (12.0) (calc. for $C_{29}H_{43}O_8^+$: 519.295), 433.283 (4.9) (calc. for $C_{26}H_{41}O_5^+$: 433.294), 417.293 (33.9) (calc. for $C_{26}H_{41}O_4^+$: 417.299), 399.303 (7.6) (calc. for $C_{26}H_{39}O_3^+$: 399.289), 381.276 (4.6) (calc. for $C_{26}H_{37}O_2^+$: 381.278), 289.204 (17.8) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.240 (88.0) (calc. for $C_{20}H_{31}^+$: 271.242).

Nicotianoside V. HPLC/ESI(+)-TOF/MS, t_r = 21.27 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 1095.486 (<1) [M+H $^+$] (calc. for $C_{50}H_{79}O_{26}^+$: 1095.4485), 1117.456 (6.4) [M+Na^+] (calc. for $C_{50}H_{78}O_{26}Na^+$: 1117.467), 1112.502 (19.8) [M+NH_4^+] (calc. for $C_{50}H_{78}O_{26}NH_4^+$: 1112.511). Fragmentation: *m/z* (rel.int. (%)) = 829.429 (5.4) (calc. for $C_{41}H_{65}O_{17}^+$: 829.421), 683.364 (13.6) (calc. for $C_{35}H_{55}O_{13}^+$: 683.361), 665.342 (5.5) (calc. for $C_{35}H_{53}O_{12}^+$: 665.353), 417.296 (20.6) (calc. for $C_{26}H_{41}O_4^+$: 417.299), 399.181 (47.2) (calc. for $C_{26}H_{39}O_3^+$: 399.289), 289.250 (9.0) (calc. for $C_{20}H_{33}O^+$: 289.252), 271.235 (39.6) (calc. for $C_{20}H_{31}^+$: 271.242).

Nicotianoside VI. HPLC/ESI(+)-TOF/MS, t_r = 19.16 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 1025.473 (8.2) [M+H $^+$] (calc. for $C_{47}H_{77}O_{24}^+$: 1025.479), 1047.454 (41.7) [M+Na^+] (calc. for $C_{47}H_{76}O_{24}Na^+$: 1047.461), 1042.500 (82.3) [M+NH_4^+] (calc. for $C_{47}H_{76}O_{24}NH_4^+$: 1042.506). Fragmentation: *m/z* (rel.int. (%)) = 1007.464 (9.4) (calc. for $C_{47}H_{75}O_{23}^+$: 1007.469), 845.413 (24.5) (calc. for $C_{41}H_{65}O_{18}^+$: 845.416), 827.403 (5.8) (calc. for $C_{41}H_{63}O_{17}^+$: 827.405), 699.348

(9.3) (calc. for $C_{35}H_{55}O_{14}^{+}$: 699.358), 683.360 (10.4) (calc. for $C_{35}H_{55}O_{13}^{+}$: 683.361), 665.353 (8.2) (calc. for $C_{35}H_{53}O_{12}^{+}$: 665.353), 647.336 (2.5) (calc. for $C_{35}H_{51}O_{11}^{+}$: 647.342), 537.300 (12.9) (calc. for $C_{29}H_{45}O_{9}^{+}$: 537.305), 519.294 (9.1) (calc. for $C_{29}H_{43}O_{8}^{+}$: 519.295), 417.296 (23.2) (calc. for $C_{26}H_{41}O_{4}^{+}$: 417.299), 399.276 (4.4) (calc. for $C_{26}H_{39}O_{3}^{+}$: 399.289), 289.247 (7.3) (calc. for $C_{20}H_{33}O^{+}$: 289.252), 271.238 (100) (calc. for $C_{20}H_{31}^{+}$: 271.242).

Nicotianoside VII. HPLC/ESI(+)-TOF/MS, t_r = 20.10 min, capillary exit = 130 V, *m/z* (rel.int. (%)) = 1111.468 (8.9) $[M+H^{+}]$ (calc. for $C_{50}H_{79}O_{27}^{+}$: 1111.480), 1133.455 (30.5) $[M+Na^{+}]$ (calc. for $C_{50}H_{78}O_{27}Na^{+}$: 1133.455), 1128.501 (58.9) $[M+NH_4^{+}]$ (calc. for $C_{50}H_{78}O_{27}NH_4^{+}$: 1128.506). Fragmentation: *m/z* (rel.int. (%)) = 1093.464 (2.8) (calc. for $C_{50}H_{77}O_{26}^{+}$: 1093.469), 931.434 (1.7) (calc. for $C_{44}H_{67}O_{21}^{+}$: 931.416), 913.367 (1.5) (calc. for $C_{44}H_{65}O_{20}^{+}$: 913.406), 845.418 (24.5) (calc. for $C_{41}H_{65}O_{18}^{+}$: 845.416), 827.405 (6.4) (calc. for $C_{41}H_{63}O_{17}^{+}$: 827.405), 699.355 (7.4) (calc. for $C_{35}H_{55}O_{14}^{+}$: 699.358), 683.363 (11.6) (calc. for $C_{35}H_{55}O_{13}^{+}$: 683.361), 665.350 (9.6) (calc. for $C_{35}H_{53}O_{12}^{+}$: 665.353), 537.305 (14.7) (calc. for $C_{29}H_{45}O_{9}^{+}$: 537.305), 519.292 (9.3) (calc. for $C_{29}H_{43}O_{8}^{+}$: 519.295), 417.295 (23.0) (calc. for $C_{26}H_{41}O_{4}^{+}$: 417.299), 399.269 (4.5) (calc. for $C_{26}H_{39}O_{3}^{+}$: 399.289), 289.235 (6.6) (calc. for $C_{20}H_{33}O^{+}$: 289.252), 271.238 (100) (calc. for $C_{20}H_{31}^{+}$: 271.242).

Time	Compound	P value
1 h	Lyciumoside I	**0.039**
4 h		**0.025**
14 h		**0.011**
3 d		**0.023**
5 d		0.219
7 d		0.266
1 h	Lyciumoside IV	**0.024**
4 h		0.340
14 h		0.149
3 d		0.111
5 d		**0.002**
7 d		0.159
1 h	Nicotianoside I	0.337
4 h		0.909
14 h		**<0.001**
3 d		**<0.001**
5 d		**0.002**
7 d		**<0.001**
1 h	Nicotianoside II	0.085
4 h		0.409
14 h		**0.050**
3 d		**0.009**
5 d		**0.014**
7 d		**0.007**
1 h	Nicotianoside III	0.280
4 h		0.863
14 h		0.072
3 d		0.661
5 d		**0.006**
7 d		0.147
1 h	Nicotianoside IV	0.108
4 h		0.456
14 h		**<0.001**
3 d		**<0.001**
5 d		**0.001**
7 d		**0.008**

Five leaves per plant were treated and pooled or the same leaves were harvested and pooled for each untreated plant (N = 5).
Statistically significant P values are in **bold** (*P ≤ 0.05, ** P < 0.01, *** P < 0.001).

Table S3 – Results of Student's t-tests Comparing Individual HGL-DTGs at Several Time Points in Rosette Control Leaves or Leaves Elicited with Wounding and *Manduca sexta* Oral Secretions (W+OS).

		ANOVA		Bonferroni*
Genotype	**Compound**	**$F_{3,16}$**	**P value**	**P value**
IR*lox3*	Lyciumoside I	0.485	0.697	—
IR*coi1*		5.14	**0.011**	0.113
IR*sys*		21.7	**<0.001**	**<0.001**
IR*lox3*	Lyciumoside IV	5.92	**0.006**	**0.026**
IR*coi1*		10.4	**<0.001**	**0.002**
IR*sys*		15.2	**<0.001**	**<0.001**
IR*lox3*	Nicotianoside I	104.5	**<0.001**	**<0.001**
IR*coi1*		2.87	0.069	0.245
IR*sys*		39.6	**<0.001**	**<0.001**
IR*lox3*	Nicotianoside II	59.6	**<0.001**	**<0.001**
IR*coi1*		6.38	**0.005**	0.76
IR*sys*		48.3	**<0.001**	**<0.001**
IR*lox3*	Nicotianoside III	3.78	**0.032**	0.212
IR*coi1*		5.96	**0.006**	**0.026**
IR*sys*		15.9	**<0.001**	**<0.001**
IR*lox3*	Nicotianoside IV	131	**<0.001**	**<0.001**
IR*coi1*		2.06	0.146	—
IR*sys*		39.4	**<0.001**	**<0.001**
IR*lox3*	Nicotianoside V	39.1	**<0.001**	**<0.001**
IR*coi1*		0.618	0.613	—
IR*sys*		102	**<0.001**	**<0.001**
IR*lox3*	Attenoside	51.3	**<0.001**	**0.007**
IR*coi1*		29.8	**<0.001**	0.126
IR*sys*		73.6	**<0.001**	1.00
IR*lox3*	Nicotianoside VI	7.13	**0.003**	0.84
IR*coi1*		15.9	**<0.001**	1.00
IR*sys*		70.9	**<0.001**	**<0.001**
IR*lox3*	Nicotianoside VII	n.d.	n.d.	n.d.
IR*coi1*		n.d.	n.d.	n.d.
IR*sys*		17.9	**<0.001**	**0.006**

HGL-DTGs were measured in control S1 leaves of elongated plants or S1 leaves treated on d 41 with W+OS, Lan+MJ, or lanolin only (Lan) (N = 5, Experiment 1). *Bonferroni-corrected comparisons; n.d. = not determined; statistically significant P values are in **bold** (*P ≤ 0.05, ** P < 0.01, *** P < 0.001). Bonferroni-corrected P values are shown for Lan versus Lan+MJ and have been corrected for additional comparisons (values were also compared within genotypes among treatments).

Table S4 – Results of ANOVAs Comparing Individual HGL-DTGs in WT Versus IR*lox3* and IR*coi1* Leaves.

		ANOVA		Bonferroni*			
Treatment	**Compound**	**$F_{3,15}$**	**P value**	**P value**			
				Control	W+OS	Lan	Lan+MJ
Control	Lyciumoside I	3.72	**0.035**	—			
W+OS				**0.040**	—		
Lan				1.00	n.d.	—	
Lan+MJ				1.00	0.163	1.00	—
Control	Lyciumoside IV	19.7	**<0.001**	—			
W+OS				**0.001**	—		
Lan				1.00	n.d	—	
Lan+MJ				**0.001**	1.00	**<0.001**	—
Control	Nicotianoside I	18.5	**<0.001**	—			
W+OS				1.00	—		
Lan				1.00	n.d	—	
Lan+MJ				**<0.001**	**0.001**	**<0.001**	—
Control	Nicotianoside II	12.5	**<0.001**	—			
W+OS				**0.036**	—		
Lan				1.00	n.d	—	
Lan+MJ				**<0.001**	0.411	**0.002**	—
Control	Nicotianoside III	14.2	**<0.001**	—			
W+OS				**0.048**	—		
Lan				0.134	n.d	—	
Lan+MJ				0.099	1.00	**<0.001**	—
Control	Nicotianoside IV	20.6	**<0.001**	—			
W+OS				1.00	—		
Lan				0.198	n.d	—	
Lan+MJ				**<0.001**	**<0.001**	**0.001**	—
Control	Nicotianoside V	67.4	**<0.001**	—			
W+OS				0.614	—		
Lan				1.00	n.d	—	
Lan+MJ				**<0.001**	**<0.001**	**<0.001**	—
Control	Attenoside	3400	**<0.001**	—			
W+OS				1.00	—		
Lan				**<0.001**	n.d.	—	
Lan+MJ				**<0.001**	**<0.001**	**<0.001**	—
Control	Nicotianoside VI	28.7	**<0.001**	—			
W+OS				**<0.001**	—		
Lan				1.00	n.d	—	
Lan+MJ				0.954	**<0.001**	1.00	—
Control	Nicotianoside VII	10.7	**0.001**	—			
W+OS				**<0.001**	—		
Lan				0.907	n.d	—	
Lan+MJ				**<0.001**	1.00	**0.001**	—

HGL-DTGs were measured in control S1 leaves of elongated plants or S1 leaves treated on d 41 with W+OS, Lan+MJ, or Lan (Supplemental Table 5), N = 5, Experiment 2.P values have been corrected for additional comparisons (values were also compared among genotypes within treatments).

*Bonferroni-corrected comparisons; n.d. = not determined; statistically significant P values are in **bold** (*P ≤ 0.05, ** P < 0.01, *** P < 0.001).

Table S5 – Results of Student's t-tests Comparing Individual HGL-DTGs in WT Versus IR*sys* Leaves.

			ANOVA		Bonferroni*
Treatment	**Line**	**Compound**	**$F_{2,12}$**	**P value**	**P value**
Control	WT	Lyciumoside I	2.76	0.104	—
	IR*lox3*				**—**
	IR*coi1*				**—**
W+OS	WT		15.8	**<0.001**	—
	IR*lox3*				**0.024**
	IR*coi1*				**<0.001**
Lan+MJ	WT		50.1	**<0.001**	—
	IR*lox3*				1.00
	IR*coi1*				**<0.001**
Control	WT	Lyciumoside IV	32.2	**<0.001**	—
	IR*lox3*				0.819
	IR*coi1*				**<0.001**
W+OS	WT		36.8	**<0.001**	—
	IR*lox3*				**<0.001**
	IR*coi1*				1.00
Lan+MJ	WT		4.41	**0.037**	—
	IR*lox3*				0.201
	IR*coi1*				1.00
Control	WT	Nicotianoside I	42.6	**<0.001**	—
	IR*lox3*				**0.021**
	IR*coi1*				**<0.001**
W+OS	WT		11.58**	**0.003**	—
	IR*lox3*				**<0.001**
	IR*coi1*				**<0.001**
Lan+MJ	WT		341	**<0.001**	—
	IR*lox3*				1.00
	IR*coi1*				**<0.001**
Control	WT	Nicotianoside II	11.58**	**0.005**	—
	IR*lox3*				0.117
	IR*coi1*				0.090
W+OS	WT		9.50**	**0.009**	—
	IR*lox3*				**<0.001**
	IR*coi1*				**<0.001**
Lan+MJ	WT		20.0	**<0.001**	—
	IR*lox3*				**0.027**
	IR*coi1*				**<0.001**

HGL-DTGs were measured at 3 d in control S1 leaves (first stem leaf) of elongated plants or S1 leaves treated on d 41 with W+OS, 150 µg methyl jasmonate in 20 µL lanolin paste (Lan+MJ), or lanolin only (Lan, Supplemental Table 5), N = 5, Experiment 1. P values have been corrected for additional comparisons (values were also compared within genotypes among treatments).
*Bonferroni-corrected comparison with WT; **Chi-squared value, DF = 2; statistically significant P values are in **bold** (*P ≤ 0.05, ** P < 0.01, *** P < 0.001).

Table S6 – Results of ANOVAs Comparing Individual HGL-DTGs in IR*lox3*, IR*coi1*, or IR*sys* plants After Different Treatments.

Treatment	Compound	P value
Control	Lyciumoside I	**<0.001**
W+OS		0.118
Lan+MJ		1.00
Control	Lyciumoside IV	1.00
W+OS		0.407
Lan+MJ		1.00
Control	Nicotianoside I	0.397
W+OS		1.00
Lan+MJ		1.00
Control	Nicotianoside II	1.00
W+OS		1.00
Lan+MJ		0.280
Control	Nicotianoside III	1.00
W+OS		1.00
Lan+MJ		1.00
Control	Nicotianoside IV	**0.003**
W+OS		1.00
Lan+MJ		0.210
Control	Nicotianoside V	0.684
W+OS		1.00
Lan+MJ		**0.013**
Control	Attenoside	1.00
W+OS		1.00
Lan+MJ		1.00
Control	Nicotianoside VI	0.309
W+OS		1.00
Lan+MJ		1.00
Control	Nicotianoside VII	1.00
W+OS		0.924
Lan+MJ		**0.033**

HGL-DTGs were measured in control S1 leaves of elongated plants or S1 leaves treated on d 41 with W+OS, Lan+MJ or Lan (Supplemental Table 5), N = 5, Experiment 2. P-values have been corrected for additional comparisons (values were also compared within genotypes among treatments).
Statistically significant P values are in **bold** (*P ≤ 0.05, ** P < 0.01, *** P < 0.001).

Table S7 – Results of ANOVAs Comparing Individual HGL-DTGs in WT Leaves After Different Treatments.

			ANOVA		Bonferroni*
Treatment	**Line**	**Compound**	**F****	**P value**	**P value**
Lan	WT	Lyciumoside I	9.94	**0.003**	—
	IR*ggpps* Line 1				**0.033**
	IR*ggpps* Line 2				**0.004**
Lan+MJ	WT		31.6	**<0.001**	—
	IR*ggpps* Line 1				**<0.001**
	IR*ggpps* Line 2				**<0.001**
Lan	WT	Lyciumoside IV	30.2	**<0.001**	—
	IR*ggpps* Line 1				**0.012**
	IR*ggpps* Line 2				**<0.001**
Lan+MJ	WT		7.10	**0.012**	—
	IR*ggpps* Line 1				**0.049**
	IR*ggpps* Line 2				**0.017**
Lan	WT	Nicotianoside I	9.43	**0.004**	—
	IR*ggpps* Line 1				**0.030**
	IR*ggpps* Line 2				**0.005**
Lan+MJ	WT		26.9	**<0.001**	—
	IR*ggpps* Line 1				**<0.001**
	IR*ggpps* Line 2				**<0.001**
Lan	WT	Nicotianoside II	2.52	0.126	—
	IR*ggpps* Line 1				—
	IR*ggpps* Line 2				—
Lan+MJ	WT		3.82	0.058	—
	IR*ggpps* Line 1				—
	IR*ggpps* Line 2				—
Lan	WT	Nicotianoside III	25.5	**<0.001**	—
	IR*ggpps* Line 1				0.200
	IR*ggpps* Line 2				**<0.001**
Lan+MJ	WT		2.70	0.116	—
	IR*ggpps* Line 1				—
	IR*ggpps* Line 2				—
Lan	WT	Nicotianoside IV	9.63	**0.004**	—
	IR*ggpps* Line 1				**0.041**
	IR*ggpps* Line 2				**0.004**
Lan+MJ	WT		26.5	**<0.001**	—
	IR*ggpps* Line 1				**0.001**
	IR*ggpps* Line 2				**<0.001**
Lan	WT	Nicotianoside V	2.23	0.154	—
	IR*ggpps* Line 1				—
	IR*ggpps* Line 2				—
Lan+MJ	WT		4.36	**0.044**	—
	IR*ggpps* Line 1				0.310
	IR*ggpps* Line 2				**0.044**

The first fully-expanded rosette leaf (position +1) on 35 d old plants was treated with Lan+MJ or Lan and harvested after 3 d.

*Bonferroni-corrected comparison with WT; **DF_{Lan} = 2,11; DF_{Lan+MJ} = 2,10; statistically significant P values are in **bold** (*P ≤ 0.05, ** P < 0.01, *** P < 0.001).

Table S8 – Results of ANOVAs Comparing Individual HGL-DTGs in WT Versus IR*ggpps* Lines 1 and 2.

Measure	Day	Treatment	P value
Rosette diameter	33	Lan	0.239
		Lan+MJ	0.227
	37	Lan	0.637
		Lan+MJ	0.334
	40	Lan	0.212
		Lan+MJ	0.686
	47	Lan	0.222
		Lan+MJ	0.330
Stalk length	37	Lan	**0.008**
		Lan+MJ	0.112
	40	Lan	**0.011**
		Lan+MJ	0.118
	47	Lan	**0.003**
		Lan+MJ	0.371
	50	Lan	**0.012**
		Lan+MJ	0.289
	54	Lan	**0.044**
		Lan+MJ	0.472
	61	Lan	0.160
		Lan+MJ	0.825
Axial branches		Lan	0.797
		Lan+MJ	0.510
Buds		Lan	0.595
		Lan+MJ	0.257
Flowers		Lan	0.388
		Lan+MJ	0.104
Unripe capsules		Lan	0.778
		Lan+MJ	0.952
Ripe capsules		Lan	0.075
		Lan+MJ	0.370
Seeds per capsule		Lan	0.073
		Lan+MJ	**0.017**

Statistically significant P values are in **bold** (*P ≤ 0.05, ** P < 0.01, *** P < 0.001).

Table S9 – Results of Paired t-tests Comparing Growth and Fitness Measurements in IRggpps Line 1 and Empty Vector Control Plants Grown in Competition With Lan or Lan+MJ Treatment.

CHAPTER 5

Herbivory-induced volatiles function as defenses increasing plant fitness in nature

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This manuscript has been revised following review at *Science*, and has been resubmitted to *Science*.

5.1 Abstract

From an herbivore's first bite, plants release herbivory-induced plant volatiles (HIPVs) which can attract enemies of herbivores. However, other animals and competing plants can intercept HIPVs for their own use, and it remains unclear whether HIPVs serve as an indirect defense by increasing fitness for the emitting plant. In a two-year field study, HIPV-emitting *N. attenuata* plants produced twice as many buds and flowers as HIPV-deficient plants, but only when native *Geocoris* predators reduced herbivore loads (by 50%) on HIPV-emitters. In concert with HIPVs, plants also employ antidigestive trypsin protease inhibitors (TPIs), but TPI-producing plants were not fitter than TPI-silenced plants. TPIs weakened a specialist herbivore's behavioral evasive responses to simulated *Geocoris* attack, indicating that TPIs function against specialists by enhancing indirect defense.

5.2 Main text

Plant indirect defenses are traits that disable or remove herbivores by manipulating tri-trophic interactions to the advantage of the plant *(1)* . They attract and inform the third trophic level, predators or parasitoids, resulting in increased attacks on herbivores *(2)*. Indirect defenses are widespread and include domatia, extrafloral nectar, and food bodies which provide shelter and nutrition for predators and parasitoids, as well as herbivory-induced plant volatiles (HIPVs) which convey information about feeding herbivores *(3)*. Field studies with the native tobacco *Nicotiana attenuata*, a desert annual, and with maize have shown that HIPVs can reduce herbivore loads by 24% to more than 90%, by both increasing predation and parasitization of herbivores *(4-8)* and deterring herbivore oviposition *(7)*.

If HIPVs really function as defenses, they should increase Darwinian fitness, defined as successful reproduction, for plants under herbivore attack *(9)*. But because HIPVs can be perceived by many other members of the ecological community - from herbivores, pollinators, predators and parasitoids to competing or parasitic plants - it is not clear whether HIPVs increase plant fitness in nature *(10, 11)*. The field studies described above have either spanned too short a time to reveal Darwinian fitness benefits, or have not reported fitness data at all *(4-8)*. Two laboratory studies showed that parasitization of herbivores can increase plant reproduction *(12, 13)*, but the parasitization in these studies was not mediated by HIPVs. Hence three decades after their description, it remains unclear whether HIPVs are really indirect defenses.

Long-term field studies comparing HIPV-emitting versus -deficient plants are required to demonstrate a defensive function for HIPVs. Experimental additions of pure volatiles or mixes to plants growing in nature has worked well to test short-term effects of specific compounds *(6, 7)*, but only endogenously produced HIPV emissions can ensure specific, lasting and consistent differences under field conditions. *(14)* the inducibility of HIPV emission, which is likely essential for HIPV function, is difficult to engineer *(15)*. Engineered constitutive HIPV emissions have been used, either on predators and parasitoids trained to associate target volatiles with prey in short-term laboratory experiments *(16, 17)*, or in set-ups in which target volatiles are always associated with prey *(4, 8)*. When plants are engineered to constitutively emit HIPVs, they no longer provide accurate information about the location of feeding herbivores, and predators will not associate these signals with prey in nature. Genetically silencing the biosynthesis of HIPVs, however, permits naturally inducible wild-type (WT) plants to serve as HIPV emitters, for comparison with transformants lacking specific volatile components *(5, 18)*. Furthermore, field experiments that manipulate the production of HIPVs which not only attract the third trophic level, but also influence the second trophic level (e.g. as feeding stimulants and host location cues), require additional experimental manipulations to preserve the plant-herbivore part of the tritrophic interaction.

When HIPVs do attract the third trophic level, how can herbivores adapt? Many herbivores can outgrow their vulnerability to predators and parasitoids, but plant direct defenses can slow herbivore growth and prolong vulnerability as postulated by the slow growth-high mortality hypothesis *(7, 19-22)*. The solanaceous specialists *Manduca sexta* and *M. quinquemaculata* (Lepidoptera, Sphingidae) are resistant to the potent alkaloid toxin nicotine *(23)*, but sensitive to the nutritional value of plant tissue *(24)*. Non-toxic protease inhibitor (PI) proteins, which inhibit protein digestion and thus decrease the availability of organic nitrogen in the form of amino acids *(25)*, are widespread in flowering plants *(26)*, and trypsin protease inhibitors (TPIs) slow the growth

of *M. sexta* on *N. attenuata (25)*. However, herbivores can overcome PIs by producing insensitive or desensitized proteases, inactivating or degrading PIs, eating more plant tissue, and eating more nutritious young tissue *(25, 27, 28)*. In the latter two cases, PIs could reduce plant fitness. TPI-producing *N. attenuata* plants produce more seeds than TPI-deficient plants *M. sexta* under controlled glasshouse conditions *(24)*, whether TPIs function as a direct defense in nature is unknown.

