Trichomes in plant-herbivore interactions:

The first line of defense of the wild tobacco *Nicotiana attenuata* against attacking insects

Dissertation

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Chapter 1: General introduction

1.1 Motivation

When the first man in space looked down at the Earth, he described it as a ”blue planet“. Even though oceans are cover a major part of the Earth, shaping its blue appearance, most of the landmasses appear green. Therefore it is not wrong to speak of a green planet. There are between 380.000 and 500.000 plant species causing this “greenness” and, more importantly, there are even more species of herbivores with the sole interest of feeding on green leaf tissue. This makes the existence of a green planet quite remarkable. In addition this illustrates that plants have to face an incredible number of enemies and that there would be no green planet if plants would not have evolved an enormous plethora of defense strategies against attacking herbivores.

Plant defenses have been intensively studied in the last 50 years in the field of chemical ecology **(Fraenkel 1959)** since it became more and more apparent, that understanding these defenses would have a strong impact on agriculture. Nowadays, over 50 percent **(Oerke 2006)** of the world’s crops are lost to insects or pathogens. The resulting the loss of valuable food in a world of exponential population growth will lead to increasing food shortages and conflicts. Hence the understanding of plant defenses is crucial for the breeding of crops that are resistant or less susceptible to herbivores and diseases. Many plant resistance traits are mediated by secondary metabolites. These molecules are not directly involved in plant growth and primary plant processes. Until the discovery of their important functions in plant-plant or plant-insect interactions, these compounds were regarded as “waste-products” of metabolism that may have evolved a secondary ecological function. Today the idea that different secondary metabolic pathways have evolved through evolution is widely accepted and many examples for their ecological functions are given in the literature. For instance, volatile compounds like terpenes and oxylipin-derived alcohols, aldehydes and esters attract natural enemies of herbivores or are incorporated in the defense against pathogens **(Prost, et al. 2005)**. Alkaloids with different structures are effective toxins against herbivores (for a review of plant responses to herbivory see **(Howe, et al. 2008)**). Many of these metabolites are produced and deployed within leaves, but the first tissues that come into contact with chewing herbivores, are the leaf hairs of the plant.

These leaf hairs, known as trichomes, have long attracted the interest of researchers on different plant species. In the beginning trichomes, were only seen as a useful character for elucidating the taxonomy of plants **(Spring 2000)**, but their functionality in plant defenses generated further interests in studying them. Already in the late 1890s Ernst Stahl, one of the founders of chemical ecology, described the function of trichomes of the genus *Onagraceae* which he referred to as "acid hairs" **(Hartmann 2008)**.

Stahl was the first, who reported about the acid fluids that are exuded by theses hairs and his observations that snails would avoid plants protected by acid hairs can be considered as the first report of a trichome-based plant defense. Since then, many studies have contributed to the evidence that trichomes can act as defense systems, either as mechanical obstacles or as well as chemical factories, which protect plants through the biosynthesis of secondary metabolites.

There are reports, which indicate that leaf hairs are able to synthesize their metabolites independently from the surrounding leaf tissue, even when they are excised from the leaf **(Kroumova, et al. 2003)**. Therefore it is necessary to elucidate the function of theses leaf hairs and their metabolites in a model system, like the wild tobacco *Nicotiana attenuata.* This system has been shown to be an important ecological model for investigating the role of secondary metabolites in plant defenses and offers the opportunity of investigating the role of trichomes in plant defense in its natural habitat. Hence I would like to introduce this system in the following section.

1.2 The Model System Nicotiana attenuata:

Nicotiana attenuata is native to the Great Basin Desert of southwestern U.S.A. As an annual plant it germinates after induction by smoke cues, derived from the seasonal bushfires in this region, and can be considered as a pioneer plant species. The nitrogen rich soils in the post-fire environment allow *N. attenuata* to grow rapidly in monocultures without much competition from other plant species. On the other hand, every freshly grown plant has to face the attack of a large and unpredictable herbivore community, like the larvae of the tobacco hornworm (*Manduca sexta*), a specialist herbivore of *N. attenuata*. Therefore the plant's survival is subject to a strict trade-off between growth and chemical defense biosynthesis. Since modern agricultural plants have lost most of their defensive traits through selective breeding, there is a need to study plant defenses in nature with plants in their native ecological system. The knowledge acquired from natural systems can be used for reintegration of specific defenses into crop breeding. For instance, it has been shown that the emissions of E-β-caryophyllene by maize plants increases the resistance against the larvae of *Diabrotica virgifera* by the attraction of nematodes that infest the larvae **(Rasmann, et al. 2005)**. Most of the North American maize cultivars have lost the ability to emit this volatile and are therefore prone to Diabrotica attacks **(Köllner, et al. 2008)**.

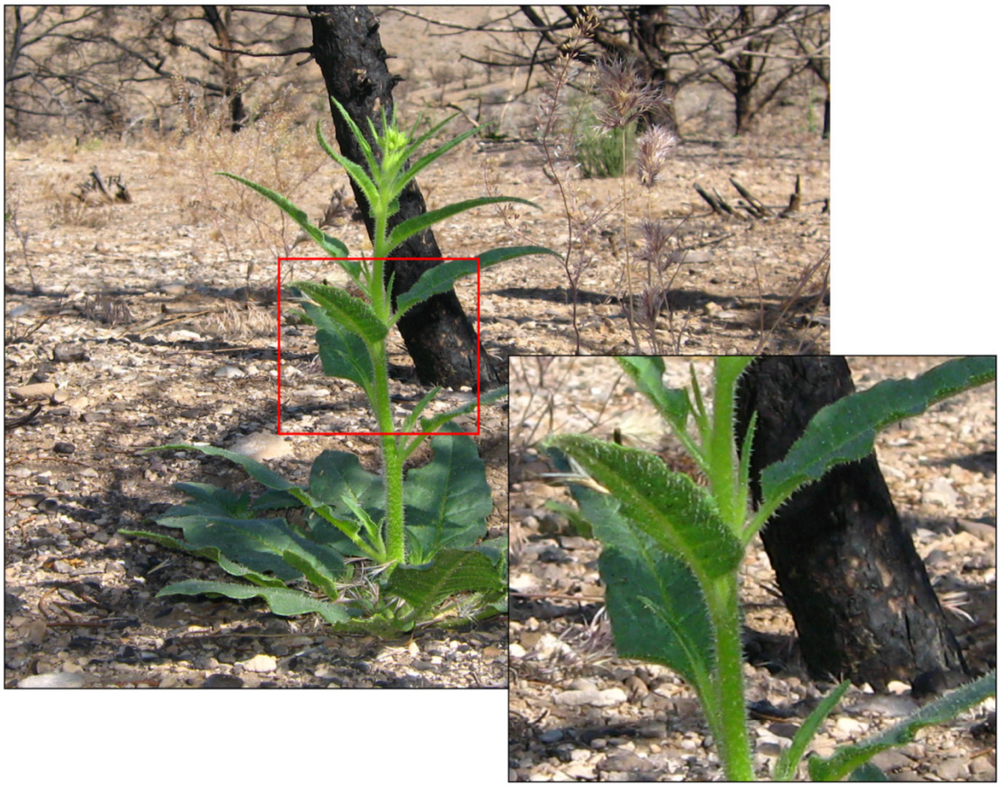

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Fig 1-1: The wild tobacco *Nicotiana attenuata* in it's natural habitat in the Great Basin Desert in the Southwest USA. The trichomes covering the leaf and stem surface are clearly visible.
(Picture taken by Danny Kessler)

Hence studies of how plant defense are function in nature, could provide answers to the problems of dealing with agricultural pests of crop plants. In the past decade, a large molecular tool box was developed for *N. attenuata*, allowing the stable transformation of this plant. This enables us to test hypotheses about the function of defenses in field experiments making *N. attenuata* a valuable model plant in chemical ecology. The trichomes of the genus Nicotiana were extensively characterized by Godspeed **(Goodspeed 1955)**, who described the different trichome types (**Fig. 1-2)** that can be found in the various *Nicotiana* species. With the established molecular, chemical and ecological tool boxes at hand, *Nicotiana attenuata* is the perfect study object with which to understand the role of trichomes in plant herbivore interactions

The scope of this thesis is to understand the versatile roles that trichomes play in plant herbivore interactions. This will be done in the plant-herbivore model system *Nicotiana attenuata* – *Manduca sexta*. Chapter 2 will give a short introduction into plant defenses against herbivores and chapter 3 will explain the role of plant surface chemistry and the role of trichomes in this context. Chapter 5 will show that previously characterized defensive metabolites from trichomes, *O*-acyl sugars, can have different defensive functions from those which have so far been proposed for these compounds. Chapter 6 continues to elucidate the function of *O*-acyl sugars in plant defenses, while Chapter 7 will deal with the different trichome constituents of *N. attenuata* and their effect on specialist and generalist herbivores. Finally, Chapter 8 will give an overview of the variability in *O*-acyl sugar structures in the wild tobacco *N. attenuata*.

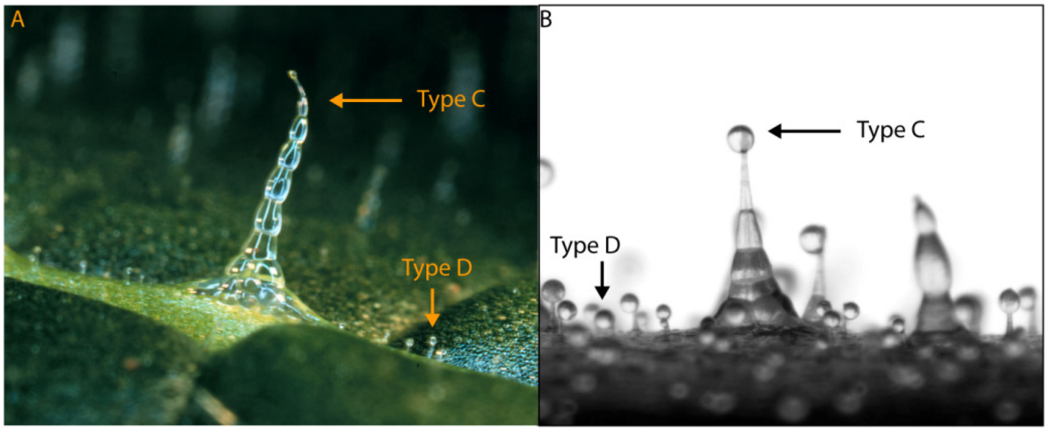

Fig. 1-2: Trichomes on the leaf surface of *Nicotiana attenuata* and their assignment according to Godspeed. **B** droplets of exuded *O*-acyl sugars are clearly visible. (Picture A taken by Danny Kessler

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Chapter 2: Plant defenses

2.1 Interactions between plants and their environment

In their native ecological environment plants interact with many different biotic as well as abiotic factors. These interactions are complex, and the plants' adaption to them led, among other reasons like reproductive isolation **(Widmer, et al. 2009)**, to the speciation of different plant species. An example of abiotic factors is the availability of light, which is essential for plants to do photosynthesis. Plants have evolved phototropism to direct their growth towards a light source **(Farré 2012)**. Additionally the circadian clock of plants and therefore, many different circadian metabolic pathways, relies on light perception. On the other hand high, energetic wavelengths in the UV- range can cause DNA damage and can lead to mutations in the genome. To counter the harming effects of light plants have developed pigments (for an overview see **(Ueda, et al. 2011)**) to absorb radiation and avoid these damages. Another important abiotic, and especially in arid regions, limited factor, is the availability of water. Water is essential for all living organisms and is needed by the plants to maintain the transport of primary and secondary metabolites between different plant tissues. Since water is not uniformly distributed among the different habitats, different strategies have been developed by plants to cope with the lack or the excess of water. The formation of thick cuticles **(Seo, et al. 2011a, Seo, et al. 2011b)** or the storage of water in specialized tissues (e.g. succulent plats) are only some of the morphological adaptations to tolerate water deficient conditions.

Biotic factors that influence plant fitness are no less important and are also a field of intense research. We can discriminate between three main biotic factors which plants have to face. First, there are competitors in the same habitat, which compete for light, water and nutrients. This competition can either be between individuals of the same species (intraspecific) or between individuals of different species (interspecific), as seen in the competition for light in a forest. Second, there are pathogens like fungi, bacteria and also viruses infecting plants. The third factor is the attack of herbivores, which can belong to diverse taxa such as insects or mammals. Although mentioned here separately, these biotic factors may also overlap, for instance when pathogens use insect as vectors **(Weintraub, et al. 2006)** to infect their specific host plants.

When confronted with competitors for light, plants may respond either by elongated growth or by changes in flowering time, as well as in changes of the apical dominance. This so-called shade avoidance response is common in the plant kingdom and well-studied **(Franklin 2008)**. Plants are even able to distinguish between competitors and inanimate objects like rocks. This is achieved by sensing changes in far-red/red light ratio, caused by the absorption of red light by the photosynthetic pigments of shading plants.

As an adaption to herbivory, plants have evolved a plethora of different defense strategies. The majority of plant defenses can be classified in two different ways, on one hand we can distinguish between direct and indirect defenses and on the other hand between constitutive and induced defenses after herbivore or pathogen attack. In the following chapters I would like to give a short introduction into these different defense mechanisms and highlight the differences and similarities between them.

2.2 Constitutive vs. induced defense

Constitutive defensive strategies of plants can be quite simple and manifest themselves as mechanical barriers that protect against herbivores. Examples of mechanical barriers include leaves that become tougher by producing a thicker cuticle to make it less easy for herbivores to feed on them, trees that protect their trunks with a thick bark. Other plant species have developed thorns and spines in order to repel herbivores, like species of the genus *Acacia*, which have evolved long spines that protect them against the attack of large mammals **(Gowda 1996)**.

In addition, constitutive defensive strategies do not have to be only of a mechanical nature. For instance, glucosinolates **(Chen, et al. 2001)** are present in almost every family of the order *Brassicales.* These nitrogen- and sulfur-containing compounds are derived from glucose and different amino acids. Every glucosinolate contains a central carbon atom, which is bound via a sulfur atom to the thioglucose group (making a sulfated ketoxime) and via a nitrogen atom to a sulfate group. When a plant is attacked and cell tissues are disrupted, the glucose is cleaved from its aglycone by an enzyme, myrosinase; subsequently the free aglycones react to form isothiocyanates, nitriles or thiocyanates, which are the active molecule species that act against the attacking herbivore **(Fahey, et al. 2001)**.

A very common defense mechanism in plants is the chemical defense against herbivory by the production of toxic substances, e.g. the above mentioned glucosinolates. The production of these substances is costly and requieres an allocation of resources which cannot be invested in other plant functions. For example, the biosynthesis of many alkaloids is strongly dependent on the availability of nitrogen. This means that an increase in alkaloid production leads to a decrease in the nitrogen pools available for the primary metabolism **(Lynds, et al. 1998)**. Since nitrogen is already a major limiting factor for normal plant growth, the allocation to defenses can consequently affect plant growth negatively. Hence it is more beneficial for the plant not to invest in the production of secondary metabolites constitutively, but only when they are needed, in the time of an herbivore attack. These defensive traits that are triggered after herbivore attack are called induced defenses. An example of this type of defenses is the production of nicotine. In the wild tobacco *N. attenuata*, nicotine remains at basal levels as long as the plant is not injured. Upon herbivore attack, a signaling cascade is triggered that leads to the production of nicotine in the plant roots and the subsequent transport to the leaf tissues **(Howe, et al. 2008, Leon, et al. 2001)**. The nicotine accumulation peaks at day 3 after herbivore attack and is therefore a good example of induced defenses. Another defense mechanism in *N. attenuata* that affects the herbivore directly utilizes hyroxygeranyllinalool-diterpeneglycosides (HGL-DTGs). These molecules consist of a hyroxygeranyllinalool diterpene backbone that is connected to a glucose moiety. The sugar residues can have further substituents like rhamnose or malonic acid. It has been shown that feeding on HGL-DTG containing plants dramatically reduces the growth of *M. sexta* when compared to HGL-DTG free plants **(Heiling, et al. 2010)**. Like nicotine, these metabolites are constitutively present in plant tissue but increase in their accumulation after induction by herbivory, also making them an induced defense.

Volatile organic compounds (VOCs) that are emitted after wounding are an interesting example of an inducible defense. In *N. attenuata* we can discriminate between two different types of volatiles that are released after herbivore attack. On the one hand there are green leaf volatiles (GLVs), six carbon unsaturated aldehydes and alcohols and their esters, and on the other hand we have the emission of terpenoids, mono- and sesquiterpenes **(Halitschke, et al. 2000, Turlings, et al. 1998)**. While GLVs are released just after the disruption of the plant tissue, terpenoids are released in the next photoperiod, after herbivore attack. This means they are *de novo* produced by biosynthetic processes that are triggered by the herbivore's attack. Trans-α- bergamotene is a good example **(Halitschke, et al. 2003)** of this induction pattern. The emission of this sesquiterpene peaks in the morning hours after herbivore attack **(Halitschke, et al. 2000)**. A study has shown that this herbivore induced volatile attracts predators and increases the predation of the tobacco hornworm *Manduca sexta* **(Halitschke, et al. 2008)**.

Leaf hairs or trichomes can additionally be constitutive defenses, by being simple obstacles or containing toxic metabolites. It has been demonstrated that after simulated herbivore attack with by use of the phytohormone methyl jasmonate, the densities of trichomes increase on newly developing leaves **(Boughton, et al. 2005)**. Therefore the chemical as well as the mechanical defense by the trichomes can be inducible. This also highlights the importance of these epidermal protuberances for the whole plant defense.

2.3 Direct defense vs. indirect defense:

Direct defenses can be simple mechanical barriers, but besides the purely mechanical defenses, plants can also effectively use chemical defenses; compounds with an origin in the plants' secondary metabolism, which act as a chemical barrier against attackers. The name-giving compound of the genus *Nicotiana*, nicotine, is an inducible direct defensive metabolite **(Steppuhn, et al. 2004)**. Nicotine acts as a neurotoxin and can stimulate human acetylcholine receptors resulting in higher heart rates and an increase in the blood pressure. It is one of the most prominent members of the alkaloids, a class of substances that is widely spread in the plant kingdom, with around 10.000 different known compounds. Since all of these compounds are derived from the plants' secondary metabolism and are not directly essential for plant growth, they are considered to have evolved as an evolutionary adaption to counteract herbivory.

In response to this large defensive arsenal that plants contain, herbivores have evolved strategies to cope with these toxins. This evolutionary arms race can lead to the specialization of herbivores, for instance, *Manduca sexta* **(Murray, et al. 1994)** is able to detoxify most of the nicotine consumed from its host plant *N. attenuata* or to excrete it in the frass **(Snyder, et al. 1994, Wink, et al. 2002)**. As a counter adaption, *N. attenuata* has developed additional defensive traits against *M. sexta*. When the caterpillars start feeding on leaf tissue, the plant releases a bouquet of different volatile organic compounds (VOCs).

These VOCs mainly consist of unsaturated C_6 aldehydes or alcohols **(Gaquerel, et al. 2009)** and their esters. In keeping with the saying “the enemy of my enemy is my friend”, these volatiles are able to attract predatory bugs, like the big eyed bug *Geocoris spp.*, that prey on first and second instar larvae and eggs of *M.sexta* **(Allmann, et al. 2010)**. This kind of defensive mechanism that involves another trophic level is referred to as indirect defense. This type of indirect plant defenses have been reported for several plant species, apart from the genus *Nicotiana*. Cotton and maize plants emit specific volatile blends after attack by *Heliothis virescens* and *Helicoverpa zea* which are then perceived by the predatory wasp *Cardiochiles nigriceps* **(De Moraes, et al. 1998)**. The release of VOCs is an efficient way to attract predators, but has disadvantages, like the dispersion of the alarm signal in the air and it also requires the presence of suitable receivers of the signal, in the plant’s vicinity. In this light it would, be more effective for a plant not only to call for help but also to make sure that potential helpers are present at the scene of the attack.

Many plant species attract ants to protect themselves against herbivores. These “ant-loving” myrmecophytes provide food and shelter for the ant species. For instance, the Southeast Asian pioneer tree *Macaranga tanarius*, has extrafloral nectaries on its leaves, from which a sugar rich solution is exuded **(Heil, et al. 2001)**. Ants of the genus *Crematogaster* **(Linsenmair, et al. 2001)** are attracted to this additional food source and will defend it against herbivores. Ants are also used by some *Acacia* species as guardians against attackers. In this case not only extrafloral nectars are exuded, but in addition so called food bodies are provided by the plants **(Heil 2008)**. These protein- and fat-rich spheres are readily consumed by ants, which will then defend the plants against attackers. But *Acacia* plants do not only provide food, they can also provide shelter for the ants in hollow thorns, known as domatia **(Heil 2008)**. Since the ants build their nest within these domatia, they aggressively defend it against every intruder and therefore protect the plant. Together these examples show the enormous complexity and variability of plant defenses. Given the advances in plant transformation - the elucidation of the mechanisms of plant defenses might help to improve the breeding of agricultural plants.

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Chapter 3: Trichomes in plant defenses

3.1 Plant surface interactions:

The leaf surface of plants is the “crossroads of interactions with the environment” **(Shepherd, et al. 2007)**. At this barrier, plants fight against water loss, UV-radiation, microorganisms, fungi and herbivores. The development of the plant cuticle, consisting of waxes like cutin **(Kolattukudy 1996)**, is thought to be a major adaption against water loss, but on the other hand it is also a well suited substrate for fungi spores and microorganisms. Therefore plants have evolved different defensive strategies to hold this first line of defense.

Defensive proteins - secreted on the phylloplane (phylloplanins) - are one way, plants defend themselves against herbivores. Phylloplanins are quite diverse in terms of their mode of action against attackers. For instance, a cationic peroxidase involved in resistance against *Xanthomonas oryzae* is found to be secreted on the leaf surface of rice (*Oryza sativa*) **(Young, et al. 1995)** and pathogen-related proteins can be found on leaves of barley (*Hodeum vulgare*) **(Grunwald, et al. 2003)**. PIN2, a protein inhibitor is found in *Solanum americanum* **(Komarnytsky, et al. 2000)**, while in the cultivated tobacco, *Nicotiana tabacum*, phylloplanins are excreted and have been shown to reduce the plant’s susceptibility against blue mold (*Peronospera tabacina*) **(Shepherd, et al. 2005)**. These examples emphasize the importance of leaf surface proteins against attackers and in particular pathogens. There are different mechanisms proposed that allow plants to deploy phylloplanins on the leaf surface. They can be provided by apoplastic diffusion **(Schreiber 2005)**, in which after being synthesized in underlying epidermal cells, they are transported to the surface either by hydrophilic pathways in the cuticle or by capillarity via open stomata. Another path seems to be the deployment via guttation fluid excreted from hydathodes (HT) **(Shepherd, et al. 2005)**. After the evaporation of the guttation fluid the proteins will be deployed all over the leaf. The third exudation pathway utilizes glandular trichomes as sources for phylloplane exudates. To put it in a nutshell, the leaf surface of plants has an important role in plant defenses, and specialized surface structures, like trichomes, will be therefore discussed in the following section.

3.2. The multiple functions of trichomes

Trichomes, or leaf hairs, are epidermal protuberances that occur on more than 30 % of all vascular plants. After being only considered a morphological character for taxonomy, they are now attracting the interest of researchers more and more. Not only do they harbor and exude defensive proteins, but also accumulate a wide array of secondary metabolites. In addition, trichomes may also function as mechanical obstacles for attacking herbivores. Trichomes can be divided in two groups, glandular secreting trichomes (GST) and non-glandular simple trichomes (ST).

Non-glandular trichomes can act in a direct and constitutive defensive way to protect the plant by being an obstacle for insects and therefore slowing down insect movement on the plant surface as well as insect feeding **(Cardoso 2008, Kennedy 2003)**. They can even have spine- or thorn-like structures that are able to injure and trap herbivores **(Pillemer, et al. 1976)**. Glandular trichomes can serve an important defensive function, like those of tomato plants which exude *O*-acyl sugars and store sesquiterpenes. Both compound classes have been shown to be active against white flies and spider mites **(Buta, et al. 1993, van Schie, et al. 2007)**. The versatile roles of trichomes in plant defenses and their other roles in plant-environment interactions are summarized in **Table 3-1**. Glandular trichomes can also be storage organs for substances, which are toxic for the plants. It is not unusual that heavy metals like cadmium are secreted via trichomes **(Choi, et al. 2001)**. Apart from these "waste depositing" processes, it is highly interesting to have a look at the great variety of chemicals produced in glandular trichomes. The most commonly known trichomes can be found on the stinging nettle (*Urtica dioica*) **(Pullin, et al. 1989)**, whose leaf hairs of contain a mixture of acetylcholine, histamine, serotonin, moroidin, leukotrienes, and possibly formic acid **(Flury 1927)**. When trichomes of *U. dioica* are disrupted by an herbivore they form needle like structure that are able to penetrate the herbivores skin and to inject the defensive cocktail into the attacker

Table 3-1: Modified from**(Wagner, et al. 2004)** functions of trichomes in nature (bold letters represent plant defense functions)

Apparent Function	Trichome type*	Reference
Reduced insect movement	ST and GST	**(Levin 1973)**; **(Johnson 1975)**; **(Kessler, et al. 2002)**; **(Kennedy 2003)**
Temperature regulation	ST and GST	**(Johnson 1975)**; **(Dell, et al. 1978)**; **(Ehleringer 1984)**
Increased light reflectance (including UV)	ST and GST	**(Ehleringer 1984)**
Decreased water loss through reflection	ST and GST	**(Ehleringer 1984)**
Reduced mechanical abrasion	ST and GST	**(Uphof 1962)**; **(Kessler, et al. 2002)**
Protection of phylloplane organisms	ST and GST	**(Beattie, et al. 1999)**
Reduced leaf humidity	ST and GST	**(Brewer, et al. 1997)**
Reduced photosynthesis through reflectance	ST and GST	**(Ehleringer 1984)**
Seed dispersal and establishment	ST and GST	**(Uphof 1962)**; **(Werker 2000)**
Epidermal Ca++ homeostasis	ST and GST	**(Lionel, et al. 2001)**
Pollen collection and dispersal	ST and GST	**(Uphof 1962)**; **(Werker 2000)**
Provide insect and herbivore deterrence	GST	**(Johnson 1975)**; **(Kelsey, et al. 1984)**.;**(Wollenweber 1984)**; **(Bennett, et al. 1994)**; **(Berenbaum 1995)**
Insect immobilization	GST	**(Kennedy 2003)**
Fungal and bacterial toxicity/nutrition	GST	**(Johnson 1975)**; **(Behnke 1984)**
Pollinator attraction	GST	**(Werker 2000)**
Ion and pollutant metal secretion	GST	**(Choi, et al. 2001) (Salt, et al. 1995)**; **(Kupper, et al. 2000)**
Water retention and decreased desiccation in seeds	GST	**(Werker 2000)**
Allelopathy	GST	**(Macias, et al. 1999)**; **(Werker 2000)**
Assist in climbing, and seed establishment	ST	**(Uphof 1962)**; **(Werker 2000)**
Absorb water and nutrients	ST	**(Sakai, et al. 1980, Uphof 1962, Werker 2000)**
Guide pollinators	ST	**(Johnson 1975)**
Water and ion secretion	HT	**(Johnson 1975)**; **(Aloni, et al. 2003)**

*ST, simple trichomes; GST, glandular secreting trichomes; HT, hydathode trichomes

Glandular trichomes can also be storage organs for substances, which are toxic for the plants. It is not unusual that heavy metals like cadmium are secreted via trichomes **(Choi, et al. 2001)**. Apart from these “waste depositing” processes, it is highly interesting to have a look at the great variety of chemicals produced in glandular trichomes. The most commonly known trichomes can be found on the stinging nettle (*Urtica dioica*) **(Pullin, et al. 1989)**, whose leaf hairs of contain a mixture of acetylcholine, histamine, serotonin, moroidin, leukotrienes, and possibly formic acid **(Flury 1927)**. When trichomes of *U. dioica* are disrupted by an herbivore they form needle like structure that are able to penetrate the herbivores skin and to inject the defensive cocktail into the attacker. This example nicely shows how complex the chemical composition of trichomes in a single species can be. Therefore it is not surprising that trichomes of different plant species are also described as self-containing chemical factories. There are reports indicating that leaf hairs are able to synthesize their metabolites independently from the surrounding leaf tissue, i.e. even when **(Kroumova, et al. 2003)** they are excised from the leaf. These facts and the variety of trichome-borne compounds have also attracted the attention of natural products researchers. For instance, the trichomes of *Artemisia annua* produce a sesquiterpene-like compound, called artemisinin **(Duke, et al. 1994)**. This trichome-derived compound is now commonly used as an antimalarial drug **(Ro, et al. 2006)**.

3.3. O-Acyl sugars in the trichome exudates of Solanacea

Sugar esters or *O*-acyl sugars belong to one class of trichome specific metabolites that have been the object of intensive research in the last years, especially in the genus *Nicotiana.* These compounds have been described for many *Nicotiana* species, but are also present in other Solanaceous species like tomato (*Solanum pennellii* **(Slocombe, et al. 2008)**, angel’s trumpet (*Datura wrightii*). **(Van Dam, et al. 1998)** or petunia **(Chortyk, et al. 1997)**. They consist of a sugar backbone, either a monosaccharide like glucose or a disaccharide like sucrose, which is then esterified on the free hydroxyl group by aliphatic carboxylic acids. The nature of the sugar core molecule can vary between different species. While in tobacco mainly esters of sucrose are known, the majority of sugar esters in tomato are made up by glucose esters.

Similar to the variation in sugar backbone, there is also a great variation in the acid **(Arrendale, et al. 1990, Van Dam, et al. 1998)** residues that are esterified to the sugars. The chain length of the acids varies from C_2 to C_{12} and branched and unsaturated acid moieties can befound, depending on the plant species. *O*-acyl sugars in general, and the acid moieties in particular, can also influence tobacco flavor **(Severson, et al. 1985)**. Furthermore, *O*-acyl sugars have been postulated to be a defense mechanism against herbivores. One important feature of these metabolites is their high viscosity and glue-like appearance. Therefore they might slow down caterpillar movement and may even be able to entrap small insects. They have been shown to be active against white flies **(Buta, et al. 1993)**. Other studies indicate that they could function similarly to insecticidal soap, by making the insect's cuticle more permeable and causing desiccation **(Puterka, et al. 2003)**. In addition to these findings, *O*-acyl sugars can also influence the oviposition of moths of the tobacco budworm *Heliothis virescens* **(Jackson, et al. 1991)** and sugar ester from *Datura wrightii* can negatively affect the larvae performance of the tobacco hornworm *Manduca sexta* **(Van Dam, et al. 1998)**. Nevertheless most of the described studies were conducted under highly artificial conditions. Assays to support these studies were performed on artificial diets or with concentrations that did not reflect the natural abundance of *O*-acyl sugars. In addition *O*-acyl sugars used in some studies were synthetic and do not mirror the naturally occurring structural features of these trichome-borne chemicals. In particular the work of **(Puterka, et al. 2003)** gave highly interesting results in regard of the structural and function relationship of these compounds but was lacking the comparison to natural occurring structures. Because of their many different proposed roles in plant defenses, *O*-acyl sugars are a well suited target for increasing plant resistance against pests, there is a need for more applied studies in a more natural experimental set-up. The well established plant-herbivore model system *Nicotiana attenuata* – *Manduca sexta* will help to answer more questions about *O*-acyl sugars like: How do these surface-borne compounds affect herbivores in an ecology driven experimental set-up? How big is the structural diversity of *O*-acyl sugars, and in which way are different structural features contributing to plant defenses? Within the following chapters I will provide answers for these questions and present a novel twist in the story of plant-herbivore interactions involving *O*-acyl sugars.

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Chapter 4: Manuscript overview

Manuscript I: Trichome-derived *O*-acyl sugars are a first meal for caterpillars that tags them for predation

Alexander Weinhold, Ian T. Baldwin

Published in

Proceedings of the National Academy of Science of the United States of America 2011
Volume 108 Issue 19 7855-7859 doi:10.1073/pnas.1101306108

In this manuscript we showed that trichomes represent a first meal for neonate larvae of the tobacco hornworm *Manduca sexta* and we explored the functions of their exudates in terms of plant defense. We could demonstrate that trichome exudates contain *O*-acyl sugars that are consumed by the larvae and thereby cause a release of branched chain aliphatic acids from either the larval frass or body. Furthermore we could show that these volatile acids in *M. sexta's* body odor are utilized by the omnivorous ant *Pogonomyrmex rugosus* to locate its prey and forage on *M. sexta.* These results underline the important defensive function of trichomes and their exudates in nature.

Contributions:

Alexander Weinhold: Participated in the experimental design, in conducting field and laboratory caterpillar assays and in the writing of the manuscript; conducted chemical assays.

Ian T. Baldwin: Conceived of the study, designed the experiments, conducted the field studies, and wrote the manuscript.

Manuscript II: Trichomes as dangerous lollipops: Do lizards also use caterpillar body and frass odor to optimize their foraging?

William F.J. Stork, Alexander Weinhold, Ian T. Baldwin

Published in

Plant Signaling and Behavior 2011

Volume 6 Issue 12 1893-1896 doi:10.4161/psb.6.12.18028

In this manuscript we demonstrated based on our previous findings (Manuscript I) that not only omnivorous ants but most likely lizards also use *M. sexta's* body and frass odor to locate their prey. Our studies showed that caterpillars of later instars are preyed upon in the field on a regular basis. Since all previously known predators in our field site are not capable of preying on caterpillars of this size, we hypothesized that lizards native to the field site could be responsible for the loss of these caterpillars. In different bioassays we demonstrated that lizards are attracted by the volatile acids in *M. sexta's* body and frass odor, thereby underlining once more the importance of trichomes and their exudates for the defense of plants in nature.

Contributions:

William F.J. Stork: Conducted field assays and participated in the experimental design and the writing of the manuscript

Alexander Weinhold: Participated in the writing of manuscript and made chemical solutions, used in the field studies.

Ian T. Baldwin: Conceived of the study, designed the experiments, conducted the field studies, and wrote the manuscript

Manuscript III: Phaseoloidin, a homogentisic acid glucoside from *Nicotiana attenuata* trichomes, contributes to the plant's resistance against Lepidopteran herbivores

Alexander Weinhold, Kamel Shaker, Michael Wenzler, Bernd Schneider, Ian T. Baldwin,

Published in
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In this manuscript we described the identification of a trichome metabolite novel to the Solanaceae. Comparative NMR- and MS studies revealed the presence of phaseoloidin - a homogentisic acid glucoside - in the trichomes of the wild tobacco *N. attenuata*. We could show that besides *O-acyl sugars* (Manuscript I, II), phaseoloidin and nicotine are present in the different trichome types of *N. attenuata* and that their abundance varies between different trichome parts. In addition we could demonstrate that phaseoloidin negatively affects the growth of specialist *Manduca sexta* and generalist *Spodoptera littoralis* larvae. These results highlight once again the defensive role of trichome-based plant defenses.

Contributions:

Alexander Weinhold: Designed experiments, conducted HPLC-MS analysis and caterpillar assays; drafted the manuscript

Kamel Shaker: Designed and conducted NMR-experiments and caterpillar assays; contributed to the writing of the manuscript

Michael Wenzler: Designed and conducted NMR-experiments

Bernd Schneider: Designed and conducted NMR-experiments; contributed to the writing of the manuscript

Ian T. Baldwin: Designed experiments and wrote the manuscript

Appendix: Characterization of trichome-derived *O*-acyl sugars in *Nicotiana attenuata*

Alexander Weinhold

Unpublished Data

In this Appendix I will show that the composition of *O*-acyl sugars in *Nicotiana attenuata* is even more complex than previously described (Manuscript I, II). Fractionation of crude plant extracts and subsequent HPLC-ToFMS analysis revealed the presence of at least 15 different compounds. In addition, I will show by MS^2 experiments that *O*-acyl sugars can be classified into three different structural classes and that different *O*-acyl sugar classes vary in their substitution pattern of the sucrose core molecule.

Chapter 5: Manuscript I

Trichome-derived O-acyl sugars are a first meal for caterpillars that tags them for predation

Alexander Weinhold, Ian T. Baldwin

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In this manuscript we showed that trichomes represent a first meal for neonate larvae of the tobacco hornworm *Manduca sexta* and we explored the functions of their exudates in terms of plant defense. We could demonstrate that trichome exudates contain *O*-acyl sugars that are consumed by the larvae and thereby cause a release of branched chain aliphatic acids from either the larval frass or body. Furthermore we could show that these volatile acids in *M. sexta's* body odor are utilized by the omnivorous ant *Pogonomyrmex rugosus* to locate its prey and forage on *M. sexta.* These results underline the important defensive function of trichomes and their exudates in nature.

Trichome-derived *O*-acyl sugars are a first meal for caterpillars that tags them for predation

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Edited by Jerrold Meinwald, Cornell University, Ithaca, NY, and approved March 31, 2011 (received for review January 27, 2011)

Plant glandular trichomes exude secondary metabolites with defensive functions, but these epidermal protuberances are surprisingly the first meal of Lepidopteran herbivores on *Nicotiana attenuata*. *O*-acyl sugars, the most abundant metabolite of glandular trichomes, impart a distinct volatile profile to the body and frass of larvae that feed on them. The headspace composition of *Manduca sexta* larvae is dominated by the branched chain aliphatic acids hydrolyzed from ingested *O*-acyl sugars, which waxes and wanes rapidly with trichome ingestion. In native habitats a ground-hunting predator, the omnivorous ant *Pogonomyrmex rugosus*, but not the big-eyed bug *Geocoris* spp., use these volatile aliphatic acids to locate their prey.

indirect defenses | dangerous body odors

Trichomes, or leaf hairs, have long been considered a plant's first line of defense. These epidermal protuberances are known to function as physical and chemical barriers against attacking herbivores and pathogens (1, 2), as well as protecting plants against UV-B radiation, water loss, and heat stress (3). Glandular trichomes can be found on ≈30% of all vascular plants. These secretory hairs contribute to a plant's resistance in numerous ways: as obstacles that thwart movement across plant surfaces (4), as entrapment devices (5), and sources of volatile and nonvolatile secondary metabolites (6, 7) or proteins (8) that poison or repel herbivores.

The native tobacco, *Nicotiana attenuata,* as it is common among Solanaceous plants, produces glandular trichomes on both adaxial and abaxial leaf surfaces (Fig. 1*A* and Fig. S1 *A* and *B*). These trichomes contain as a minor constituent, nicotine (Fig. S1*C*), a secondary metabolite known to inhibit growth even of adapted larvae (6, 9). *O*-acyl sugars (AS), viscous liquids that consist of aliphatic acids of different chain lengths esterified to sucrose, are the most abundant secondary metabolites in the glandular trichomes of Solanaceous plants (10, 11). They dominate aqueous extracts of *N. attenuata* leaf surfaces and can be readily detected after Rhodamine B staining (Fig. 1 *A* and *B*) and by HPLC-ToFMS (Fig. S1). AS, particularly those esterified to aliphatic acids with alkyl chains longer than eight carbons, are effective defenses against psyllids (12), aphids (13), white flies, and spider mites (14), likely by speeding the desiccation of entrapped or anointed herbivores (15). AS from *Datura wrightii*, although differing in their sugar moiety and aliphatic acid residues, decrease growth of *Manduca sexta* larvae when applied to artificial diets (16).

Results and Discussion

Neonate Lepidopteran Larvae Consume Trichomes as a First Meal. Given the established defensive value of trichomes and their chemistry, we were surprised to observe that these trichomes and their exudates were the first feeding choices of both generalist (*Spodoptera littoralis* and *S. exigua)* and specialist (*M. sexta)* Lepidopteran herbivores on *N. attenuata* (Fig. 1*C*, Fig. S2*A*, and Movie S1). More than 70% of all larvae chose to feed on trichomes for their first meal after hatching. Moreover, larvae did not suffer from this choice. When placed on *N. attenuata* sepals, which are entirely covered in glandular trichomes, larvae feeding on sepals survived and grew as well as larvae fed on previously washed and AS-free wild-type (WT) sepals as well as sepals from plants transformed to silence nicotine production (Fig. S2 *C* and *D*). Observations of neonate *M. quinquemaculata* and *S. littoralis* feeding behavior on native *N. attenuata* growing in natural habitats in Utah confirmed these laboratory observations and led us to conclude that trichomes and their exudates, rather than being directly defensive, provide a sugary first meal for these Lepidopteran herbivores. Interestingly, trichome feeding has been reported for other Lepidopteran species and may be common (4, 17).

Ingestion of *O*-Acyl Sugars Dramatically Alters *M. sexta* Body Headspace. Although the ingestion of trichomes and their AS contents may not be directly deleterious for larvae, it dramatically alters larval body and frass volatile profiles. Trapping and GC-MS analysis of the headspace surrounding larvae that had fed on *N. attenuata* leaves and trichomes revealed that four branched chain aliphatic acids (BCAA), namely 2-methyl butanoic acid (I), 3-methyl butanoic acid (II), 3-methyl pentanoic acid (III), and 4-methyl pentanoic acid (IV) were the most abundant constituents (Fig. 1 *B* and *D*). These 4 BCAAs found in larval body headspace are exactly those found from an alkaline hydrolysis of *N. attenuata* AS (Fig. S3), and we hypothesized that BCAAs in the larval headspace originate from AS hydrolysis in the alkaline midgets (18) of these larvae. AS are readily removed by washing leaves with water and do not occur in a species of *Nicotiana* that lacks trichomes, *N. glauca* (Fig. S4*A*). When fed on water-washed *N. attenuata* or *N. glauca* leaves, larvae lose their characteristic body or frass volatile profile. Similarly, when fed on an artificial diet amended with AS from *N. attenuata* leaves, larvae produce a body headspace similar to that of larvae feeding on WT plants (Fig. 2 *A* and *B*). The effect of AS ingestion on larval body headspace rapidly waxes and wanes with diet; when switched from the AS-free *N. glauca* to *N. attenuata* diets, larvae rapidly emit the characteristic BCAAs, and these volatile acids disappear again when larvae are returned to AS-free *N. glauca* leaves (Fig. 2*C*). We conclude that consuming AS from trichomes has a similar effect for larvae as the consumption of asparagus has for humans: It conveys a strongly dynamic and distinct olfactory signature to bodies and excretions. Although only a potential social annoyance for humans, these emissions, which approach 5 µg of single BCAAs from larval bodies and 30 µg from frass over 2 h (Fig. 2), may have more serious consequences for a caterpillar.

The natural enemies of caterpillars are known to use fecal odors to locate their prey. For example, parasitic wasps can learn to associate frass volatiles with prey location under laboratory

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Author contributions: A.W. and I.T.B. designed research, performed research, analyzed data, and wrote the paper.

The authors declare no conflict of interest.

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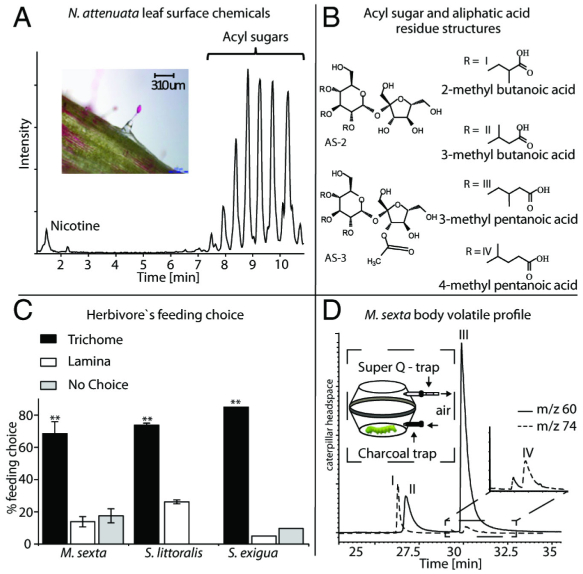

Fig. 1. Trichomes and their exudates are commonly the first meal of neonate larvae of the three main Lepidopteran species that feed on *N. attenuata*. (*A*) HPLC-ToFMS analysis of an *N. attenuata* leaf surface wash. *Inset* shows a single trichome with the *O*-acyl sugars stained by Rhodamine B dye. (*B*) Structures of the two classes of *O*-acyl sugars (ASII, III; ref. 10) found in *N. attenuata* trichomes and the four most abundant BCAA substituents. (*C*) Feeding choices of neonate larvae of the three most abundant Lepidopteran herbivores of *N. attenuata* in nature: *M. sexta, S. littoralis*, and *S. exigua*. Choice is expressed as the mean (±SE) percent of feeding choices of larvae in four (*M. sexta*, $n = 55$), three (*S. littoralis*, $n = 43$), and one (*S. exigua*, $n = 20$) experiments. Asterisks indicate significant differences by χ^2 analysis: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (*D*) Headspace of *N. attenuata*-fed *M. sexta* larvae trapped on Super-Q traps (*Inset*) is dominated by the four BCAAs esterified to the sucrose moiety within AS-2 and AS-3. Dashed line represents ion-trace *m/z* 74 and the solid line *m/z* 60 (Fig. S3).

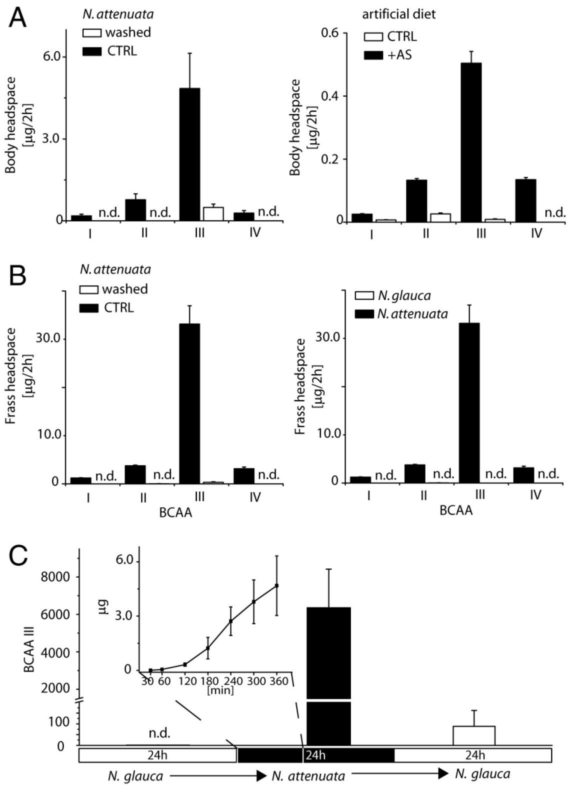

Fig. 2. Ingestion of *O*-acyl sugars determines the body and frass headspace of *M. sexta* larvae. (*A*) Mean ± SE of four BCAAs trapped from *M. sexta* body headspace from caterpillars feeding on plants with AS (CTRL) or without AS (washed) as well as artificial diets supplemented with *N. attenuata* AS (+AS) or without AS (CTRL). (*B*) Mean ± SE of four BCAAs trapped from *M. sexta* frass headspace from larvae feeding on plants with (CTRL) or without AS (washed) as well as AS-containing *N. attenuata* plants and AS-free *N. glauca* plants. Values are expressed as micrograms per 2-h trapping interval. (*C*) Mean ± SE of BCAA III (3-methyl pentanoic acid) from *M. sexta* body headspace from larvae feeding sequentially on AS-free *N. glauca*, AS-containing *N. attenuata*, and AS-free *N. glauca*. *Inset* shows mean ± SE of BCAA III released from larvae reared on AS-free *N. glauca* and switched to AS-containing *N. attenuata* leaves at time 0.

conditions (19, 20), and many insects have developed sophisticated means of distancing themselves from their potentially traitorous excrements. The larvae of the silver-spotted skipper (*Epargyreus clarus*), a shelter-building insect, for example, goes to lengths to remove its frass from its hideouts to avoid detection by predators (21). Plants have exploited the proclivity of carnivorous insects to use plant volatiles to locate their prey and have evolved means of amplifying and modifying these emissions in response to herbivore attack to provide reliable information about the location of herbivorous prey to hunting carnivores (22).