We tested the hypotheses that HIPVs and TPIs defend plants in nature by increasing herbivore predation and thereby plant fitness. To do so, we monitored the performance and predation of *Manduca* from wild-type *N. attenuata* plants and RNAi lines silenced for the production either of a specific group of HIPVs, or of TPIs *(29)*, and compared the resulting plant reproductive output. *N. attenuata* is an annual, opportunistic out-crosser seeds are produced within one growing season mostly *(29, 30)*, we can relate bud and flower production to lifetime seed production, which is commonly accepted as a measure of fitness *(12, 13, 31)*.We hypothesized that HIPVs would increase plant reproduction by increasing predation of herbivores, and that TPIs alone would not increase reproduction under herbivore attack, but would either increase predation or increase herbivores' susceptibility to predators. We then assembled a toolbox of wild-type and transgenic lines carefully chosen to best test these hypotheses.

We chose a genotype of *N. attenuata* native to the Great Basin Desert of southwestern Utah. In many years, *Manduca* larvae cause the most defoliation of *N. attenuata* plants in this area *(7)* and thus the *N. attenuata* " UT" genotype is likely adapted to defend against *Manduca*. *Manduca* eggs and young larvae are predated by *Geocoris* spp. (big-eyed bugs) which occur naturally in the Utah habitat and are attracted to components of *N. attenuata*'s HIPV bouquet *(5, 7, 18)*. Specifically, Utah *Geocoris* predators are attracted to the sesquiterpene (*E*)-α-bergamotene as well as green leaf volatiles (fatty acid-derived C6 aldehydes, alcohols and esters) *(5, 7, 32)*. Green leaf volatiles, or GLVs, can be silenced via a single upstream 13-lipoxygenase, Na*LOX2*, which specifically supplies lipid hydroperoxides for their production *(33)*. Although GLVs are released upon mechanical damage, an isomerase in *Manduca* oral secretions (OS) converts (*Z*)-GLVs to their (*E*)-isomers, resulting in greater *Geocoris* predation than the damage-induced isomer ratio *(6)*. GLVs are released immediately upon damage *(6)* and may therefore be a " first line of defense."

Like GLVs, many other HIPVs are also released after mechanical damage, but change in amount or ratio upon herbivory, and thus GLVs mirror the functional complexity of the total HIPV blend. Furthermore, GLVs prime or directly regulate responses in neighboring plants *(34, 35)*, attract herbivores as well as predators *(5)*, and are important cues for pollinating and ovipositing moths *(7, 36-38)*, thus performing several roles which may harm or benefit plant fitness in addition to their role in attracting predators. Perhaps most significantly, GLVs also stimulate *Manduca* feeding, and silencing plant GLV production results in reduced herbivore damage *(39, 40)*. All these qualities made the manipulation of GLV emissions an ideal means to rigorously test the fitness consequences of HIPV emissions and evaluate whether these emissions can truly be considered defensive.

We chose a line of inverted repeat (ir)-*PI* plants with no detectable TPI activity *(28)*, and a line of ir-LOX2 plants with GLV emissions $<$20% of WT *(33)*; both have been characterized previously, and non-target defense metabolites are not affected in either line, including emission of (*E*)-α-bergamotene measured in a glasshouse characterization of all lines prior to field release (supplementary online text) *(28, 29, 33)*. Because of the importance of GLVs for the plant-

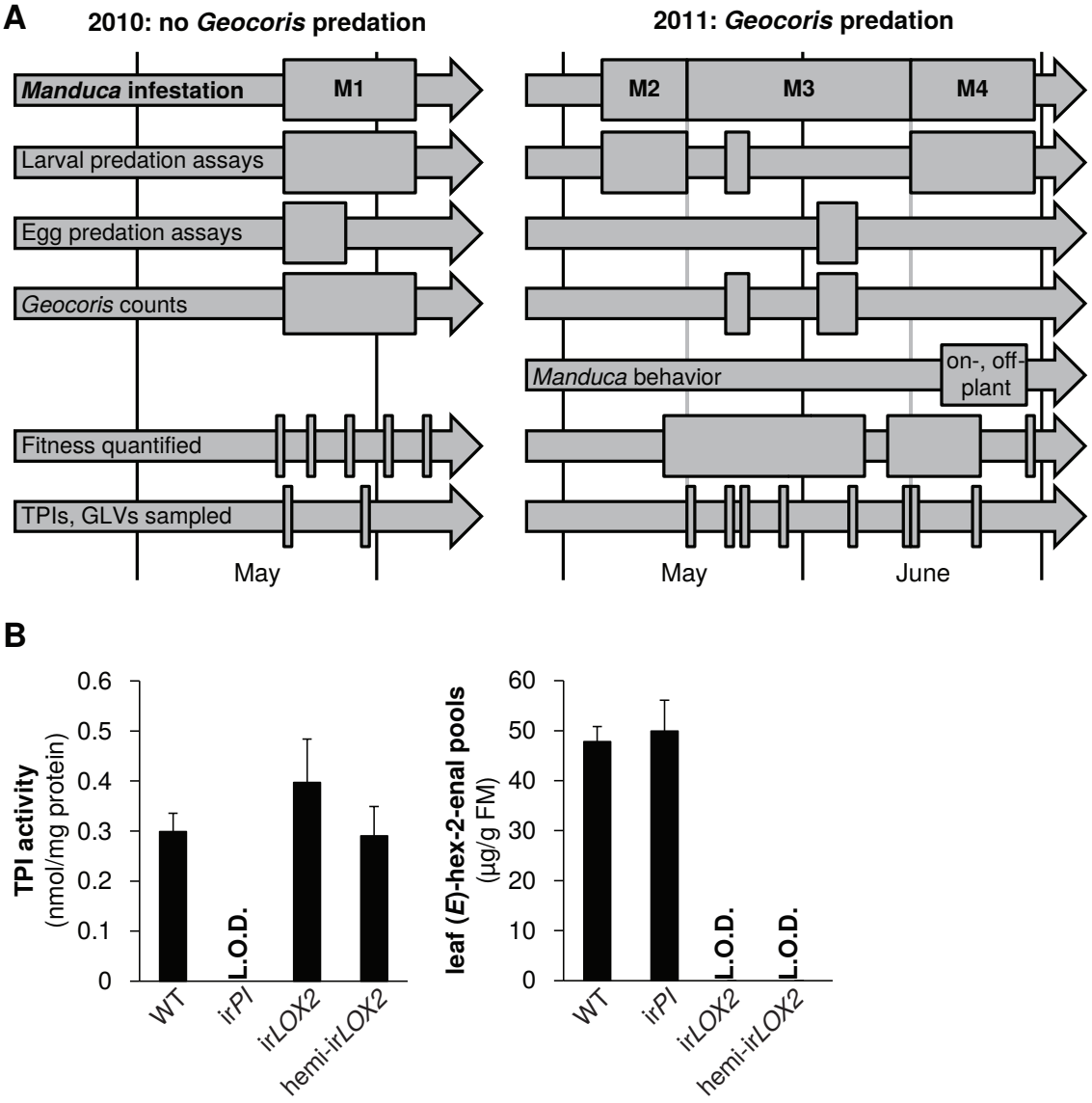

Figure 1 – Experimental timeline and silencing of TPI or GLV production in transformed lines. (**A**) Timeline of field experiments in 2010 and 2011. Different assays and measurements are represented by individual arrows, and rectangles span the time frame of each assay or measurement; narrow rectangles represent single days. Four experimental *Manduca* infestations (M1-M4) structure the overall experimental design: M1-M3, with laboratory *Manduca*, and M4, with wild *Manduca* larvae. (**B**) TPI activity (left panel, N=11-17) and tissue GLV pools (right panel, N=10) in leaves harvested from *Manduca*-infested field-grown plants on May 28th, 2011, in the middle of M3 *(29)*. The dominant GLV in tissue extracts was (*E*)-hex-2-enal, and others were not present in quantifiable amounts in these samples (supplementary online text) *(29)*. Additional data is shown for TPI activity, GLVs in tissue and headspace samples, and transcript accumulation of target genes in figs. S1 and S2. L.O.D., below limit of detection for measurement.

herbivore interaction, we used both homozygous *(33)* and hemizygous inverted repeat (ir)-*LOX2* plants to provide different levels of GLV silencing (figs. S1, S2) *(29)*. Hemizygous (hemi-) ir *LOX2* plants were created by crossing homozygous ir*LOX2* and ir*PI* plants, but the ir*PI* construct was not active in this cross (figs. 1, S2) (supplementary online text) *(29)*.

WT, ir*PI*, ir*LOX2* and hemi-ir*LOX2* plants were planted in 40-50 quadruplets into one of two experimental fields: a first-year plot, or an older plot across a river from the first plot *(29)*. Because GLVs influence *Manduca* oviposition *(7, 36, 37)*, and the timing and extent of *Manduca* oviposition varies from year to year, we created even, synchronous oviposition events by distributing *Manduca* neonates and eggs, either from a lab-reared culture or from wild collections (fig. 1A) *(29)*. We monitored the predation of *Manduca* larvae and eggs daily*(7)*, and counted *Geocoris* individuals around plants every 2-3 d (*Geocoris* counts, fig. 1A) *(29)*. In 2010, we planted into the first-year plot; no *Geocoris* were observed on either plot through May, and *Geocoris* first arrived and began to predate *Manduca* eggs on the older plot on June 9^{th}. In 2011, we planted into the older plot, where we observed *Geocoris* in May prior to the first *Manduca* infestation (M2, fig. 1A).

During infestation M2 (fig. 1A), we allowed *Geocoris* to associate all four plant genotypes with the presence of prey: we infested half of all plants with equal numbers of first-instar *Manduca* larvae from the laboratory strain (supplementary online text) *(29)* and, because *Geocoris* predates more from GLV-emitting or-perfumed plants *(5-7)*, we supplemented GLV emission from ir*LOX2* and hemi-ir*LOX2* plants by placing cotton swabs with lanolin paste containing GLVs representative of the *Manduca*-fed *N. attenuata* headspace (table S1) adjacent to *Manduca*-infested leaves; swabs containing lanolin with solvent were placed next to ir*PI* and WT as a control *(6, 29)*. *Manduca* larvae were predated at a rate of 12-37% over two 2- to 3-d trials. *Geocoris* to predate more larvae from GLV-supplemented plants (Fisher's exact tests, 35-37% versus 22-27% 5^{th}-6^{th} May, P=0.066, 17-21% versus 12% 13th-15^{th} May, P=0.069, combined trials, Bonferroni-corrected P=0.0063) (fig. S3).

We removed the cotton swabs and the remaining larvae, and monitored predation of newly-infested larvae and eggs without GLV supplementation during infestation M3 (fig. 1A) *(29)*. Predation of both larvae and eggs without GLV supplementation was two to four times as great on GLV-emitting WT and ir *PI* plants: 43%/60% (WT/ir*PI*) for larvae and 34%/39% for eggs, versus 17%/33% (ir*LOX2*/hemi-ir*LOX2*) for larvae and 9%/20% for eggs (fig. 2), and associated with a steady *Geocoris* population of 16-23 individuals/day within a 5 cm radius around plants (table S2). However, there was no difference among plant genotypes in the number of *Geocoris*, indicating that *Geocoris* regularly survey all plants and use GLVs as a short-distance cue. Fig. 2 shows larval predation rates at the beginning of the assay, when the *Manduca* load was comparable across plant genotypes. Over the following week, *Geocoris* predated a total of 80% of these larvae from WT and ir*PI* versus 47% from ir*LOX2* (P=0.015, Fisher's exact test) and 67% from hemi-ir*LOX2* (P=0.382, Fisher's exact test).

Lower predation of *Manduca* from ir*LOX2* and hemi-ir *LOX2* in 2011 correlated with the reduced growth and reproduction of both genotypes, by 30-50% for ir*LOX2* and 20-30% for hemi-ir *LOX2* versus WT during infestations M2 and M3, although this reduction was also apparent in plants not infested with *Manduca* (figs. 1A, S4). In 2010 however, in the absence of predation, there was no difference in stem growth, branching, or bud and flower production among genotypes (fig. S5, table S3) *(29)*. *Manduca* feeding did not significantly reduce growth or reproductive output

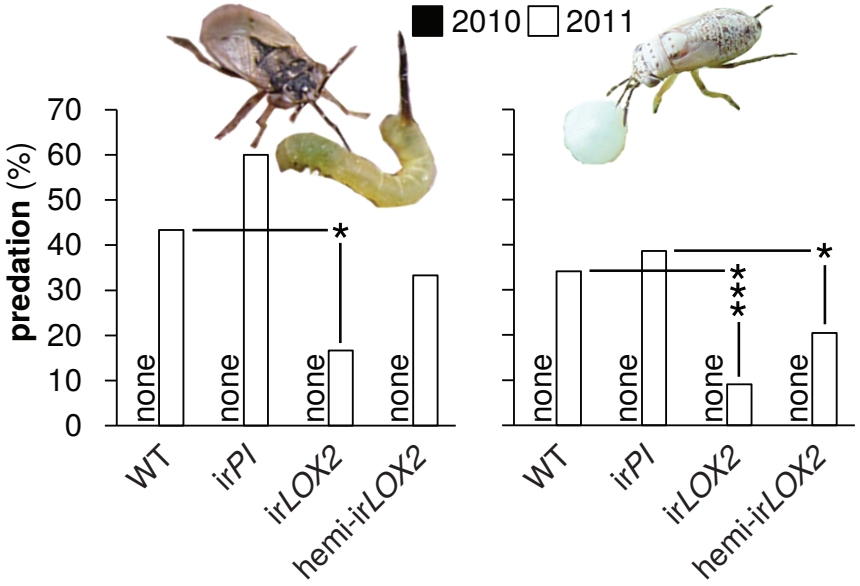

Figure 2 – Predation of *Manduca* larvae and eggs by *Geocoris*. Total percentage of *Manduca* larvae (left panel, N=51 larvae 2010, 30 larvae 2011) and eggs (right panel, N=50 eggs 2010, 88 eggs 2011) predated during infestations M1 in 2010 (none = no predation) or in two separate trials *(29)* during M3 in 2011 (fig. 1A). Pictures are of a *Geocoris* adult predating a first-instar *Manduca* larva (left) and a 5^{th}-instar *Geocoris* nymph predating a *Manduca* egg (right picture, S. Allmann). *P<0.05, ***P<0.001 in Bonferroni-corrected Fisher's exact tests against WT (ir*LOX2*) or ir *PI* (hemi-ir*LOX2*, which also contains the ir*PI* construct *(29)*).

of ir*LOX2* or ir*PI* plants in either year (figs. S4, S5; tables S3, S4). For ir*PI*, this was likely due to reduced feeding damage (fig. S6) resulting from a lack of TPI-induced compensatory feeding *(28, 29)*; although GLVs have been shown to stimulate *Manduca* feeding *(39)*, we did not measure reduced feeding damage on ir*LOX2* or hemi-ir*LOX2* plants (fig. S6), and hemi-*irLOX2* plants still suffered reduced growth and reproduction from *Manduca* feeding (figs. S4, S5, tables S3, S4).

We hypothesized that the 50% lower predation rates of *Manduca* from GLV-deficient plants, combined with *Manduca*'s negative effect on growth and reproduction, would result in reduced reproduction for GLV-versus matched GLV-producing plants if *Geocoris* were present. Homozygous ir *LOX2* plants were excluded from these "matched" experiments because they did not suffer reduced growth or reproduction from *Manduca* feeding, and because they were too small in comparison to other lines in 2011 (fig. S4). We therefore selected triplets of WT, ir*PI* and hemi-ir *LOX2* plants similar in size, previous reproductive output, apparent health, and prior *Manduca* damage; damage from naturally occurring herbivores did not differ among these genotypes (fig. S6). In 2010, these triplets were part of infestation M1 (fig. 1A) and received 3 lab strain larvae per plant to a lower stem leaf *(29)*. In 2011, hemi-ir*LOX2* , ir*PI* and WT plants were matched prior to infestation M4 to exclude differences in growth, reproduction and damage arising during infestations M2 and M3 (figs. 1A, S7). We removed all reproductive meristems from matched plants in 2011 follow plant reproduction over *Manduca* larval development without incurring ripe transgenic seed capsules (supplementary online text) *(29)*; because matched plants had similar numbers of buds and side branches (fig. S7), removing reproductive meristems at bud clusters caused similar damage to all plants. We then and placed one wild *Manduca* neonate per plant on a lower stem

leaf to mimic natural oviposition rates *(41)*.

We recorded the mortality of *Manduca* larvae and the new reproductive output of plants until they began to set seed. In the absence of *Geocoris* in 2010, genotypes did not differ in *Manduca* mortality or plant reproduction (fig. 3A). In 2011, during the first to third larval instars, in which larvae are vulnerable to *Geocoris* predation *(7)*, wild *Manduca* mortality reached 38% on hemi-ir*LOX2* versus 62-76% on WT and ir*PI* (fig. 3B); the overall mortality of larvae on all three lines was significantly different (Bonferroni-corrected pairwise comparisons by Friedman tests, $P \leq 0.005$). Although *Manduca* mortality on hemi-ir*LOX2* jumped to 70% in the fourth and semi-final larval instar, this was likely due to predation by whiptail lizards (*Cnemidophorus* spp.) which were present on the field plot: these lizards predate late-instar *Manduca* and are attracted to short-chain fatty-acid volatiles produced by the larvae due to ingestion of acyl sugars in plant trichomes *(42, 43)*.

Thus hemi-ir*LOX2* plants bore twice as many *Manduca* larvae through the third instar as WT or ir*PI*, and they produced 40-50% fewer buds and flowers than WT or ir*PI* (fig. 3B). This reduced bud and flower production was not due to accelerated seed set: unripe seed capsules on hemi-ir*LOX2* plants were also reduced by 50% (fig. S7). These data demonstrate that herbivore-induced GLV emissions function as indirect defenses by increasing predation of a specialist herbivore twofold, resulting in a twofold increase in bud and flower production for a wild plant in its native habitat.

TPIs had a less consistent negative effect on *Manduca* predation (figs. 2, 3)there was no positive effect of TPIs on plant growth and reproduction (figs. S4, S5) and no significant effect of TPIs on *Manduca* growth under natural conditions (fig. S8). We hypothesized that the TPI-producing plants might nevertheless affect *Manduca* behavior independently of larval size. Indeed, wild *Manduca* larvae feeding on WT plants (infestation M4, fig. 1A) reacted more sluggishly to experimental provocation than size-matched larvae on ir*PI* plants: they were 75% less likely to attack when lifted off of the leaf (fig. 4A, videos S1, S2). We were careful not to harm wild larvae so that we could monitor their natural mortality and consequences for plant reproduction (fig. 3B). To more accurately imitate *Geocoris* attack, we developed an off-plant assay with larvae from the laboratory *Manduca* strain feeding on detached leaves from field-grown plants *(29)*, in which size-matched larvae were poked, pierced and lifted using an insect pin to mimic the *Geocoris* beak (fig. 4B, videos S3, S4). Similarly to the on-plant assay, larvae fed on WT leaves were 50% less likely to successfully attack the insect pin, either when initially poked, or poked and lifted with the pin (fig. 4B). We also monitored recovery post-trial *(29)* and found that WT-fed larvae ceased to grow for at least 24 h after simulated attack, while ir*PI*-fed larvae continued to grow (fig. 4B); mortality did not differ (fig. S8). Thus TPIs did not increase plant reproduction under attack from a specialist in nature, but may support indirect defense by weakening the response of larvae to predator attack. higher predation rates ir*PI* than from WT plants reflect *Geocoris*' preference if ir*PI*-fed larvae are more nutritious than WT-fed larvae *(19)*.

By indicating the long-sought defensive function of HIPVs, these data set the stage for the of HIPVs integrated pest management strategies (IPM) which rely on recruiting biological control agents to reduce pesticide use *(44)*. These agents are usually naturally occurring generalist parasitoids and predators, such as *Geocoris* spp. *(6, 14, 45, 46)*. HIPVs are produced by genotypes of most, if not all crop plants and IPM would benefit from selective breeding or engineering of HIPV emission *(15)* rather than relying on alternatives such as controlled release dispensers, which have

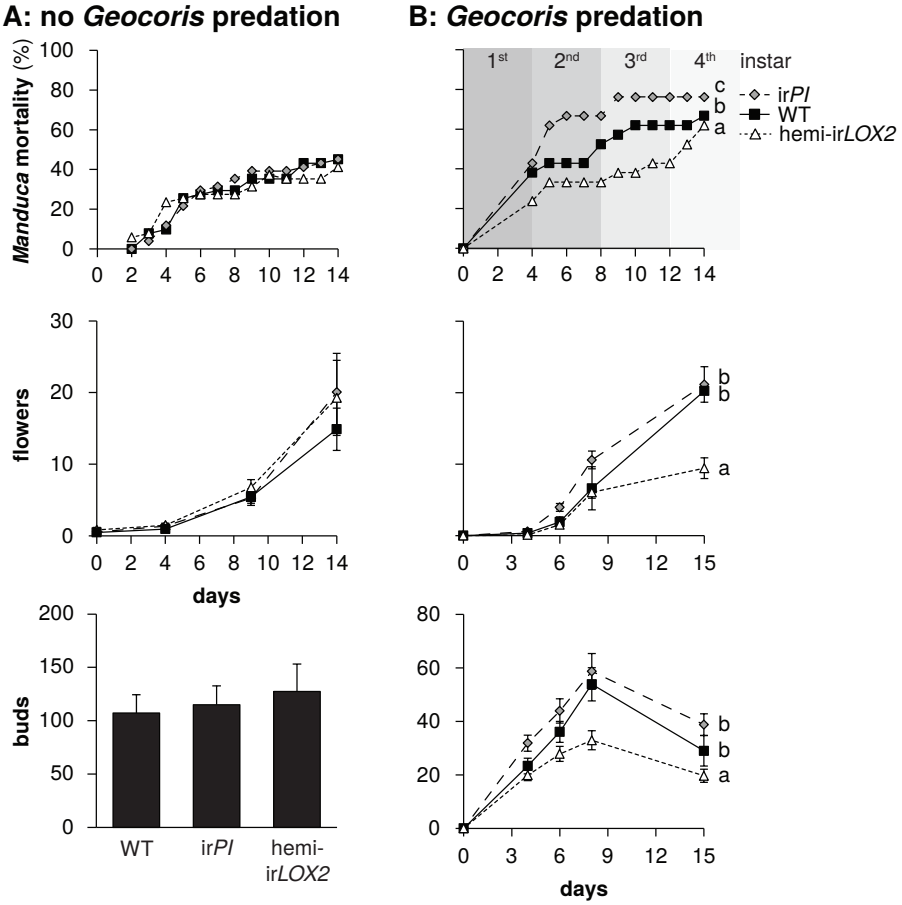

Figure 3 – Mortality of *Manduca* larvae and numbers of reproductive units produced by infested plants in 2010, in the absence of *Geocoris* predation, and in 2011, when *Geocoris* were active predators of *Manduca*. (**A**) In 2010, flowering plants matched for size (N=17) were each infested with three *Manduca* neonates from a laboratory culture (N=51 larvae), which were allowed to reach the final instar on plants *(29)*. The upper panel shows larva mortality over time, which reached a maximum of 40% by the 5^{th} instar, after 12 d. Flower production (lower panel) did not differ, nor did any other parameters of plant size and reproduction (fig. S5 and table S3). (**B**) In 2011, plants (N=21) were matched for size, prior reproduction, health, and previous damage by *Manduca* and other herbivores (figs. S6, S7) following the end of infestation M3 (fig. 1A), and reproductive meristems were removed. Matched plants were infested with one wild *Manduca* neonate each (M4 in fig. 1A), and *Manduca* were allowed to reach the fourth (penultimate) instar *(29)*. Larval mortality (upper panel) reached a maximum of 76% after larvae transitioned from the second to third instar (days 9 and 10, fig. S7D), at which time larval mortality on hemi-ir*LOX2* was only half as great as on WT or ir*PI*; larvae beyond this stage are not susceptible to *Geocoris (7)*. Flower and bud production (lower panel) was twice as great in WT and ir*PI* as in hemi-ir*LOX2*, and numbers of flowers and buds correspond to numbers of seed capsules: hemi-ir*LOX2* plants also produced fewer unripe seed capsules than WT or ir*PI* plants (fig. S7E). ^{a,b,c}Different letters indicate significant differences ($P<0.05$) in Bonferroni-corrected pairwise Friedman tests (*Manduca* mortality), or Scheffe *post-hoc* tests of hemi-ir*LOX2* versus WT and ir*PI* flowers and buds following a repeated-measures MANOVA over all flower and bud counts shown (results of Greenhouse-Geisser-corrected univariate tests for the interaction of line and day: buds, $F_{4.988}=5.297$, $P<0.001$; flowers, $F_{3.722}=4.403$, $P=0.003$).