Hemipteran Predator *Geocoris* spp. Is Not Attracted by Larval Body Headspace. Herbivory-induced plant volatiles released from *N. attenuata* plants have been shown to attract and optimize the foraging behavior of *Geocoris* hemipteran predators in nature (23, 24), and we hypothesized that this predator might also use the volatile BCAAs released from AS-feeding larvae. Because the release of BCAAs occurs rapidly from AS-feeding larvae (Fig. 2*C*), we imagined that the BCAAs might complement the rapid isomerization of green leaf volatiles (GLVs) that is elicited by *M. sexta* oral secretions to provide *Geocoris* predators with rapid and spatially explicit information about the whereabouts of their prey on plants (23). This however, was not the case. Although the native *Geocoris* predators at our field station in Utah responded to the perfuming of plants with GLV blends produced by *M. sexta*-attacked plants in an egg predation assay, they did not respond to perfuming with either BCAA III or a mixture of four BCAAs in amounts equivalent to that released from frass (Fig. S5 and Table S1). However, when early instar larvae were perfumed with BCAAs in either lanolin pastes or water sprays, perfumed larvae tended to be preyed on more frequently than control larvae reared on AS-free *N. glauca* leaves. These larvae were placed on *D. wrightii* plants that differ from *N. attenuata* in AS composition, on AS-free plants (*N. glauca*), and on *N. attenuata* plants transformed to silence their ability to release herbivore-induced volatiles (*irlox2/3*) (Fig. S6). Although none of the differences in predation rates were statistically significant (lanolin paste: $\chi^2 = 3.2$, $P = 0.073$; $\chi^2 = 2.57$, $P = 0.109$; spray: $\chi^2 = 2.67$, $P = 0.102$), the tendency toward higher predation rates of perfumed larvae motivated us to examine the responses of other ground-hunting predators.

***Pogonomyrmex rugosus* Is Attracted by Larval Body Headspace.** Ants are ground-hunting insects that are common in *N. attenuata's* habitat and are frequently observed carrying larvae of various species, including *M. sexta* and *M. quinquemaculata*, back to their nests. These social insects are capable of associating particular volatiles with potential prey (25) and might therefore be able to associate BCAAs from *M. sexta* larvae or frass with the presence of larvae on a plant. To determine which of the many different ant species that occur in *N. attenuata*'s native habitat might respond to larval BCAAs, we scented cooked rice grains with frass quantities of the BCAA mixture, and counted the number of scented and unscented rice grains removed in 15 min from each of 24 nests. With this rice pickup assay, we identified five BCAA-responsive ant nests (Fig. S7*E*, colony A–E) that all belonged to the same species: *P. rugosus*, the rough harvester ant.

P. rugosus is a seed-harvesting ant that is also known to be an opportunistic predator of cicadas during outbreaks of this prey species (26). *P. rugosus* were occasionally observed climbing elongated *N. attenuata* plants in the field and carrying cutworm larvae back to their nests. They were also found to readily climb 20-cm wooden sticks to retrieve *M. sexta* larvae from pieces of leaves affixed to the top (Fig. S7*A*). To exclude the possibility that the ants climbed the sticks in response to visual cues from the caterpillars at the top of the sticks, we conducted trials without larvae or leaves and placed ≈3 cm^3 of either oven dried, and hence BCAA free, *M. sexta* frass or fresh frass from *M. sexta* larvae-fed *N. attenuata* leaves at the base of the sticks. Ants showed a strong preference to climb sticks with fresh, BCAA redolent frass, at the base compared with sticks with oven-dried frass at their base (Fig. S7*F*).

BCAA are rapidly volatilized from frass excreted by the larvae, particularly when it falls onto the hot desert soil from larvae that commonly feed on the undersides of leaves, as *Manduca* larvae tend to do. To determine how long BCAAs would be retained in the headspace, we measured the recovery of the BCAA mixture from a 45 °C surface (Fig. S8*A*). Frass quantities of aqueous BCAA mixtures were found to completely dissipate within 15 min. We monitored the tendencies of *P. rugosus* ants from one nest to climb sticks scented at their base either with a BCAA mixture or a detergent control. At both morning and evening activity periods of the ants, 73–82% of all ants that climbed sticks within 15 min of treatment, climbed sticks that were scented at their base with BCAAs (Fig. S7 *G* and *H*).

To determine the consistency of *P. rugosus* responses to BCAA from different nests, we repeated the assay with five nests that differed in their proximity to *Manduca*-infested *N. attenuata* and *D. wrightii* plants (Fig. 3). The AS of *D. wrightii* trichomes include sucrose esterified with hexanoic acid (H) and *Manduca* larvae that feed on *Datura* leaves have body and frass volatile profiles that are dominated by H (Fig. S8*B*). We conducted two sequential tests of each of the five colonies: with the BCAA mixture characteristic of the ingestion of *N. attenuata* AS followed by a test of the same BCAA mixture amended with H. Colonies A–C were located in an area with *Manduca*-infested *N. attenuata* and *D. wrightii* plants, and in both trials, ants from two of three colonies significantly preferred to climb sticks scented with BCAA or BCAA+H. Colony D, which likely only foraged within *N. attenuata* plants, showed a strong response to the BCAA treatment, but responded less strongly when H was added to the mixture. In contrast, colony D, which foraged in an area in which only *D. wrightii* plants grew, did not respond to the BCAA treatment, but responded strongly to the BCAA+H treatment (Fig. 3). These results demonstrate

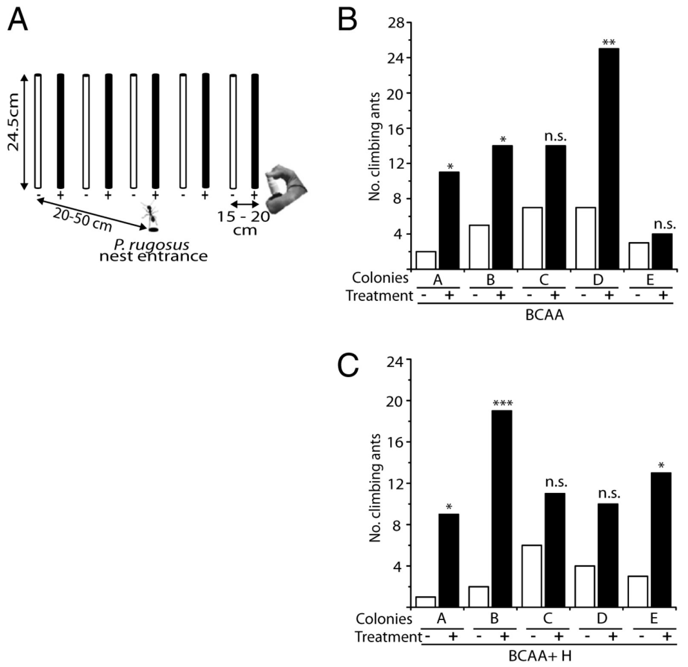

Fig. 3. *P. rugosus* foragers are attracted by larval body headspace BCAA. (*A*) Experimental setup for the multiple colony assays. The base of bamboo sticks were sprayed with either 100 μL of an aqueous BCAA mixture (+) or a Tween-20 control (-), which did not differ from a water control. In a second experiment, hexanoic acid (H) (*B*) was added to the BCAA mixture (*C*). Ten sticks with alternating treatments were placed in a line 20–50 cm from the nest entrance. Shown are the number of climbing ants 15 min after treatment at 5 different ant nests (A–E). Asterisks indicate significant differences between choices by χ^2 analysis: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (*B*) A: $\chi^2 = 6.23$, $P = 0.013$; B: $\chi^2 = 4.26$, $P = 0.039$; D: $\chi^2 = 10.12$, $P = 0.002$. Colony C did not show a significant difference but a clear trend toward BCAAs ($\chi^2 = 2.33$, $P = 0.127$). Colony E did not show a preference to any of the aliphatic acids present in *N. attenuata*-fed headspace ($\chi^2 = 0.143$, $P = 0.706$). (*C*): A: $\chi^2 = 6.4$, $P = 0.011$; B: $\chi^2 = 13.76$, $P = 0.0001$; Colony C and D did not show a significant difference but a clear trend toward aliphatic acids (C: $\chi^2 = 1.47$, $P = 0.225$; D: $\chi^2 = 2.57$, $P = 0.109$). Colony E, when hexanoic acid, present in *D. wrightii*-fed headspace, was added to the BCAA mixture, responded significantly ($\chi^2 = 6.25$, $P = 0.012$).

that *P. rugosus* foragers respond strongly to BCAA from larval and frass volatile emissions and may even adjust their responsiveness to the particular BCAA produced by locally available prey. Ants are known to use cuticular hydrocarbons (27, 28) and aliphatic acids, albeit with different chain lengths and structures, in nest mate recognition. To our knowledge, there is only one report of a free carboxylic acid that acts as a ant trail pheromone, namely nerolic acid, found in the hindgut of *Camponotus floridanus* (29). *P. rugosus* might learn to associate prey availability with BCAAs, but this hypothesis will require additional work to place on solid experimental footing.

Trichomes Function as "Dangerous Lollipops." Despite their well-documented function as direct defenses in other Solanaceous plants, the trichome-associated AS of *N. attenuata* combine aspects of nutritional- and information-based indirect resistance traits. These abundant trichome exudates do not directly defend the plant against attack by *M. sexta* larvae but rather are the first feeding choice of neonate larvae. As an exuded sugar-rich compound, AS have similarities with the production of extra floral nectars (EFNs) that function as indirect defenses by providing nutritional rewards for carnivorous insects (30). In contrast to EFNs, however, AS function as a dangerous lollipop that tags caterpillars with a distinctive odor that complements the information-based indirect defenses of plants that provide spatially and temporally explicit information about the location of feeding larvae to predators. To determine whether AS increase plant fitness and, thereby, can be justifiably considered an indirect "defense" will require the engineering of plants that do not esterify aliphatic acids to their trichome-sugar exudates, a potentially manageable task given the recent advances in our molecular understanding of acyl sugar biosynthesis (11).

Materials and Methods

Plant Material and Insect Rearing. We used an isogenic line, obtained after 30 generations of inbreeding, of *N. attenuata* Torr. *ex* Watson derived from field-collected seeds. Seeds of *N. glauca* also originated from a field collection from the campus of the University of California, Davis. Additionally, we used the following *N. attenuata* RNAi lines that have been characterized: irPMT (31) and irLOX2/3 (32, 33). irPMT plants are impaired in nicotine production because of silencing in the putrescine-methyltransferase (PMT) activity. irLOX2/3 plants are silenced in both LOX2 and LOX3 and have dramatically lower levels of GLVs (LOX2) (32) and of the signaling molecule, JA, and its conjugates (LOX3) (32, 33).

Seed germination was performed as described in Krügel et al. (34). All plants were grown in the glasshouse in 1-L individual pots at 26–28 °C under 16 h of light supplied by Philips Sun-T Agro 400- or 600-W sodium lights. Artificial diet for feeding experiments was prepared according to Waldbauer et al. (35). *M. sexta* larvae were derived from an in-house colony. Larvae of *S. littoralis* and *S. exigua* (*Lepidoptera, Noctuidae*) were supplied as egg clutches by BayerCropScience (40789). *M. sexta* frass was collected daily from fourth to fifth instar caterpillars feeding on either of the *Nicotiana* species and stored at −80 °C until used for experiments.

Trichome Density Determinations. Trichome density (Fig. S1 *A* and *B*) was measured, as described by Boughton et al. (36), by counting trichomes on the adaxial and abaxial sides of leaf discs of three different laminar positions that span the length of the leaf. Trichomes were stained with a 0.5% m/v solution of Rhodamine B in water as described in Lin and Wagner (37), and subsequently counted under a Zeiss Stereomicroscope Discovery.V8 (Carl Zeiss). Leaf areas were calculated by using the SigmaScanPro 5 (SPSS Inc.) software, and trichome densities were calculated as the number of trichomes per leaf area.

Glasshouse Bioassays. Freshly hatched neonates of *M. sexta*, *S. littoralis*, and *S. exigua* were transferred to the abaxial side of freshly excised leaves of *N. attenuata* and observed under a Zeiss stereomicroscope SV11 (Carl Zeiss). The caterpillars were allowed to move freely, their first feeding choice within 60 s was monitored, and the first feeding choice was recorded (Fig. 1*C*).

To evaluate the effects of *N. attenuata O*-acyl sugars on caterpillar performance, we used sepals subtending immature capsules (Fig. S2). These tissues are characterized by their high trichome densities so that larvae cannot consume the lamina without first consuming the trichomes that cover the lamina and, hence, are ideally suited for trichome feeding assays. To dissect the roles of the trichome specific metabolites, we used *N. attenuata* wild-type plants and irPMT plants, which do not contain nicotine in their trichomes. Additionally, *O*-acyl sugars were removed from sepals by gently shaking leaves or sepals three times in 30 mL of distilled water for 10 s. Freshly hatched *M. sexta* larvae were placed on excised sepals or unripe seed capsules of either washed or unwashed irPMT and WT plants. Caterpillars were placed in single plastic containers with one bud and caterpillar and allowed to feed freely for 3 h. Afterward, the plant tissue was removed, larvae were starved for 18 h, and the survival rate was recorded.

***O*-Acyl Sugar Analysis.** Rosette leaves were harvested by cutting the petiole with a scalpel, washed with 10 mL of methanol (VWR International), and spiked with 10 μg of sucrose monolaureate as an internal standard. The washing solution was filtered through paper filter (Whatman) and gently evaporated to dryness under a stream of nitrogen. Before HPLC-ToFMS analysis, the residue was resolved in 1 mL of methanol and transferred to a 2-mL LC-vial.

One-microliter aliquots of AS extract were separated by using an Agilent HPLC 1100 Series system, combined with Gemini NX C18 column (150 × 2 mm). Eluted compounds were detected by a Bruker MicroToF mass spectrometer (Bruker Daltronik) equipped with an ion spray source in positive-ion mode. The instrument was operated with parameters according to Heiling et al. (38).

The composition of the aliphatic acids moieties was studied by analysis of aliphatic acid methyl esters. The dichloromethane phase was collected, the remaining water was removed by filtering through a Pasteur pipette filled with anhydrous sodium sulfate, and 1 μL was analyzed by GCxGC-ToFMS according to Gaquerel et al. (39). Identification was performed by comparison of spectra and retention times with those of authentic standards.

For the alkaline hydrolysis, 500 μL of KOH (0.2 M) were added to 500 μL of the methanolic extract and sealed in a vial for 24 h. The mixture was neutralized by addition of HCL (0.2 M), and the aliphatic acids were extracted and measured as described above (Fig. S3). For additional information, see *SI Materials and Methods*.

***M. sexta* Body and Feces Headspace Analysis.** An alkaloid-free mixture of *N. attenuata* and *D. wrightii* AS was extracted by following the method described in ref. 16. A small portion was dissolved in MeOH and analyzed by HPLC-ToFMS to verify the extraction.

The alkaloid-free mixture of AS obtained from leaves of *N. attenuata* was dissolved in diethylether and applied to artificial diet (1 mg/g diet). The diethylether was allowed to evaporate, and afterward, caterpillars were allowed to feed on either AS-free or AS-containing diet. AS were removed from excised leaves of *N. attenuata* by gently soaking leaves in MeOH for 30 s and carefully drying them with paper tissue (Fig. S4 *B* and *C*).

Caterpillars of second to third instar were enclosed in two 50-mL food-quality plastic containers (Huhtamaki) containing either artificial diet (±AS) or leaf tissue (washed/unwashed *N. attenuata, N. glauca*, or *D. wrightii*). Volatile emissions from the enclosed caterpillars were trapped for 2 h. Immediately after collection, traps were eluted by spiking each with 400 ng of tetralin (Sigma Aldrich) as an internal standard and flushing the trap with 150 μL of dichloromethane into a GC vial containing a glass insert. The sample was subjected to GC-MS analysis on a Varian 3800 system (Varian) equipped with a 30-m DB-Wax column (ID 0.25 mm, df 0.25 μm; Supelco) connected to a Saturn 2000 ion trap. One microliter was injected into a 230 °C injector. The flow was constant and kept at 1 mL/min throughout the following temperature gradient: 3 min at 40 °C, 4 °C/min until 180 °C and 10 °C/min until 240 °C. Transfer line was maintained at 250 °C and ionization was performed in EI-mode.

Field Bioassays. We choose five colonies of which two were spatially separated from the remaining three (Fig. S7*E*). We placed wooden sticks near to the nest entrance but without leaflets or caterpillars attached. BCAA in water (0.05% Tween-20) or water controls were sprayed at the bases of the sticks. We used five sticks for each treatment and counted the number of climbing ants within a 15-min interval (Fig. 3). Additionally we added hexanoic acid (H), which is the major aliphatic acid within the body odor of *D. wrightii*-

fed caterpillars (Fig. S8 *B* and *C*), to the previously used BCAA mixtures (BCAA+H). The same assays as before were performed on the previously described colonies spraying BCAA+H to the base of the sticks and using water +0.05% Tween-20 as a control.

Statistical Analysis. All statistical analyses were performed with Excel (Microsoft Corporation) or StatView (SAS Institute). Data were transformed, if necessary, to meet the requirements for homogeneity of variance.

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Supporting Information

Weinhold and Baldwin 10.1073/pnas.1101306108

SI Materials and Methods

***O*-Acyl Sugar Analysis.** Rosette leaves were harvested by cutting the petiole with a scalpel, washed with 10 mL of methanol (VWR International), and spiked with 10 µg of sucrose monolaureate as an internal standard. The washing solution was filtered through paper filter (Whatman) and gently evaporated to dryness under a stream of nitrogen. Before HPLC-ToFMS analysis, the residue was redissolved in 1 mL of methanol and transferred to a 2-mL LC vial.

One microliter of AS extract were separated by using an Agilent HPLC 1100 Series system, combined with Gemini NX C18 column (150 × 2 mm) applying the following binary gradient: 0–6 min, isocratic 70% A [deionized water, 0.1% (vol/vol) acetonitrile (Baker HPLC grade) and 0.05% formic acid], 30% B (acetonitrile and 0.05% formic acid); 6–13 min, isocratic 20% A, 80% B; and 70% A, 30% B; 13–18 min, isocratic 70% A, 30% B. Flow rate was 200 $\mu L \cdot min^{-1}$. Eluted compounds were detected by a Bruker MicroToF mass spectrometer (Bruker Daltronik) equipped with an ion spray source in positive-ion mode. The instrument was operated with parameters according to Schuman et al. (1). Electrospray ionization conditions the following: 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 by using sodium formiate (50 mL of isopropanol, 200 mL of formic acid, and 1 mL of 1 M NaOH in water) (Fig. S2).

For the total AS analysis, leaf tissue (Fig. S1*C*) was ground in liquid nitrogen and extracted with a buffer containing a 60% solution 1 (2.3 mL/L acetic acid, 3.41 g/L ammonium acetate adjusted to pH 4.8 with 1 M NH_4OH) and 40% methanol (MeOH). Two microliters were injected into the Agilent HPLC 1100 Series system combined with a Phenomenex Gemini NX 3u (150 × 2.0mm) 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 flow rate was 200 $\mu L \cdot min^{-1}$ and electrospray conditions as described above.

The composition of the aliphatic acids moieties was studied by analysis of aliphatic acid methyl esters. Therefore, 1% vol/vol sulfuric acid (98%) was added to a 500-µL aliquot of the methanol extract. The methanol sample was heated to 65 °C for 90 min, and the methyl esters was partitioned by adding 300 µL of water and 1.6 mL of dichloromethane (DCM; VWR International). The DCM phase was collected, the remaining water was removed by filtering through a Pasteur pipette filled with anhydrous sodium sulfate, and 1 µL was analyzed by GCxGC-ToFMS according to Gaquerel et al (2). Identification was performed by comparison of spectra and retention times with those of authentic standards.

For the alkaline hydrolysis, 500 µL of KOH (0.2 M) were added to 500 µL of the methanolic extract and sealed in a vial for 24 h. The mixture was neutralized by addition of HCL (0.2 M), and the aliphatic acids were extracted and measured as described above (Fig. S3).

***M. sexta* Body and Feces Headspace Analysis.** An alkaloid-free mixture of *N. attenuata* and *D. wrightii* AS was extracted by following the method described in ref. 3. Plant material was harvested by excising leaves as well as cutting stems into suitable pieces. Both tissues were soaked in $CHCl_3$ (VWR Normapur) (4.5 mL/g FW) for 1 h and subsequently filtered over Na_2SO_4 (Sigma Aldrich). The mixture was reduced to dryness by using a rotary evaporator. The yellow-green residue was suspended in CH_3CN (VWR Normapur) (100 mL/g) and sonicated. The suspension was subsequently separated three times against hexane (VWR Normapur) (1:1.3). The acetonitrile phase was removed under vacuum, and the brown residue was dissolved in DCM (VWR Normapur) (40 mL/g). This mixture was separated three times against 0.5 M tartaric acid and once against distilled water. DCM was removed under vacuum resulting in a brown-red highly viscous residue. The yield was ≈1 mg/g FW. A small portion was dissolved in MeOH and analyzed by HPLC-ToFMS to verify the extraction.

Caterpillars of second to third instar were enclosed in two 50-mL food-quality plastic containers (Huhtamaki) containing either artificial diet (±AS) or leaf tissue (washed/unwashed *N. attenuata, N. glauca*, or *D. wrightii*). Plastic containers were secured with miniature clawstyle hair clips. After 24 h of feeding, the leaf tissue was removed and larvae and frass were transferred to separate plastic containers. Ambient air flowed into the cage primarily through a charcoal trap secured in a shortened P1000 pipette tip inserted into the bottom container and was pulled out through a glass tube (ARS) containing glass wool and 20 mg of Super Q (Alltech) and secured in a second shortened P1000 pipette tip inserted into the top (Fig. 1*D Inset*). Airflow was created by a manifold vacuum pump (model DAA-V114-GB; Gast Manufacturing) as described by Halitschke et al. (4).

To evaluate the persistence of the BCAAs under temperatures found in the field (Fig. S8*A*), we used 20-mL scintillation vials containing two gas inlets inserted into the cap. The vials were placed in a heating block at 45 °C, the mean daytime soil temperature at the field station in June. Afterward, 100 µL of an aqueous BCAA mixture of similar concentrations to that used in the field were added to the vial and the cap was closed. The BCAA bouquet was allowed to equilibrate for 10 min. Then a Super-Q trap was placed on one inlet while the other one was connected to a nitrogen supply. The vial was then flushed with nitrogen at a flow rate of 300 mL/min and the aliphatic acids were trapped. After removal of the Super-Q trap, the vial was purged for 10 min. Finally, the remaining aliphatic acid bouquet was trapped for 5 min. Super-Q traps were then eluted and the samples were analyzed by GC-MS as described above. The experiment was conducted with three replicate vials.

Field Bioassays. If not otherwise stated, *M. sexta* larvae from our own 1 1/2-y-old inbred colony in Jena or eggs from a 40-y-old inbred colony from the Binghamton University (Biological Sciences Department), kindly provided by Carol Miles, were hatched and raised on excised leaves of *N. glauca* placed into plastic containers containing no more than 15 larvae in each box. To maintain an adequate level of humidity, the boxes were kept within larger plastic containers with wet paper tissue. Leaves were changed every 2 d or earlier if necessary, and caterpillars of suitable instars were used for field experiments.

Egg predation assay. To test whether *Geocoris* spp. are attracted to the BCAAs, two *M. sexta* eggs per plant were glued to the underside of a lower stem leaf of 20 *N. attenuata* plants on the field plot as described in Kessler and Baldwin (6). A neutral α-cellulose glue that is known not to affect predation rates or the plants' VOC emissions was used. Plants were of the same size and developmental stage. A cotton swab was dipped into lanolin paste containing different odor-mixtures and placed immediately adjacent to the leaf with the *M. sexta* eggs. The numbers of eggs predated were counted after 12 and 24 h. Eggs predated by *Geocoris* spp. are emptied through a characteristic feeding hole and are thus easy to distinguish. For all experiments, we used eggs either from our colony in Jena or from a colony from the Binghamton University (Biological Sciences Department), but never mixed eggs from the different sources within an experiment.

On June 16 and June 17 of the 2010 field season, we used 10 *N. attenuata* planted on the main field plot for all of the three following treatments. A mixture of *trans*-2-hexenyl acetate and *cis*-3-hexenyl acetate (1:1) (GLV) (Fig. S5) was used as a positive control because this mixture was known to be attractive to *Geocoris* (7). A mixture of BCAAs in quantities equal to the amounts that can be trapped from 1 g of fresh frass in 2 h (Table S1) was used to examine the potential attractiveness of BCAAs to the native *Geocoris* spp. community. Pure lanolin (CTRL) was used as negative control.

From June 18 to June 20, we used 11 replicate *N. attenuata* plants growing on the smaller field plot (Snow plot, see Fig. S7*E*) for the three following treatments. Amounts, equal to the amount of BCAA released from 1-g frass of the BCAA mixture as well as only 3-methyl pentanoic acid (III) (Table S1) was used to test for a potential attractiveness to *Geocoris* spp. 3-methyl pentanoic acid was chosen because it is the most abundant BCAA detected in the body and frass headspace of *M. sexta* larvae (Fig. 2*A*). Pure lanolin (CTRL) was used as negative control.

***Datura wrightii* assay.** We selected 10 size-matched *D. wrightii* plants located on the field plot at the Lytle Preserve, near Santa Clara, Utah. First to second instar larvae were labeled with lanolin paste ≈5–10 µL containing either a mixture of BCAA (Table S1) or a solvent control. Larvae were labeled by gently wiping the lanolin paste with a toothpick on their back and placing the larvae on leaves on opposite sides of each plant (Fig. S6). Wooden sticks were used to mark the location of the larvae. Experiments were conducted on the 20/5 and 21/5 during the 2010 field season and started in the morning at 8.30:00 AM; plants were examined every 4 h until 17.30:00 PM. To determine whether missing larvae were predated, we inspected the entire plant and the surrounding 3-m area for larvae. Because caterpillars of these early instars remain on their first host plant and do not survive very long off the plant, we estimated predation rates from the disappearance of caterpillars. We also observed that after predation by *Geocoris* spp., caterpillar carcasses desiccate and are readily blown from plants. Therefore, the disappearance of larvae likely only marginally overestimates actual predation events. The results of both days were summarized and analyzed by χ^2 test.

***N. attenuata* assay.** Second instar larvae were sprayed with ≈100 µL of either a mixture of BCAA (Table S1) in water (0.05% Tween-20) or a water control (0.05% Tween-20). Additionally, a mixture of BCAA in lanolin (Table S1) or pure lanolin was added to the *N. glauca* plants by applying 20 µL to the base of the plant (Fig. S6). The larvae were placed singly on alternating perfumed and control plants growing on either the Snow place or the main field plot (Fig. S7*E*). Predation was monitored as described above for 2 h. The results of both plots were summarized and analyzed by χ^2 test (n = 28). In addition, we performed a similar assay on irLOX2/3 *N. attenuata* plants. These transformed plants are silenced in the expression of two different lipoxygenases that supply fatty acids hydroperoxides for GLV and JA biosynthesis (5) and, hence, do not emit either GLVs or terpenoids in response to herbivore attack. These RNAi plants allowed us to examine the function of BCAA independently of jasmonate signaling or GLV emission. Sprayed caterpillars were again placed singly on plants. After 30 min, the plants were carefully examined for larvae and missing caterpillars were regarded as predation event. The results were also analyzed by a χ^2 test (n = 12). The higher preference for the BCAA sprayed larvae (Fig. S6) within this short timeframe lead us the hypothesis that *Geocoris* might be attracted by the caterpillar's body odor.

Ant trials. We examined the response of 24 ant colonies in the surroundings of native or planted *Nicotiana* or *Datura* populations to BCAAs with a rice pickup assay. We perfumed cooked and dried rice grains with BCAA suspended in water (0.05% Tween-20) and monitored the ant's response to 10 rice grains placed within 40 cm of the nest entrance within a 10-min period. From eight responding colonies, we further examined the speed with which first instar *M. sexta* larvae reared on *N. attenuata* leaves were carried back to the next and selected five for further trials (see Fig. S7*E* for nest locations). Foragers from each of the five colonies were collected and all were identified as *Pogonomyrmex rugosus* by Paul Frandsen at Brigham Young University.

We used a stick climbing assay to measure the response of foraging ants to larvae, frass, and BCAA. The number of ants climbing past a 20-cm mark on the stick during a defined time interval was counted. In a first trial, we tested the response of three colonies on the main field plot to fresh frass from *M. sexta* fed on *N. attenuata* leaves in comparison with 80 °C oven-dried frass from caterpillars fed on *N. glauca* leaves. We placed 10 wooden bamboo sticks of 24.5 cm height in a line at a distance of 20–50 cm from the nest entrances. The bamboo sticks were heated in a microwave oven for 3 min to remove any trace volatiles. We pinned excised leaflets of *N. glauca* on the top of the sticks as well as second instar *N. glauca* reared larvae. Afterward, we applied either fresh or dried frass in alternating order at the base of 10 sticks and counted the individual ants climbing up the sticks in a 5-min interval (Fig. S7*F*). The experiment was repeated three times on the same day. The results of all three trials were combined, and a χ^2 test was performed.

To exclude potential volatile or visual cues from the frass, caterpillars, or leaflets, we tested one colony's responses to the spraying of ≈100 µL of a mixture of BCAA in water (0.05% Tween-20) or water control to the base of the sticks where the stick was inserted into the soil (Fig. S7*G*). Before the spraying, we counted the number of ants climbing all 10 untreated sticks for 15 min, to detect any position or stick associated preferences of the ants. Sticks with significantly higher climbing rates during this period were excluded from later statistical analysis. We used four replicates for each treatment. The experiment was conducted six times in either the morning and three times in the evening. The results of all morning trials were combined as well as all evening trials were combined separately. χ^2 test was performed.

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7. Allmann S, Baldwin IT (2010) Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. *Science* 329:1075–1078.

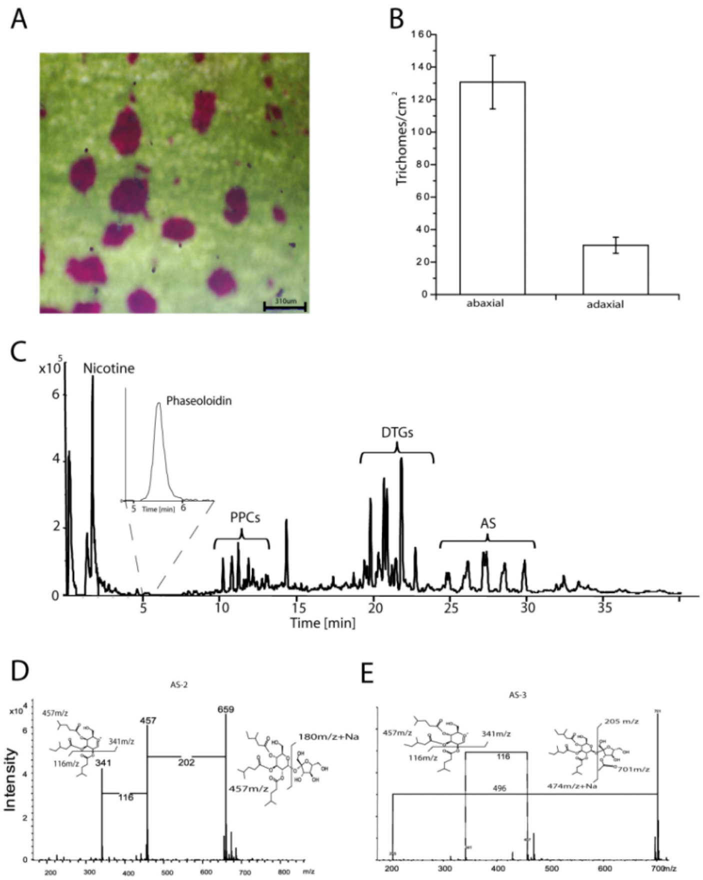

Fig. S1. Trichome abundance, *N. attenuata's* metabolome and characterization of *N. attenuata O*-acyl sugars (AS). (*A*) *O*-acyl sugars on the leaf surface of *N. attenuata* were stained with a solution of Rhodamine B in water (0.02%). The tips of trichomes are recognizable as well as the stained bases. (*B*) Mean ± SE of trichome numbers per cm^2 on either the adaxial or abaxial side of a *N. attenuata* leaf. (*C*) HPLC-ToFMS analysis (TIC+) of a *N. attenuata* whole leaf extract. The leaf surface metabolome is dominated by nicotine, phenylpropanoid polyamine conjugates (PPCs), diterpene glycosides (DTGs), and AS. *Inset* shows ion trace of a negative mode *m*/*z* 329.05 and the minor metabolite phaseoloidin, which was recently shown to occur in trichomes and able to affect caterpillar growth of *M. sexta* and *Spodoptera littoralis*. (*D*) Mass spectra of the AS with MW 678 showing the two characteristic fragmentations. Fragment of *m*/*z* 205 corresponds to an acetylated fructose moiety while fragment of *m*/*z* 116 corresponds to a C_6 aliphatic acid. (*E*) Mass spectra of acyl sugar with the MW of 636 showing the two characteristic fragmentations. Loss of *m*/*z* 202 corresponds to a nonacetylated fructose moiety while fragment of *m*/*z* 116 corresponds to a C_6 aliphatic acid.

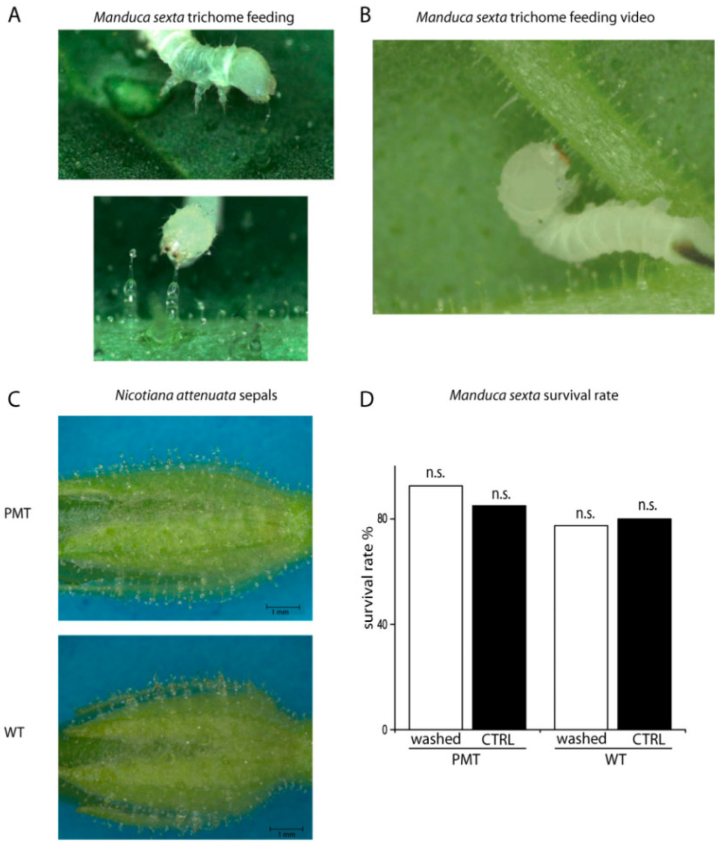

Fig. S2. Caterpillar sepal feeding. (*A*) Freshly hatched neonates of *M.* sexta feeding on trichomes of different leaf tissues. (*B*) Freeze frame from the video footage of a *M. sexta* neonate larvae feeding on trichomes (Movie S1). (*C*) Sepals subtending immature capsules of *N. attenuata* irPMT (32) and WT plants. High density of trichomes in these tissues can be clearly seen. (*D*) Survival rate of *M. sexta* neonates feeding on either washed or unwashed sepals subtending immature capsules of *N. attenuata* irPMT or WT plants. Neonates were transferred to sepals immediately after hatching and were allowed to feed for 3 h. The sepals were removed and caterpillars were starved for 18 h. The number of larvae used for each treatment was 40.

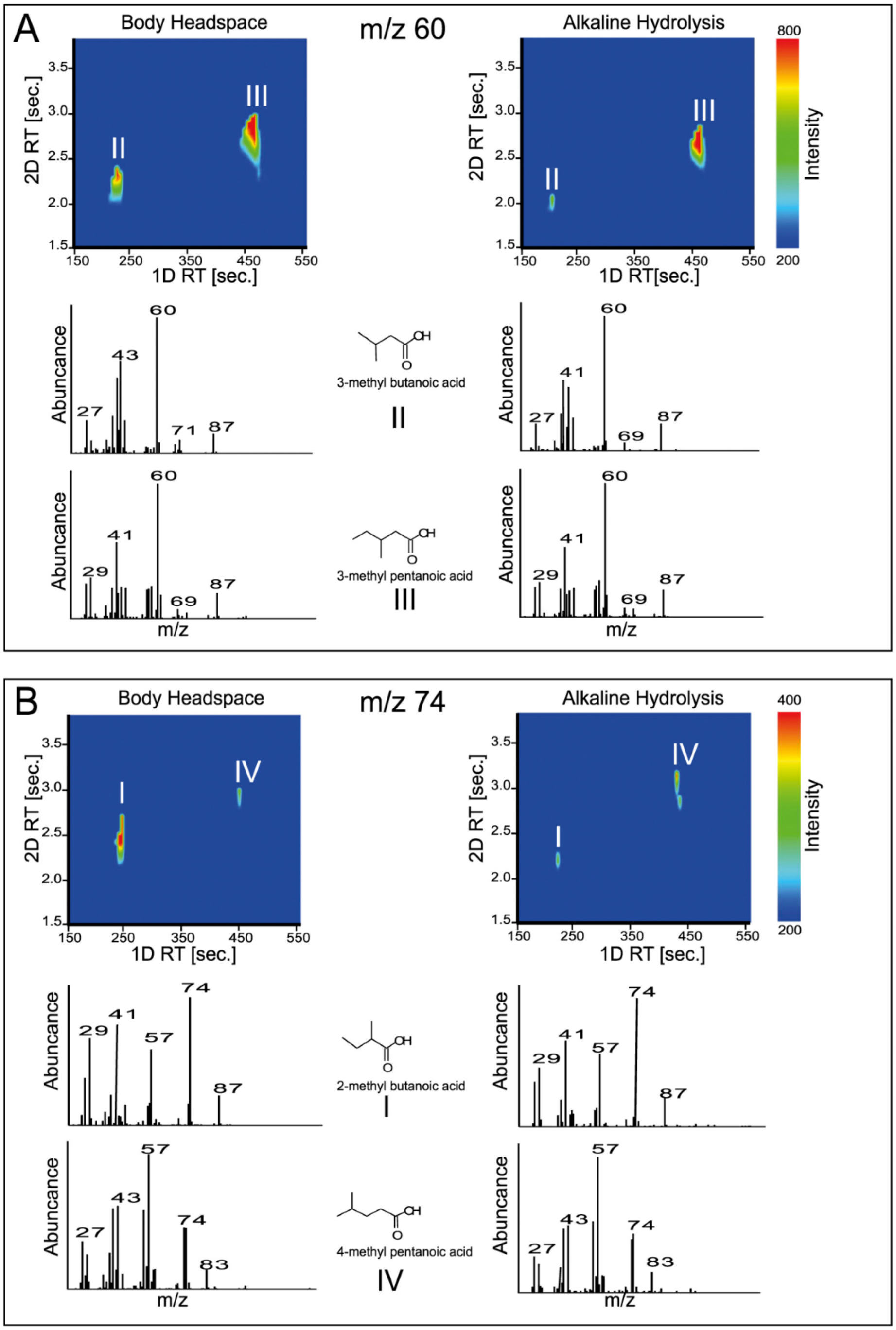

Fig. S3. *M. sexta* larval body headspace and alkaline hydrolysis of *N. attenuata* AS. (*A*) Ion trace *m/z* 60 from GCxGC-TofMS analysis of aliphatic acids emitted by caterpillars (body headspace) and aliphatic acids derived from the alkaline hydrolysis of *O*-acyl sugars (alkaline hydrolysis). 3-methyl butanoic acid (II) as well as 3-methyl pentanoic acid (III) are present in both samples as seen by the similar retention times and mass spectra. (*B*) Ion trace *m/z* 74. 2-methyl butanoic acid (I) as well as 4-methyl pentanoic acid (IV) are present in both samples as seen by the similar retention times and mass spectra.

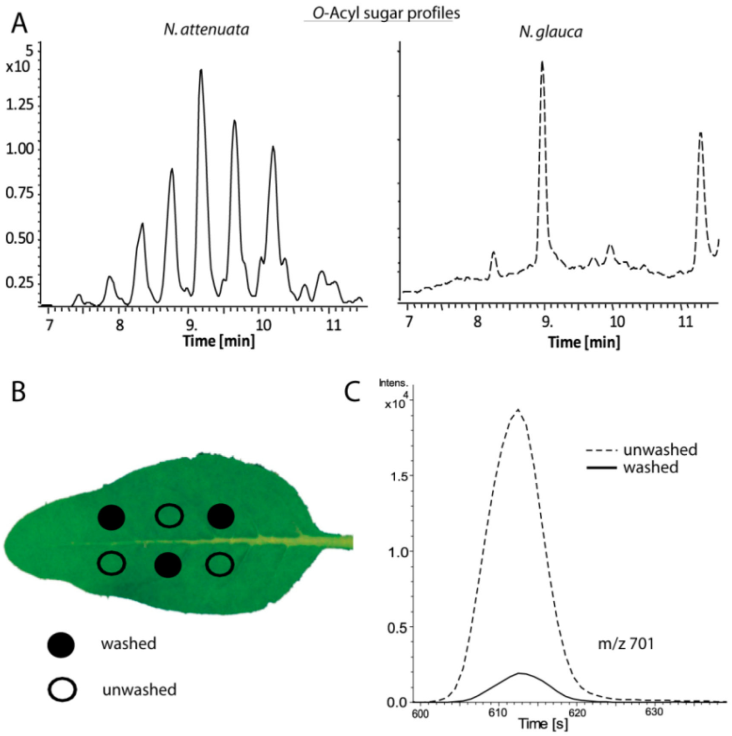

Fig. S4. Acyl sugar abundance on *N. attenuata* vs. *N. glauca* or washed vs. unwashed *N. attenuata* leaves. (*A*) HPLC-TofMS analysis (+TIC) of *N. attenuata* leaf surface wash shows the presence of *O*-acyl sugars on the leaf surface, whereas they are missing in a *N. glauca* leaf surface wash. (*B*) Experimental setup for validating the washing efficiency. Three leaf discs were taken from different positions a *N. attenuata* leaf and soaked in water for 30 s (washed). Washed leaf discs and additional three unwashed leaf discs were then rinsed with MeOH. The methanolic leaf surface extract was analyzed by HPLC-ToFMS. (*C*) Ion trace *m*/*z* 701 shows the almost complete removal of an acyl sugar (MW 678 M+Na 701 *m*/*z*) from previously washed leaves (solid black line) in comparison with unwashed leaves (dashed black line).

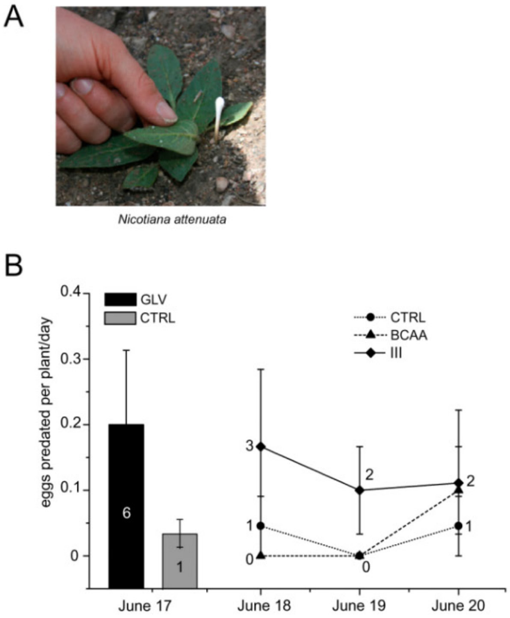

Fig. S5. *Geocoris* predators do not use SFCAs to guide their egg predation behavior. (*A*) Experimental setup of an egg predation assay. Cotton swaps with either lanolin (CTRL), lanolin + BCAA (BCAA) mixture, lanolin + GLVs 5 μg/mL (trans-2-hexenyl acetate and cis-3-hexenyl acetate 1:1) (GLV) and lanolin + BCAA III (III) were placed adjacent to a rosette leaf of 11 *N. attenuata* plants. Two eggs of *M. sexta* were glued to the abaxial side of the leaf with α-cellulose glue. Predated eggs were counted after 12 h. (Image courtesy of D. Kessler.) (*B*) Mean ± SE of the number of eggs predated per plant per day and total number of predated eggs per treatment on 1 d and within a 3-d interval during the 2010 field season. None of the odors presented showed a significance difference although the GLVs showed the best trend ($P = 0.167$, two-tailed Student's *t* test).