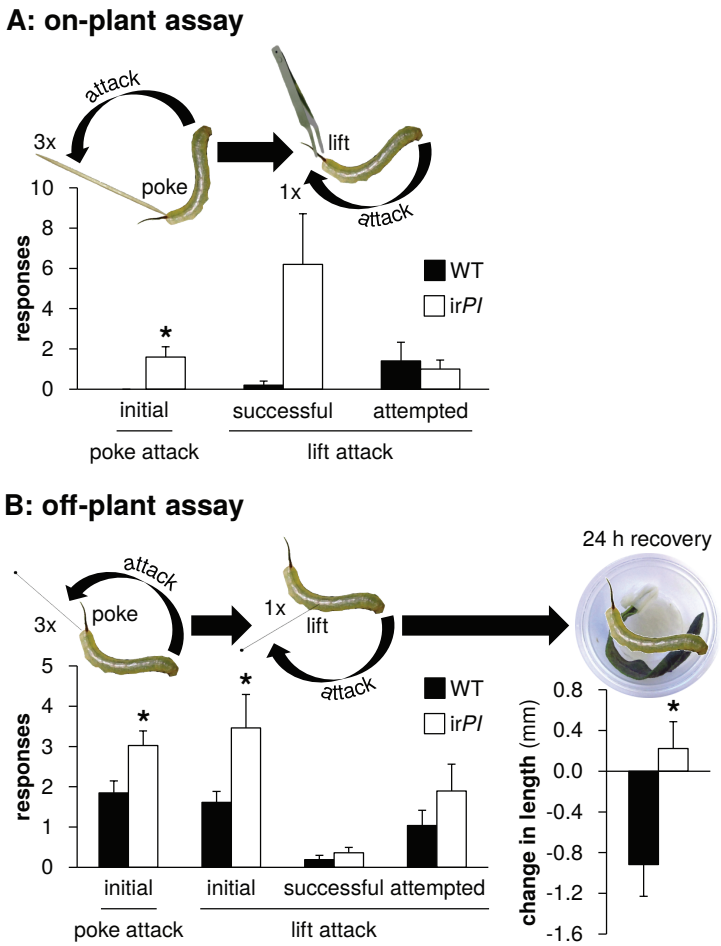

Figure 4 – Mock *Geocoris* predation assays with *Manduca* larvae fed on WT or ir*PI* plants. (A) Response of wild *Manduca* (fig. 1A) on plants in the field to poking with a toothpick and lifting with a featherweight forceps (N=5 second-instar larvae matched for size). We first poked larvae below the horn three times, 3 s apart, with the end of a toothpick and counted how often they attacked the toothpick, defined as the larva whipping its head around toward the toothpick and making contact. We then lifted larvae from the plant using the forceps and counted how often they attempted to attack, or succeeded in attacking the forceps over 15 s. In an attempted attack, the larvae moved from hanging at a 180º angle below the forceps vertically toward the forceps; and in a successful attack, the front end of the larva made contact with the forceps, before returning to its original position. All individuals were recorded and responses were counted from videos (see videos S1, S2) *(29)*. *P<0.05 in a paired t-test. (**B**) Left, response of *M. sexta* from a laboratory strain raised for 48 h in boxes on either WT or ir*PI* leaf tissue (N=20 first-instar larvae matched for size) to being poked, pierced and lifted with an insect pin. Right, growth of larvae in the following 24 h. The procedure was identical to that for the on-plant assay described above, except that larvae were poked with an insect pin rather than a toothpick, and then pierced in the rear flank and lifted with the same insect pin (see videos S3, S4). *P<0.05 in a paired t-test. The length of each larva was measured prior to poking and lifting. Afterward, larvae were placed in individual cups, each with a moist paper towel round and fresh WT or ir*PI* leaf tissue, and length of the larvae in mm was again measured after 24 h *(29)*; mortality did not differ between WT- and ir*PI*-fed larvae (fig. S8B). *P<0.05 in a Student's t-test.

mixed success and require large amounts of synthetic HIPVs *(47)*. PIs may be employed to enhance the efficiency of indirect defense, especially combined with toxins like Bt that directly target herbivores and are safe for biological control agents. With growing concerns about field-evolved

Bt resistance *(48)*, indirect defenses promise an effective " first line of defense" against agricultural pests, to which not even specialist herbivores are likely to rapidly evolve resistance.

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Acknowledgements

Supported by the Max Planck Society. We thank S. Allmann for conducting egg predation assays in 2010; C. Diezel, M. Erb, M. Kallenbach, D. Kessler, D. Marciniak, M. Stanton, and A. Steppke for help with field work; M. Erb, J. Gershenzon, D. Heckel, M. Kallenbach, A. Kessler, A. Steppke, and A. Weinhold for comments on the manuscript; Brigham Young University for the use of their Lytle Preserve field station; and APHIS for constructive regulatory oversight.

Supplemental

GLVs are strongly reduced in ir*LOX2* and hemi-ir*LOX2* plants

We assessed GLV production by hexane extraction of GLVs from frozen leaf tissue, and GLV emission by GC analysis of leaf headspaces. The dominant GLV in hexane extracts was (*E*)-hex-2-enal, and (*Z*)-hex-3-en-1-ol was additionally quantifiable as a minor component (figs. S1, S2). Only (*E*)-hex-2-enal was quantifiable in extracts from field-grown plants on May 28th, 2011, and was below quantifiable levels in ir *LOX2* and hemi-ir*LOX2* plants, but detectable in pooled samples from hemi-ir*LOX2* (figs. 1B, S1). Extracts from later in the season also contained quantifiable amounts of (*Z*)-hex-3-en-1-ol and hemi-ir*LOX2* extracts contained up to 50% as much of this alcohol as WT and ir*PI* extracts (fig. S2). Headspace measurements from field- and glasshouse-grown plants detected a similar 80-100% reduction in GLV emissions from ir*LOX2* and hemi-ir*LOX2* plants compared to WT and ir*PI* (fig. S2), and transcript accumulation of *LOX2* was 2% of WT levels in these lines but unaffected in ir*PI* (fig. S2).

TPI activity is not detectable in ir*PI* plants

The ir*PI* plants *(1)* had no detectable TPI activity in the glasshouse or throughout the field experiment in 2011 (figs. 1B, S2), and *PI* transcripts accumulated to only 0.3% of WT levels in ir *PI* (fig. S2). In contrast, TPI activity and *PI* transcripts were similar to WT plants in ir*LOX2* and hemi-ir*LOX2* (figs. 1B, S2).

Non-target defense metabolites are not affected in ir*LOX2*, hemi-ir *LOX2* or ir*PI* plants

Other than GLVs, HIPVs in *N. attenuata* are elicited via jasmonate signaling *(2, 3)*. The ir*PI* line A-04-186-1 *(1)* and ir*LOX2* line A-04-52-2 *(4)* have been characterized previously, and neither is affected in jasmonate signaling. Particularly, the emission of (*E*)-α-bergamotene, the best-characterized HIPV in *N. attenuata* apart from GLVs *(3, 5-7)*, does not differ significantly among the lines used [N=4 measured 24-32 h after W+OS treatment *(7)* and normalized as a percentage of the internal standard peak: WT, 67.9±17.1%; ir*PI*, 30.2±13.2%; ir*LOX2*, 26.6±5.8%; hemi-ir*LOX2*, 42.7±23.3%; ANOVA: F_3=1.338, P=0.308]. The transformation process itself does not affect plant fitness or competitive ability *(8)*, TPI production or volatile emission (fig. S2).

Different Manduca loads during predation assays do not bias *Manduca* predation and plant reproduction data

During M2 (fig. 1A) we staggered initial *Manduca* infestations to accommodate differences in plant growth: WT and ir*PI* plants were initially larger and therefore went into the field on average earlier than ir *LOX2* and hemi-ir*LOX2* plants, so that all plants were planted at a similar size, which is important for even establishment. All plants were simultaneously infested in M2, but we first re-infested WT and ir *PI* plants in M3, to allow ir*LOX2* and hemi-ir*LOX2* plants to catch up in their growth to WT and ir*PI* before re-infestation. However, we then left *Manduca* larvae on ir *LOX2* and hemi-ir*LOX2* as long as on WT and ir*PI*, and we made several control measurements to ensure that differences in *Geocoris* predation were not due to our staggering of infestation: we counted *Geocoris* populations around all genotypes and saw that they were not different, indicating that *Geocoris* continued to explore ir *LOX2* and hemi-ir*LOX2* plants but not to predate for them over a week of *Manduca* infestation followed by 5 d of *Manduca* egg predation assays (during which *Manduca* eggs were simultaneously applied to all genotypes); and we followed predation of *Manduca* larvae from all four genotypes over 1 week, during which ir*LOX2* and hemi-ir*LOX2* were infested with more larvae than WT and ir *PI* due to sustained higher predation rates on WT and ir*PI* (80% from WT and ir*PI* versus 47% from ir*LOX2* and 67% from hemi-*irLOX2*).

As a result, *Geocoris* had the same opportunity to locate *Manduca* larvae and eggs on all genotypes, and ir*LOX2* and hemi-ir *LOX2* plants suffered, in total, a similar amount of *Manduca* damage to WT plants; only ir*PI* plants suffered significantly less *Manduca* damage (fig. S6C). We took the different number of " days in field" for each plant into account in our comparison of growth and reproduction among genotypes and therefor the staggered planting does not affect this comparison (fig. S4, table S4).

Finally, to ensure that the correlated differences we observed in plant reproduction and *Manduca* mortality were due to plant GLV emission and not to different timing and amounts of *Manduca* damage, we conducted a *Manduca* predation and plant performance assay during infestation M4 (figs. 1A, 3B) for which all plants used were matched for size as well as former damage and reproduction (fig. S7) and infested simultaneously with one *Manduca* larva each.

Flower and bud counts are our best measures of plant reproduction for transgenic plants in the field, and correlate with plant Darwinian fitness

Although the production of viable offspring is the accepted definition of Darwinian fitness, we are not permitted to allow transgenic plants to disperse ripe seed in the field, and measures to prevent seed dispersal, such as bagging meristems, strongly affect production of buds and flowers and can also affect seed viability by increasing temperature, and decreasing respiration and photosynthesis of reproductive tissue and associated green tissue.

For field-grown *N. attenuata* plants, fewer than 5% of buds and flowers (in total) are aborted by healthy (not diseased) plants, and abortion seems always to be due to damage by insects (M. Schuman and I. T. Baldwin, personal observation). Plants are self-compatible and more than 70% of seed set from plants in native populations results from fertilization via self-pollen *(9)*. Thus numbers of buds and flowers correlate to lifetime seed capsule production, which in turn correlates to lifetime seed production, which has been used as a proxy measure of Darwinian fitness *(10-12)*.

The transgenic lines used do not vary in seed mass or their seedling viability under laboratory conditions.

Manduca damage reduces growth and reproduction of WT and hemi-ir *LOX2*, but not of ir*PI* and ir*LOX2*

Manduca feeding reduced flower production rates by about 50% in WT and by about 30% in hemi-ir*LOX2* plants in 2010, although the overall reduction was only significant in WT (fig. S5, table S3); and reduced bud production significantly for both WT and hemi-ir*LOX2* by 25-30% in 2011 (fig. S4, table S4).

Damage from naturally occurring herbivores other than *Manduca* cannot explain differences in plant fitness

We monitored herbivore attack to determine whether GLV-silenced plants suffered different amounts of herbivore damage, which could influence the fitness measurements. All genotypes were attacked by naturally occurring mirid (*Tupiocoris notatus*) and noctuid herbivores which caused similar amounts of damage across genotypes and years (ca. 15 and 3% of total canopy area, respectively) although ir*LOX2* plants suffered 60% less mirid and noctuid damage in 2011 (fig. S6). However, reduced herbivore damage on ir *LOX2* in 2011 cannot explain why ir*LOX2* plants were less fit than WT. Plants in 2011 were also damaged by flea beetles and grasshoppers ($<$ 3% of canopy area, fig. S6).

We cannot exclude that reduced growth and reproduction of ir*LOX2* -silenced control plants in 2011 (fig. S4, table S4) might have been due to non-herbivory-related factors (e.g. differences in root health corresponding to GLV antimicrobial properties) which did not play a role in 2010. Because of this uncertainty, the growth and reproduction data for plants in *Manduca* infestations M2 and M3 are presented in supplemental figures and not in the main figures. The reproduction data shown in the main figures (fig. 3) is from plants which were carefully matched for size and prior reproduction (fig. S7), and this " triplet" experiment is the more robust basis for our argument that GLV-mediated indirect defense increases plant reproduction.

Meristem removal from plants in the " triplet" experiment (fig. 3) was necessary despite matching, and affected all plants similarly

To rigorously test the consequences of GLV-mediated predation of *Manduca* on plant reproduction, we selected triplets of WT, ir*PI* and hemi-ir*LOX2* plants similar in size, previous reproductive output, apparent health, and prior *Manduca* damage (fig. S7); damage from naturally occurring herbivores did not differ among these genotypes (fig. S6). We removed all reproductive meristems from matched plants in 2011 to set initial reproductive units to zero, and to allow us to follow plant reproduction over full *Manduca* larval development without incurring ripe transgenic seed capsules (see above section on bud and flower counts as best measures of reproduction).

The hemi-ir*LOX2* plants chosen in 2011 had produced more flowers than WT - but not more than ir*PI* - prior to the start of the triplet experiment (fig. S7B). This did not correspond to more cuts

on average for hemi-ir*LOX2* when removing floral meristems prior to the start of the experiment: floral meristems were cut at the base of clusters of buds, and the number of these did not differ for the plants chosen, and neither did the number of side branches (fig. S7B) which bore most of the floral meristems.

Therefore, in the absence of additional effects during the triplet experiment, the reproduction of the matched hemi-ir*LOX2* plants should have been similar to that of WT and ir*PI*. The meristem cutting at the beginning of the experiment.

Materials and Methods

Plants, growth conditions and field plantations

Seed germination, glasshouse growth conditions, and the *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure have been described previously *(13)*. Seeds of the 31^{st} generation of the inbred " UT" line of *Nicotiana attenuata* (Torr. ex S. Wats.) were used as the wild-type plant in all experiments. For the field experiment, seedlings were transferred to 50-mm peat pellets (Jiffy) 15 d after germination and gradually hardened to the environmental conditions of high sunlight and low relative humidity over 10 d. Small, adapted, size-matched rosette-stage plants were transplanted into a field plot in a native habitat in Utah and watered thoroughly once at planting and as needed over the first two weeks until roots were established; all plants received the same watering regime in each year. Plants were arranged in quadruplets of one plant per genotype, with individuals 0.5 m apart, a distance sufficient to allow predators and herbivores to distinguish volatiles from neighboring plants *(14)*. Quadruplets were arranged so that no two adjacent plants were of the same genotype (fig. S9). In 2010, the field plot was located at latitude 37.141, longitude 114.027; in 2011, plants were planted at a second field site located at latitude 37.146, longitude 114.020. Field plantations were conducted under APHIS permission numbers 06-242-3r-a3 (2010 and 2011) and 10-349-102r (2011).

We used previously characterized, homozygous, inverted-repeat RNAi transformants of the second transformed generation (T_2) to silence GLV biosynthesis and emissions: ir*LOX2* line number A-04-52-2 *(4)*, and TPI activity: ir*PI* line number A-04-186-1 *(1)*. Vector construction and the pSOL3 plasmid have been described previously *(15)* . Hemi-ir*LOX2* plants were created in an ir*PI* background by crossing ir*LOX2* and ir*PI* homozygous lines. However, the hemizygous ir*PI* construct did not silence TPI activity or transcripts (fig. 1B, fig. S2), and these plants therefor served as vector controls for comparison with ir*PI* but were phenotypically intermediate between WT and ir*LOX2* with regard to GLV emissions (fig. S1, S2).

Manduca eggs and larvae

Eggs from laboratory-reared *Manduca sexta* used in the field were kindly provided by Dr. Carol Miles at SUNY Binghampton. Wild *Manduca* eggs were collected when available from natural ovipositions. Eggs were allowed to hatch in well-aerated boxes on fresh *N. attenuata* leaf tissue over a moistened paper towel. *Manduca* used to elicit glasshouse-grown plants, or to collect oral

secretions (OS) for plant treatments, were taken from an in-house colony at the Max Planck Institute for Chemical Ecology in Jena.

Plant treatments

Plants in field experiments were either infested with *Manduca* larvae as described, or left uninfested (control). For measuring headspace GLVs in the field and for glasshouse assays, plants were treated with wounding and *Manduca* OS (W+OS) as a standardized method to mimic *Manduca* feeding. Pure OS collected from 4^{th}-5^{th} instar *Manduca* larvae from the Jena colony fed on WT plants was diluted 1:5 with distilled water before use (even 1000-fold diluted OS is still sufficient to cause most OS-elicited responses *(16)*). For field-grown plants, a similar, mature, non-senescent leaf was chosen from each plant; for glasshouse-grown plants, the two adjacent older leaves (nodes +1, +2) to the leaf undergoing a source-sink transition (node 0) on rosette-stage plants were used for *PI* and *LOX2* transcript quantification, and the +2 node of a separate set of bolting plants was used for measuring headspace volatiles. The leaf chosen for treatment was wounded by using a fabric pattern wheel run over the adaxial surface to make six rows of holes in the lamina, three rows on either side of the midvein. Twenty µL of 1:5 diluted OS were deposited on the adaxial surface and gently rubbed over the holes with a gloved finger. Control plants were left untreated.

Plant tissue harvests and sample handling

For field-grown plants and glasshouse-grown *Manduca*-fed plants, a similar, mature, non-senescent systemic leaf was chosen from each plant; for glasshouse-grown plants, the leaves at nodes +1 and +2 were harvested. Leaves were cut at the petiole and wrapped in a double layer of aluminum foil. In the field, harvested leaves were immediately frozen on dry ice insulated with ice packs frozen at -20 °C; samples were stored at -20 °C until transport to Jena on dry ice, where they were kept at -80 °C until analysis. Leaves harvested from glasshouse-grown plants were flash-frozen in liquid nitrogen and kept at -80 °C until analysis. All sample processing was carried out over liquid nitrogen until the addition of the extraction solvents. Prior to analysis, entire leaves were ground in a mortar and pestle and transferred to a microcentrifuge tube for storage. For specific measurements, aliquots were weighed into microcentrifuge tubes containing two steel balls and finely ground in a GenoGrinder (SPEX Certi Prep) prior to extraction.

Quantification of *PI* and *LOX2* transcripts

Leaf samples were from control plants or plants treated with W+OS and harvested at the peak of transcript accumulation for *PI (17)* and *LOX2 (4)*. Total RNA was extracted from leaves using the TRIzol reagent (Invitrogen). A 0.5 µg aliquot of total RNA of each sample was reverse-transcribed using oligo$(dT)_{18}$ and RevertAid H Minus reverse transcriptase (Fermentas) following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed with a Mx3005P Multiplex qPCR system (Stratagene) and the qPCR Core kit for SYBR Green I (Eurogentec). Transcripts were quantified using external standard curves for each gene. Elongation factor 1A (*EF1A*) transcript abundance in each sample was used to normalize total cDNA concentration variations. Samples of RNA used to make cDNA were pooled to the same dilution as

in cDNA samples and run alongside cDNA in qPCRs to control for gDNA contamination; no contamination was detected. The sequences of primers used for qPCR *(18, 19)* are provided in table S5.

Quantification of GLV pools in tissue

To qualitatively asses GLV pools in leaf tissue from field-grown plants, and to determine appropriate amounts of leaf tissue and internal standard (IS) for GLV extraction, we extracted pooled samples from leaves collected June 6^{th} from *Manduca*-infested plants during the infestation M3. Each sample was pooled from all leaves collected from one genotype. Three hundred µL hexane was added to 100 mg tissue spiked with 3 µg tetralin as an IS and incubated by rotating at RT overnight. Samples were allowed to settle and 100 µL of water- and tissue-free hexane was transferred to a GC vial containing a 250 µL microinsert. Individual analytes were analyzed by a Varian CP-3800 GC-Saturn 4000 ion trap MS connected to a ZB5 column (30 m0.25 mm i.d., 0.25 µm film thickness, Phenomenex). One µL of samples was injected by a CP-8400 autoinjector (Varian) onto the column in with a 1/10 split ratio; the injector was returned to a 1/70 split ratio 2 min after injection through the end of each run. The GC was programmed as follows: injector held at 250 °C, initial column temperature at 40 °C held for 5 min, then ramped at 5 °C min^{-1} to 185 °C and finally at 30 °C min^{-1} to 300 °C, held for 0.17 min. Helium carrier gas was used and the column flow set to 1 mL/min. Compounds eluted from the GC column were transferred to the MS for analysis. The MS was programmed as follows: transfer line at 250 °C, trap temperature 110 °C, manifold temperature 50 °C, source heater 200 °C and scan range from 40 to 399 m/z at 1.33 spectra per second as described *(20)*. The identification of compounds was conducted by GC retention time and mass spectra compared to mass spectra databases, Wiley version 6 (Wiley) and NIST (National Institute of Standards and Technology) spectra libraries.

For the quantification of GLV pools in leaf tissue from field-grown plants, (*Z*)-3-hexenyl acetate was chosen as an internal standard because it was not detected in preliminary qualitative GC-MS analyses of tissue samples pooled from each line (fig. S1A), and because its chemical similarity to (*E*)-hex-2-enal, and (*Z*)-hex-3-en-1-ol made it a good choice of internal standard for normalization and calculation of yield from extracts. Three hundred µL hexane were added to 50 mg tissue spiked with 15 µg (*Z*)-hex-3-enyl acetate as an IS and extracted as described above (N=10). Analytes were separated by Varian CP-3800 GC-FID connected to a ZB-Wax column (30 m0.25 mm i.d., 0.25 µm film thickness, Phenomenex). One µL of samples was injected by a CP-8400 autoinjector (Varian) onto the column in a splitless mode; the injector was returned to a 1/70 split ratio 2 min after injection through the end of each run. The GC was programmed as follows: injector held at 230 °C, initial column temperature at 40 °C held for 7 min, then ramped at 5 °C min^{-1} to 115 °C and finally at 30 °C min^{-1} to 250 °C, held for 0.5 min. Helium carrier gas was used and the column flow set to 1 mL/min. Compounds eluted from the GC column were transferred to a Varian FID set at 250 °C for analysis (airflow 300 mL/min, hydrogen 30 mL/min, nitrogen (make-up gas) 5 mL/min). Individual volatile compound peaks were quantified by peak areas using MS Work Station Method Builder and Batch Report software (Varian) and normalized to the peak area of the internal standard [(*Z*)-hex-3-enyl acetate] in each sample. Peak identification and quantification was done by comparison to standard curves of pure compounds in hexane. Compounds present in quantifiable amounts in hexane extracts were (*Z*)-hex-3-en-1-ol, (*E*)-hex-2-enal and the IS (*Z*)-hex-3-enyl acetate.

Relative quantification of GLVs and (E)-α-bergamotene in the plant headspace

For measurement of GLVs in the headspace of field-grown plants, intact leaves were harvested (N=3) and kept fresh by placing the petioles in microcentrifuge tubes filled with water. Immediately before each measurement, one leaf was treated with W+OS, and a 1 cm^2 disc was stamped out and placed in a 4 mL GC vial. After 15 min, the headspace in the vial was measured with a ZNose® 4200 portable gas chromatograph with a 1 m DB5 column (Electronic Sensor Technology, Newbury Park, CA, USA) by inserting the ZNose inlet needle through the septum of the GC vial into the headspace. The program was as follows: valve set at 165 °C , inlet at 200 °C, trap at 250 °C; 30 s sampling time, column ramped from 30 to 190 °C at 4 °C/s, data collection for 20 s. Genotypes were analyzed in an alternating order within each replicate: first replicate 1 of all genotypes, then replicate 2, then replicate 3. Retention times of GLV aldehydes and alcohols, the most abundant GLV headspace components, were determined using pure standards.

For the analysis of GLVs in the headspace of glasshouse-grown plants, the +2 leaf was enclosed immediately after W+OS elicitation in a food-quality 50 mL plastic container (Huhtamaki) connected to self-packed Poropak Q filters [20 mg of Poropak (Sigma-Aldrich) packed with silanized glass wool and Teflon tubing in the column bodies (ARS, Inc.) as described *(7)*]. The ambient air was pulled from the trapping container through the tubing connected to a Poropak Q filter using a vacuum pump connected to a manifold as described *(20)* for 3 h. After trapping, sampled leaves were excised at the base of the petiole, scanned, and the leaf area was measured in comparison to a 1 cm^2 standard (SigmaScan 5.0, Systat Software Inc.) for normalization of volatile emission to cm^2 leaf area. Poropak Q filters were wrapped in aluminum foil and stored at -20 °C until elution of volatiles with 250 µL dichloromethane. Immediately prior to elution, each filter was spiked with 320 ng of tetralin internal standard in hexane (Sigma-Aldrich). Filters were eluted into a GC vial containing a 250 µL glass insert. Samples were analyzed by a CP-3800 GC Varian Saturn 2000 ion trap MS (Varian) connected to a polar ZB-wax column (30 m0.25 mm i.d., 0.25 µm film thickness, Phenomenex). One µL of samples was injected by a CP-8200 autoinjector (Varian) onto column in a splitless mode; the injector was returned to a 1/70 split ratio, 2 min after injection through the end of each run. The GC was programmed as follows: injector held at 230 °C, initial column temperature at 40 °C held for 3 min, then ramped at 5 °C min^{-1} to 180 °C and finally at 10 °C min^{-1} to 240 °C, held for 1 min. Helium carrier gas was used and the column flow set to 1 mL/min. Eluted compounds from GC column were transferred to the MS for analysis. The MS was programmed as follows: transfer line at 230 °C, trap temperature 150 °C, manifold temperature 80 °C and scan range from 40 to 399 m/z at 1.33 spectra per second as described *(20)*. Individual volatile compound peaks were quantified by peak areas of two specific and abundant ion traces per compound using MS Work Station Data Analysis software (Varian) and normalized by the 104+132 ion trace peak area of the internal standard (tetralin) in each sample. The identification of compounds was conducted by GC retention time and mass spectra compared to mass spectra databases, Wiley version 6 (Wiley) and NIST (National Institute of Standards and Technology) spectra libraries. In 3 h headspace samples we detected (*Z*)-hex-3-en-1-ol, (*Z*)-hex-3-en-1-ol, (*E*)-hex-2-en-1-ol (forms from (*E*)-hex-2-enal on filters over trapping periods longer than 20 min), (*Z*)-hex-3-enyl acetate, (*Z*)-hex-3-enyl butanoate, (*Z*)-hex-3-enyl isobutyrate, and (*Z*)-hex-3-enyl propanoate .

The collection of (*E*)-α-bergamotene from the headspace of glasshouse-grown plants and its extraction from Poropak Q filters was carried out as for GLVs, except that (*E*)-α-bergamotene was collected 24-32 h after treatment of the leaf. Eluted samples were analyzed by an HP 6890 GC-5973

quadropole MS (Hewlett-Packard) connected to a nonpolar DB-5ms column (30 m0.25 mm i.d., 0.25 μm film thickness, Agilent). One μL of samples was injected by a HP 7683 autoinjector (Hewlett-Packard) onto column in a splitless mode; the injector was purged at 50 mL/min 1.5 min after injection and switched to gas saver mode (20 mL/min) from 10 min through the end of each run. The GC was programmed as follows: injector held at 230 °C, initial column temperature at 40 °C held for 2 min, then ramped at 5 °C min^{-1} to 165 °C and finally at 60 °C min^{-1} to 300 °C, held for 2 min. Helium carrier gas was used and the column flow set to 2 mL/min. Eluted compounds from GC column were transferred to the MS for analysis. The MS was programmed as follows: source at 230 °C, quad temperature 150 °C, and scan range from 33 to 350 m/z at 4.49 spectra per second. (*E*)-α-bergamotene was quantified by peak area using the ion trace 119 m/z in Chemstation software (Agilent) and normalized by the 104 ion trace peak area of the internal standard (tetralin) in each sample. The identification of (*E*)-α-bergamotene and tetralin was conducted by GC retention time and mass spectra compared to mass spectra of known standards as described *(20)*.

Quantification of *TPI* activity

TPI activity was quantified in 100 mg of leaf tissue using a radial diffusion assay as described *(21)*.

Plant growth and reproduction

Plant size (rosette diameter, stem length and branching) was monitored at the end of infestation M1 in 2010 and from the beginning of infestation M2 in 2011 (fig. 1A): rosette diameter was measured as the maximum diameter found by gently laying a ruler over the rosette; stem length was measured from the base of the stem to the tip of the apical inflorescence by placing a ruler beside the stem; and all side branches 5 cm or longer were counted. Reproductive output was monitored by counting the number of closed flowers removed every 2-3 d (before they opened) from the beginning of flowering, and by counting numbers of closed flowers and buds 2 mm or larger at the end of infestation M1 in 2010, and during all *Manduca* infestations in 2011. All growth and reproduction data were analyzed for differences in control versus *Manduca*-infested plants within each genotype (tables S3, S4). Because size-matched plants had been planted over one week in 2011, growth and reproduction data from plants in 2011 were organized by the number of days since planting for comparison between genotypes (fig. S4).

Herbivore damage and plant health

Photographs were taken of entire plants and *Manduca*-damaged leaves during infestations M2 and M3 in 2011. Damage caused by *Manduca* larvae was rated from photographs by an independent observer with no knowledge of plant identity. Total percent canopy damage due to *Manduca* was rated as 1, 2, 3, or 4 using the damage index in fig. S6.

We monitored herbivore attack to determine whether GLV-silenced plants suffered different amounts of herbivore damage, which could influence the fitness measurements. The naturally occurring

herbivore community on plants in 2010 and 2011 comprised mirids (*Tupiocoris notatus*), and noctuid larvae; in 2011 grasshoppers (*Trimerotropis* spp.), and flea beetles (*Epitrix* spp.) were also present. Total canopy damage due to herbivores occurring naturally on the field plot was quantified prior to *Manduca* infestations in 2010 and 2011 and at again during M3 in 2011. Damage was calculated by identifying damage from specific herbivores by their characteristic feeding patterns, counting the number of leaves per plant (small leaves were counted as 1/5 to 1/2 of a leaf based on leaf area and large leaves were counted as 1 leaf), estimating the total percentage of leaf area damage due to each herbivore, and dividing the total leaf area damage from each herbivore by the total number of leaves. Leaf area damage was estimated in categories of 1%, 5%, 10%, 15%, and so on, in steps of 5%. All such damage estimates were made by M. Schuman or K. Barthel, who first practiced quantifying damage together until they consistently arrived at the same numbers.

As part of matching plants in triplets prior to infestation M4 in 2011, plant health was rated on a scale of 1 (dead) to 5 (healthy) using the index in fig. S7.

Manduca bioassays

Manduca behavior, predation, and growth assays were conducted with 1 st- and 2nd-instar *Manduca* larvae, except infestations M1 2010 and M4 in 2011, in which larvae were reared from the 1st through 5th instars on plants. Larvae used for plant infestations were placed as neonates on a rosette or lower stem leaf which was at a standardized position for each assay, and monitored mornings and evenings, during times outside of the main period of *Geocoris* activity that occurs at midday. One or two larvae were placed on plants at a time for each assay, depending on the number available, and were equally distributed among plants.

Larvae used in the off-plant mock predation assays were hatched on the appropriate *N. attenuata* genotype (WT or ir*PI*) and hatching was monitored three times per day (morning, noon, evening) so that the mock predation assay could be timed to 48 h after larvae hatched. A protocol of the mock predation assays is given in fig. 4, and supplemental videos S1-S4 depict on-plant (S1, S2) and off-plant (S3, S4) behavioral assays. Larvae for off-plant mock predation assays were kept in aerated plastic boxes on cut leaves over moist paper towels. Leaves were refreshed twice daily and were kept fresh by placing the petioles in water in 1.5 mL microcentrifuge tubes which were closed around the petiole with Parafilm (Pechiney Plastic Packaging Company). Larval growth was measured as increases in body length (mm) using calipers or a small, flexible, transparent plastic ruler.

Predation assays

Predation assays were conducted with larvae placed on plants as described above, or with two eggs per plant fixed with droplets of α-cellulose glue *(14)* to the underside of a rosette or lower stem leaf at a standardized position; for the egg predation assay, one larva per plant was clip-caged and allowed to feed on a remote leaf to ensure continual HIPV emission. Predation was monitored mornings and evenings. Larvae were considered to be predated when either the larva was missing and confirmed missing over multiple days, but clear *Manduca* feeding damage was present, or when the predated larval carcass was found (fig. S3B). Mortality was defined as the total number

of missing larvae. Eggs were considered predated when the eggshell was empty but intact except for a small hole which characterizes the typical damage caused by *Geocoris* feeding; eggs occasionally collapse during *Geocoris* predation, but collapsed eggs were not counted unless the eggs were mostly or fully empty and with a visible hole (fig. S3B).

GLV supplementation

During infestation M2, GLVs were added back to ir*LOX2* and hemi-ir *LOX2* headspaces by placing a cotton swab adjacent to the *Manduca*-infested leaf and adding ca. 20 µL of lanolin paste containing pure GLVs dissolved in hexane (table S1) *(22)* to the cotton swab. Cotton swabs bearing 20 µL of lanolin paste with hexane as a control were placed next to *Manduca*-infested leaves of WT and ir*PI* plants. Lanolin pastes were regularly refreshed by adding 20 µL in the early afternoon and in the morning. Placing GLVs next to, rather than on the leaf ensured that the supplemented headspace would not be altered by plant metabolism, and that we could terminate the supplementation by removing the cotton swabs.

Geocoris counts

Field plots were monitored daily for *Geocoris* presence during the experiments in 2010 and 2011. Soon after the first *Geocoris* sightings in May 2011 (before the first *Manduca* infestation, M2), *Geocoris* populations in the immediate vicinity of experimental plants were monitored every 2-3 days by counting individuals. Counts were conducted during the main period of *Geocoris* activity in the early afternoon, by at least two observers in parallel, in order to complete the count around all *Manduca*-infested and control plants within 20-30 min. Each observer proceeded by looking at a focal plant and its immediate vicinity for 15s and then quickly inspecting the rosette leaves; all *Geocoris* adults and nymphs seen on, under, or within 5 cm around the rosette of the plant during this time were counted. Observers moved in synchrony with each other from one end of the field plot to the other, in this way counting predators around plants which had not yet been disturbed.

Statistical analyses

Fisher's exact tests were conducted using a macro (J. H. Macdonald, http://udel.edu/mcdonald/statfishers.html) for Excel (Microsoft). All other statistical analyses were conducted with SPSS 17.0 (IBM). Count data were analyzed either by Fisher's exact tests (independent values) or by Friedman tests (repeated measures). Levene's test for homogeneity of variance was performed prior to all t-tests and ANOVAs and when necessary, data were $\log_2$ transformed (volatile and transcript data), square root transformed (count data) or arcsin transformed (herbivore damage data) to meet requirements for homogeneity of variance. Parametric data were compared using ANOVAs, MANOVAs, or repeated-measure ANOVAs followed by Scheffe post-hoc tests. If variance was not homogeneous following transformation, data were compared using Kruskal-Wallis tests (for multiple comparisons) or Mann-Whitney U-tests (for two-way comparisons) and Bonferroni P-value corrections were used to correct for nonparametric multiple comparisons. For Kruskal-Wallis

tests and Mann-Whitney U-tests, a Monte Carlo algorithm was used with 10000 permutations and a 95% confidence level.

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Supplemental Video S1. On-plant assay, plant 7u, WT, 18th June 2011.

`https://www.dropbox.com/s/yqgeotanxf81t8r/Video\%20S1.AVI`

Supplemental Video S2. On-plant assay, plant 2o, ir*PI*, 18th June 2011.

`https://www.dropbox.com/s/efe6h6ha4yy3zg3/Video\%20S2.AVI`

Supplemental Video S3. Off-plant assay, replicate 3, WT, 24th June 2011.

`https://www.dropbox.com/s/uttz1j0t7cbutsy/Video\%20S3.AVI`

Supplemental Video S4. Off-plant assay, replicate 3, WT, 24th June 2011.

`https://www.dropbox.com/s/seue1m6etwoxhr5/Video\%20S4.AVI`

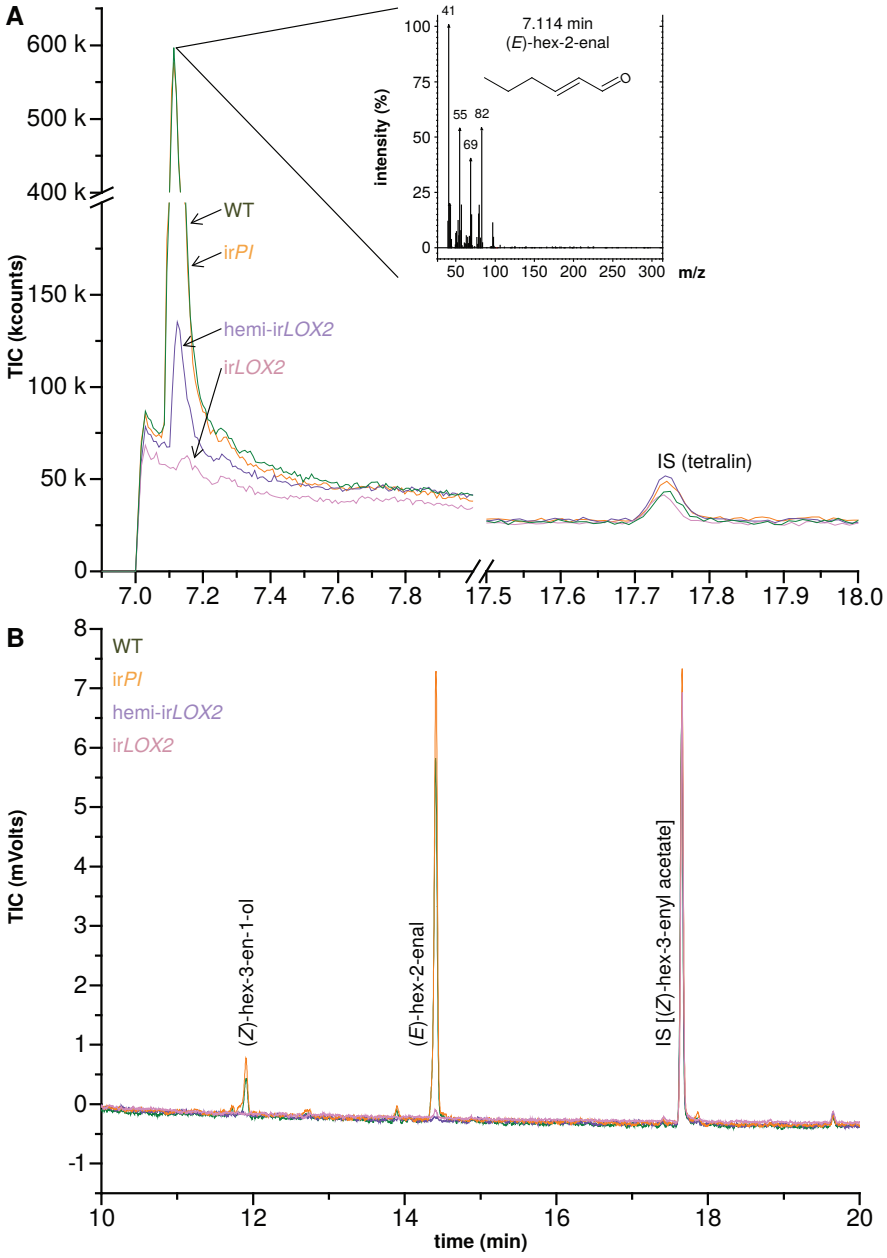

Figure S1 – Hexane extracts of leaves from field-grown plants. (A) Hexane extracts from pooled leaf samples of field-grown plants for a qualitative assessment of GLV pools, analyzed by GC-MS with a split ratio of 1/100 onto a non-polar column; only (*E*)-hex-2-enal was identified due to poor resolution of (*E*)-hex-2-enal and (*Z*)- hex-3-en-1-ol on the nonpolar column; no ester peaks were detected. (B) Example chromatograms from hexane extracts of individual leaf samples from field-grown plants, analyzed by GC-FID on a wax column. The dominant compound was (*E*)-hex-2-enal; (*Z*)-hex-3-en-1-ol was also present in quantifiable amounts. (*Z*)-3-hexenyl acetate was chosen as an internal standard because no esters were detectable in the preliminary qualitative GC-MS analysis (1A), and because its chemical similarity to (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol made it a good choice of internal standard for normalization and calculation of yield from extracts. IS, internal standard.

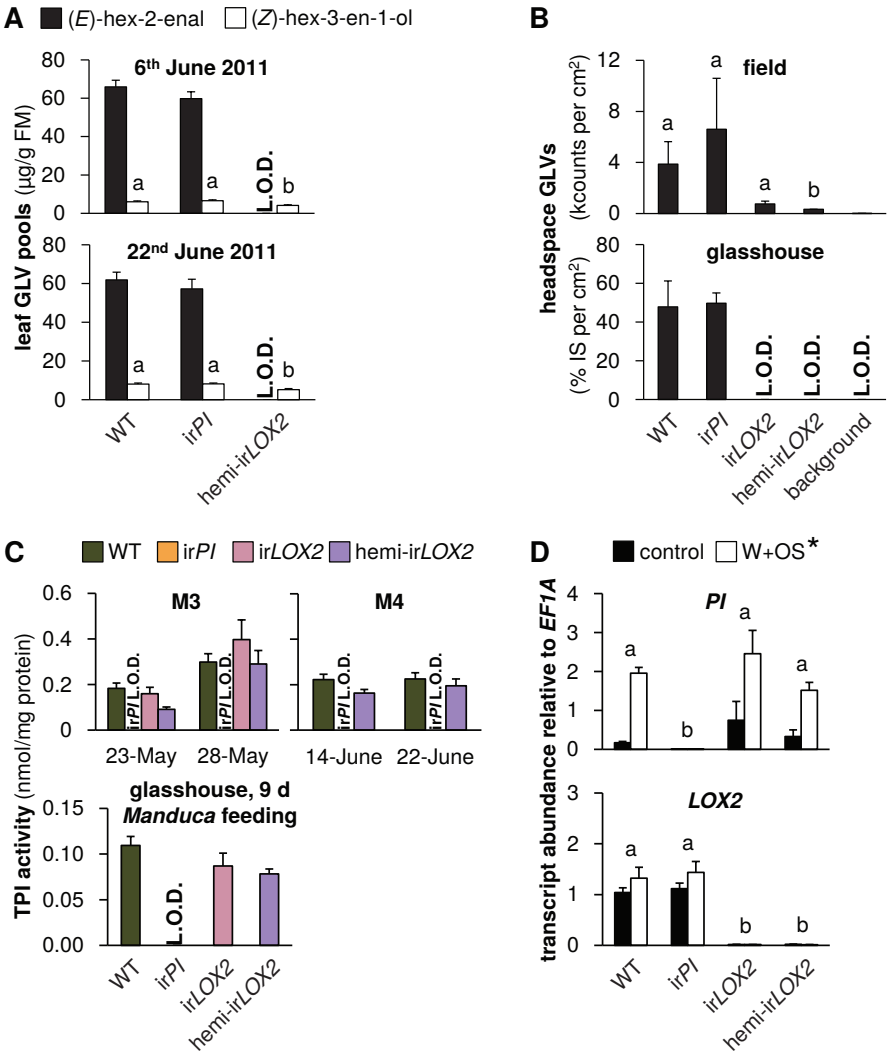

Figure S2 – Genetic manipulation of GLV and TPI production; graphs show means+SEM. (**A**) GLVs extracted with hexane from leaf tissue of field-grown WT, ir*PI*, and hemi-ir*LOX2* plants grouped in triplets for infestation M4 in 2011(see fig.1A). Leaves were harvested from every plant at the beginning (6^{th} June) and in the middle of M4 (22^{nd} June) and leaves from plants in 10 randomly chosen triplets were analyzed. Only (*E*)-hex-2-enal and (*Z*)-hex-3-en-1-ol were quantifiable in leaf extracts. Different letters indicate significant differences (P≤0.05) in Scheffe *post-hoc* tests following one-way ANOVAs for (*Z*)-hex-3-en-1-ol (top panel, $F_{2,26}$ =9.556, P=0.001; bottom panel, $F_{2,26}$=12.196, P<0.001). (**B**) GLVs measured in headspace samples of leaves from field-grown (top panel, N=3) or glasshouse-grown plants (bottom panel, N=4). For field-grown plants, leaves were harvested and measured on May 21^{st} (just before M3). Intact leaves were kept fresh by placing petioles in water. Immediately before each measurement, one leaf was treated with wounding and *Manduca* oral secretions (W+OS); a 1 cm^2 disc was stamped out and placed in a 4 mL GC vial. After 15 min the headspace in the vial was measured with a Z-Nose™ 4200 and total alcohols and aldehydes were quantified. Different letters indicate significant differences (P<0.05) in Scheffe *post-hoc* tests following one-way ANOVA ($F_{3,8}$=7.346, P=0.011). (continued on p. 181)

Continued Figure S2 – For glasshouse-grown plants, leaves were left on plants, treated with W+OS, and enclosed in padded, 50 mL food-quality plastic containers for 3 h while the headspace was pulled over a Poropak Q filter. Filter eluents were measured by GC-MS. Three h headspace samples contained (*Z*)-hex-3-en-1-ol, (*E*)-hex-2-en-1-ol (forms from (*E*)-hex-2-enal on filters over trapping periods longer than 20 min), (*Z*)-hex-3-enyl acetate, (*Z*)-hex-3-enyl butanoate, (*Z*)-hex-3-enyl isobutyrate, and (*Z*)-hex-3-enyl propanoate, all of which showed the pattern shown for the total amount. (**C**) TPI activity measured in systemic leaves of field-grown (top two panels, 2011, N=11-14 for panel 1 and N=21 for panel 2) or glasshouse-grown plants (bottom panel, N=10) attacked by *Manduca* larvae. Only WT, ir*PI* and hemi-ir*LOX2* plants were used in the wild *Manduca* assay. For a timeline of *Manduca* infestations M1-M4 see fig. 1A. (**D**) Transcripts of *PI* and *LOX2* in unelicted leaf tissue (control), and at the point of maximum accumulation in W+OS-elicited leaf tissue in glasshouse-grown plants (N=5). *W+OS treatment had a significant effect on *PI* ($P<0.001$) transcript accumulation. ^{a,b,c} Different letters indicate significant differences between genotypes ($P<0.001$) in Scheffe *post-hoc* tests following two-way ANOVAs on $\log_2$ -transformed data with factors treatment and genotype (*PI*, genotype $F_{3,29}=174.077$, $P<0.001$, treatment $F_{1,29}=75.909$, $P<0.001$; *LOX2*, genotype $F_{3,32}=635.477$, $P<0.001$, treatment $F_{1,32}=0.021$, $P=0.887$). L.O.D., below limit of detection for measurement.

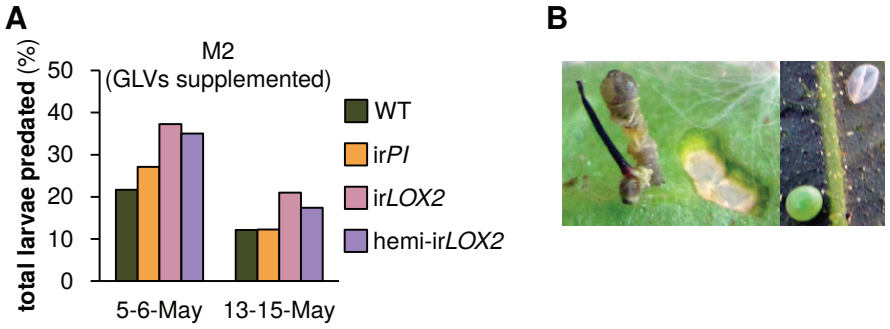

Figure S3 – Total predation of *Manduca* larvae per trial over two trials during infestation M2 (see fig. 1A). GLVs were supplemented externally by placing cotton swabs next to *Manduca*-infested leaves (1/plant). Cotton swabs next to ir*LOX2* and hemi-ir*LOX2* plants received 20 µL of a GLV mixture in lanolin paste (table S1); those next to WT and ir*PI* plants received lanolin with hexane as a control because hexane was used to dissolve GLVs before mixing with lanolin. N=59-60 larvae on May 5^{th}-6^{th} and 92-100 larvae on May 13^{th}-15^{th}. Differences were not significant in Fisher's exact tests. (**B**) Examples of predated *Manduca* larva (left panel) and egg (right panel). Left, the carcass of a predated first-instar *Manduca* larva and typical feeding damage from early-instar *Manduca* larvae. Right, an intact (lower left) and a predated (upper right) *Manduca* egg. In this case, the predated egg collapsed during predation.