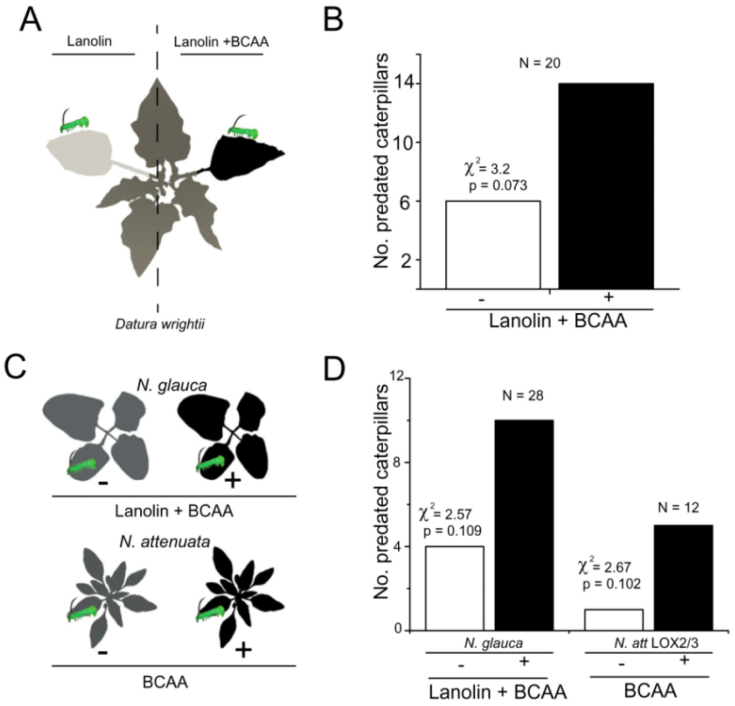

Fig. S6. BCAA predation assays with perfumed larvae placed on different types of plants. (*A*) Experimental setup for predation assays on *D. wrightii*. Two neonates of *M. sexta* perfumed with either lanolin paste + BCAA or pure lanolin were placed on size-matched leaves on opposite sides of 10 *D. wrightii* plants. (*B*) Number of predated lanolin (-) or lanolin + BCAA (+)-treated caterpillars. χ^2 statistics revealed a trend toward preference to BCAA-tagged larvae. (*C*) Experimental setup for predation assays on *N. glauca* and irLOX2/3 iRNA *N. attenuata* plants, which are unable to release herbivore-induced plant volatiles after herbivore attack. Single neonates were sprayed with a mixture of BCAA in water and placed on leaves of both plant species. Leaves of *N. glauca* were additionally treated on their petioles with either lanolin (-) or lanolin + BCAA (+). (*D*) Number of predated lanolin (-) or lanolin + BCAA (+)-treated caterpillars on *N. glauca* and irLOX2/3 *N. attenuata*. χ^2 statistics show a trend toward preference for BCAA-tagged larvae.

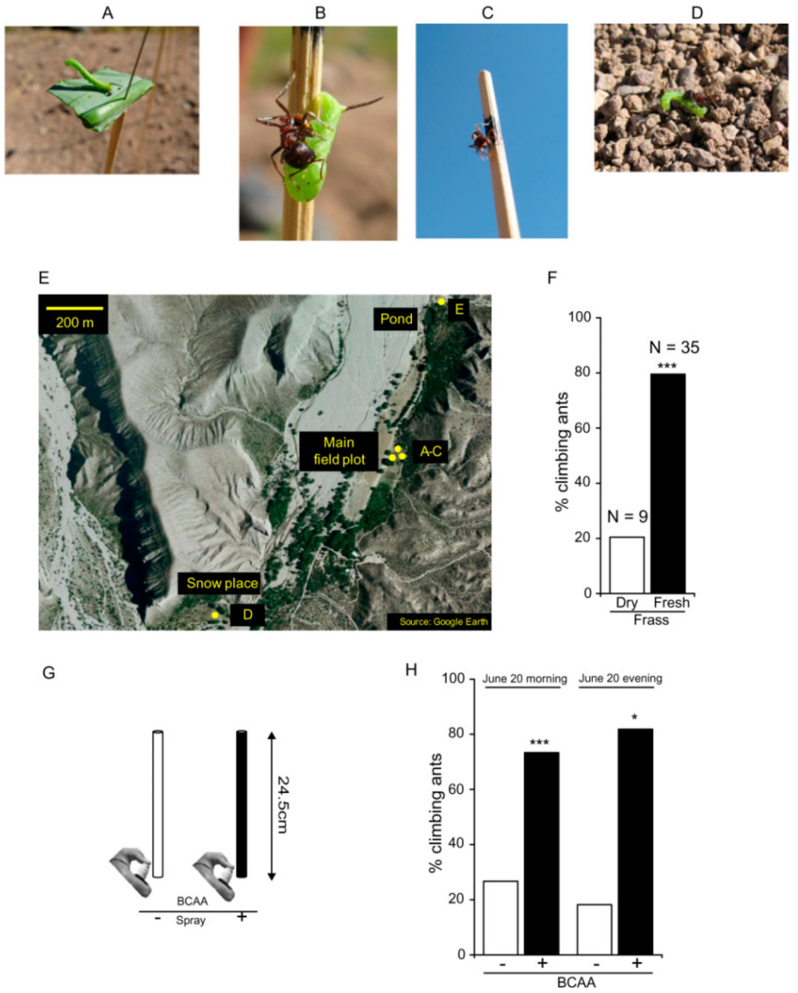

Fig. S7. Preliminary experiments on ant attraction and localization of ant colonies: Dried and fresh frass of *N. attenuata* reared *M. sexta* larvae were placed at the base of bamboo stick. (*A*) Second instar *M. sexta* neonates were placed on excised leaf pieces of *N. glauca* on the top of bamboo sticks. (*B*) Ants climbing up the bamboo sticks and attacking neonates and returned them to their nest (*D*). (*E*) Ant colonies used in bioassay were located on different sites at the Utah field station. (*F*) Percentage of climbing ants attracted by either dried or fresh frass of *N. attenuata*-reared *M. sexta* larvae placed at the base of bamboo stick within a 5-min period on colonies A–C. Total number of ants of all three colonies was summed. Asterisks indicate significant differences between choices by χ^2 analysis $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. ($\chi^2 = 15.36$). (*G*) Experimental setup for preliminary ant attraction assays. See Table S1 for the composition of the 100-uL BCAA mixture in water + 0.05% Tween-20 (+) that was sprayed at the base of the bamboo sticks. Water + 0.05% Tween-20 was the control (-). (*H*) Percentage of ants climbing the stick past the 20-cm marker (*C*) for a mixture of BCAA (+) ($n = 5$) or the control (-) ($n = 5$) on the morning and the evening of June 20 in 2010 field season. Asterisks indicate significant differences between choices by χ^2 analysis $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. ($\chi^2 = 13.06$ morning, $\chi^2 = 4.45$ evening).

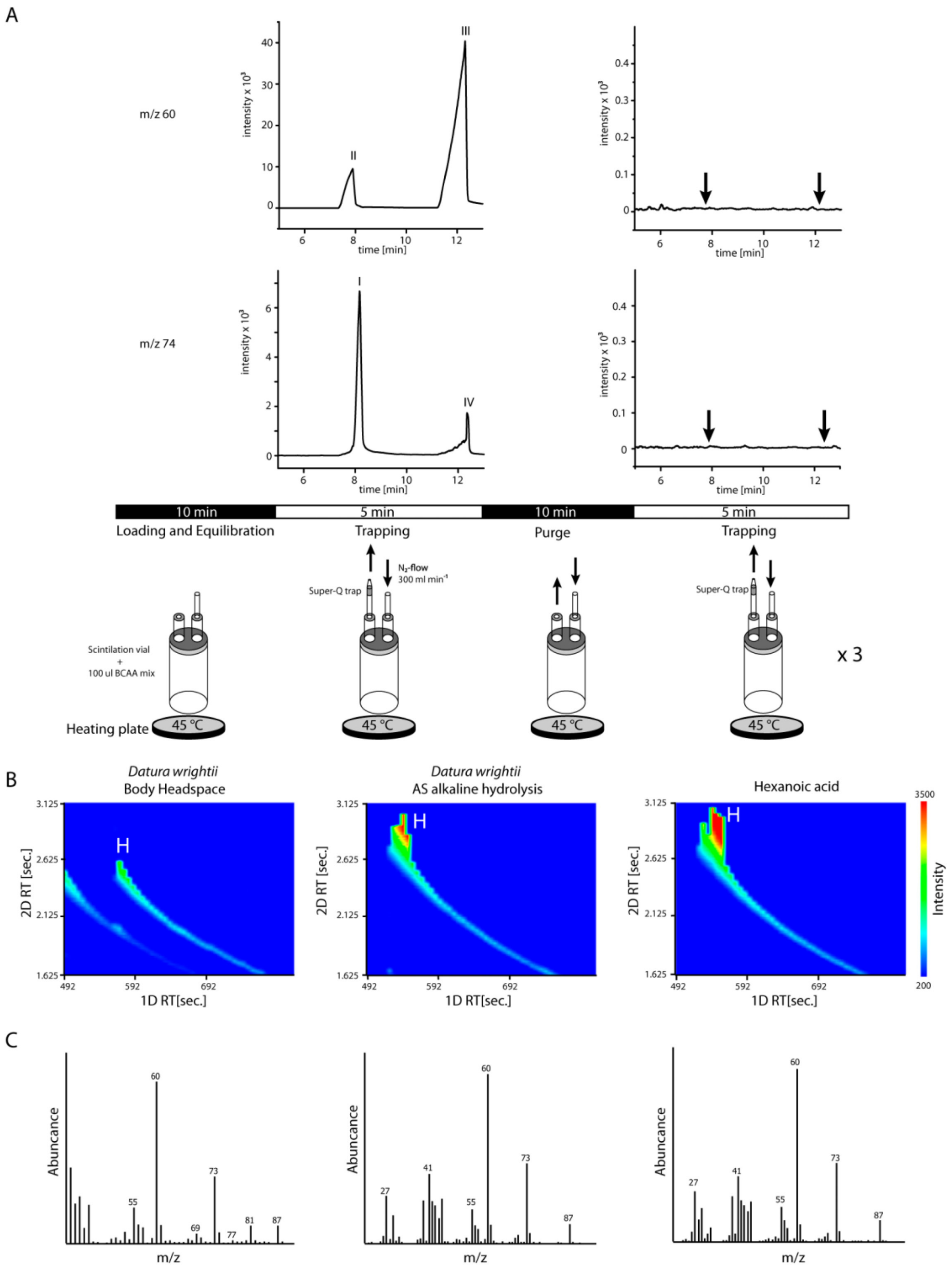

Fig. S8. Evaluation of BCAA volatilization and *Datura wrightii*-related BCAA emissions. (*A*) Evaluation of BCAA volatilization: 100 μL of a BCAA mix containing concentrations equivalent to those used in the field were added to a 20-mL scintillation vial within a heating block set at 45 °C—the average midday soil temperature during the month of June at our field station among *N. attenuata* plants. After 10 min of equilibration, nitrogen was purged through the vial at a flow rate of 300 mL/min. On the exit we placed a super-Q trap for 5 min. Afterward the Super-Q trap was removed and the vial was purged for an additional 10 min followed by another 5-min trapping period. Trapped BCAA were analyzed by GC-MS. After 15 min of purging the entire BCAA bouquet had volatilized from the vial, and this formed the basis for the 15-min monitoring interval for the field assays. *D. wrightii* related body headspace: (*B*) Contour plots of the 60 *m/z* ion trace from a GCxGC-ToFMS analysis of the headspace of *M. sexta larvae* of the third to fourth instar feeding on *D. wrightii* for 2 d as well as the products of the alkaline hydrolysis of *D. wrightii O*-acyl sugars (AS) and a commercial standard of hexanoic acid show the presence of hexanoic acid. (*C*) Mass spectra confirm the identity of hexanoic acid within the *M. sexta* body headspace and the products of the alkaline hydrolysis of *D. wrightii O*-acyl sugars.

Table S1. BCAA concentrations used in bioassays

Measurement	2-methyl butanoic acid	3-methyl butanoic acid	3-methyl pentanoic acid	4-methyl pentanoic acid
CAS	116–53-0	503–74-2	105–43-1	646–07-1
Molecular weight	102.13	102.13	116.15	116.15
Laboratory				
Trapped from caterpillars within 2 h, ng	44.06*	212.32	1210.57	37.17
Trapped from 1 g of frass within 2 h, ng	199.96	660.12	5110.20	248.72
Field				
Amount in 20 µL of lanolin, ng	199.96	660.12	5110.20	248.72
Concentration in lanolin, mM	0.098	0.323	2.200	0.107
Amount in 100 µL of water + 0.05% Tween-20, ng	199.96	660.12	5110.20	248.72
Concentration in water + 0.05% Tween-20, µM	19.579	64.635	439.966	21.414

Concentrations were calculated from a GCxGC-ToFMS measurement of a third instar *M. sexta* larvae. Samples were eluted as described in *Materials and Methods*. Tetralin was used as an internal standard (ISTD) for calculating recoveries (formula A). The total amount trapped was calculated by comparison with the direct injection of BCAA standards.

$$^{*}Recovery = \frac{Area_{\mathrm{ISTD}}\,eluted}{Area_{\mathrm{ISTD}}\,injected} \quad Amount_{\mathrm{eluted}} = \frac{\dfrac{Area_{\mathrm{analyte}} \times amount_{\mathrm{ISTD}}}{Area_{\mathrm{ISTD}}}}{Recovery} \times Volume_{\mathrm{Eluentsolvent}} \quad c = \frac{\dfrac{Amount_{\mathrm{eluted}}}{MW}}{V_{\mathrm{solvent}}}$$

Table S2. *N. attenuata O*-acyl sugars summary: Table of major AS using the nomenclature of Arrendale et al. (1) and ordered by their retention time (RT)

AS	RT	MW	M+Na	Major MS ions	Predicted aliphatic acid substitution: Glucose	Predicted aliphatic acid substitution: Fructose
AS-II	7.3	580	603	603(100) 401(50) 313(25) 299(12) 285(6)	C_5	—
	7.8	594	617	617(100) 415(50) 299(20) 313(15) 327(10)	C_4-C_6	—
	8.2	608	631	631(100) 429(60) 313(20) 327(15)	C_5-C_6	—
	8.7	622	645	645(100) 443(80) 327(20) 341(10)	C_5-C_6	—
	9.2	636	659	659(100) 457(95) 341(50)	C_6	—
AS-III	9.1	650	673	673(100) 429(25) 313(10) 341(10) 205(15)	C_4,C_6	C_2
	9.6	664	687	687(100) 443(20) 327(5) 341(2) 205(10)	C_5,C_6	C_2
	10.1	678	701	701(100) 457(10) 341(5) 205(10)	C_6	C_2

MW, molecular weight; M+Na, mass of the molecular ion sodium adduct.

1. Arrendale RF, et al. (1990) Characterization of the sucrose ester fraction from Nicotiana Glutinosa. *J Agric Food Chem* 38:75–85.

Movie S1. Video of *M. sexta* neonate feeding on the trichomes of *N. attenuata*.

Movie S1

Chapter 6: Manuscript II

Trichomes as dangerous lollipops: Do lizards also use caterpillar body and frass odor to optimize their foraging?

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In this manuscript we demonstrated based on our previous findings (Manuscript I) that not only omnivorous ants but most likely lizards also use *M. sexta's* body and frass odor to locate their prey. Our studies showed that caterpillars of later instars are preyed upon in the field on a regular basis. Since all previously known predators in our field site are not capable of preying on caterpillars of this size, we hypothesized that lizards native to the field site could be responsible for the loss of these caterpillars. In different bioassays we demonstrated that lizards are attracted by the volatile acids in *M. sexta's* body and frass odor, thereby underlining once more the importance of trichomes and their exudates for the defense of plants in nature.

SHORT COMMUNICATION

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Trichomes as dangerous lollipops

Do lizards also use caterpillar body and frass odor to optimize their foraging?

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Key words: *O*-acyl sugars, *Nicotiana attenuata*, *Manduca sexta, Manduca quinquemaculata*, lizards, body odor, frass odor,

Abbreviations: BCAA, branched chain aliphatic acids

When attacked by herbivores, plants produce toxic secondary metabolites that function as direct defenses, as well as indirect defenses that attract and reward predators of the offending herbivores. These indirect defenses include both nutritive rewards such as extra floral nectar, as well as informational rewards, such as the production and release of volatile compounds that betray the location of feeding herbivores to predators. Herbivory of *Nicotiana attenuata* by the tobacco hornworm (Manduca larvae) alters the volatile profiles of both the plant and larval headspace. Herbivory-elicited specific changes in the volatile profiles are detected by arthropod predators of Manduca larvae. The known predators that perceive volatile cues induced by Manduca herbivory of *N. attenuata* are insects that target Manduca at early developmental stages, when the larvae are still small; large, late-instar larvae may have outgrown these predation risks. However, here we offer evidence that branched chain aliphatic acids derived from the digestion of plant *O*-acyl sugars from trichomes may betray Manduca larvae to lizard predators during late developmental stages as well.

Acyl Sugars are Part of Plants' Defensive Arsenal

Recently we showed that volatile aliphatic acids from the headspace of the tobacco hornworm (*Manduca sexta,* Lepidoptera, Sphingidae) are used by Pogonomyrmex ants to locate their prey.[1] These branched chain aliphatic acids (BCAA) originate from the digestion of plant *O*-acyl sugars consumed by Manduca larvae. Secreted by trichomes on the phylloplane of the wild tobacco *Nicotiana attenuata*, *O*-acyl sugars serve as a first meal for larvae but tag these herbivores with a distinctive body odor that is detected by the rough harvester ant, *Pogonomyrmex rugosus*, as it forages along the ground. *O*-acyl sugars, the most abundant metabolite of trichomes, are thus a potent indirect defense against herbivores that combines both nutritional as well as informational components. Ants are not the only threat to emerging Manduca larvae; for example, the big-eyed bug Geocoris spp detects changes in the Z/E ratio of the isomers of green leaf volatiles to locate Manduca eggs and early instar caterpillars.[2] Additionally, predatory insects use fecal odors, such as the BCAAs from *M. sexta* larvae, which are emitted from frass, to optimize their prey foraging behavior.[3,4] Previously, the risk of predation was thought to be negligible for large, late-instar Manduca larvae. Here, we provide evidence that desert lizards prey upon large, late-instar, Manduca larvae and suggest that these reptilian predators also use plant-derived larval body and frass odors to enhance their foraging success.

Observation of Lizard Predation in the 2009 Field Season

During field experiments in 2009, we monitored the daily movements of 3rd-instar *M. sexta* and *M. quinquemaculata* larvae among *N. attenuata* plants growing in an irrigated field plot as well as among plants growing in a native population. In the field plot, Manduca larvae (*M. sexta,* n = 30; *M. quinquemaculata*, n = 30) were distributed among pairs of *N. attenuata* plants (n = 60, 1 larva per plant pair). In the native population, *M. quinquemaculata* (n = 11) and *M. sexta* (n = 19) were distributed among 428 plants growing in a roadside wash.

We observed high lizard activity (mostly *Aspidoscelis tigris*, *Uta stansburiana* and *Sceloporus magister*) during the twice-daily censuses. In the field plot, Manduca larvae frequently disappeared between the two observation intervals. The experimental design allowed us to determine that lost larvae were not present on either of the plants in each pair and extended searches of all other plant pairs and other plant species in a 100 m radius of the focal pair were conducted when larvae disappeared. After 3 d,

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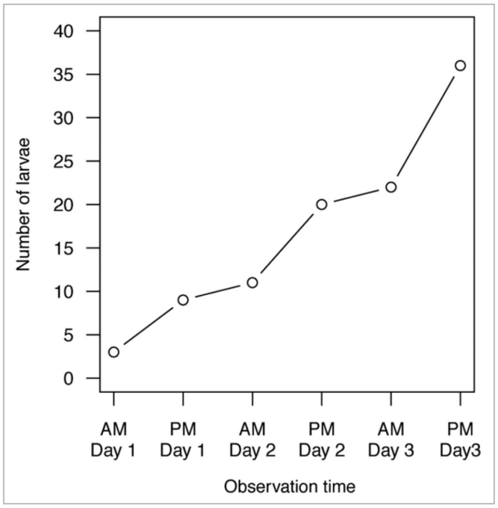

Figure 1. Predation of Manduca larvae by diurnally active lizards. Manduca larvae that were unaccounted for during an observation time during field experiments during the 2009 field season were subject to repeated, expanded searches in each subsequent observation time. Most individuals that were lost and never recovered initially disappeared between daytime observation points, correlating with lizard activity. Later baiting experiments indicated predation of Manduca by lizards in both the field plot and a native population.

36 individuals had disappeared. Most disappearances (n = 29) occurred between the two daytime observation intervals, when lizards are active (**Fig. 1**). Manduca larvae in the native population showed a similar pattern of disappearance over a longer (4 week) observation time.

Initially, we could not rule out loss of larvae due simply to extensive long-distance movement. However during the 2009 field season, we twice witnessed lizards preying upon Manduca in the native population. Furthermore, baiting with exposed *M. sexta* larvae in the field plot revealed that lizards would readily take Manduca larvae as prey. We videotaped 3rd- and 4th-instar *M. sexta* (n = 10) placed on *N. attenuata* plants in the native population. After 2 h, three larvae had disappeared, and the videotape revealed a lizard crawling up the stem of a *N. attenuata* plant and preying upon the larva (**Video S1**). After 6 h, half of the larvae were gone.

These observations demonstrated that lizard predation can be a major threat to Manduca larvae, and the characteristic tongue flicking behavior of *Aspidoscelis tigris* lizards as they forage along the ground before climbing *N. attenuata* stalks to search for larvae, suggested that they might use the BCAA volatiles from freshly deposited frass to locate prey. Hence we designed experiments for the following field season to evaluate whether lizards might use olfactory cues to assist their clearly visually-guided foraging behavior.

Observation of Lizard Predation in the 2010 Field Season

During the 2010 field season numerous predation experiments were conducted with 1st- and 2nd-instar *M. sexta* larvae reared on *Nicotiana glauca*, a plant that completely lacks trichomes and consequently produced larvae that lack the telltale BCAA-redolent body and frass odors.[1] These larvae were reared on *N. glauca* foliage to produce 3rd- to 4th-instar larvae with BCAA-free body odor and frass. We selected an experimental arena adjacent to an *N. attenuata* field plantation, where whiptail (*Aspidoscelis tigris)* and spiny (*Sceloporus magister*) lizards were commonly seen foraging among *N. attenuata* plants. In order to determine if natively foraging lizards also used BCAAs to inform their foraging behavior, we created an experimental arena in which large, scentless *N. glauca*-reared *M. sexta* larvae were placed individually on 10 dried Datura branches which were tied to a string to keep them in an upright position. The larvae were placed on these branches approximately 15–20 cm above a cement pad so that self recruited lizards would have to climb the stems to capture the larvae (**Fig. 2A**). Two types of experiments were conducted in which (1) equal volumes (approximately 8 cm^3) of either fresh, redolent frass from *N. attenuata*-fed larvae or oven-dried odorless frass (in alternating order) was placed at the base of the branches that touched the cement pad or (2) the base of the branches were sprayed with BCAA mixtures in frass-equivalent amounts.[1] While the native lizard community quickly learned to locate the larvae on the branches by vision in subsequent replicates, the first trials of both experimental setups (conducted almost a month apart with two weeks of no experimentation in the arena) revealed a strong preference for lizards to climb branches and consume larvae that had been scented with fresh frass or BCAA sprays (**Fig. 2B**). These results, which will require further experimental support with experimental designs that minimize the ability of lizards to use vision to locate prey, strongly suggest that lizards use BCAA odors to locate larval prey.