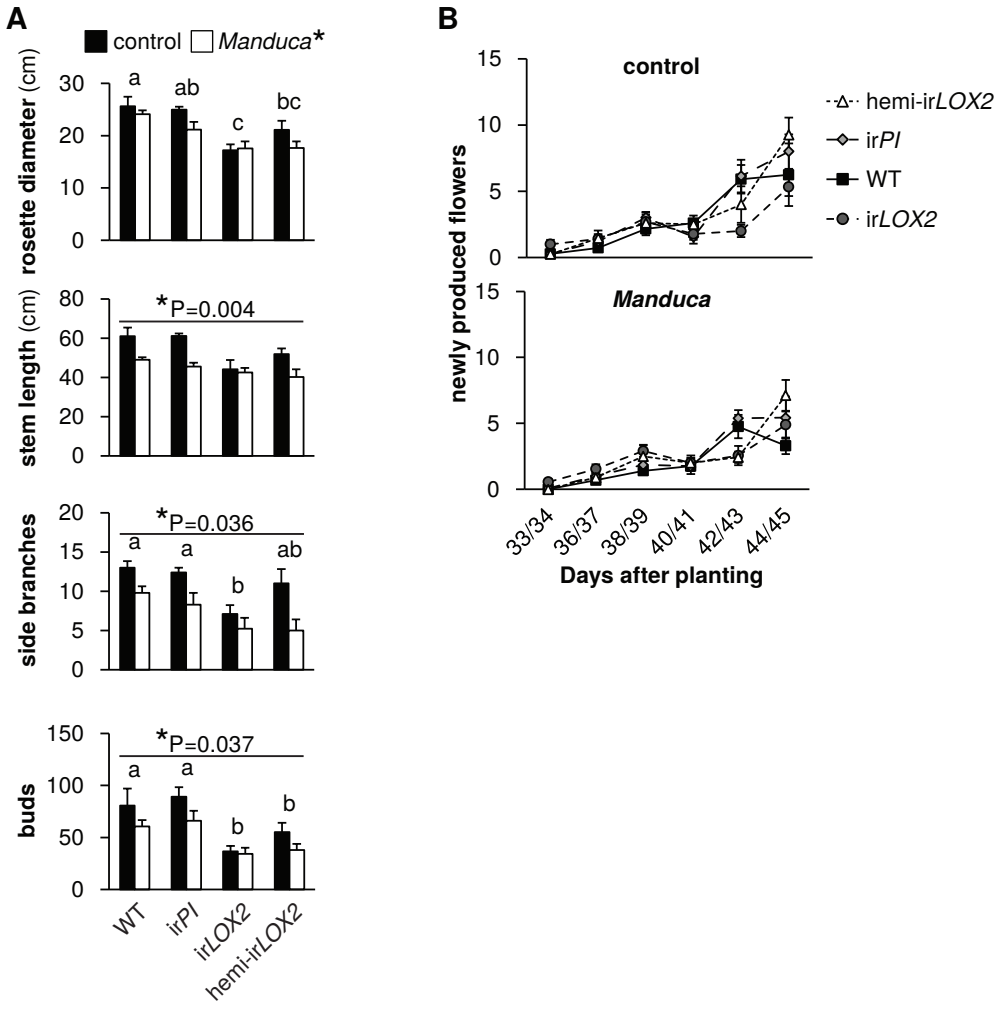

Figure S4 – Growth and reproduction of plants during the 2011 field season, during infestations M2 and M3 (see fig. 1A); graphs show means±SEM. (**A**) Final growth measurements for *Manduca*-infested and uninfested control plants of each genotype (44-45 d after planting, N=11-17). *P<0.05 for Wilks' Lambda test of the effect of *Manduca* feeding on growth and reproduction, day 44-45, in a two-way MANOVA with factors genotype and treatment ($F_{6,52}$=2.287, P=0.049). *P-values above individual graphs denote the significance of *Manduca* feeding over all genotypes for the measurement shown in the MANOVA, or in a separate Mann-Whitney U-test for side branches (stem $F_{1,57}$=9.155;side branches, U = 270; buds $F_{1,57}$=4.572); values for individual genotypes are in table S4. ^{a,b,c}Different letters denote significant (P<0.05) differences between genotypes in Scheffe *post-hoc* tests (rosette diameter $F_{3,57}$ =8.791, P<0.001, stem length $F_{3,57}$=4.192, P=0.009, number of buds $F_{3,57}$=9.876, P<0.001) or Bonferroni-corrected P-values for Mann-Whitney U-tests following a Kruskal-Wallis test (side branches χ^2 = 10.958). (**B**) Flower production for *Manduca*-infested and uninfested control plants from the beginning of flowering. Flowers were counted and removed at the time points shown: each time point represents new flower production.

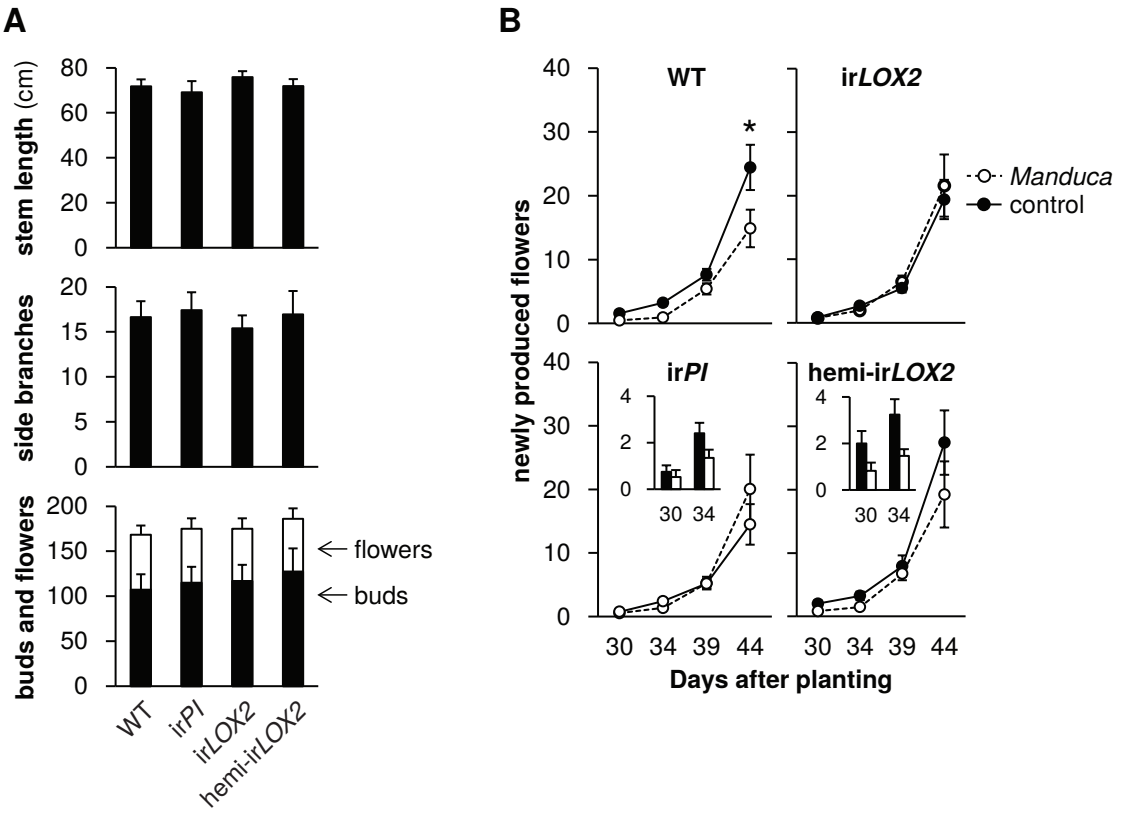

Figure S5 – Growth and reproduction of plants during the 2010 field season in the absence of *Geocoris* activity (means±SEM). (**A**) Final growth and reproduction measurements for *Manduca*-infested plants (M1 in fig. 1A). Measurements were made on June 6th, 2010, 2 d after the removal of 5th-instar *Manduca* larvae. There were no significant differences between genotypes in the parameters shown with or without *Manduca* infestation (table S3). Bud numbers are also shown in fig. 3A. (**B**) Flower production for *Manduca*-infested and uninfested control plants from the beginning of flowering leading up to the final measurements in (**A**). Flowers were counted and removed at the time points shown: each time point represents new flower production. Insets show the first two time points for ir*PI* and hemi-ir*LOX2*. *P<0.05 for the main effect of *Manduca* treatment in a repeated-measures ANOVA with $\log_2$-transformed data (see table S3).

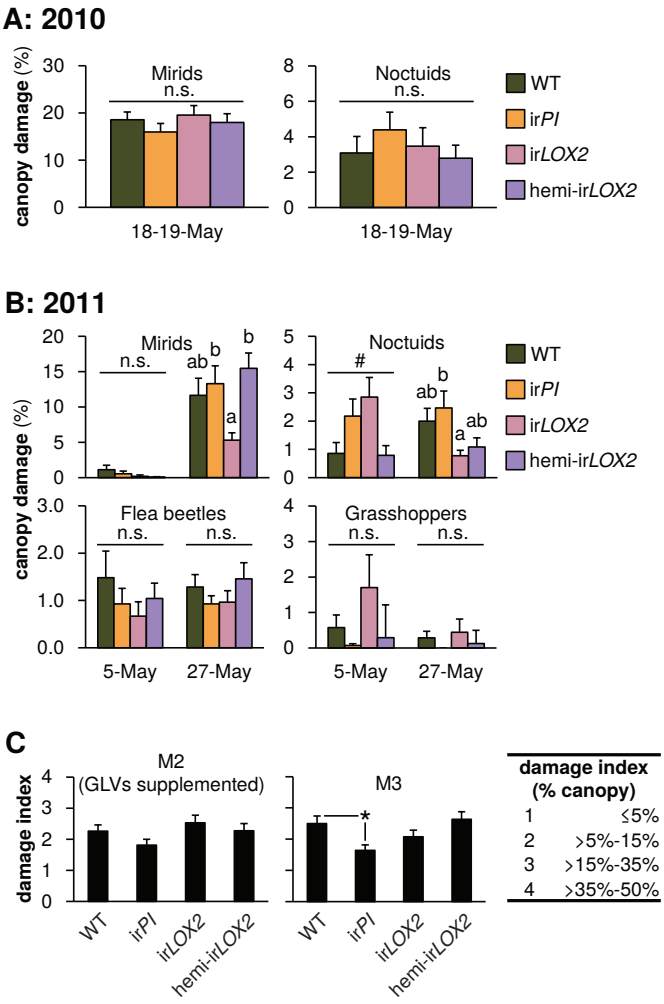

Figure S6 – Herbivore damage to plants during the 2010 and 2011 field seasons (means+SEM). For a timeline of *Manduca* infestations M1-M4, see fig. 1A. (**A**) Total canopy damage due to naturally occurring herbivores before the start of infestation M1 in 2010. (**B**) Total canopy damage due to naturally occurring herbivores before infestation M2 (May 5 th) and near the end of M3 (May 27th) in 2011. ^{a,b,c} Different letters denote significant (P<0.05) differences between genotypes in Scheffe *post-hoc* tests following one-way ANOVAs for arcsine-transformed data at each timepoint (mirids May 27th $F_{3,103}$=5.291, P=0.002; May 27th $F_{3,103}$=3.503, P=0.018); n.s., not significantly different. $^{\#}$P<0.05 for the main effect of genotype on noctuid damage in a Bonferroni-corrected Kruskal-Wallis test, May 5th (χ^2 = 11.239, P=0.027). (**C**) Damage in 2011 from *M. sexta* larvae used in the predation assays in M2 (left panel) and M3 (right panel). GLVs were externally supplemented to plants in infestation M2 and not in M3. Total canopy damage was estimated, using the index, by an independent observer without knowledge of plant identity (N=11-17). *P<0.05 in a Mann-Whitney U-test between ir*PI* and WT on May 28th (U= 54, P=0.046); the difference on May 15th was not significant (P>0.1). **Note that scales differ.**

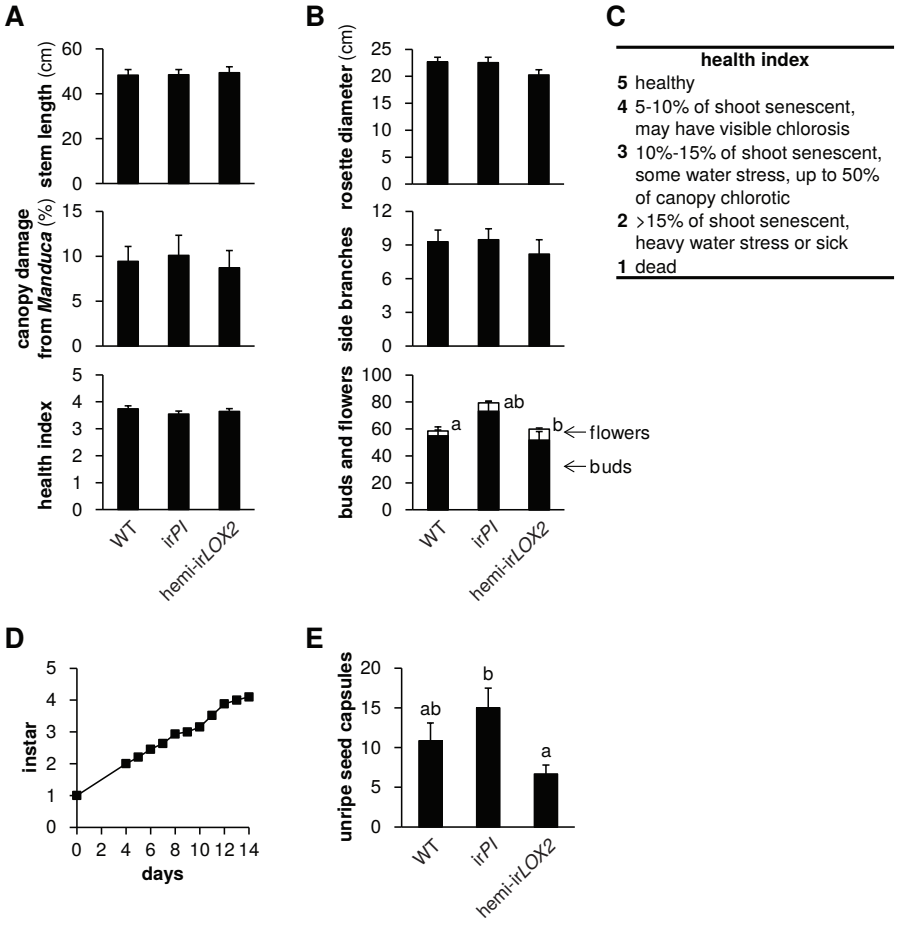

Figure S7 – Comparison of plants used in triplets for infestation M4 in 2011 (see fig. 1A); graphs show means+SEM (N=21 plants). (**A**) Parameters used to match plants in triplets. Measurements and assessments are from the first day of M4. (**B**) Final measurement of prior growth and reproduction for plants used for triplets; data are from the final two measurements during infestation M3 (see fig. 1A). Different letters denote significant differences ($P<0.001$) for flower number in Scheffe *post-hoc* tests following a MANOVA with all measurements and genotype as the factor ($F_{2,60}=8.668$, $P<0.001$). (**C**) Health index used in (**A**). (**D**) Progression of larval instars over time for wild *Manduca* during infestation M4. (**E**) Unripe seed capsules at the final measurement of plant reproduction in figure 3B (day 15) show the same pattern as bud and flower production and therefore cannot explain differences in bud and flower production among genotypes. ^{a,b,c}Different letters denote significant differences ($P<0.05$) for in Scheffe *post-hoc* tests following an ANOVA with genotype as the factor ($F_{2,60}=4.142$, $P=0.021$).

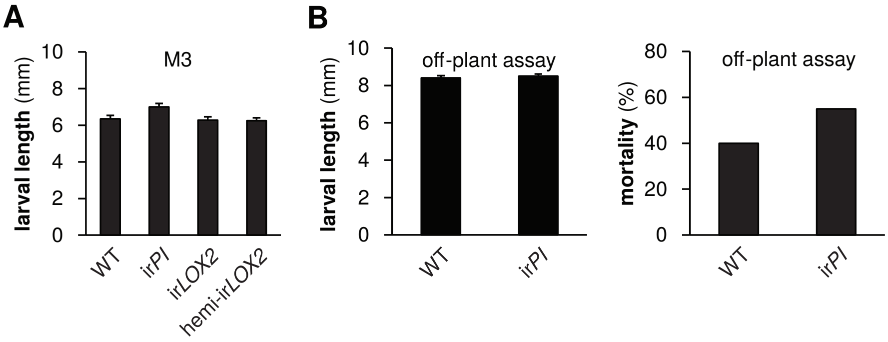

Figure S8 – *Manduca* larval growth and mortality during the first instar. (**A**) Larval length in the first instar after 2 d on plants in the field. Length of surviving larvae was measured in a predation assay during infestation M3 (fig. 2, see fig. 1A), N=13-26 larvae. Length was not significantly different in a one-way ANOVA. (**B**) Left, length of first instar larvae fed for 2 d on WT or ir*PI* tissue and size-matched for use in an off-plant behavioral assay mimicking *Geocoris* attack (fig. 4B); right, mortality of first instar larvae 24 h after mock *Geocoris* attack. Larval length was recorded immediately prior to the assay. Following mock attack, larvae (N=20) were placed in individual cups with WT or ir*PI* leaf tissue (the same genotype which they had been feeding on previously) and allowed to feed for 24 h, after which mortality and size were recorded. Mortality was not significantly different in a Fisher's exact test.

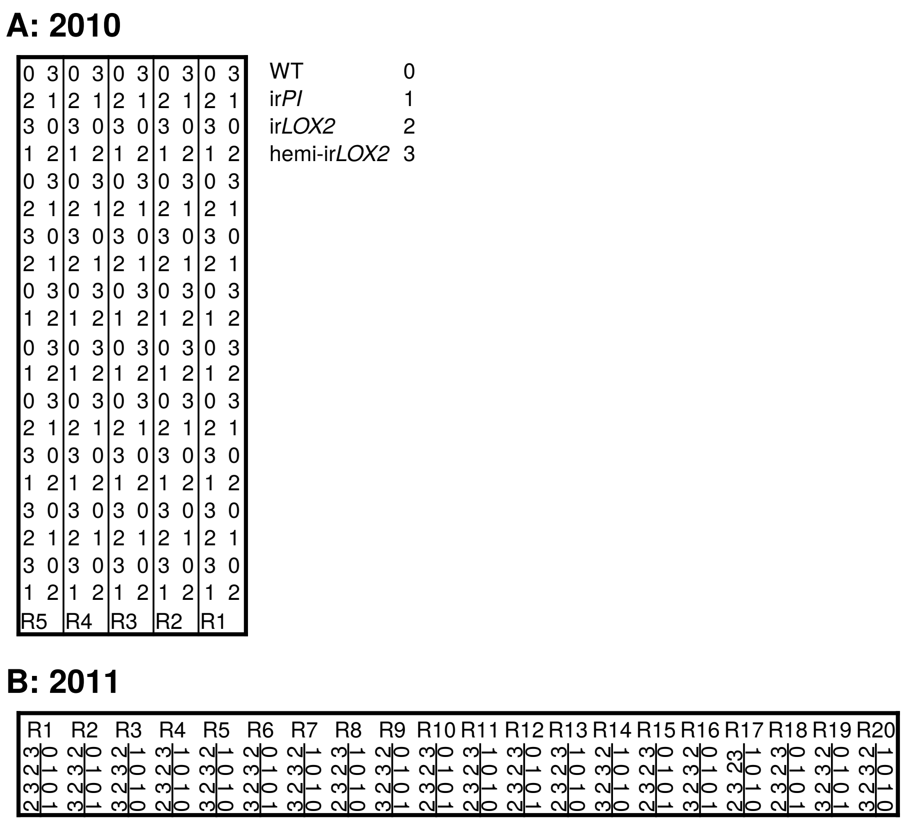

Figure S9 – Layouts of field plots in (**A**) 2010 and (**B**) 2011. Thick lines denote the borders of the experiment, thin lines denote irrigation lines [vertical borders of plot were also irrigation lines in (**A**) 2010], and R# denotes row number (used for identifying replicates during the experiment). The genotype key in (**A**) applies to both (**A**) and (**B**).

Table S1 – GLV mix used to externally supplement plant GLV emission in M2 (see fig. 1A) *(22)*. Pure GLVs were diluted in 1 mL of hexane and mixed into 14 mL of lanolin to yield the amount shown per 20 µL, representing the emission per g leaf material within the first 20 minutes of W+OS elicitation. Lanolin containing an equivalent amount of hexane was used as a control.

component	ng/20 µL lanolin
(*Z*)-hex-3-enal	3525.29
(*E*)-hex-2-enal	2693.12
(*Z*)-hex-3-en-1-ol	1776.39
(*E*)-hex-2-en-1-ol	2444.62
(*Z*)-hex-3-enyl acetate	46.56
(*E*)-hex-2-enyl acetate	35.46
(*Z*)-hex-3-enyl propanoate	9.00
(*E*)-hex-2-enyl propanoate	8.08
(*Z*)-hex-3-enyl butanoate	97.00
(*E*)-hex-2-enyl butanoate	35.62

Table S2 – Numbers (#) of *Geocoris* individuals (nymphs and adults) within 5 cm radii around plants used for predation experiments, counted within half an hour during the main period of *Geocoris* activity. Numbers are shown as subtotals for each plant genotype and grand totals per day (**bold**).

experiment	**genotype**	***Geocoris* per day (#)**				**plants (#)**
larval predation	*dates*	*May*	*21st*	*22nd*		
21st-23rd May, 2011	WT		3	4		19
	ir*PI*		6	6		24
	ir*LOX2*		6	4		20
	hemi-ir*LOX2*		8	2		20
	total		**23**	**16**		**83**
egg predation	*dates*	*June*	*3rd*	*4th*	*5th*	*7th*
2nd-6th June, 2011	WT		2	5	2	1	18
	ir*PI*		3	7	1	5	21
	ir*LOX2*		4	2	0	3	21
	hemi-ir*LOX2*		1	1	2	2	24
	total		**10**	**15**	**5**	**11**	**84**

Table S3 – Results of Mann-Whitney U-tests, Kruskal-Wallis tests and ANOVAs for control versus *Manduca*-infested plants of each genotype grown in the field in 2010 (fig. S5). Numbers of side branches (Mann-Whitney, Kruskal-Wallis), stem length, and final numbers of buds and flowers (MANOVA) were recorded in a single measurement at the end of M1 (fig. 1A). Numbers of newly produced flowers were counted repeatedly upon flower removal, and F values for the main effect of *Manduca* feeding are shown from repeated-measures ANOVAs across all measurements; Wilks' F values for the *Manduca*-by-time interaction were not significant. Significant P values are **bold**.

		branches (fig. S5A)			**stem, buds, flowers** (fig. S5A)		
comparison	**genotype**	df	χ^2	P*	df	F	P
treatment	all	1	0.022	1.000	3, 148	0.463	0.709
genotype	all	3	2.909	0.802	9, 360.344	1.186	0.303

		flowers (fig. S5B)			**units**
comparison	**genotype**	df	F	P	branches, buds, flowers (#)
treatment	WT	1, 37	4.869	**0.034**	stem length (cm)
	ir*PI*	1, 36	0.075	0.786	
	ir*LOX2*	1, 38	0.030	0.863	
	hemi-ir*LOX2*	1, 36	0.167	0.685	

Table S4 – Results of Student's t-tests and repeated-measures ANOVAs of control versus *Manduca*-infested plants of each genotype grown in the field in 2011 (fig. S4). Because many plants had few or no side branches before the final measurement, and rosette diameters did not change over the period that plants were measured, t-tests are shown for the final measurement of these parameters in M3 (fig. 1A). For stem lengths, numbers (#) of buds and numbers of flowers, Wilks' lambda F values for the *Manduca*-by-time interaction are shown from repeated-measures ANOVAs across all measurements. Significant P values are **bold**.

	branches (#)			**rosette d. (cm)**			**stem length (cm)**			**buds (#)**			**flowers (#)**		
genotype	df	t	P	df	t	P	df	F	P	df	F	P	df	F	P
WT	26	1.696	0.102	26	-0.870	0.932	5, 22	3.871	**0.011**	5, 22	3.188	**0.026**	3, 24	1.213	0.326
ir*PI*	26	1.024	0.315	26	-0.161	0.873	5, 22	0.991	0.446	5, 22	0.656	0.660	5, 22	0.525	0.755
ir*LOX2*	25	1.112	0.277	25	-0.058	0.954	5, 21	0.606	0.696	5, 21	0.535	0.748	5, 21	0.540	0.744
hemi-ir*LOX2*	22	1.753	0.094	22	1.140	0.267	5, 18	1.118	0.386	5, 18	3.001	**0.038**	4, 19	0.723	0.587

Table S5 – Primers used for quantitative PCR (SYBR Green).

gene	**forward primer sequence (5'-3')**	**reverse primer sequence (5'-3')**	**citation**
PI	TCAGGAGATAGTAAATATGG	ATCTGCATGTTCCACATTGC	*(14)*
LOX2	TTGCACTTGGTGTTTGAGATGGT	TTAGTAGAAAATGAGCACCACAA	*(15)*

Discussion

I will begin this discussion with an overview of reverse genetics techniques commonly used in plant functional genetics, then outline plant defense theory post-Fraenkel (1959), and finally discuss the results of this thesis in light of technical considerations and current theory.

How is genetic modification (GM) used to discover functions of plant metabolites?

There are three main approaches to **genetic modification (GM)** which have commonly been used in plant **reverse genetics**, i.e., altering expression of a gene to determine its function in an organism. The **gene of interest (goi)** may be "knocked out" by disrupting its sequence in the **genomic DNA (gDNA)** such that the functional gene product is no longer produced; an **antisense (AS)** or **inverted-repeat (IR)** fragment of the goi may be inserted into plant cells, causing a reduction in functional gene transcripts via **RNA interference (RNAi)**; or a functional copy of the gene may be **ectopically expressed**; RNAi and ectopic expression may be **stable**, inserted into the plant gDNA and germline gDNA, or **transient**, temporarily expressed by viruses or bacteria (Somerville and Somerville 1999, Hannon 2002, Voinnet *et al.* 2003).

Knocking out the goi may be in some ways the "cleanest" option, because the only alteration to the gDNA is in the goi, and there is no residual expression of the functional goi. However, the generation of targeted gene knockouts requires either mutagenesis followed by extensive screening and often back-crossing to generate single-gene knockouts, or extensive genomic sequence information. The genomic sequence allows researchers to specifically target the goi, and to confirm that the correct site in the goi, and no other site has been affected (Hannon 2002, Lloyd *et al.* 2005, Tzfira and Citovsky 2006).

The RNAi and ectopic expression approaches are especially valuable in the absence of genomic information, or for the manipulation of essential genes which cause lethality when knocked out. However, both techniques, when used to create stable transformants, involve the insertion of a gene construct into the gDNA outside of the goi sequence, in an untargeted location (Tzfira and Citovsky 2006). When using these techniques, care must be taken to exclude that observed effects are due to disruption of the genomic sequence into which the gene or RNAi construct was inserted. Also, the transcription of the gene construct will be more or less active depending on its insertion

site. These are collectively called **insertion effects**, and must be eliminated beyond a reasonable doubt as explanations for the novel phenotype of an RNAi or ectopic expression line. Furthermore, if an RNAi construct has 21 or more nt in common with a non-target gene, that gene may also be silenced, so nonhomologous regions of the goi must be targeted (Elbashir *et al.* 2001, Tang and Galili 2004). Ectopic expression can also cause post-transcriptional silencing of the endogenous gene, which is how RNAi was first discovered in plants (Napoli *et al.* 1990).

Researchers usually control for insertion effects by demonstrating the same phenotype in two or more independently transformed lines, and additionally looking for signs of off-target effects in plant growth, health, and metabolite pools (Schwachtje *et al.* 2008). Even if one compares only two lines, it is statistically impossible that both lines would have the same insertion site in a genome comprising millions to billions of base pairs, and thus phenotypes consistent between both lines can be attributed with confidence to the manipulated gene. Once such a multi-line characterization has established a robust phenotype, and comparison to **wild-type** (**WT**) or **empty vector** (**EV**) control plants has demonstrated no off-target effects, it is reasonably safe to draw further conclusions from experiments with only a single transformed line per construct. The use of a single line is often necessary to reduce the labor and space costs of large experiments, especially if those experiments must be planted and established in a field plot – which involves significantly more effort than establishment in a laboratory or glasshouse.

Furthermore, effects other than insertion site can also drive differences among, or even within single GM lines. It is common molecular biology knowledge that functional RNAi constructs may be rendered ineffective as a result of insufficient gene dosage, e.g. Travella *et al.* (2006) and references therein; this may occur when an RNAi construct is present in the hemizygous state (García-Pérez *et al.* 2004). The 35S promoter which drives the transcription of the RNAi construct may also be methylated: an epigenetic effect which can reduce the dose of RNAi in individual plants *within* a single transformed line (A. Weinhold *et al.*, in preparation). Thus, the only way to ensure the suitability of an RNAi line is to regularly monitor its phenotype, and the same holds for ectopic expression lines.

Discovering the "raison(s)" d'être *of plant specialized metabolites*

Shortly after Fraenkel's seminal paper, Ehrlich and Raven (1964) proposed that a tight "arms race" of plant defense evolution and herbivore counter-adaptation is responsible for the diversity of plant defense metabolites in nature. However, ensuing research has indicated that the arms race metaphor is too simple a view of plant-herbivore interactions (Stamp 2003 and references therein). Myriad plant defense hypotheses have emerged to codify aspects of the complexity of plant-herbivore interactions, and to provide explanatory power within ecological time (as opposed to the geological timescale of Ehrlich and Raven's **Coevolutionary Hypothesis**). These plant defense hypotheses focus on defense against insect herbivores, but it must be kept in mind that many specialized metabolites also have antimicrobial activity, or protect against abiotic stress factors (e.g. Khosla and Keasling 2003, Holopainen and Gershenzon 2010).

Most plant defense hypotheses start from the assumption that plants must balance metabolic and ecological costs of producing metabolites against the potential benefits of employing those metabolites. **Cost** is generally defined as a decrease in Darwinian fitness, and **benefit** as a gain in Darwinian fitness. **Darwinian fitness** is usually defined as the number of grandchildren produced by an individual, but there are correlate measures, such as total biomass, number of reproductive

structures, or number of offspring produced. **Metabolic costs** refer either to the diversion of nutrients away from growth and reproduction to specialized metabolism, or autotoxicity of metabolites and the resources which must be allocated to avoid it. **Ecological costs** comprise effects of metabolites on other members of the ecological community: if these effects are detrimental to the plant, e.g. attracting herbivores or deterring pollinators, they are costly (Steppuhn and Baldwin 2008).

The four most-cited plant defense hypotheses (Stamp 2003) are the Optimal Defense Hypothesis (McKey 1974, 1979, Feeny 1975, 1976, Rhoades and Cates 1976, Rhoades 1979), the Carbon:Nutrient Balance Hypothesis (Rhoades 1979, Bryant *et al.* 1983, Tuomi *et al.* 1988), the Growth Rate (or Resource Availability) Hypothesis (Coley et al. 1985), and the Growth-Differentiation Balance Hypothesis (Loomis 1932, 1953, Herms and Mattson 1992). The latter three hypotheses all propose explanations for *how* plants distribute nutrients and photosynthate between growth/development and specialized metabolism – i.e., how plants pay metabolic costs – but do not address *why* specialized metabolites are produced. In contrast, the **Optimal Defense Hypothesis (ODH)** supposes that specialized metabolism also has benefits. The ODH proposes reasons *why* patterns of specialized metabolites occur as they are found in nature, based on the optimization of these benefits and costs, both metabolic and ecological.

Thus among the most-cited plant defense hypotheses, only the ODH addresses the "why" of specialized metabolism: the *raison* in the *raison d'être*. There are two different levels at which the question of "why" (as opposed to "how") a trait exists can be answered (Sherman 1988). The ultimate answer to "why" questions in biology is always evolution, and the proximate answer is function. Evolution operates over geological time, whereas function can be demonstrated in ecological time. The two are related through the ecological definition of **function**: an effect which increases Darwinian fitness. There are of course fitness-neutral, or even fitness-decreasing events which co-determine phenotypes, such as genetic bottlenecks, founder effects, and genetic drift. However, evolutionary theory predicts that at least conserved, perpetuated, and convergent traits have evolved through natural selection, and are therefore functional in the conditions under which organisms evolved.

The ODH is actually a framework comprising multiple hypotheses (Stamp 2003). The basic hypothesis, which could be seen as the framing hypothesis, is very broad and mixes evolutionary and functional reasons: "Organisms evolve and allocate defenses in a way that maximizes individual inclusive fitness" (Rhoades 1979). In other words, the evolution and deployment of defense is optimized. That is close to a tautology, and it is acknowledged that the basic hypothesis is so general as to be untestable. However, the more specific hypotheses of the ODH can be tested, and these are: that "organisms evolve defenses in direct proportion to their risk from predators and in inverse proportion to the cost of defense", also called the **Plant Apparency Hypothesis**; that the distribution of defenses within a plant is in direct proportion to the fitness value and vulnerability of a tissue, and in inverse proportion to the cost of maintaining defenses in that tissue; that defenses are lower in the absence of enemies and higher in their presence; and that plants which are otherwise stressed are less well-defended (Rhoades 1979, Stamp 2003). The distribution of **17-hydroxygeranyllinalool-diterpene glycosides** (**HGL-DTGs**) in flowering *N. attenuata* plants is according to the "tissue value" prediction of the ODH, as is the distribution of nicotine (Ohnemeiss and Baldwin 2000).

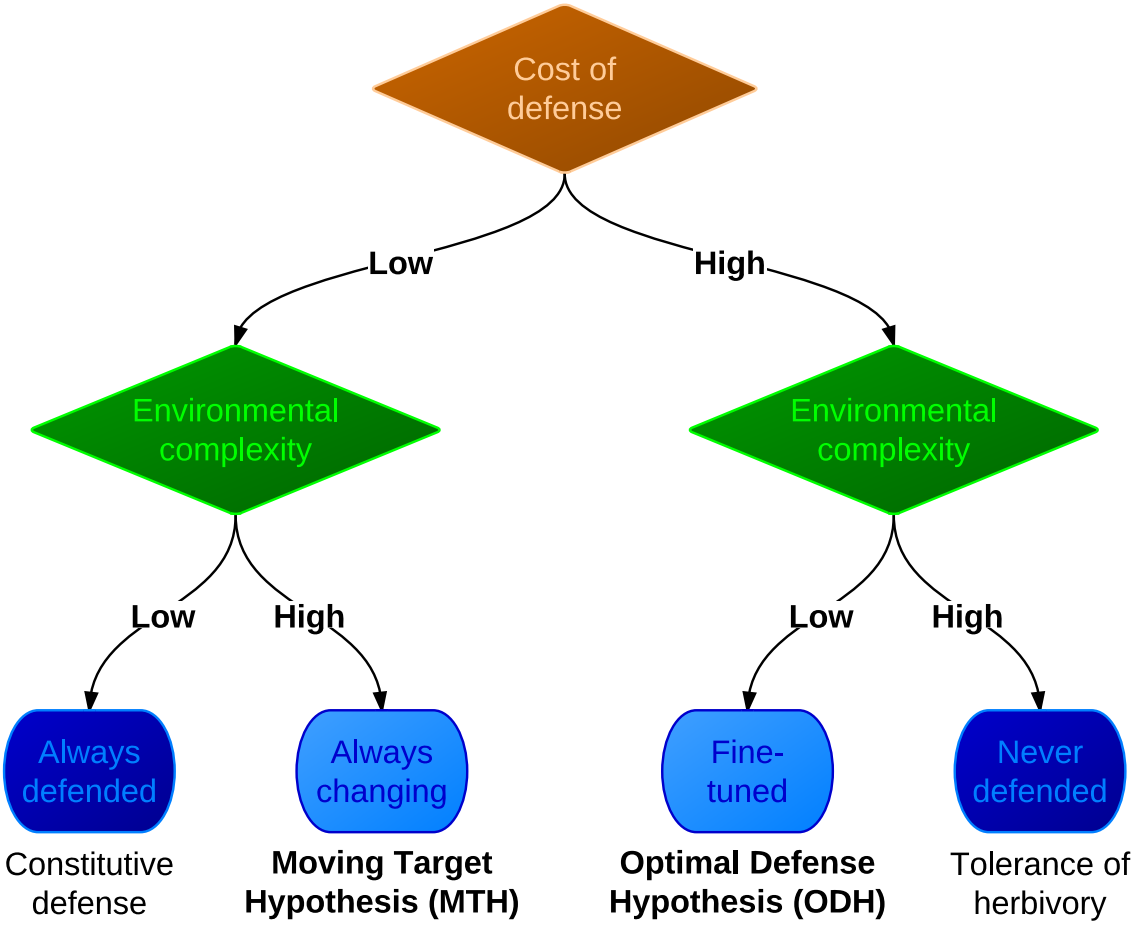

Figure 1 – A flow chart of plant defense strategies based on the reasoning behind the **Optimal Defense Hypothesis (ODH)** and the **Moving Target Hypothesis (MTH)**. Depending on the costs of employing defenses and the complexity of the environment, e.g. the complexity and dynamics of the herbivore community, different defense strategies may result in the greatest Darwinian fitness. In particular, constitutive phenotypes (dark blue boxes) are most adaptive either if costs of defense are low and the herbivore community is predictable, in which case the production of constitutive defenses is prudent; or if costs of defense are prohibitively high, in which case plants would do best to invest in tolerance strategies to protect their resources and endure herbivory. Inducible phenotypes (light blue boxes) are favored either if defenses are costly and plants face predictable attack by one or a few herbivores, in which case the optimal strategy may be to induce a set of defenses fine-tuned to those herbivores; or if defense is cheap and the herbivore community is unpredictable, in which case plants may benefit most from simply altering their production of defense metabolites in response to herbivore attack, without employing a strategy which is predictable from the herbivore's perspective. The fine-tuned approach is the typical scenario envisioned from the ODH, and the basis for Ehrlich and Raven's brand of tight plant-herbivore coevolution. The approach of simply changing defense status in response to herbivore attack is the behavior proposed by the MTH.

Induction of plant defense metabolites

The ODH provides the hypothesis most often cited as the *raison d'ĕtre* of induced defense: that, by only producing defenses in the presence of (specific) enemies, plants can optimize their investment in defense (e.g. Karban and Baldwin 1997). Plants must, however, be able to induce metabolites sufficiently quickly so as to minimize the damage incurred prior to their employment. In fact, sometimes the minimization of resources lost to herbivory, or tolerance, is more important than defense (**Fig. 1**). In reality, plants often seem to employ multiple strategies at once (Schwachtje and Baldwin 2008). In *N. attenuata*, herbivore attack induces the increased transport of sugar form leaves to roots, concurrently with the upregulation of defense (Schwachtje *et al.* 2006).

Herbivore-induced plant volatiles (HIPVs) are often the "first responders" in plant defense against herbivory. After herbivore damage, *N. attenuata* emits a blend of methanol and ethylene, which have direct effects on plant defense (von Dahl *et al.* 2007, Körner *et al.* 2009); as well as C6 aldehydes, alcohols and esters (**green leaf volatiles**), mono- and sesquiterpenes (**Chapter 1 Fig. 1**). These volatiles are common to most plants (see **Chapter 1**). Although there is evidence that the green leaf volatiles (**GLVs**), mono- and sesquiterpenes can affect plant defenses (Kessler *et al.* 2006, Paschold *et al.* 2006, Heil and Silva-Bueno 2007, Frost *et al.* 2008), they can also attract predators and parasitoids of herbivores (reviewed in Dicke and Baldwin 2010). Ethylene, methanol, and GLVs are emitted immediately upon tissue damage (von Dahl *et al.* 2007, Körner *et al.* 2009, Allmann and Baldwin 2010). In *N. attenuata*, GLV ester emission increases with time and persists over more than 24 h (M. Schuman *et al.*, in preparation). Monoterpene emission is induced within h of wounding or herbivory, and the emission of induced sesquiterpenes is detectable within h after herbivore attack, but peaks in the following photosynthetic period (Halitschke *et al.* 2000, Arimura *et al.* 2008, M. Schuman *et al.*, in preparation). In contrast, elevated levels of non-volatile induced defenses are usually first detectable after 12-24 h, and peak after 2-5 d or more in *N. attenuata* (**Chapter 4**, Wu *et al.* 2006, Onkokesung *et al.* 2012) and the closely related native tobacco *N. sylvestris* (Baldwin and Ohnemeiss 1994).

The **Moving Target Hypothesis (MTH)** proposes an alternative prediction for plant metabolite induction in cases where the cost of defense is low, which are not uncommon (Adler and Karban 1994). In *N. attenuata*, although total jasmonate-mediated defense is costly (Baldwin 1998), individual defenses, e.g. GLVs and TPIs, may not be (**Chapter 5**). The MTH states that for an attacked plant, simply changing its defensive state in response to herbivore attack is likely to be adaptive if the change in defensive state does not bear significant costs, especially if the herbivore community is sufficiently complex that plants may not succeed in employing optimized defenses against each herbivore, which is likely often the case (**Fig. 1**). For plants employing the "Moving Target" strategy, herbivore-induced phenotypes should be highly variable both for individuals measured concurrently, as well as within a single individual. In *N. attenuata*, both predictions have been shown to be true for jasmonate accumulation (**Chapters 2, 3**, Stork *et al.* 2009) and for nicotine concentrations in floral nectar (D. Kessler *et al.*, in review).

Plant volatiles (**PV**)s emitted after herbivore or jasmonate elicitation vary greatly among concurrently assayed individuals of *N. attenuata* (**Chapters 2 and 3**) and in this way, behave consistently with the MTH. However, it seems unlikely that HIPV emission should follow "Moving Target" behavior, because there are likely to be high ecological costs associated with inconsistent HIPV emission. Most data on predator and parasitoid responses to HIPVs is from insects which first learn to associate HIPVs with prey (Allison and Hare 2009). The stark change in emission of

HIPVs upon herbivory is vital for conveying this association. From this reasoning, it seems that HIPVs are more likely to behave like Optimal Defenses: emitted only upon herbivore attack, fine-tuned to the attacking herbivore, and with low variation among individuals in a population.

Fine-tuning of HIPVs to the attacking herbivore has been shown in many plant systems (e.g. De Moraes *et al.* 1998), but in other systems different herbivores can elicit similar HIPVs (e.g. Kessler and Baldwin 2004). Additionally, variation among conspecific individuals even within a single population is not unique to *N. attenuata* and has also been demonstrated e.g. in wild sage (*Salvia fruticosa*, Skoula *et al.* 2000) and horsenettle (*Solanum carolinense* L., Delphia *et al.* 2009). However, it is possible that PV emissions are highly variable, but herbivore attack alters the constitutive PV blend in a predictable way; or, that natural variation in PVs does not prevent predators or parasitoids from learning to associate herbivore attack with a few, consistent HIPVs which are likely controlled by jasmonate signaling (**Chapter 3**).

Defense induction versus activation: costs and benefits

Defense activation, like induction, is an alternative to constitutive defense. Ecologically, the difference between activation and induction is the speed with which plants are able to employ defenses upon attack. Herbivore-activated metabolites are constitutively produced and stored in an inactive form, and first activated upon herbivory. This allows plants to avoid autotoxicity and ecological costs of active defenses, but reduces the time lag between herbivore damage and defense deployment. However, because precursors must be present in resting tissue, activation does not save on biosynthetic costs. Likely the most famous example of activation in plant-herbivore interactions is the glucosinolates produced by plants of the order *Brassicales*, which are sulfur- and nitrogen-containing compounds derived from glucose and an amino acid. In intact cells, glucosinolates and their activating enzyme myrosinase are stored in separate compartments. Activation occurs upon tissue disruption by herbivores, which brings glucosinolates into contact with myrosinase, resulting in cleavage of sugars to free reactive isothiocyanates, thiocyanates, and nitriles (Wentzell and Kliebenstein 2008).

In fact, it might be appropriate to refer to GLVs as "Herbivory-Activated" PVs, rather than HIPVs. (This distinction is important for considering biosynthetic costs, but does not matter for the ecological function of GLVs: what is important for the ecological function of HIPVs and "HAPVs" is that they carry an herbivory-specific signature.) In *N. attenuata*, transcripts of the GLV biosynthetic enzyme LOX2 are constitutively present at high levels (**Chapter 5 Fig. S2**) and the GLV *trans*-2-hexenal is present in undamaged tissue in μg/g FM (**Chapter 5** figs. S1, S2; M. Schuman *et al.*, in preparation). Upon damage, there is a nearly instantaneous isomerization of the aldehyde and oxidation of both aldehyde isomers to alcohols; esters are also emitted in much lower amounts (Allmann and Baldwin 2010). The ratio of GLV isomers is altered by a putative isomerase in *Manduca sexta* oral secretions (**OS**), creating an herbivore-specific signature which consists of a greater proportion of *trans*-GLVs; the altered *trans*:*cis* ratio increases predation of *Manduca* (Allmann and Baldwin 2010). The malonylation of HGL-DTGs upon herbivore attack in *N. attenuata* might also be a form of activation, although it is induced over a period of h to d (**Chapter 4**): malonylation may retain HGL-DTGs in the tissue which is currently being eaten (Bouzayen et al. 1989, Morris and Larcombe 1995, Martinoia *et al.*, 2000).

Natural and metabolic diversity in plant responses to herbivory

A large body of literature discussed in **Chapter 1** demonstrates that individual HIPVs, as well as volatile blends which may include both HIPVs and constitutively emitted "background volatiles," can attract or repel host-seeking herbivores as well as attract predators and parasitoids, and have myriad other effects besides (see **Table 1** in the **Introduction**). Thus it seems that the natural diversity of HIPVs is associated with functional diversity: the MTH also proposes that Moving Target behavior is likely to occur in cases where phenotypes are a result of a combination of stresses, although this also undermines the argument that HIPVs are reliable indicators of herbivory. Few studies (reviewed in **Chapters 1 and 5**) have tried to dissect HIPV functional diversity. **Chapter 5** describes an attempt to demonstrate one function in nature: indirect defense, for one group of HIPVs: the GLVs.

The GLVs were chosen for manipulation in **Chapter 5** because their functional complexity mirrors that of total HIPVs, and because it is easy to genetically manipulate total GLV biosynthesis independently of other traits. As discussed above, although they are commonly considered HIPVs, GLVs might more accurately be considered Herbivory-Activated Plant Volatiles; but the distinction between induction and activation does not matter for the functional test conducted in **Chapter 5**. The ecologically important character of HIPVs is that they carry an herbivory-specific signature, and the same is true of "HAPVs". The differences between induction and activation lie in the speed and metabolic cost of the defense. The instantaneous nature of GLV emission upon herbivory (Allmann and Baldwin 2010) likely makes them a reliable indicator of current herbivore feeding, and the metabolic cost of synthesizing and storing *trans*-2-hexenal seems low (**Chapter 5**). Because GLVs comprise a relatively small class of PVs (see table 1 in the **Introduction**), it might be argued that they cannot mirror natural variation in total PVs; however, the data in **Chapter 3** demonstrate that several GLVs do in fact vary significantly among accessions from a single *N. attenuata* population, and thus in this respect they are also an appropriate test group – although isogenic lines were used to make controlled comparisons in the experiments described in **Chapter 5** and thus the experiments did not incorporate this natural variation.

It is also speculated in **Chapter 3** that HIPVs correlated with jasmonate levels – which the GLVs generally are not (**Chapter 3**, Allmann *et al.* 2010) – are also likely to serve defensive functions. In that respect, it would be interesting to test *trans*-α-bergamotene, and this will require identification and manipulation of the native sesqiterpene synthase. An approach to manipulate community dynamics of *trans*-α-bergamotene emission is described in **Chapter 1**. These first experiments, which also incorporate different levels of emission among plants within populations, have been conducted and are in preparation for publication (M. Schuman *et al.*). The correlated differences in *trans*-α-bergamotene emission and *M. sexta* OS-induced signaling in Utah and Arizona accessions of *N. attenuata* described in **Chapter 2**, as well as the correlation between *trans*-α-bergamotene and OS-induced jasmonates across accessions in **Chapter 3**, indicate that *trans*-α-bergamotene emission by wild plants may be a reliable marker of the magnitude of their defense response to herbivore attack.

How can we test the ecological functions of plant metabolites in nature?