Conclusion

Late-instar Manduca larvae are clearly vulnerable to lizard predation in their native habitats in southwest Utah. In an experimental arena with self-recruited lizards, native lizards appeared to use fresh (BCAA-containing) caterpillar frass as well as BCAA-scented branch bases to locate prey located above them on the branches, before they learned the experimental setup and located prey purely by vision. Since larvae are commonly hidden from visually hunting ground-foraging predators by foliage, we propose that BCAAs in fresh frass deposited on the ground by canopy feeding larvae are used by ground-foraging lizards to select plants to climb and locate prey. Because the fatty acid bouquet vanishes quickly under field conditions,[1] these volatile fatty acids are a strong and rapid signal that betrays the location of larvae to predators. Since the BCAA body and frass odor of Manduca larvae is strongly dependent on the ingestion of trichome-produced *O*-acyl sugars, this compound class seems to be an important component of *N. attenuata*'s arsenal of indirect defenses. To more

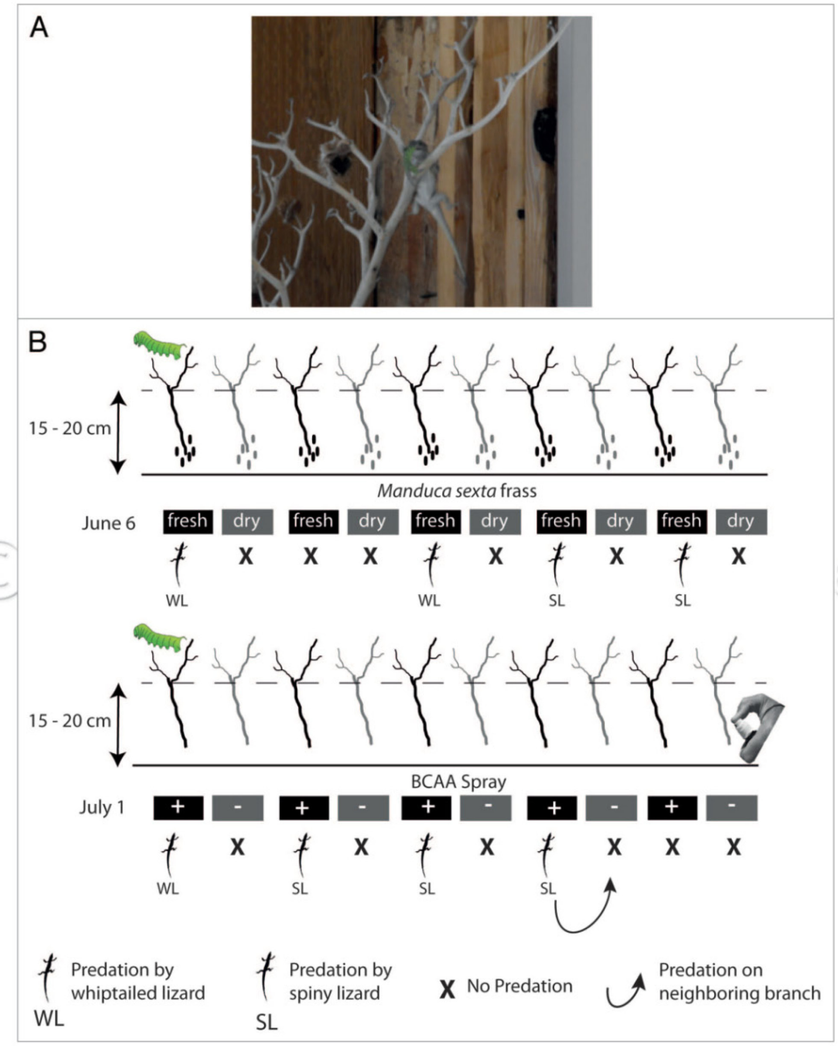

Figure 2. Lizard predation of *M. sexta* larvae was highest on branches whose bases were treated with fresh BCAA-redolent frass or scented with BCAA volatiles. Predation of 3rd- to 4th-instar *M. sexta* larvae: (A) Spiny lizard (SL) predating a *M. sexta* larvae from one of the 10 replicate Datura branches of the experimental arena. (B) Experimental set up and results of the first trials (conducted on June 6 and July 1, 2010). Ten dried wooden *D. wrightii* branches were affixed to a string that stretched across a slab of concrete across on which native lizards of at least two species regularly foraged. Fresh (black) or oven-dried (gray) *M. sexta* frass (8 cm^3) was applied to the base of the branches that touched the cement slab (upper part). Larvae (previously reared on *N. glauca* leaves) were placed 15–20 cm above the ground in a fork of each branch with an affixed piece of *N. glauca* leaf. Predation by spiny (SL) and whiptailed (WL) lizards is depicted during the subsequent hour. The data from the first trial on June 6 revealed a strong preference of lizards to climb branches with fresh frass at their base compared with branches tagged with dried frass. In four subsequent replicates, conducted on consecutive days, the lizards climbed the first branch they arrived at and consumed larvae on adjacent branches without returning to the ground, clearly finding larvae by vision. On July 1, after 14 d of no experimentation, the experiment was repeated but instead of placing frass at the base of the branches, branches were either sprayed with 100 μL water + BCAA (0.05% Tween-20) (+) or with a water control (-). Lizard predation was monitored for an hour; BCAA-sprayed branches were preferred over water-sprayed branches. A SL after first predating the larvae of the 4th BCAA-perfumed replicate, crossed over to the adjacent control branch to consume the larvae on this branch.

rigorously test these inferences, we are currently attempting to engineer plants with morphologically normal trichomes but lacking in *O*-acyl sugars.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:
www.landesbioscience.com/journals/psb/article/18028/

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Chapter 7: Manuscript III

Phaseoloidin, a homogentisic acid glucoside from *Nicotiana attenuata* trichomes, contributes to the plant's resistance against Lepidopteran herbivores

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In this manuscript we described the identification of a trichome metabolite novel to the Solanaceae. Comparative NMR- and MS studies revealed the presence of phaseoloidin - a homogentisic acid glucoside - in the trichomes of the wild tobacco *N. attenuata*. We could show that besides *O-acyl sugars* (Manuscript I, II), phaseoloidin and nicotine are present in the different trichome types of *N. attenuata* and that their abundance varies between different trichome parts. In addition we could demonstrate that phaseoloidin negatively affects the growth of specialist *Manduca sexta* and generalist *Spodoptera littoralis* larvae. These results highlight once again the defensive role of trichome-based plant defenses.

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Phaseoloidin, a Homogentisic Acid Glucoside from *Nicotiana Attenuata* Trichomes, Contributes to the Plant's Resistance against *Lepidopteran* Herbivores

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Michael Wenzler · Bernd Schneider · Ian T. Baldwin

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Abstract Plant trichomes are known for their capability to produce and store secondary metabolites that protect plants from biotic and abiotic stresses. ^{1}H NMR studies on intact individual trichomes located on the leaf surface of *Nicotiana attenuata* revealed the presence of two major secondary metabolites: nicotine, the signature metabolite of the genus, and phaseoloidin, a homogentisic acid glucoside. This glucoside was reported originally from the seeds of *Entada phaseoloides*, and this is the first report of its occurrence in a Solanaceous plant. Artificial diet feeding bioassays with *Manduca sexta* and *Spodoptera littoralis* larvae, two important herbivores of *N. attenuata*, revealed that the ingestion of phaseoloidin negatively influenced caterpillar performance. This effect was more pronounced for the generalist, *S. littoralis,* than for the specialists, *M. sexta*.

Key Words Phaseoloidin · Nicotine · Trichome · *Nicotiana attenuata* · *Manduca sexta* · *Spodoptera littoralis* · Homogentisic acid

Introduction

Trichomes are often considered a plant's "first line of defense" against attacking herbivorous insects. These epidermal protuberances act as mechanical defenses by hindering insect movement on plant surfaces (Cardoso, 2008). Several studies have reported that trichomes function as obstacles to caterpillar movement, and that glandular trichomes can entrap herbivorous arthropods, particularly soft bodied insects such as aphids and white flies (Chortyk et al., 1997). In addition to presenting a formidable physical barrier for small insects, trichomes serve as miniature self-contained factories for the biosynthesis of secondary metabolites that function as defenses against herbivores (Schilmiller et al., 2008). For example, trichomes of *Artemisia annua* produce the defense compound, artemisinin (Duke et al., 1994), and tomato trichomes produce terpenoids that protect plants against spider mites (van Schie et al., 2007).

In the genus *Nicotiana,* trichomes have been categorized by their morphology (Goodspeed, 1955), and two different types of glandular trichomes have been described from *Nicotiana attenuata*: the longer stalked type C and the smaller type D. These glandular hairs not only differ in their morphology but also in their distribution across the leaf surface. While type D trichomes can be found all over the leaf, the longer stalked type C trichomes are present mainly on midribs. These glandular hairs, which can release exudates onto the leaf surface, can profoundly influence the chemistry of the leaf surface. One of the most abundant and well-studied classes of trichome metabolites are the *O*-acyl sugars (Arrendale et al., 1990), which are excreted by glandular trichomes from several Solanaceae species and contribute to resistance against aphids, spider mites, and other soft-bodied insects (Puterka et al., 2003; McKenzie

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Alexander Weinhold, Kamel Shaker, and Michael Wenzler contributed equally to the results presented in this paper.

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and Puterka, 2004). In addition to acyl sugars, nicotine, caffeoylputrescine, and several derivatives of the flavonoid, quercetin, are produced in trichomes and contribute to the leaf surface chemistry of *N. attenuata* (Roda et al., 2003). Many important steps in the biosynthesis of nicotine-related compounds take place in the trichomes. For example, nornicotine in *Nicotiana stocktonii* is converted in trichomes to *N*-hydroxyacylnornicotine, which subsequently is rapidly excreted onto the leaf surface (Zador and Jones, 1986). Such *N*-acylnornicotines (NacNN) have been shown to be a much more potent defense against the specialist herbivore *Manduca sexta* than nicotine itself (Jones et al., 1985). In *Nicotiana repanda,* the production of NacNN from nornicotine and its exudation to the leaf surface is rapidly elicited by methyl jasmonate (Laue et al., 2000). All these studies make inferences about trichome chemistry and function not by directly examining trichomes but rather by washing the leaf surface or even extracting complete leaf tissue.

In recent years, advances in non-destructive techniques such as NMR-spectroscopy have improved our ability to study intact tissues and plant organs. Hence NMR is the method of choice to gain a better understanding of the role of trichomes in the wild tobacco, *Nicotiana attenuata.* We examined wild type plants and a line of plants transformed with an RNAi construct harboring a fragment of the subunit of a SnRK1 (SNF1-related kinase) protein kinase (Schwachtje et al., 2006). This particular transformed line produced large trichomes with large basal cells (Supplemental Fig. S1E). Since only one of more than 20 transformed lines produced this phenotype, we infer that the trichome phenotype results from a t-DNA insertional mutagenesis event, rather than the RNAi of the SnRK1. These enlarged trichomes allowed us to harvest sufficient trichome mass to conduct NMR experiments and HPLC analysis to profile the secondary metabolites in the different cell types of type C trichomes and in the trichome heads of type D trichomes.

NMR spectroscopy and LC-TOF-MS analysis revealed for the first time the presence of phaseoloidin in the trichomes of a Solanaceous plant. This homogentisic glucoside was described previously from the seeds of *Entada phaseoloides* (Fabaceae) (Barua et al., 1988). Additionally, we showed in bioassays with artificial diets that phaseoloidin reduces larval growth of the specialist larvae *Manduca sexta* and the generalist larvae *Spodoptera littoralis,* suggesting a strong defensive function comparable to that of nicotine.

Methods and Materials

Plant Growth We used an isogenic line, obtained after 30 generations of inbreeding, of *Nicotiana attenuata* Torr. *ex* Watson originally derived from field-collected seeds. For trichome collection, a transformed line, (irGAL83; transformation construct described in Schwachtje et al. (2006)) containing a tDNA insertion mutation that resulted in the production of large trichomes. Seed germination was performed as described by Krügel et al. (2002). All plants were grown individually in 1 L pots in the glasshouse at 26–28°C under 16 hr of light supplied by Philips Sun-T Agro 400- or 600-W sodium lights.

Trichome Collection For the NMR studies, the two distal cells with the glandular vesicle of 20 trichomes (C tip, as indicated in Supplemental Fig. S1, panel A) or the following three to four cells (C middle) of flowering plants containing the insertional mutation were collected with tweezers. Approximately 150 heads of type D trichomes of flowering plants were collected with a glass capillary (D head). All samples were dissolved in 600 μl deuterated methanol directly after collection. Cells were sonicated before ^{1}H NMR measurements at 500 MHz using a cryogenic probe.

To estimate the amount of phaseoloidin and nicotine present in trichomes (Fig. 1), we harvested 60 type C trichomes from the midvein of rosette stage wild type *N. attenuata* leaves. Trichomes were excised with a razor blade and rinsed from the blade by dipping into a 4-ml vial containing 2 ml of methanol; a process that was repeated three times. Afterwards the samples were sonicated and the solvent was removed under a constant flow of nitrogen. The residue was then dissolved in 70 μl deuterated methanol, and 7.5 μg phloroglucinol (Fluka) were added as internal standard (ISTD) for quantification. Total amounts of phaseoloidin and nicotine were calculated according to formula 1–2, described in Fig. 1.

Isolation of Phaseoloidin Seeds of *Entada phaseoloides* (90 g) were obtained from Urban Jungle (Munich, Germany). The seeds were ground and defatted with *n*-hexane (200 ml each) × 3 and extracted with 80% aqueous ethanol (300 ml) × 3. The alcoholic extract was concentrated, and subsequently fractionated with *n*-hexane, ethyl acetate, *n*-butanol/water. The ethyl acetate fraction (5.0 g) was subjected to two silica gel 60 (40–63 μm, *Merck*, Darmstadt, Germany) column chromatographic separations and eluted with *n*-hexane/ethyl acetate (9:1, 8:2, 7:3, 6:4). The fractions were monitored by TLC on silica gel 60 F_{254} plates (*Merck*), using a mixture of n-hexane/ethyl acetate 13:7, and visualized under a UV lamp (254 nm) after heating and spraying with phosphomolybdic acid in ethanol. The similar fractions were combined. Ten fractions (200.0 mg) contained crude phaseoloidin and were subjected to a second column chromatography separation to produce 150.0 mg semi-pure phaseoloidin, which was

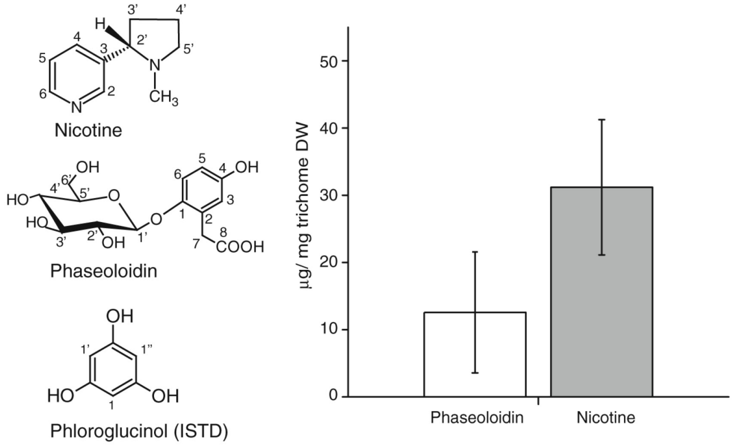


Fig. 1 Quantification of phaseoloidin and nicotine content in *Nicotiana attenuata* wild type trichomes was achieved by ^{1}H NMR. Total amounts of both metabolites were calculated relatively to phloroglucinol (ISTD) according to Formulae 1–2 (below). The integrals of the following proton signals were summed: phaseoloidin: No. of nuclei 3; H-6, H-3, H-5; nicotine: No. of nuclei 4; H-2, H-6, H-4, H-5; phloroglucinol: No. of nuclei 3; H-1, H-1′, H-1″. Error bars indicate standard errors (N=3)

Formula 1: Calculation of the absolute phaseoloidin content

$$m_{\text{phaseoloidin}} = \frac{\text{No. of nuclei}_{\text{ISTD}} \times n_{\text{ISTD}} \times \text{Area}_{\text{phaseoloidin}}}{\text{No. of nuclei}_{\text{phaseoloidin}} \times \text{Area}_{\text{ISTD}}} \times M_{\text{phaseoloidin}}$$

Formula 2: Calculation of the absolute nicotine content

$$m_{\text{nicotine}} = \frac{\text{No. of nuclei}_{\text{ISTD}} \times n_{\text{ISTD}} \times \text{Area}_{\text{nicotine}}}{\text{No. of nuclei}_{\text{nicotine}} \times \text{Area}_{\text{ISTD}}} \times M_{\text{nicotine}}$$

Abbreviations: m—mass; M—molar mass; n—amount of substance; Area—Integrated area of the NMR peak; ISTD—internal standard (Phloroglucinol)

further purified by preparative HPLC (LiChrospher RP18, 5 µm, 250×10 mm, respectively, *Merck*, Darmstadt, Germany) with the following solvents A (0.1% trifluoroacetic acid in H_2O) and B (100% MeOH) and a linear gradient from 3% B to 30% B in 15 min. The flow rate was 5 ml min^{-1} and a total of 50.0 mg pure phaseoloidin were isolated.

NMR—Experiments ^{1}H NMR, ^{13}C NMR, APT, ^{1}H,^{1}H-COSY, ROESY, HMBC, and HSQC spectra were recorded on a Bruker AVANCE 500 spectrometer (Bruker, Rheinstetten Germany) operating at a ^{1}H frequency of 500.13 MHz and a ^{13}C frequency of 125.76 MHz at 300 K using a TCI Cryoprobe™ (5 mm). Presaturation was used for suppression of the HDO signal in the 1D ^{1}H NMR spectra. Chemical shifts were referenced to internal tetramethylsilane (TMS) for ^{1}H and ^{13}C resonances.

LC-TOF-MS Analysis Prior to NMR measurements, the samples were separated using an Agilent HPLC 1100 Series system, with a Gemini NX C18 column (150×2 mm) and the following binary gradient: 0 to 6 min, isocratic 70% A (deionized water, 0.1% [v/v] acetonitrile [Baker HPLC grade] and 0.05% formic acid), 30% B (acetonitrile and 0.05% formic acid); 6 to 13 min, isocratic 20% A, 80% B; and 70% A, 30% B; 13 to 18 min, isocratic 70% A, 30% B. The flow rate was 200μl min^{-1}. Eluted compounds were detected by a Bruker MicroToF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ion spray source operated in negative ion mode. The instrument was operated with parameters described in (Heiling et al., 2010). Electrospray ionization conditions: TOF 2100 V, capillary voltage 4500 V, capillary exit −130 V, dry temperature 200°C, dry gas flow of 8 Lmin^{-1}. Mass calibration was performed using sodium formiate (50 ml isopropanol, 200 ml formic acid, and 1 ml 1 M NaOH in water).

Phaseoloidin ^{1}H NMR (MeOH-d_4), 500.13 MHz, internal standard TMS): δ 7.03 (1H, *d*, J=8.8 Hz, H-6), 6.70 (1H, *d*, J=3.0 Hz, H-3), 6.58 (1H, *dd*, J=8.8, 3.0 Hz, H-5), 4.68 (1H, *d*, J=7.5 Hz, H-1′), 3.87 (1H, *dd*, J=12.1, 5.4 Hz, H-6′ a), 3.68 (1H, *dd*, J=12.1, 2.2 Hz, H-6′b), 3.56 (1H, *d*, J= 14.4 Hz, H-7a), 3.46 (1H, *d*, J=14.4 Hz, H-7b), 3.45 (1H, *m*, H-2′), 3.44 (1H, *m*, H-3′), 3.42 (1H, *m*, H-4′), 3.33 (1H, *m*, H-5′); ^{13}C NMR (MeOH-d_4, 125.13 MHz, internal standard TMS): δ 179.4 (C-8), 153.7 (C-4), 150.1 (C-1), 130.0 (C-2), 119.0 (C-6), 118.7(C-3), 114.9 (C-5), 104.8 (C-1′), 78.1 (C-5′), 78.0 (C-2′), 75.1 (C-3′), 70.0 (C-4′), 62.7 (C-6′), 39.7 (C-7); HREIMS: *m/z* 331.101 [M + H]$^+$, 329.089 [M–H]$^-$.

Nicotine ^{1}H NMR (MeOH-d_4, 500.13 MHz, internal standard TMS): δ 8.51 (1H, *dd*, J=2.2, 0.9 Hz, H-2), 8.44 (1H, *dd*, J=5.0, 1.6 Hz, H-6), 7.86 (1H, *ddd*, J=7.9, 2.2, 1.6 Hz, H-4), 7.43 (1H, *ddd*, J=7.9, 5.0, 0.9 Hz, H-5), 3.24 (1H, *ddd*, J=9.6, 7.9, 2.3 Hz, H-5′a), 3.20 (1H, *dd*, J=9.1, 8.0 Hz, H-2′), 2.37 (1H, *ddd*, J=9.6, 9.3, 8.6 Hz, H-5′b), 2.26 (1H, *m*, H-3′a), 2.17 (3H, *s*, N-CH_3), 1.97 (1H, *m*, H-4′ a), 1.89 (1H, *m*, H-4′b), 1.77 (1H, *m*, H-3′b); ^{13}C NMR (MeOH-d_4, 125.13 MHz, internal standard TMS): 149.82

(C-2), 149.15 (C-6), 140.14 (C-3), 137.41 (C-4), 125.40 (C-5), 70.13 (C-2′), 57.90 (C-5′), 40.58 (N-CH_3), 35.73 (C-3′), 23.28 (C-4′).

Caterpillar Bioassays Larvae of *Manduca sexta* (Lepidoptera) were derived from an in-house colony. Larvae of *Spodoptera littoralis* were from Bayer CropScience (Monheim, Germany). *Spodoptera littoralis* larvae were fed prior to the experiments on an artificial diet consisting of 300 g L^{-1} agar, 250 gL^{-1} bean flour, 4.5 g ascorbic acid, 4.5 g ethyl *p*-hydroxybenzoate, 2.5 g vitamin E (all supplied by Sigma, Milan, Italy) dissolved in 17 ml of seed oil and 2 ml formaldehyde.

Artificial diet for feeding experiments was prepared according to (Waldbauer et al., 1964). While the diet was still liquid and cooling, phaseoloidin, homogentisic acid, and nicotine were dissolved in 5 ml MeOH and added to achieve a final concentration of 0.16 $\mu g\ mg^{-1}$ diet. As a control, 5 ml of pure MeOH were added. The diet was sealed with plastic wrap and aluminum foil and kept at 5°C until use. Freshly hatched *M. sexta* larvae were transferred to plastic containers containing pieces of artificial diet. Ten caterpillars were placed in one box. Larval mass was measured every 3 day, and the diet was refreshed on these days as well.

Spodoptera littoralis were fed for 5 day on bean flour-containing diet and then transferred to artificial diet amended with either phaseoloidin or nicotine. Because *Spodoptera* larvae are cannibalistic, larvae were kept in single plastic cups with small cubes of diet and wet tissue paper to maintain humid conditions. Caterpillars were weighed and the diet was changed every 3 day.