Central to this thesis is the importance of discovering evolutionary functions of plant metabolites by manipulating traits in wild plants growing in their native habitats. There are several challenges to be overcome if this approach is to yield clear and useful data. One of these is the fact that plant

metabolites potentially have many effects, as is perhaps best elaborated in research on HIPVs (**Chapter 1** and **Table 1** of the **Introduction**). Thus, how can one hope to test specific functions of metabolites, or groups of metabolites? This is especially a challenge when working with plants that are released into a wild environment, where the experimenter has little control over the fate of those plants – and indeed, this reduced control is one justification for conducting field experiments at all. Field experiments are meant to let nature take its course (albeit in a targeted way).

The solution is to clearly and precisely state the functional hypothesis to be tested, and then set up the field experiment so that the comparison between treatment and control addresses this one function, as illustrated in **Chapter 1**. Put that way, it may sound either obvious, or as though there is little advantage to conducting such a controlled experiment in the field rather than in a glasshouse or laboratory. The advantage of field research for experiments designed to test specific functions is subtle, but significant: it tells the researcher whether the hypothesized function can in fact be detected in nature, where all factors *other than* the experimental design are not under the researcher's control. The results of such "mostly controlled" field trials may therefore be more robust than results from fully controlled laboratory trials, in which the researcher controls not only the experimental design, but also the environment. However, fully controlled laboratory trials are vital to elucidate the mechanisms behind phenomena demonstrated in field trials.

In **Chapter 4**, a very simple approach to the field trial was taken by pairing WT plants with GM plants reduced in HGL-DTG production. The goal was to determine whether HGL-DTGs protect plants from herbivore damage in their natural habitat in terms of total canopy damage. The results show that HGL-DTGs do indeed reduce damage by native herbivores, consistent with glasshouse data. In this experiment, we did not attempt to demonstrate consequences for plant reproduction.

Chapter 5 describes the design of, and results from a field experiment to test for an indirect defense function of GLVs, i.e., whether GLVs can increase predation and thus reduce herbivore load, and whether this results in increased net plant reproductive output in nature. HIPVs (and HAPVs), and their ambiguous ODH/MTH behavior, are a good example of the experimental challenges presented by induced defenses. Herbivore inducibility or activation of HIPVs and HAPVs is likely vital to their function (Kos *et al.* 2009). It is very difficult to engineer inducible PV emission, but easy to either engineer constitutive emission, or silence emission by RNAi. Experiments which use constitutive emission to test predator and parasitoid responses to HIPVs must be constructed so as to obviate the need for herbivore induction of the HIPVs, and if such plants were to be simply released into nature, predators and parasitoids would likely not learn to associate their volatile emissions with prey, or would rapidly learn to ignore the HIPV as it no longer provides useful information about the location of prey. The comparison of WT, inducible HIPV emissions with plants transformed to lack one component of their HIPVs circumvents this problem.

There are two field studies using plants which constitutively emit a root HIPV in which the experimental design eliminated the need for HIPV induction. In Rasmann *et al.* (2005); and Degenhardt *et al.* (2009), *Zea mays* plants constitutively emitting or not emitting (-)-β-caryophyllene from their roots were grown in fields and infested at the roots with *Diabrotica virgifera virgifera* larvae. Field plots were then seeded with nematodes. In this design, *D. virgifera virgifera* larvae (the prey) were associated with both treatments (emitters and non-emitters), but all plants were infested, and therefore all (-)-β-caryophyllene-emitting plants were associated with prey. The implication is that a set-up in which target volatiles are always associated with prey permits predators

and parasitoids to learn to associate constitutively emitted volatiles and prey, which they might not learn to do if prey were not always associated with the constitutive volatiles.

In the experimental design described in **Chapter 5**, half of all plants (GLV-emitters and non-emitters) were not infested with *Manduca* larvae during the first experiments (M in 2010, M1, M2; **Fig. 1A**), in which we were nevertheless able to show twofold greater predation from GLV-emitting genotypes (WT and ir*PI*), which only emitted GLVs upon damage.

The goal of the experiments in **Chapter 5** was not to investigate the total contribution of GLVs to plant reproduction in nature, but rather to determine whether the role of GLVs *as indirect defenses* could increase plant reproduction under natural conditions, where the GLVs were free to have all of their other effects – as opposed to a laboratory setting in which the only interactions which occur are those created by the researchers. This hypothesis required us to artificially create even and synchronous *Manduca* oviposition (given that natural oviposition is neither) so that we could measure the effect on plant reproduction of the GLV-mediated removal of herbivores, from the same starting load on each plant. Simultaneously, we measured other natural effects of GLVs by e.g. monitoring damage to the plants from naturally occurring herbivores (**Chapter 5 Fig. S6**) and indexing plant health (**Chapter 5 Fig. S7**). This also allowed us to separate these GLV effects from differences in plant reproduction resulting from differential predation of *Manduca* larvae from GLV-emitting and non-emitting plants.

Data from separate field experiments with *N. attenuata* allow us to extrapolate how our data might have looked had we also been able to integrate a test of the direct effect of GLVs on *Manduca* oviposition. It is known that GLVs can affect *Manduca* oviposition (Kessler and Baldwin 2001), and of course herbivores can only be predated from plants if they are there in the first place – thus differences in oviposition are one "uncontrolled" effect that the field trial would allow us to test; and it would be meaningful to integrate the effect of GLVs on herbivore oviposition with the consequences for plant reproduction of GLV-mediated predation of herbivores. The blend of GLVs produced by *Manduca* larval feeding deters oviposition by the moths on *Datura* (Allmann *et al.*, in preparation). A laboratory study has shown that GLVs in the nectar headspace may attract or repel nectaring *Manduca* moths (Kessler and Baldwin 2006). Thus in years of heavy *Manduca* infestation, the combination of decreased oviposition and increased larval predation resulting from the larvae-produced GLV blend might create a greater increase in plant fitness than we measured when only testing the impact of larval predation.

We did in fact measure was in fact monitor *Manduca* oviposition during the field experiments described in **Chapter 5**, but in the two years of the experiment we did not have sufficient oviposition loads on the experimental plants to draw statistically robust conclusions, nor would that oviposition have given us sufficient replicates for predation assays. This is not because *Manduca* rarely oviposit on *N. attenuata*, but rather because our experimental plants grow amongst hundreds to thousands of conspecifics and near native *Datura* plants, as do plants in a native post-fire monoculture. *Datura* plants receive many *Manduca* eggs per plant per oviposition, whereas *N. attenuata* plants receive one egg per plant when moths oviposit. Thus it is relatively unlikely that, except during a *Manduca* outbreak year, there will be a sufficient replicate number within a small focal group of experimental plants to draw conclusions about *Manduca* oviposition.

During the experiments described in **Chapter 5**, there was only nominal natural oviposition on experimental plants in 2011 (fewer than 5 eggs in total), and in 2010 there were a total of 18

Manduca eggs naturally oviposited at the end of the caterpillar assay (M1, oviposition 29^{th}-30^{th} May) when experimentally oviposited *Manduca* larvae were in the fourth instar. (However, native oviposition on nearby *Datura* plants had been observed prior to oviposition on the experimental plants.) These eggs were distributed 11 on control plants and 7 on *Manduca*-infested plants; numbers of eggs on individual genotypes were too low to have any statistical meaning.

Uncontrolled ecological effects can and should be measured in field experiments. There is however another category of uncontrolled effect which must be considered in functional genetics research, and that is the possible pleiotropic effects of single genes.

How can we deal with pleiotropic effects in GM plants?

All GM lines, including knockouts, may display pleiotropic effects which complicate experimental comparisons, but are not due to insertion or other non-target effects. Such pleiotropic effects were present in the data set presented in **Chapter 5**, and had to be dissected and factored out in a series of experiments.

The reproduction of homozygous and hemizygous ir*LOX2* plants in **Chapter 5** (**Figs. S3, S4**; **Table S3, S4**) was reduced both for control and *Manduca*-attacked plants. This could be due to the positive impact of *Geocoris* predating other naturally occurring herbivores other than *Manduca*: because *Geocoris* predation is decreased on ir*LOX2*-silenced plants, this would result in a greater impact of all types of herbivory on their growth and reproduction (naturally occurring herbivores were not excluded from control plants). However, if that were the case, we would expect to observe greater cumulative herbivore damage on hemi-ir*LOX2* and ir*LOX2*, which we generally did not (**Chapter 5 Fig. S6**). Thus we could not exclude that the reduced growth and reproduction of control plants might have been due to another factor (e.g. differences in root health corresponding to GLV antimicrobial properties, see **Table 1** of the **Introduction** and **Chapter 1**) which did not play a role in 2010. Because of this uncertainty, those reproduction data are presented in supplemental figures and not in the main figures. The reproduction data shown in the main figures (**Chapter 5 Fig. 3**) are from plants which were carefully matched for size and prior reproduction (**Chapter 5 Fig. S7**), and this "triplet" experiment was the main basis for our argument that GLV-mediated indirect defense increases plant reproduction.

When discussing the supplemental growth and reproduction data in **Chapter 5**, I focused on the effect of *Manduca* feeding on growth and reproduction overall and within individual genotypes; these comparisons are independent of differences between genotypes, but acknowledge the basal difference among genotypes.

Can we extrapolate measures of bud and flower production from field studies on GM plants to Darwinian fitness, and evolutionary function?

This question may at first seem too specific for a general discussion, but it is at the heart of the validity of the approach outlined in the **Introduction**, and employed in **Chapters 1, 4 and 5** of this thesis. In the **Introduction**, it is stated that the function of individual metabolites is best investigated by comparing otherwise isogenic lines varying only in the trait of interest, and that this is usually only possible with GM lines. Ideally, the function of a trait would be determined by comparing the Darwinian fitness of GM lines having or lacking that trait, and grown in their native habitat.

However, safety restrictions on the environmental release of GM plants make traditional measures of Darwinian fitness difficult to obtain. The production of viable offspring is the accepted definition of Darwinian fitness, but researchers are generally not permitted to let GM plants disperse ripe seed in the field, and measures to prevent seed dispersal, such as bagging meristems, strongly affect production of buds and flowers and can also affect seed viability by increasing temperature, and decreasing respiration and photosynthesis of reproductive tissue and associated green tissue.

Fortunately, plant size (Glawe *et al.* 2003), bud and flower production correlate strongly to seed capsule number, especially for self-fertilizing species such as *N. attenuata* (Sime and Baldwin 2003). For field-grown *N. attenuata* plants, fewer than 5% of buds and flowers (in total) are aborted by healthy (not diseased) plants, and abortion seems always to be due to damage by insects (M. Schuman and I. T. Baldwin, personal observation). Lifetime seed capsule production in turn correlates to lifetime seed production, which has been used as a proxy measure of Darwinian fitness (Baldwin 1998; Hoballah and Turlings 2001; van Loon *et al.* 2000).

It should be pointed out that for *N. attenuata* and other plant species where seed lays dormant in a seed bank, a true measure of Darwinian fitness must include a long-term measurement of seed viability in the seed bank. In the case of *N. attenuata*, 90% of seeds lay dormant for up to 150 years before germinating post-fire (Preston and Baldwin 1999).

Conclusion: the chemical ecology of plant-insect interactions enters the "omics" era

Chemical ecology and plant biology, like the rest of biology, are moving into the omics era (Coulbourne *et al.* 2011, Hu *et al.* 2011). Soon it will be possible to sequence the genomes of individual research organisms, e.g. single plants in a wild population, and directly identify genes which might underlie natural variation. With full genome profiling, these genes can be disrupted using techniques such as zinc finger nuclease technology (Lloyd *et al.* 2005) which create knock-outs and leave no transgenic material in the altered genome. Because the gametes these plants produce will not be GM, this will permit full analysis of classical Darwinian fitness with genetically modified plants, i.e., determination of their number of surviving offspring or grandchildren in plants' natural habitats. Genome sequencing will be coupled with volatile and non-volatile metabolite profiling to generate individual profiles and gene-to-metabolite networks. The huge amount of information this will generate must be analyzed in light of an informed understanding of the study organism, its range of variation, and the adaptive pressures it faces. Field work with wild plants and plants which are genetically manipulated in single traits, coupled with controlled laboratory studies, will remain indispensable to deciphering the adaptive value of all the new traits and their regulation which will be discovered in the omics era. The omics era will provide a new comprehensive understanding of the *raison d'être* of specialized plant metabolites.

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Summary

Chemical ecology began with the study of plant-insect interactions. In the nineteenth century, biologists began to realize the profound importance of plant chemistry in determining herbivore choice. Given that plants are the primary source of food in terrestrial ecosystems, the role of plant metabolites in herbivore choice and performance translates to a role in structuring ecological communities.

This work describes natural variation in volatile and non-volatile herbivore-induced metabolites in the native tobacco *Nicotiana attenuata*, and experiments to test the defensive functions of some of these metabolites in nature. The variation described includes natural variation among accessions in herbivore-induced signaling cascades and metabolite production, as well as complex metabolic variation in a class of terpenoid specialized metabolites, the 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs), in response to herbivore-induced signaling. I dissect the contribution of herbivore-induced jasmonate signaling to the regulation of different traits, and present evidence from wild plants that jasmonate regulation is a suitable, although not exclusive criterion for identifying candidate anti-herbivore defense metabolites.

I furthermore present evidence that some of these metabolites do in fact function as anti-herbivore defenses for *N. attenuata* plants in their natural habitat. Specifically, I demonstrate that HGL-DTGs effectively reduce damage from native specialist herbivores, and that green leaf volatile emission mediates indirect defense and increases plant reproduction in nature. The determination of metabolite functions under controlled laboratory conditions is essential but can be misleading: although trypsin protease inhibitors (TPIs) have become a classic example of anti-herbivore defense, I show that they provide little or no direct benefit to plants in the field, and may instead function to support indirect defense. A combination of laboratory and field assays using both native accessions, and isogenic lines genetically modified in single traits of interest, permits the discovery of plant defense strategies, the elucidation of complex regulatory networks, and the demonstration of their importance.

Zusammenfassung

Chemische Ökologie begann mit der Forschung über die Wechselwirkungen zwischen Pflanzen und Insekten. Im 19. Jahrhundert fingen Biologen an zu erkennen, dass Pflanzenmetabolite die Wahl von Pflanzenfressern steuern. Da Pflanzen die primäre Nahrungsquelle in Festlandökosystemen sind, spielen deren Metabolite eine wichtige Rolle in der Zusammensetzung ganzer Ökosysteme.

Hier beschreibe ich natürliche Variationen in flüchtigen und nicht-flüchtigen Fraß-induzierten Metaboliten im wilden Tabak *Nicotiana attenuata* sowie Versuche zur Überprüfung der natürlichen Abwehrfunktionen mancher Metabolite. Die Variationen umfassen große Unterschiede unter wilden Akzessionen von *N. attenuata* sowie Fraß-induzierte Änderungen des Stoffwechsels spezialisierter Terpenoide, den 17-Hydroxygeranyllinalool-Diterpen-Glycosiden (HGL-DTGs). Ich habe untersucht welchen Anteil die Jasmonate an Fraß-induzierten Regulationen haben, und zeige in wilden Akzessionen, dass die Regulation durch Jasmonate ein nicht exklusives Merkmal für Abwehr gegen Insektenfraß ist.

Zusätzlich zeige ich, dass einige dieser Metabolite tatsächlich der natürlichen Abwehr von *N. attenuata* gegen Fraßfeinden dienen. Vor allem die HGL-DTGs vermindern den Schaden durch spezialisierte Fraßfeinde. Es zeigte sich, dass grüne Blattdufstoffe indirekt als Abwehr funktionieren und die Reproduktion von Pflanzen in Feldversuchen erhöhen. Metabolite und ihre Funktionen unter kontrollierten Laborbedingungen zu untersuchen ist sehr wichtig, die Ergebnisse in der Natur weichen jedoch manchmal ab. Obwohl Trypsin Protease-Inhibitoren (TPIs) mittlerweile ein sehr bekanntes Beispiel von direkter Abwehr sind zeige ich, dass die TPIs der Pflanze im Feld keinen direkten Vorteil bringen. Möglicherweise unterstützen sie jedoch die indirekte Abwehr. Eine Kombination von Labor- und Feldversuchen mit wilden Akzessionen sowie Pflanzen die in bestimmte Eigenschaften gentechnisch verändert wurden ergibt neues Wissen über Pflanzenabwehr-Strategien. Dies ermöglicht die Erläuterung von komplexen regulatorischen Netzwerken und zeigt deren Bedeutung.

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Erklärung

Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, dass ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena, Meredith Christine Schuman

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufen hatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstes Promotionsverfahren überhaupt.

Jena, Meredith Christine Schuman

Meredith Christine Schuman

Born 28th October 1982, Minneapolis, MN, USA
Nationality: USA

Research Experience

From 03/2007 MPICE Jena, DE Doctoral Thesis
Max Planck Institute for Chemical Ecology Department of Molecular Ecology
Dissertation: "The response of *Nicotiana attenuata* plants to herbivory: polymorphism and functions in nature"
Advisors: Prof. Dr. Ian T. Baldwin and Prof. Dr. Jonathan Gershenzon

09/2005-02/2007 MPICE Jena, DE Postgraduate Research
Max Planck Institute for Chemical Ecology Department of Molecular Ecology
Topic: "Silencing jasmonate signalling and jasmonate-mediated defenses reveals different survival strategies between two *Nicotiana attenuata* accessions" (results published in *Molecular Ecology*),
Advisor: Prof. Dr. Ian T. Baldwin

05-08/2005 U WI-Madison Madison, WI, USA Research Assistant
University of Wisconsin-Madison Microbial Ecology group in the Department of Soil Science
Duties: Laboratory maintenance, assistance with enzyme time-course assays, molecular analysis (PCR, gel separation and sequencing of products), scientific reports
Principle Investigator: Prof. Dr. Teri Balser

09/2004-05/2005 U WI-Madison Madison, WI, USA Bachelors Thesis
University of Wisconsin-Madison Microbial Ecology group in the Department of Soil Science
Topic: "Detection and characterization of ammonia oxidizing bacteria in acidic Hawaiian soils"
Advisor: Prof. Dr. Teri Balser

U WI-Madison Madison, WI, USA Independent Study
University of Wisconsin-Madison Robert M. LaFollette School of Public Affairs
Topic: "Application of molecular techniques to research on ammonia oxidizing bacteria: effects on the changing directions, approaches, goals and applications of nitrifier research"
Advisor: Prof. Dr. Clark Miller

05-08/2003 U MN-Twin Cities St. Paul, MN, USA Independent Study
University of Minnesota-Twin Cities Department of Ecology, Evolution, and Biology
Topic: "History and development of molecular biology and the pursuit and issuing of patents in biotechnology"
Advisor: Prof. Dr. Philip Regal

Teaching Experience

2011-2012 MPICE, IMPRS Jena, DE Co-Instructor
Max Planck Institute for Chemical Ecology Department of Molecular Ecology
International Max Planck Research School for the Exploration of Ecological Interactions with Molecular and Chemical Techniques
Co-teach annual course in analytical techniques for biologists in the IMPRS (external course) and the Molecular Ecology department (internal course)

2011-2012 MPICE, TU Freiberg Jena & Freiberg, DE Scientific Supervisor
Supervised Diplom (equivalent to Master's) thesis: planned and guided experiments and data analysis, taught theory behind experiments, served as committee member for post-defense examination, and co-evaluated (graded) the thesis and defense.

2007-2012 **MPICE** **Jena, DE** **Scientific Supervisor**
Supervised three different Seminarfacharbeite (high school senior research projects): planned and guided experiments and data analysis, taught theory behind experiments, and co-evaluated (graded) the resulting research reports.

Education

From 03/2007 **IMPRS** **Jena, DE** **Doctoral Thesis**
International Max Planck Research School for the Exploration of Ecological Interactions with Molecular and Chemical Techniques

09/2005-02/2007 **MPICE, FSU** **Jena, DE** **Postgraduate**
Fulbright fellowship (09/2005-07/2006) and Max Planck Society fellowship (07/2006-02/2007) for research at the Max Planck Institute for Chemical Ecology, with concurrent enrollment at Friedrich Schiller University

09/2001-05/2005 **U WI-Madison** **Madison, WI, USA** **Bachelors of Science**
Graduated with Comprehensive Honors

09/2003-05/2004 **U Warwick** **Coventry, UK** **Bachelors of Science**
Study Abroad exchange program at the University of Warwick completed during the third year of Bachelors studies

09/1997-06/2001 **Irondale HS** **New Brighton, MN, USA** **High School Diploma**
Graduation with Honors (in top 10% of class) from Irondale High School

Experience Abroad

From 09/2005 **MPICE, FSU** **DE and Europe** **Research and Study**
Postgraduate research and study at the Max Planck Institute for Chemical Ecology and Friedrich Schiller University, Jena, Germany; conferences and travel in Europe

12/2011 **IISER-K** **IN** **Conference**
Max Planck Partner Group conference with the Indian Institute of Science Education and Research in Kolkata, India, 1 week

09/2003-05/2004 **U Warwick** **UK and Europe** **Study**
Study abroad exchange at the University of Warwick and occasional travel in Europe

Spring 2000 **Irondale HS** **UK** **Travel**
High school class trip to England, Ireland and Wales for 1 week

Language Skills

English Native speaker
German Fluent
French Literate, basic speaking and writing

Jena, Meredith C. Schuman

List of Publications

Scientific Articles

- Schuman, M. C., Barthel, K., Baldwin, I. T. (in review). Herbivory-induced volatiles function as defenses increasing plant fitness in nature. Submitted to *Science*.
- Galis, I., Schuman, M. C., Gase, K., Hettenhausen, C., Hartl, M., Dinh, S., Wu, J., Bonaventure, G., Baldwin, I. T. (in press). The use of VIGS technology to study plant-herbivore interactions. A chapter in the series *Methods in Molecular Biology*. Humana Press, New York, NY, US.
- Schuman, M. C., Baldwin, I. T. (in press). Asking the ecosystem if herbivory-inducible plant volatiles (HIPVs) have defensive functions. In G. R. Iason, M. Dicke and S. E. Hartley (Eds.), *The ecology of plant secondary metabolites*: *genes to global processes* (Chapter 15), in the series *Ecological Reviews*. Cambridge University Press, Cambridge, UK.
- Heiling, S., Schuman, M. C., Schöttner, M., Mukerjee, P., Berger, B., Schneider, B., Jassbi, A., Baldwin, I. T. (2010). Jasmonate and ppHsystemin regulate key malonylation steps in the biosynthesis of 17-hydroxygeranyllinalool diterpene glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. *The Plant Cell*, **22**, 273-292.
- Schuman, M. C., Heinzel, N., Gaquerel, E., Svatos, A., Baldwin, I. T. (2009). Polymorphism in jasmonate signaling partially accounts for the variety of volatiles produced by *Nicotiana attenuata* plants in a native population. *New Phytologist*, **183**, 1134-1148.
- Steppuhn, A., Schuman, M. C., Baldwin, I. T. (2008). Silencing jasmonate signalling and jasmonate-mediated defenses reveals different survival strategies between two*Nicotiana attenuata* accessions. *Molecular Ecology*,**17**, 3717-3732.
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Presentations

- Schuman, M. C. Community matters: ecological effects of plant volatiles on neighbor phenotypes. Gordon Research Conference on Plant Volatiles: Ecology, Biosynthesis, Regulation and Animal Perception of Floral and Vegetative Volatiles, Plus their Roles in Human Flavor and Agriculture, Gordon Research Conferences, Ventura, CA, US, Feb 2012
- Schuman, M. C. The "talking genome"? Searching for the genomic basis of plant volatile-mediated interactions. Kick-off meeting Max Planck Partner Group Kolkata, Institute of Science Education and Research, Kolkata, IN, Dec 2011
- Baldwin, I.T.*, Schuman, M. C. Asking the ecosystem if herbivory-induced volatile organic compounds have defensive functions. British Ecological Society Annual Meeting "The Integrative Role of Plant Secondary Metabolites in Ecological Systems", British Ecological Society, University of Sussex, Brighton, GB, Apr 2010
- Schuman, M. C., Heiling S., Schöttner M., Mukerjee P.A., Berger B., Schneider B., Baldwin I.T. "Lite tobacco?" Glycoside "sugar substitutes" in *Nicotiana attenuata* lead to thinner caterpillars. 5th Plant Science Student Conference, Halle (Saale), DE, Jun 2009
- Schuman, M. C. "Light tobacco?" Glycoside "sugar substitutes" in *N. attenuata* lead to thinner caterpillars. 8th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Mar 2009
- Schuman, M. C., Heinzel N., Gaquerel E., Svatoš A., Baldwin I.T. Polymorphism in jasmonate-controlled volatile signaling in *Nicotiana attenuata*. Plant Interactions with the Environment, Universität Neuchâtel, Neuchâtel, CH, Sep 2008
- Schuman, M. C. Die (nicht sehr?) geheime Sprache der Pflanzen. Schulvortrag anlässlich MPG Hauptversammlung, Max-Planck-Gesellschaft, Kiel, DE, Jun 2007
- Schuman, M. C. Studium im Ausland. Girls' Day, MPICE, Jena, DE, Apr 2007
- Schuman, M. C. 5-minute talk at the annual Hawai'i Ecosystems Project Meeting, Hawai'i Volcanoes National Park, Big Island, 07/2005
- Schuman, M. C. 15-minute talk at the Soil Ecology Society 10 th Biennial International Conference, Argonne National Laboratory, Illinois, USA, May 2005

Posters

- Schuman, M. C. Community matters: ecological effects of plant volatiles on neighbor phenotypes. Gordon Research Conference on Plant Volatiles: Ecology, Biosynthesis, Regulation and Animal Perception of Floral and Vegetative Volatiles, Plus their Roles in Human Flavor and Agriculture, Gordon Research Conferences, Ventura, CA, US, Feb 2012
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Jena, Meredith C. Schuman

Acknowledgments

A note These acknowledgments are an ode to the people from whom I learned things vital to the completion of this thesis. Those things include techniques, approaches, and scientific ideas, but also life philosophies, survival strategies, and the support of friendship.

The acknowledgments are separated into thanks directed toward my advisors, reviewers, funding organizations, colleagues, friends at the institute, students, teachers, family, friends outside the institute, and the people who especially supported me in completing the thesis. Names may appear once, or more than once in these different sections.

Thanks My first thanks go to my **ADVISORS**, **Professor Ian T. Baldwin** and **Professor Jonathan Gershenzon**. **Ian** and **Jonathan**, I want to truly and deeply thank you for your guidance, support, wisdom, and enthusiasm! I have learned so much from you over the years, and I look forward to learning more as our professional relationship continues. Your supervision has allowed me to ripen as a scientist. Thank you for providing the soil, sunlight and water for me to grow, and teaching me to be fruitful. Thank you also for teaching me many skills in field ecology, botany, chemistry, writing, management, advising ... and survival.

Thank you to the **REVIEWERS** of this thesis and its defense: you will tell me how far I've come as a scientist, and I look forward to your wisdom.

I thank the **Max Planck Society** and **IMPRS** for **FUNDING** my doctoral work, and the **Fulbright Commission** for paying my way to Germany in the first place.

I would also like to thank my **COLLEAGUES** at the Max Planck Institute for Chemical Ecology for fruitful scientific discussions, instruction in techniques, wisdom, and guidance, and I'd especially like to thank those mentioned here (in no particular order). I am convinced that **Dr. Karin Groten**, the IMPRS scientific coordinator (among other things) is Superwoman. Thank you, **Karin**, for answering many difficult questions, for helping me to enter this Ph.D. program without a Masters degree, and for all the work you do for all of us students, on top of your research.

Dr. Jianqiang Wu, **Dr. Ivan Galis**, **Dr. Emmanuel Gaquerel**, **Dr. Gustavo Bonaventure**, and **Dr. Sang-Gyu Kim** were always ready to discuss my project and problems, and taught me several laboratory and informatics techniques. **Jianqiang** and **Dr. Christian Hettenhausen** taught me almost everything when I first came to the group as a Fulbright student, and were my first friends and scientific collaborators (Chapter 2). I look forward to more collaboration with them in the future! Extra thanks go to **Jianqiang** for advising me at the beginning, and for many delicious group cooking parties. I also especially thank **Emmanuel** for many fruitful discussions, guidance, and exciting scientific collaborations (Chapter 3, and future publications). It was co-author **Dr. Nicolas Heinzel** who corrected the identification of the α-bergamotene isomer emitted by *N. attenuata* in Chapter 3. I also thank Chapter 3 co-author **Dr. Aleš Svatoš**, who gladly provided the essential expert knowledge and intuition for the identification of α-duprezianene from mass spec data. **Professor Amir Jassbi** started the DTG project, often discussed it with me, and taught me to identify terpene skeletons; **Amir** and his wife **Simin** were always kind and generous to me, and they have a beautiful daughter. I thank **Dr. Matthias Schöttner and Sven Heiling**, for team DTG and rigorous co-authorship, as well as our other co-authors, **Purba Mukerjee**, **Dr. Beatrice Berger**, **Dr. Bernd Schneider**, **Professor Amir Jassbi**, and **Ian** (Chapter 4), and of course thanks go to **Ian** for the careful crafting and co-authorship which he gives to all publications from his department, including those in this thesis. **Dr. Anke Steppuhn** also taught me a lot about writing scientific papers, during our collaboration: my first paper in a scientific journal; and also about what it takes to get a Ph.D. – I lived across from **Anke** and I know how late her lights went off at night and how early they went on in the morning! **Dr. Matthias Schöttner** is a great teacher of analytical chemistry and methods, and is in addition a bottomless well of philosophical and moral support, friendship, crazy wonderful music, and whiskey and noodles. I thank **Dr. Stefan and Doro Meldau** also for whiskey and noodles and cakes, friendship, and **Stefan** for fruitful scientific collaboration as well as insightful comments on the introduction to the thesis. **Dr. Matthias Erb** kindly gave constructive and insightful comments on the introduction of the thesis, as well as on an earlier form of Chapter 5. The same early form of Chapter 5 was also greatly improved by comments from **Jonathan** as well as **Professor David Heckel** and **Professor Andre Kessler**. **Nico**, **Emmanuel**, **Thomas Hahn**, **Alex Weinhold** and **Mario Kallenbach** maintained many expensive and dearly-needed analytical instruments, and had the patience to teach me how to maintain those instruments, too. (Even if the metric system is sometimes difficult for me ... **Alex** and **Mario** will know what I mean here, and **Thomas** hopefully does not!) I also thank **Alex** and **Mario** for hours of discussion, chemistry advice, moral support and invaluable friendship and no, you're not getting the puma stick back, I can't walk home without it. **Alex** I especially thank for many GCxGC-Stunden and PDMS trials; and **Mario**, for many GC-Stunden, for organizing import permissions even when he didn't go to Utah, and for good scientific fun with caterpillars and magnets, and mass spectrometers, and future collaboration. (PDMS and ISS, here we come.)

More thanks go to **Dr. Klaus Gase** and **Dr. Axel Schmidt** for help with sequence searches and vector design; **Antje Wissgott** for teaching me cloning for plant transformation; and **Antje**, **Celia Diezel**, **Eva Rothe**, **Susi Kutschbach** and **Wibke Kröber** for hundreds of transformed plants and for support and friendship.

It was an honor and inspiration to share an office with **Professor Chung-Mo Park**. **Susi** and **Wibke** were also wonderful office-mates for many years until I moved to **PAO**'s old place with **Arne** and **Professor Park**, and **Antje** is an awesome lab-mistress. Thanks also to **Evelyn Claussen**, who organizes all of our (working) lives.

I thank **Danny Kessler** and **Celia Diezel** for general awesomeness, and also for teaching me many field ecology skills and showing me some of the many wonders around Lytle, and for friendship and "life support" during a couple of long (but wonderful) field seasons. Thanks also to **Brigham Young University** for supporting **Ian**'s continued use of Lytle Preserve, and to everyone else with whom I had the joy of sharing time at Lytle – it always brought us closer together, and it always impressed me how well we got along, and how we supported each other in research and life in the desert: **Ian**, his management of us and the station (often coupled to heavy physical labor), and his delicious bread, **Jonathan**, his patience with me, and his banana peels, **Danny** and his parseltongue, **Celie** and her adorable weevils, **Eva** and her nylon stocking Easter eggs, **Dr. Silke Allmann** and *GEOCORIS*!, **Melanie Skibbe** and the cow pee hypothesis, **Evie Körner** and her methanol, **Dr. Paola Gilardoni** (words fail me), **Michi Stitz** and his field nanoscope eyesight, **Arne Weinhold** and his desert penguins, **Alex Weinhold** and his fragrant *Manduca*, **Mari Stanton** and her not-beetles, **Mario** and his clear results, **Youngjoo Oh** (Son! Snake!), **Truong Son Dinh** and his WRKY 3 6 9 12 15 ... , **Melkamu Woldemariam** and his beautiful singing voice, **Ivan**, the 3 o'clock in the morning upside-down wheelbarrow hero, **Stefan M.** (Polygamy Porter!), **Stefan Schuck** and his sombrero, **Sang-Gyu**, who is a real farmer, **Maria Heinrich**, who was cold in the desert, **Lynn Ullmann-Zeunert**, who slept in the kitchen, **Matthias Erb**, who is getting to the root of the problem, **Danielle Marciniak**, who dreams of petunias, **Kathleen** (1 ... 2 ... 3 ... and poke ... 1 ... 2 ... 3 ...), **Dr. Vaiju Tamhane**, who brought field cooking to a new level of deliciousness, and **Will Stork**, who solved the mystery of the disappearing caterpillars.

Sang-Gyu, you planted a LOT of plants with me in the field – thank you, because I think I would have died without your help! **Silke**, thank you for the work we did together, although our poor plants couldn't support a publication despite all the water we lugged uphill to save them, and thanks for teaching me to make fast decisions and be confident in my abilities, and to say no, even if I still don't do it. ;-) I hope in the future we can do more work together with GLVs and *Geocoris*, and I hope you don't mind that I borrowed your fabulous Acknowledgments style! **PAO**, you were often my home away from home. You supported me so much and taught me to be strong and strategic, and I miss you terribly. **Youngjoo**, it is always wonderful to work with you. I look forward to the rest of the PDMS story, and I hope more work together in the future. To **Sang-Gyu** and the **Clockworkers** (to which I am happy to belong), I'm so excited to be starting that project, and I can't wait to see the great things we will produce! I also thank **Dr. Franzi Beran** for her great friendship and always-insightful discussion, for many long hours we shared together with the GC-PFC, and for helping me learn EAG and GC-EAD. I purified many many micromoles of *trans*-α-bergamotene, and someday I will use them. ;-) **Dr. Andreas Reinecke** was the driving force behind the purchase of the GC-PFC and getting it up and running, and together with **Dr. Isabell Uhuru**, **Andreas** also taught me EAG and GC-EAD. All of these techniques will be vital to future projects with *Geocoris*. No GC-PFC discussion would be complete without thanks to **Kerstin Weniger**, who helped me solve a number of GC-related mysteries. **Mari** and I also spent many hours caring for and feeding the GC-PFC – thank you, **Mari**, and thank you also for your support and chocolate during some of the most difficult parts of my Ph.D. Thanks to **Daniel Veit** for all his ingenuity and design skills, for help designing systems for volatile trapping, ozone fumigation, and many other things, and for always making things better. More thanks go to **Dr. Tamara Krügel** for answers to all plant cultivation- and glasshouse-related questions, and for teaching me cross-pollination, and some botany of southwestern Utah, and survival skills, and for always having an open door and a listening ear. **Andreas Schünzel** and **Andreas Weber** and

the rest of **the gardeners** accommodated all experiments and insect colonies – thank you! – and thanks go to **Jana Zitzmann** for teaching me flow cytometry.

Big thanks also go to the **directors** who steer this grand institute in good directions, the **administration** that keeps it running, the **IT department** who keeps our programs running and our data safely archived, the **librarians** who keep us intelligent, and the **workshop mechanics** who fix, maintain, and engineer our facilities. I know to treasure the very high level of scientific and logistical functioning that keeps the MPICE going, and our projects running smoothly. It's a fantastic place to work and collaborate.

To my **MPICE FRIENDS**: although many of you are already mentioned above in your role as **COLLEAGUES**, here is an extra and somewhat fluffier (it's so fluffy!) thanks. You are OOOOOO-AAAAAARRRRRRSSSSSOOOMMMMMMEEEEE. Who else would sing me Soft Kitty – in a ROUND – with AWESOME fitness room acoustics? Who else would rub VapoRub into my chest, counter-clockwise, when I'm sick? (Oh, you won't do that second thing? Dammit, I promise I don't have chest hair!) Seriously, I would never have made it without you, and maybe I helped you just a leeeeetle bit, sometimes, at least with American English (**PAOLA**). Here's a BOOMSMA! toast to the Schnapps group. We are busy beavers – there, I fixed it! – but we lay us down for each other like bridges over troubled water, bridges to to you-know-where: **Antje**, **Felipe**, **Mari**, **Mario**, **Michi**, **PAO**, **Vari**, **Weinhold and Weinhold**, **Youngjoo** ... to team UT, **Celie** and **Danny** ... to the MPICE alumni: **Alex-Girl**, I blame Canada for taking you back! **Arjen**, my late night walks home are so lonely without you! **Connie**, the hug club needs you! **Kwan**, you *are* a gift! **Nico**, our first experiment *was* the first experiment! ;-) **Silke**, no one plays *Geocoris* with me any more! **Markus**, you inspired everybody, including me. To the **Wu group**ies, **Christian, Maria, Stefan, Doro, Dahai, Hendrik** and of course **Jianqiang**, you were my first institute family and I can't wait to visit some of you in **Jianqiang**'s new home, Kunming, and play dodder with you in the field. **Sang-Gyu**, you have taught me peace. **Matthias S.**, you showed me flight without wings (but *with* a very cool electric bicycle). Thanks to all of **THE ITB GROUP** – we make such a good team, and we're not afraid of a few thousand samples – bring it on! And OF COURSE, thanks to the **OUTGROUP SAMPLES** from other departments, without which no family tree can be rooted: **Franzi, Daniel**, and **Rose**, I am so fortunate to know and work with you, and we really should get together more often; and **THE MOVIE NIGHTERS Sandy**, **Thijs**, **Maarten**, **Martin**, **Eduardo**, a friend of the Imperial March played by floppy disk drive is a friend of mine!

Thanks also to the **STUDENTS** I had the joy of mentoring as researchers, interns or HiWis, who taught me about teaching, and who contributed to work already published, or in preparation for publication. I thank **Evan Palmer-Young** for friendship, for challenging my thinking in many ways, and for valuable work on a paper that will finally be published now that this thesis is out of the way. I thank **Kathleen Barthel** for her diligent and dedicated work during her Diplomarbeit and a preceding internship, and for her co-authorship on Chapter 5, and for teaching me lessons in advising. **Stephanie Siegmund** was an energetic and inspiring friend, and her root RNA dilemma taught me how to systematically approach and teach troubleshooting. Thanks also to the HiWis and interns I've supervised before and during my Ph.D.: **Susan Schaller**, **Sandra Korte**, **Nora Petersen**, **Benni Naumann**, **Dani Schweizer**, **Steffi Spielberg**, **Jenna Olivia Miller**, **Hannah Goddard**, **Mark Kupetz**, **Isabell Schmitt**, **Danielle Marciniak**, **Gabriel Hughes**, **Anke Rauch**, **Robert Ludwig**, **Tim Sehrt** and **Amy**, **Torben**, **Christiane Schubert**, and **Magdalena Murr**; and to the

high-school students whose Seminarfacharbeite I mentored and from whom I also learned: **Hai Ly Tran**, **Lisa Stodollik**, **Julia Güpner**, **Anne-Marie Schünzel**, **Lorenz Troll**, and **Marcus Spörlein and co**.

I extend my thanks to the **TEACHERS** who have educated and inspired me. There are are few whom I would especially like to mention. In kindergarten, **Mrs. Ostlund** encouraged me to read the big kid books. My fifth-grade teacher **Mr. Sweeley** improved my writing more than anyone before or since, and made me beat my personal best every single day. In high school, **Mr. Helm** made biology even more fun than it usually is, emanated an excitement that was contagious, and emphasized the importance of learning to think like a scientist. **Mr. Meyer** constantly derived new ways to convince us that physics was fun, including one that involved revealing the secret subtext of Simon and Garfunkle's rendition of "Scarborough Fair". **Mr. Iverson** used very subtle but gut-busting humor to know which of us were awake in his chemistry class (molasses). **Mr. Schwandt** had an elaborate dry humor that made everything funny, but insisted that we all face the Apocalypse Now side of life. **Mrs. Weaver** gave us the assignment of writing an ethnography, which broadened and deepened my understanding for others, and led me to get a tattoo and several piercings (though I don't believe those latter bits were part of her intention). **Mr. Schield** might be the nicest person I ever met, and he taught us a lot of history that American history books have forgotten. **Mme. Eklund** was the coolest, Harley-drivingest teacher ever; her hair had already experienced all the various colors that mine turned during high school. She taught me enough French to be understood in Paris, and to absorb French literary works that changed my life philosophy (Albert Camus, Jean-Paul Sartre, and Antoine de St.-Éxupery). Before Madame, **Christelle** started teaching me French when I was still young enough to learn it easily, which set me up for lifelong success in learning languages. **Mrs. Haugen** gave me a voice to speak and a place to be when I most needed them, and lent me her strength and confidence until I found mine again. At university, **Professor Fleming Crim** taught me to solve the wave equation of a hydrogen atom, and made chemistry fun, even when it entailed five-hour labs starting at 7:45 a.m. twice a week and homework assignments that were never solved before 1 a.m. **Professor Gellman** kindly told me that organic chemistry sometimes doesn't make sense, and I should stop fighting it and learn to memorize reactions. **Professor Middlecamp and Ms. Omie Baldwin** taught a groundbreaking course on nuclear chemistry and its social impacts, especially for the Navajo, and showed me that science theory can be taught in real-world contexts, and scientists can be strong advocates for justice. **Professor Teri Balser** taught me that the teaching process should be well-researched, and that soil is a thing of beauty. Teri also gave me the opportunity to beat my own path into scientific research and supported my first attempts. Then almost-Dr. **Jessica Mentzer**-now-Gutknecht taught me to troubleshoot PCRs, run gels, use lab equipment, survive, etc., told me all about her cool microbial ecology experiments, and asked only a few pipetting hours in return. Then almost-Dr. **Jenny Kao-Kiffen** encouraged me to no end. **Professor Philip Regal** took the time to foster my interest in the history of molecular biology, which is both impressive and troubled, and has never ceased to fascinate me since. This resulted in **Professor Clark Miller** donating hours of his time to discuss with me how the advent of molecular biology changed perspectives and approaches in microbial ecology research. **Professor Robert Goodman** was the chair of molecular biology at UW-Madison when I studied there and at that time, carried a jar of his own crystallized virus, chaired a McKnight Foundation oversight committee, and founded his own biotech start-up of which he gave my class a tour. **Professor Goodman** taught me that the history of science is as important as its future, and that scientists can, and should, do everything.

Loving thanks go to my **FAMILY**, because what is a plant without roots? A tumbleweed. It is wonderful that we can always come home to each other. To **Dad and Mom**; to those who have left our physical presence, but continue on in our indomitable spirits: **Nana and Grandpa Derreld**, **Grandma Ann and Grandpa Phil**, and **Aunt Charlene**; and to all the other wonderful people I am fortunate to be related to: **Uncle Richard**, **Jennifer**, **Jonathan**, **Jacob**, **Tim**, **Margo**, **Beth**, **Michaela**, **Lauren**, **Corey**, **Kathleen**, **Alice**, **Ed**, **Kendra**, **Mark**, **Darcy**, **Tori**, **Renee**, **Craig**, **Aunt Berta**, **Aunt Doris**, **Uncle Lawrence**, **Lydia**, **Sam**, **Irene**, **Tim**, **Kris**, **Tony**, **Aunt Cindy**, **Uncle Bob**, **Brian**, **Scott**, **Aunt Cherie**, **Uncle Paul**, the list goes on ... thank you.

Thanks especially to **Mom and Dad**, **Nana and Grandpa Derreld**, **Grandma Ann and Grandpa Phil**: thank you for planting the seed, and nurturing the seedling. Through your care and cultivation, I have become strong enough to withstand the harsh stresses of life in the "real world". I love you so much that words are inadequate.

Thanks go to my **GERMAN FAMILY**, Mr.-Diplom-Physiker-soon-to-be-Dr. **Alexander Steppke**, and the whole extended Steppke family: **Jasmin and Markus**, **Doris and Harald**, and **Alice**, and **Archie**, and **Püpi**, and **Parodie** . . . I am so grateful to have been welcomed into your loving circle. As **Danielle** put it: "Are you sure they're German?!?" ;-) **Alex**, we fell in love almost as soon as we met, between me stomping out fires with my bare boots, and you (in two unrelated incidents) getting arrested, and quoting Star Trek at just the right time. I LOVE YOU, but that can't possibly say it all. You're the reason I applied for a Fulbright and discovered the MPICE. If it weren't for you, I might never have looked outside Minnesota. I wouldn't know what "kuschelig" means. I could go on, and I hope we do, for a long time, because we make a kick-ass team. And you're not only adorable, but so intelligent that it sometimes scares me. You have helped me and supported me so much throughout my doctoral work and now at the end with my thesis ... thank you just doesn't come close, and I only hope I support you as much in earning your doctorate.

To my **ADOPTED FAMILIES**: the **Bureks and Gasts**, the **Greenes and Welches and Ahlstroms**, the **Aarons and Betkers**, the sUUper members of **MSUS**, the **Hanninens and Shirashis**, the **Spitzers**, The **Zdunich-Brachers**, the **Smith-Auerbachs**, the **Rands** family, and **Marcia** (I'm afraid when you took care of me I was still too young to care about last names) ... you have made such awesome sisters and brothers, uncles and aunts, I just don't know who I would be without you, and I promise to make contact every once in a while now that my thesis is written! I miss you.

Ditto to my **FRIENDS** from university and after university, from school and way back before school ... Dear friends, dear friends, let me tell you how I feel: you have given me such treasures; I love you so. Some of you are already in the adopted family list :-) but I'll add you here, too: **Jon-boy**, **Nicole and Amber**, **Becks and Care**, **H and Michael**, **Shan and James**, **Chrisling and Jason**, **Laura and Lauren**, **Keji**, **Cilla**, **Mira**, **Alissa**, **Jeff**, **Sarah M.**, **Erik**, **Aaron**, **Darren**, **Alex V.**, **Chrislet**, **Colin**, **Arthur**, **Rose**, **Sara D.**, **Ted**, the sUUper **YRUUers**, **Martha**, **Laura/Clem**, **Michelle**, **Rita**, **Taimi**, **Beth**, **Devan**, **Brian**, **Bonnie**, **Dana**, **Karn**, **Carmen**, **Amy**, **Alex F.**, **Mer**, **Ava**, **Ingrid**, **Sarah G.**, **Sara-muffin and Nick and little bun in the oven**, **Tiff and John**, **Michelle and Ryan**, **Abby and Patrick**, **H-mush**, **R-mush**, **Caeli-muffin**, **Julia** (thank you for your beautiful pictures), **Michelle S.**, **Nels**, **Taylor**, **Lane and Nova**, **TaNaya and Steve**, **Chris K.**, **Brent**, **Brent R.**, **Kelly**, **Trinity United Methodist Church**, **MUUYACM**, **libraries open 24 h**, **Anja and Nick**, **Maria**, **Sabine and Lutz**, **Anne-Mareika**, **Sebastien**, **Christian P.**, **Daniel** ... it's always too long

between visits. And to my fellow **Fulbrighters**: I had a great time with you. We helped each other through those first few hard months of homesickness to move on to great accomplishments.

To my **FLATMATES**: **Benni and Nora**, we have only been flatmates for a short time, but it is fantastic to live with you. Thank you for making the flat such a nice home, and for your support and delicious food. **Anne and Chrissie**, you are an integral part of my German family; I'm really grateful that we had so much time together, and I'm excited to see how your lives develop. I look every day at the WELCOME art hanging in the kitchen and over the front door of the flat, and think of how you welcomed me into a new Chapter of my life. To the "extended" flatmates, **Christoph**, **Mitch**, and **Tanya**: the times were good. :-) And to the really old flatmates ;-) , **Anika** and **Phillip**: now I have no excuse not to get back in touch with you! **Deelee**, you were such a vital part of my first couple of years here, I'm sorry for being such a horrid correspondent, and I really miss you. I would love to have some poetry back in my life.

Last but not least, a **BIG extra-special thank you** to those who really supported me in finishing this thesis, especially after I lost my precious **Nana**; and to those among you who believed me that a proofreading party would be fun, and gave me feedback on the introduction, Thesen, and text: my **Alex S.**, **Alex W.**, **Arne**, **Daniel**, **Franzi**, **Jianqiang**, **Mari**, **Mario**, **Michi**, **Youngjoo**; and **Nora and Benni** on the homefront. **Franzi**, **Mario**, **Nora** and **Benni** helped me to convert my "German" Thesen and Zusammenfassung to real German. All mistakes that remain in this document are my fault.

THANK YOU