Results

Trichome Profiling Extracts of the two types of glandular trichomes (long stalked type C and short stalked type D) present on the leaves on *N. attenuata* (Supplemental Figs. S1A, B) were analyzed by NMR. We chose to analyze both trichomes types because of their difference in cell number and distributions on the leaf surface. An insertional mutant with enlarged trichomes allowed for the efficient harvests of type D trichome heads and the middle and tip part of type C trichomes (Supplemental Fig. S1). NMR analysis of the trichome extracts from the transformed plants showed the presence of nicotine, glucose (α, β), fatty acids, and phaseoloidin. The fatty acid and sugar signals most likely result from the well-described *O*-acyl sugars present within trichomes and their exudates (Arrendale et al., 1990). A small amount of phaseoloidin, a β-glucoside of homogentisic acid, was isolated by HPLC from *N. attenuata* trichomes and the structure was unambiguously identified by 1D- and 2D NMR spectroscopy and LC-TOF-MS. ^1H and ^{13}C-NMR data are identical with those reported in the literature (Barua et al., 1988). We could not detect phaseoloidin in MeOH/H_2O extracts of entire leaves in our studies using NMR in positive nor negative mode LC-MS-TOF analysis. ^1H NMR of trichomes revealed a variable distribution of the metabolites within the trichomes. The nicotine: phaseoloidin ratio across all trichome types and segments within wild type plants of *N. attenuata* is approximately 2: 1 (Fig. 1). There was a clear difference in the amount of nicotine between the different type of trichomes as well as the different trichome tissues dissected (Fig. 2b). Nicotine was distributed in C Tip: C Middle: D Head in an approximate 4: 1: 1 ratio, as calculated by assuming the same water content for all cells and using the water signal for normalization. While the exact ratio varied across different measurements, the overall trend of higher nicotine contents in the tip and middle of type C trichomes remained constant across all measurements. In contrast to the pattern observed for nicotine, the concentration of phaseoloidin was equivalent in the middle and the tip of type C trichomes, where it was three times less than in the head of type D trichomes. The analysis of trichome extracts by LC-TOF-MS showed a slightly different pattern because the amount of phaseoloidin is, in contrast to the NMR- results, higher in the middle part of type C trichomes. Nevertheless the overall finding of lower amounts in type D trichomes was confirmed (Fig. 3a). The ratio of nicotine: phaseoloidin in typical samples was 3: 1 in the trichome heads of type D, 1: 1 in the middle and in 4: 1 tip segments (including heads) of type C trichomes. Additional experiments were conducted on wild type *N. attenuata* plants that revealed no difference in phaseoloidin content compared to the insertional mutant, irGAL83 (Supplemental Fig. S2). Interestingly there was as well a difference in the *O*-acyl sugar distribution between both types of trichomes. The aliphatic part of the NMR- spectra showed much more intense signals corresponding to sugars and fatty acids for type D heads than for type C middle and tip (Supplemental Fig. S3).

Isolation and Identification of Phaseoloidin Due to the fact that the amount of phaseoloidin extractable from the trichomes of *N. attenuata* was insufficient for a bioassay, preparative isolation was performed from the seeds of *Entada phaseolides*. The defatted ground seeds were extracted with aqueous ethanol. The alcoholic extract was partitioned against water, *n*-hexane, ethyl acetate, and *n*-butanol. The ethyl acetate was subjected to two successive column chromatography separations using silica gel followed by semi-preparative HPLC to yield pure phaseoloidin. The structure was identified by 1D- and 2D NMR spectroscopy and HR-TOFMS at *m/z* 329.087 [M-H],

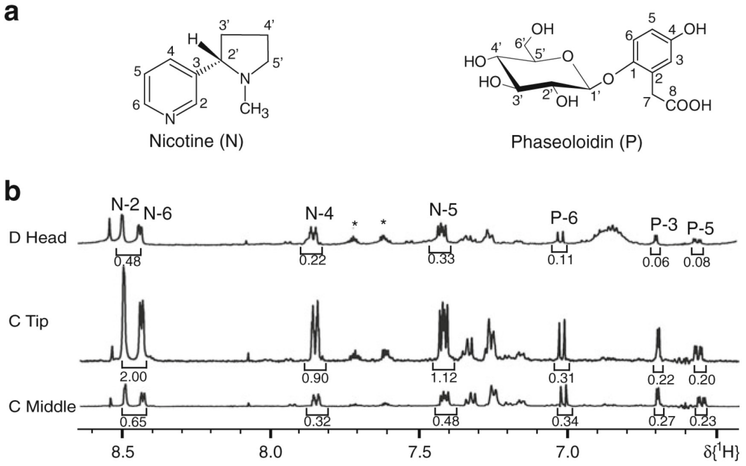


Fig. 2 **a** Structures of nicotine and phaseoloidin found in the two different trichome types of *Nicotiana attenuata*. The identity of phaseoloidin was verified by NMR and LC-ToFMS [M-H 329.0878] with a standard isolated from the seeds of *Entada phaseoloides*. **b** ^{1}H NMR spectra (500 MHz, MeOH-d_4) of head (type D), tip, and middle sections of extracts of tall trichomes (type C). Assuming the same water content of all trichome cells, the integral of the water signal (not shown) was used to determine the relative levels of phaseoloidin and nicotine in the middle and the tip (including the head) sections of type C trichomes and the head of type D trichomes. Typically the ratio of nicotine in sections C Tip: C Middle: D Head was approximately 4: 1: 1 The phaseoloidin concentrations are equivalent in the middle of type C and the head of type D trichomes, which, in turn, are three times less than in the tip of type C. Additional signals in the range of 7.4–7.1 marked with an *asterisk* (*) are from a contamination (phthalate)

corresponding to the molecular formula of $C_{14}H_{18}O_9$ (Fig. 3b, c). The ^{1}H and ^{13}C-NMR data are identical with those reported in the literature (Barua et al., 1988). The MS fragmentation pattern verified the previous results (Fig. 3b, c).

Phaseoloidin Decreases Caterpillar Growth We tested the effects of phaseoloidin and nicotine against the larvae of a generalist herbivore, *Spodoptera littoralis.* Because this insect is known be strongly affected by nicotine, we conducted an assay with this neurotoxin as a comparison. As trichomes are one of the first feeding choices of freshly hatched larvae (Weinhold and Baldwin, 2011), it is likely that trichome-derived compounds are ingested by the caterpillars. Both substances were added to an artificial diet at a final concentration of 0.16 µg mg^{-1}, a concentration approximately 100 times less than that found within the trichomes (Fig. 1). As expected, caterpillars showed decreases in growth of about 45% when fed a nicotine-containing diet. Interestingly they showed a similar decrease in growth when fed on phaseoloidin-containing diets. This response was marginally more pronounced (55% decrease) than the response to nicotine, although the differences were not statistically significant (Fig. 4).

The most damaging insect herbivore on *N. attenuata* frequently is the tobacco hornworm *M. sexta*, and we conducted the same bioassay with this insect species. In addition, we tested the aglycone of phaseoloidin, homogentisic acid. To examine possible synergistic effects of both trichome-derived compounds, we tested the combinations of nicotine and phaseoloidin and nicotine in combination with homogentisic acid. Similar to the response of the generalist, *M. sexta* larvae grew significantly less on diets with either phaseoloidin or nicotine (50 and 37% decreases in growth). Furthermore we observed the same decrease in caterpillar mass for the aglycone as well as for both combinations of phaseoloidin and the aglycone with nicotine (Fig. 4).

Discussion

Trichomes and especially trichome-derived compounds are defensive traits that protect plants from attacking herbivores or pathogens, and recent reports have shown that trichome cells are highly specialized in their ability to synthesize metabolites (Dai et al., 2010). Here, we showed that metabolites contributing to defense occur in *N. attenuata* trichomes in fairly high concentrations (1% of dry mass) and report the presence of a homogentisic acid glucoside, phaseoloidin, in two different trichome types of a Solanaceous plant. Phaseoloidin was found previously within the seeds of *Entada phaseoloides*, a putative function of this substance remained elusive even though Barua et al. (1988) showed a toxicity against fish at fairly high concentrations (125 mg ml^{-1}). Since phaseoloidin was not detectable in complete leaf extracts, we infer that this metabolite occurs primarily in the trichomes, at relatively high concentrations

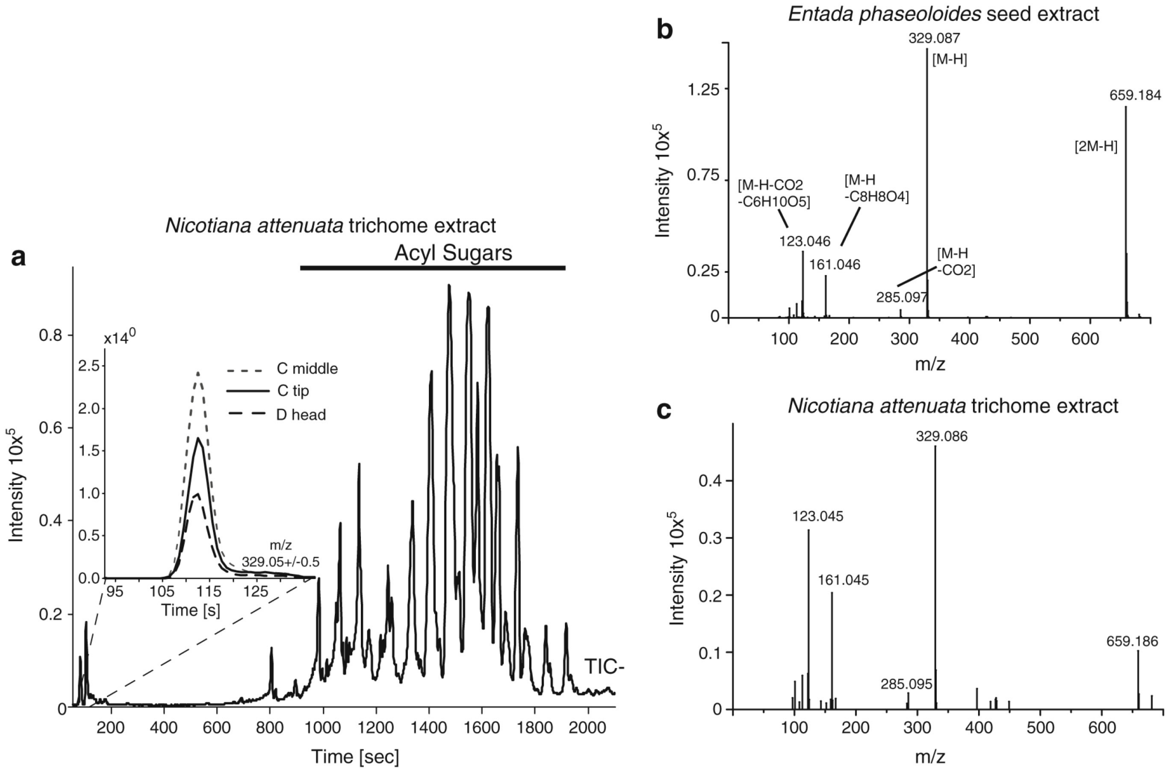


Fig. 3 **a** TIC- (negative total ion current) of a methanolic extract of excised type C and D trichomes from *Nicotiana attenuata*. The TIC is dominated by *O*-acyl sugars (MS spectra not shown) but the phaseoloidin and the differences in the amount between the different tissues can be clearly observed in the ion trace of 329.087 (Inset). **b** Negative ionization mass spectra of phaseoloidin from *Entada phaseoloides* seeds. [M-H] peak is clearly visible (*m/z* 329.087) as well as the loss of CO_2 from the carboxy group of the aglycone (*m/z* 285.097). The fragments at *m/z* 123.045 and *m/z* 161.045 originate from the cleavage of the glycosidic bond. **c** Negative ionization mass spectra of *N. attenuata* trichome extracts confirm the presence of phaseoloidin in this tissue

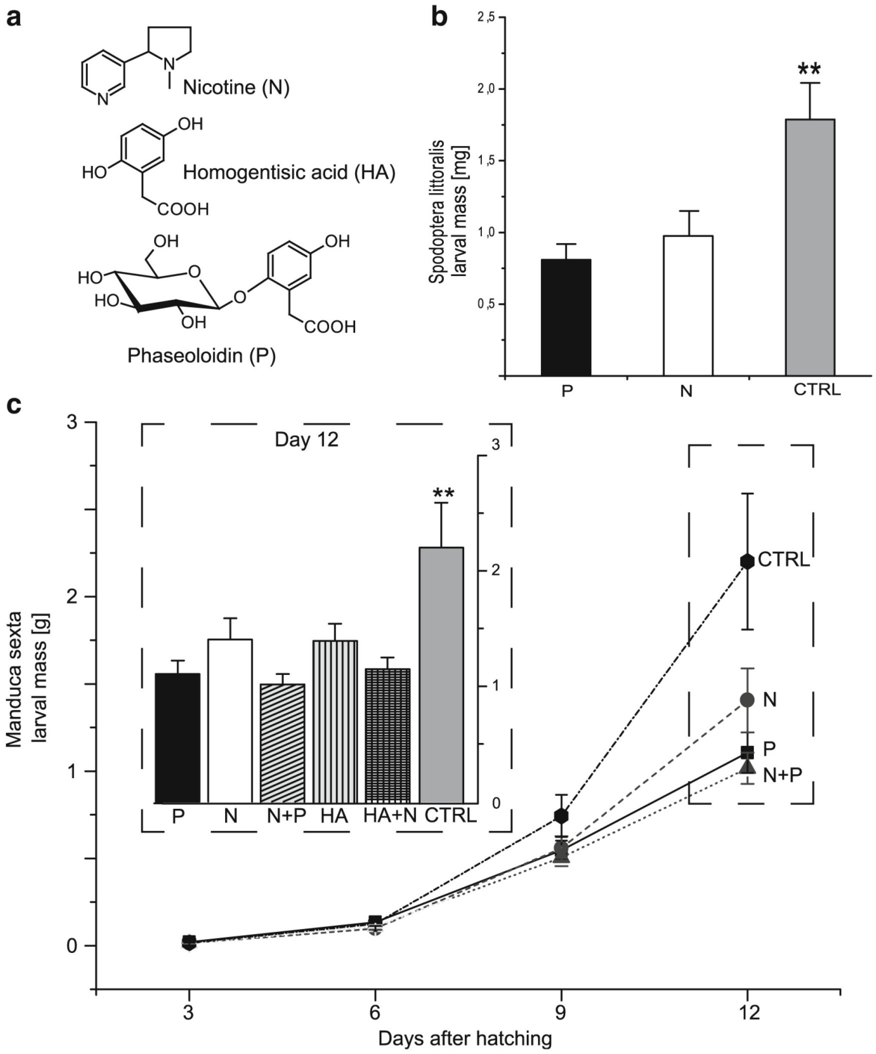
of approximately 10 µg mg^{-1} dry mass (Fig. 1). These findings suggest that phaseoloidin and nicotine occur in both trichome types present on *N. attenuata's* leaf surface.

The variation in the amounts of metabolites between both types of trichomes as well as among the different trichome parts may be due to variable cell numbers and size but could as well be a consequence of a different biosynthetic site for both metabolites. It is known that nicotine is produced within the roots (Shoji et al., 2000) of *Nicotiana* species upon attack (Baldwin, 1989) and then transported via xylem to leaves, flowers, and trichomes. This transport is mediated by a multidrug and toxic compound extrusion (MATE) transporter as shown for *Nicotiana tabacum* (Morita et al., 2009). In contrast, the biosynthetic site of the phaseoloidin remains unknown. Phaseoloidin is a glucoside of homogentisic acid, which is derived from phenylalanine or tyrosine via 4-hydroxyphenylpyruvate dioxygenase. This enzyme has been described for many plant systems, and so far has not been shown to be present in trichomes (Moran, 2005).

Since phaseoloidin could not be detected in leaf tissue, it most likely is synthesized in the trichomes. Thus, the presence of nicotine and phaseoloidin is an interesting case in which two metabolites differing in their origin are found in trichomes. This tissue type is considered to be highly specific, and there are reports of secondary metabolites specifically synthesized in the trichomes. Examples are the production of artemisinin within trichomes of *Artemisia annua* (Duke et al., 2000), cannabinoids in *Cannabis sativa* (Sirikantaramas et al., 2005), and as well the specific terpenoids in trichomes of *Solanum lycopersicon* (van Schie et al., 2007). Since recent reports show that MATE transporter might be involved in the transport of nicotine, the silencing of the transport of nicotine into the trichomes may show how phaseoloidin acts in the absence of nicotine.

Many trichome-derived compounds have been reported to play a defensive role against herbivores. Our artificial diet bioassays showed that this also holds true for phaseoloidin. This compound decreased caterpillar performance as much as the known defensive compound,

Fig. 4 **a** Structure of compounds used in the bioassay were nicotine (N), phaseoloidin (P) and its aglycone, homogentisic acid (HA). Compounds were dissolved in 5 ml methanol and mixed with freshly prepared artificial diet. As a control (CTRL) diet mixed with 5 ml pure methanol was used. Caterpillars of two different lepidopteran species were fed the amended artificial diet (Waldbauer et al., 1964) to which either nicotine or phaseoloidin in concentrations of $0.16\ \mu g\ mg^{-1}$ were added. Larval mass was measured after 8 day for *Spodoptera littoralis* **b** and after 12 day for *Manduca sexta*. Insert shows the larval mass of *M. sexta* after 12 day feeding on artificial diet **c**. Error bars indicate standard errors. *Asterisks* indicate significance levels according to a *Fischer PLSD* * <0.05 ** <0.01 *** <0.001



nicotine. We are aware that artificial diet assays do not reflect the true nature of a plant-herbivore interaction and that silencing the biosynthesis of the target compound would be a more elegant way to examine our hypothesis. Nevertheless, this experimental set-up allows us to study the influence of a single, highly abundant, metabolite without interference from other plant metabolites or proteins. The outcome of these assays shows clearly that phaseoloidin is active against Lepidopteran insects, and ingestion of this homogentisic acid glucoside significantly compromises the performance of generalist and specialist herbivores. Since the effect of phaseoloidin feeding was as pronounced as for the well-known defense metabolite, nicotine, we propose that phaseoloidin functions as a direct defense. From this finding, we hypothesize that diverse functionality of the two trichome types could additionally be responsible for the different ratios of nicotine and phaseoloidin. As has been shown previously, trichomes contribute to plant defense by exuding acyl sugars. These exudates are known to play a major part in plant defense against herbivores, e.g., tagging caterpillars with a distinct body odor that attracts predatory ants (Weinhold and Baldwin, 2011). Hence, it is not surprising that the phaseoloidin concentration is lowest in type D trichomes where the most acyl sugars are exuded (Supplemental Fig. S3). As a consequence, caterpillars may be not deterred by either phaseoloidin or nicotine and can thus feed more intensively on acyl sugars, which leads finally to their predation. In the case of the type C trichomes, the opposite situation occurs; the tips exude less acyl sugars than type D trichomes, but have 3–4 fold higher nicotine and phaseoloidin concentrations. We propose that in type C trichomes, these metabolites act synergistically as deterrents and toxins for attacking herbivores. In addition to their role in plant defenses, trichomes are known to play a role in the sequestration of phytotoxins (Sirikantaramas et al., 2008). Therefore, we cannot exclude the hypothesis that phaseoloidin might be a detoxification catabolite.

Glandular hairs or trichomes frequently function as physical obstacles and entrap insects by the exudation of glue-like substances, such as *O*-acyl sugars. We propose that caterpillars experience the deterrent effects of phaseoloidin when they navigate around these physical barriers on the leaf surface and become coated with trichome exudates or directly ingest trichome contents. Since phaseoloidin and its aglycone, homogentisic acid, had similar effects, it is possible that the aglycone, or a metabolic product, is itself the active substance. Further experiments with a bioassay-guided fractionation approach may highlight the important defensive function of this new metabolite in the Solanaceae.

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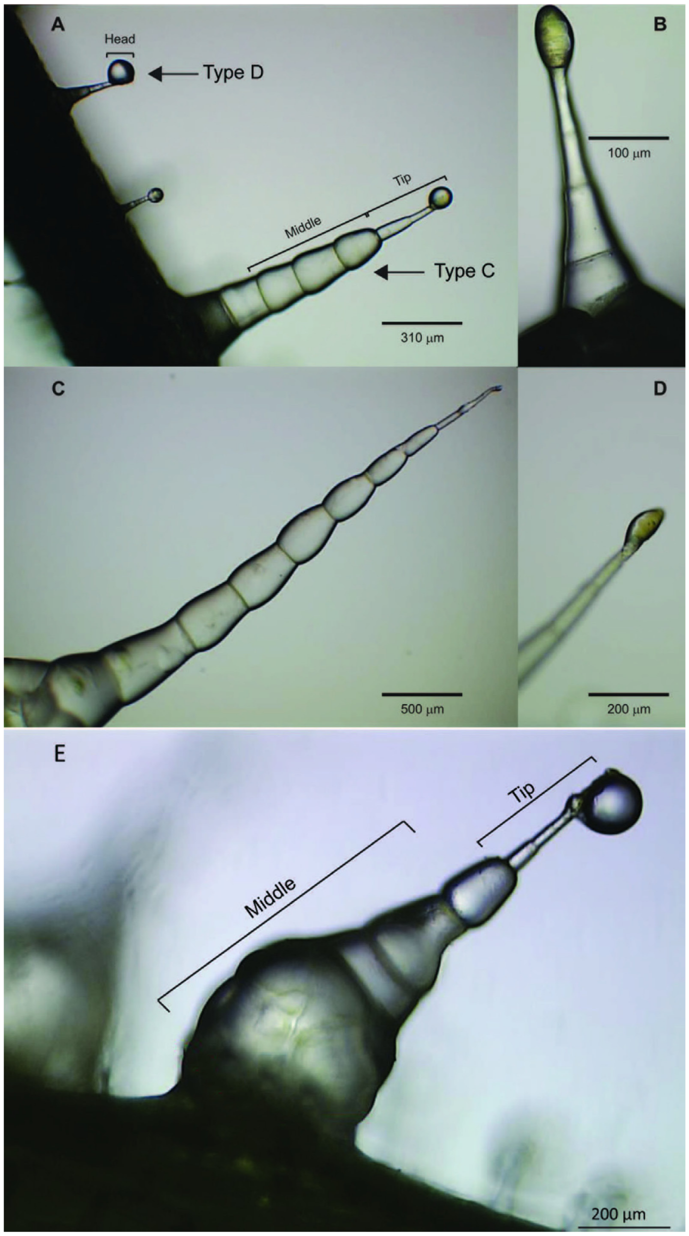
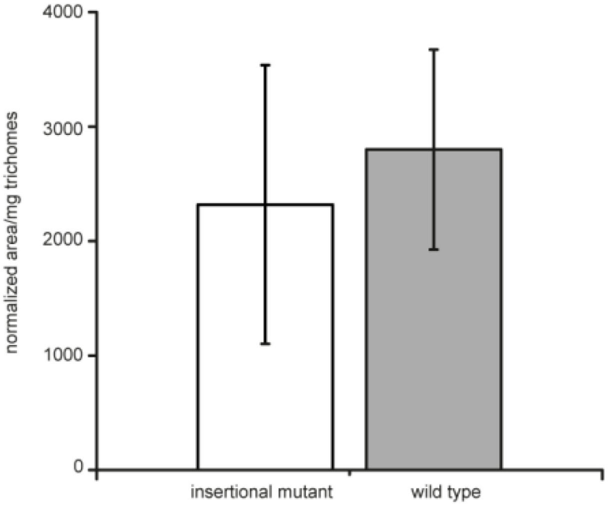
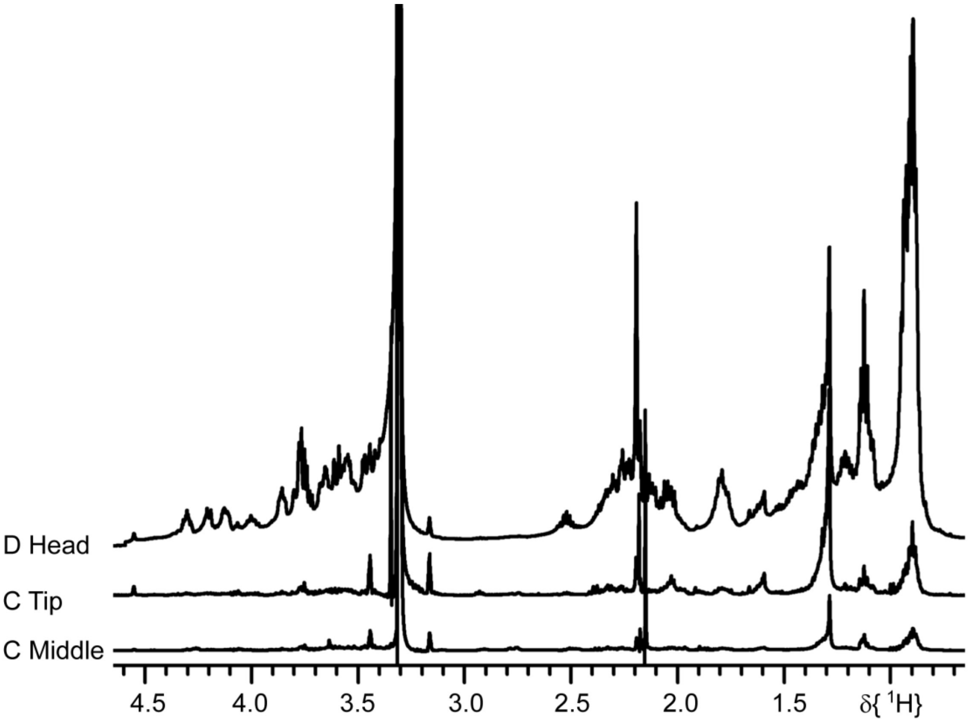
Supporting Information:



Supplemental Figure S1: Light micrographs of intact glandular trichomes of *Nicotiana attenuata* WT leaves. **A** Two different trichome types are found on the leaf surface, short trichomes with a stem consisting of two to four cells and a multi cellular head (type D), as well as tall trichomes with a multi cellular base, a stem (six to ten cells) and a small head (type C). The short trichomes are found mainly on the leaf surface whereas the tall ones are mostly located on the leaf veins shown in **C**, where they are even larger than on the surface. Magnification (200x) of a type D trichome **B** and of the head of a type C trichome (100×) **D** reveal green structures in the heads, most likely chloroplasts, as described for *N. tabacum*. **E** Trichomes of the insertional mutant irGAL83 are characterized by their enlarged basal cells



Supplemental Figure S2: Phaseoloidin contents in the trichomes of the insertional mutant irGAL83 and the *Nicotiana attenuata* wild type line do not differ. Two excised leaves were gently frozen over liquid nitrogen. The trichomes were harvested by gently scratching the leaf surface with a microscope cover slip. The plate was rinsed with 10 ml methanol. Afterwards the extract was concentrated to 1 ml under a constant flow of nitrogen. After centrifuging, the supernatant was transferred to HPLC vials. Phaseoloidin content was analyzed by LC-TOF-MS. Error bars indicate standard errors (WT *N* = 4; insertional mutant *N* = 3).



Supplemental Figure S3: Aliphatic part of the NMR spectra from the type C and D trichome samples. The spectrum of D Head contains much more sugar and fatty acid signals, belonging to acyl sugars, than the spectra of C Tip and C Middle.

Chapter 8: Appendix

Characterization of trichome derived *O*-acyl sugars of *Nicotiana attenuata*

Alexander Weinhold

Unpublished

In this Appendix I will show that the composition of *O*-acyl sugars in *Nicotiana attenuata* is even more complex than previously described (Manuscript I, II). Fractionation of crude plant extracts and subsequent HPLC-ToFMS analysis revealed the presence of at least 15 different compounds. In addition, I will show by MS^2 experiments that *O*-acyl sugars can be classified into three different structural classes and that different *O*-acyl sugar classes vary in their substitution pattern of the sucrose core molecule.

8.1. Introduction

The surface of plants (phylloplane) has attracted the interest of researchers for decades. The phylloplane is the location of the gas exchange necessary for photosynthesis. Its cuticular waxes protect the plant against insect attack as well as water loss, and epidermal extrusions, most notably trichomes, play important roles in direct and indirect defense against herbivores. These leaf hairs can act as simple mechanical obstacles, but may also contain defensive metabolites such as *O*-acyl sugars or sugar esters which are present in the trichomes of many species of the Solanaceae family **(Arrendale, et al. 1990, Slocombe, et al. 2008)**. *O*-acyl sugars have been described to be effective agents against soft-bodied insects like spider mites or whiteflies. In addition, they also influence the oviposition of *Heliothis virescens* **(Buta, et al. 1993, Jackson, et al. 1991, McKenzie, et al. 2004)**. They have been shown to be an important part of the indirect defenses of the wild tobacco *N. attenuata*, by tagging larvae of the tobacco hornworm with a distinct body odor upon ingestion **(Weinhold, et al. 2011)**. This body odor mainly consists of branched chain aliphatic acids (BCAAs) that are cleaved from a core sugar molecule. The identity of both the core sugar molecules, and the acid residues varies among different plant species. In tomato (*Solanum lycopersicon* and *Solanum pennellii*) and angel's trumpet (*Datura wrightii*) they mostly occur as glucose esters **(Van Dam, et al. 1998)**, while in tobacco species the core molecule is a sucrose moiety **(Kroumova, et al. 2003)**. The acids differ in structure and length of their aliphatic part. Because of their defensive properties described above, *O*-acyl sugars are highly valuable targets for the study of plant defenses and also interesting targets for plant engineering.

Studies of the effects of plant secondary metabolites on herbivores or pathogens rely on exact knowledge of their chemical structures, because the bioactivity of many defensive plant metabolites depends on their chemical conformation. Isomeric structures are of special importance in biology: often, either only one stereoisomer is active, or stereoisomers have different activities. For example, the emission of green leaf volatiles (GLVs) is an important indirect defense mechanism. Predators that are attracted to a plant respond to a specific ratio of different cis/trans isomers of these GLVs **(Allmann, et al. 2010)**. Especially in the field of pheromones, knowledge of structural and conformational identities is important, since many pheromones are only active as a specific stereoisomer. The bioactivity of direct defensive metabolites is determined by their stereochemistry, as well. The (S) - isomer of nicotine is a potent neurotoxin produced in many tobacco species, while the (R) - isomer is without any pathophysiological effect **(Abood, et al. 1978)**. In addition, only precise structural knowledge allows researchers to conduct reliable analytics on these compounds - including robust quantifications - and to draw conclusions about possible biosynthetic pathways or elucidate possible induction patterns **(Kallenbach, et al. 2009)**.

We analyzed the composition of the trichome exudates of *Nicotiana attenuata* for their *O*-acyl sugar constituents. In particular we focused on the substitution pattern of these metabolites. MS^2 experiments with fractions of extracted *O*-acyl sugars showed the presence of at least 15 different *O*-acyl sugars with three different patterns of substitution. These results will be highly valuable for the further investigation of these defensive metabolites and their biosynthetic pathways.

8.2. Material and methods

8.2.1 Plant material

We used an isogenic line of *Nicotiana attenuata* inbred for 31 generations from field-collected seeds. Seeds were germinated as described in **(Krügel, et al. 2002)**. All plants were grown in the glasshouse in individual 1 L pots at 26-28 °C under 16 h of light supplemented by Philips Sun-T Agro 400- or 600-W sodium lights (Philips, Turnhout, Belgium).

8.2.2 O-*Acyl sugar extraction*

We used a protocol similar to **(Van Dam, et al. 1998)**. *O*-Acyl sugars were collected by harvesting flowering *Nicotiana attenuata* plants. All plant parts except the flowers were harvested. The stem was cut into smaller pieces (approx. 10cm). Around 1 kg of tissue was combined in a 5 L glass beaker (Schott) and soaked in 3 L of chloroform for1h under stirring. Afterwards the plant tissue was removed and the solvent was dried over sodium sulfate (anhydrous Sigma Aldrich). The chloroform was removed in a rotary evaporator and the residue was resolved in acetonitrile (ACN) (VWR International) and sonicated. The ACN phase was partitioned 3 times against n-hexane (VWR International) (ACN: n-Hexane 1:2). The hexane phase was discarded and the ACN was removed in a rotary evaporator. The residue was solved in dichloromethane (DCM) (VWR) and partitioned 3 times against 1N tartaric acid (Sigma Aldrich) 2 times against distilled water. Afterwards the DCM was removed and the glue-like, brownish yellow residue was kept under argon at 4°C until further use.

8.2.3 O-Acyl sugar purification

Extracted *O*-acyl sugars were solved in acetonitrile at a concentration of 1mg/mL. We used an Agilent 1100 HPLC system equipped with a DAD detector. Separation was achieved on a preparative Luna 5n C18 column (250 x 10 mm, 5µm, Phenomenex) connected to a Luna 5n C-18 guard column (50 x 10 mm, 5 µm) with a mixture of deionized water (0.1 % [v/v] formic acid + 0.1% [v/v] ammonia) (solvent A) and methanol (solvent B) at a flow rate of 3 ml/min. We used an isocratic gradient with 80 % of solvent B for 20 min and then increased to 95% of solvent B in 5 min. The post-run time was 7 min. We collected fractions with a Foxy fraction collector (Isco) in 20 ml glass reaction tubes (Schott). 40 fractions of 30 s were cut starting 5 min. after injection. The fractions were transferred to scintillation vials and the solvent was evaporated in a vacuum centrifuge (Eppendorf). The single fractions were then analyzed for their content by injection in to a Bruker MicroToF LC system with conditions described in **(Weinhold, et al. 2011)**.

8.2.4 MS^2 Experiments

One microliter of each fraction was separated using a Dionex RSLC system (Dionex, Sunnyvale, USA) with a Dionex Acclaim RSLC 120 C-18 column (150 x 2.1 mm, 2.2 µm). The following binary gradient was applied: 0 to 1 min isocratic 90% A (deionized water, 0.1% [v/v] acetonitrile [Baker, HPLC grade], and 0.05% formic acid), 10% B (acetonitrile and 0.05%formic acid); 1 to 9 min linear gradient to 80% B; isocratic for 2 min. The flow rate was 400 µL/min. MS detection was carried out with a MicroToFQ ESI-qTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) operated in positive electrospray mode. Typical instrument settings were as follows: capillary voltage, 4500 V; dry gas temperature, 180 °C; dry gas flow, 10 L/min. Ions were detected from m/z 50 to 1400 at a repetition rate of 1 Hz. The instrument was operated in autoMS/MS mode. Mass calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid). Elemental formula and masses were calculated with the ACD/Labs 12 ChemSketch calculating tool (ACD/Labs, Frankfurt, Germany).

8.2.5. Analysis of O-acyl sugar acid composition

One mg of a crude *O*-acyl sugar mixture, obtained as described above, was saponified by adding 1 mL of a 0.2 M aqueous potassium hydroxide solution (Sigma Aldrich). The solution was sonicated and kept in a sealed vial for 24 hours. The mixture was neutralized by adding 1 mL of a 0.2 M hydrochloric acid solution and then partitioned against 1.5 mL dichloromethane (VWR). One μL of the dichloromethane phase was then injected into a Varian 3800 gas chromatograph equipped with a ZB-Wax-plus column (30m x 0.25mm x 0.25μm, Restek) and a flame ionization detector (Agilent). The injector temperature was set to 230°C and the flow was 1 mL/min (constant flow). The oven was kept at 40°C for 5 min, than heated to 185°C at a rate of 5°C/min and finally with a rate of 30°C/min to 250 °C. The FID was operated at 250°C with 25 mL/min make up gas flow and 30 mL/min hydrogen and 300 mL/min compressed air flow. The identities of the carboxylic acids were verified by the injection of authentic standards at a concentration of 50ng/μL. Retention indices were calculated in reference to an alkane standard mixture (C8-C20, Sigma Aldrich).

8.3. Results and discussion

8.3.1 Isolation of O-acyl sugars

The different *O*-acyl sugars of *N.attenuata* vary greatly in their abundance (**Fig. 8-1**). As previously shown **(Weinhold, et al. 2011)** there are at least 8 different *O*-acyl sugars that can be detected in the leaf washes of wild tobacco plants. They share the same core molecule, sucrose, but differ in their substitution pattern with different branched chain aliphatic acids. Since knowledge about the different substitution patterns might be highly valuable for the generation of further hypotheses regarding their ecological function, we decided to isolate the whole *O*-acyl sugar "community" present in *N. attenuata.* Out of approx. 1 kg (fresh mass) of plant tissue we could isolate 1.5 g of a crude *O*-acyl sugar mix, i.e. 1.5mg/g (FM). The abundance of these surface liquids is therefore comparable to the alkaloid nicotine that is also present in a range of 1 mg/g (FM) in the plant tissue, and HGL-DTGs (2.5 mg/g FM) **(Snook, et al. 1997)**. This makes *O*-acyl sugar one of the most abundant groups of secondary metabolites in *N.attenuata*.

This crude mix was then separated by preparative HPLC resulting in 30 fractions containing different *O*-acyl sugars. The separation did not resolve all *O*-acyl sugars, i.e., some fractions did contain more than one *O*-acyl sugar, and not all were present in a unique fraction. Nevertheless, we could obtain at least 14 fractions containing 3 or less *O*-acyl sugars suitable for further MS^2 experiments.

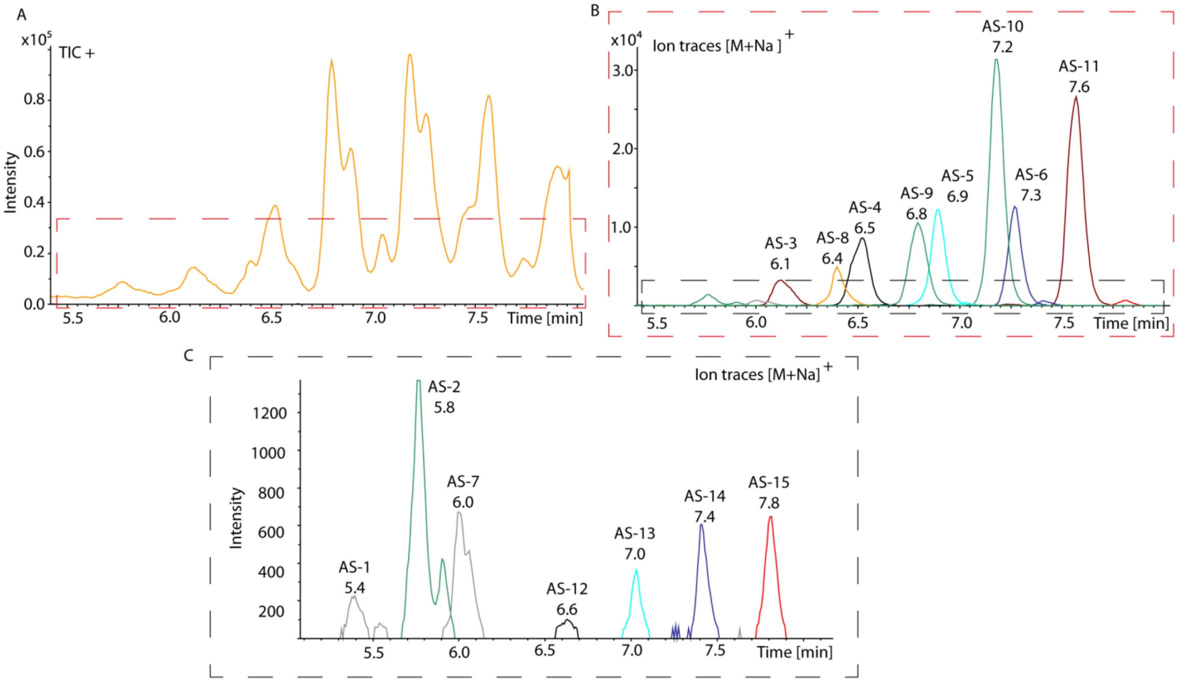


Figure 8-1: Overview of *O*-acyl sugars in *N. attenuata*. **A** Total ion current of a positive mode HPLC-ToFMS run (TIC+) from a whole leaf extract. **B** Single ion traces for the sodium adduct [M+Na]$^+$ of the different *O*-acyl sugars. **C** Single ion traces for the sodium adduct [M+Na]$^+$ of the different low abundant *O*-acyl sugars. Peak annotations are according to **Table 8-3;8-4;8-5**.

8.3.2 MS^2 reveals different acetylation patterns and fatty acid moieties

The single fractions were injected into a MicroTOFq (Bruker Daltronic, Bremen, Germany) system. The quadrupole – time-of flight mass spectrometer assembly allowed us to perform MS^2 experiments. Since the applied electrospray ionization conditions favored the formation of single sodium adducts we performed MS^2 experiments on the sodium adducts of the molecular ions $[M+Na]^+$. The CID-MS^2 spectra clearly showed two different kind of neutral losses, which could be attributed to the loss of a hexose (m/z 162.0528 $C_6H_{10}O_5$) and an acetylated hexose (m/z 204.0633 $C_8H_{12}O_6$) (**Fig. 8-2**).

In addition, the fragment peaks of an acetylated hexose (m/z 205.0707 $C_8H_{13}O_6^+$) and of the sodium adduct of a non-acetylated hexose (m/z 185.0420 $C_6H_{10}O_5Na^+$) could be observed in the spectra.

The identity of the hexose cannot be explained by the masses of the neutral losses. Since previous reports have shown that the core molecule of *O*-acyl sugars is a sucrose molecule **(Simonovska, et al. 2006)**, the observed peaks could correspond to either fructose or glucose. In addition, several branched aliphatic acids (BCAAs) have been described, which are esterified to the core molecule. **(Arrendale, et al. 1990)**have shown that the BCAAs are substituted to the glucose part of the *O*-acyl sugar.

We could also observe sodium adducts of tri- or tetra-ester hexoses e.g. m/z 469.2403 (AS 4/AS 10 **Fig. 8-2**) or m/z 469.2050 (AS 12 **Fig. 8-2**). Additionally there are no peaks present that one would expect from a mixed substitution pattern on both sugar moieties of the sucrose core molecule. Therefore it is most likely that the observed losses correspond to an either acetylated or non-acetylated fructose and branched chain carboxylic acids are bound to only one of the sugars, most likely the glucose. From these esterified sugars there are three neutral losses of different branched chain acids observable at m/z 116.0837 $C_6H_{12}O_2$, m/z 102.0681 $C_5H_{10}O_2$, and m/z 88.0525 $C_4H_8O_2$. The first mass corresponds to 3-methyl and 4-methyl pentanoic acid and the second mass to either 2-methyl or 3-methyl butanoic acid, as we had reported previously **(Weinhold, et al. 2011)**.

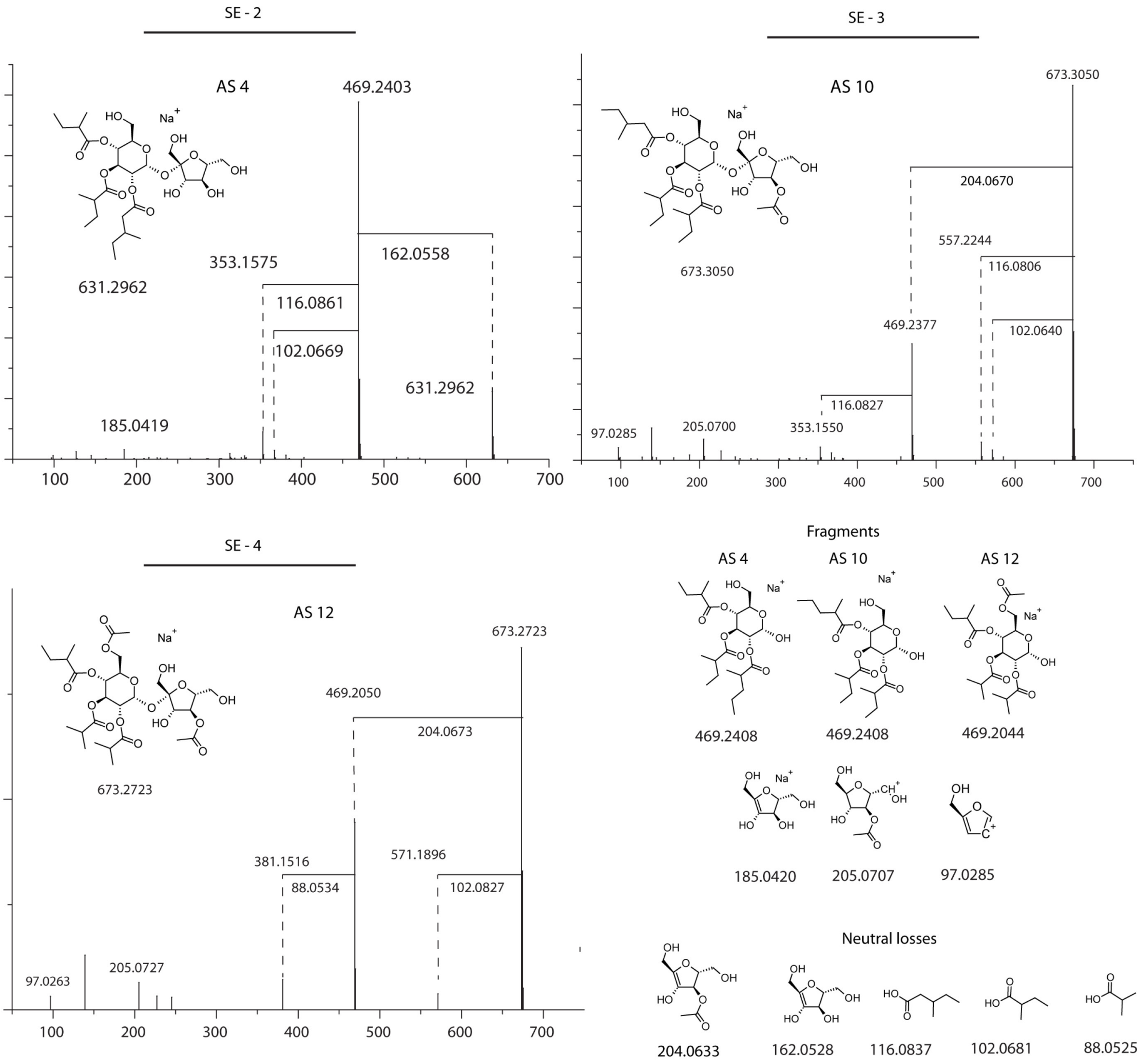


Figure 8-2: MS^2 spectra of 3 O-acyl sugars sharing the same fragment ion 469 (nominal mass) but belonging to two different classes according to Arrendale et al. 1990 (SE-2; SE-3) and one class described by **(Ding, et al. 2006)** (SE-4). Annotation of the main fragment peaks (Main peaks) and as well neutral losses. The position of the fatty acids could not be verified and may vary.

Table 8-1: MS^2 annotation of three O-acyl sugars sharing the same fragment ion 469 (nominal mass) but belonging to three different classes of which are according to **(Arrendale, et al. 1990)**(AS-4, AS-10) and one class described by **(Ding, et al. 2006)** (AS-12). The table shows the annotation of the *O*-acyl sugar, the monoisotopic mass and elemental formula of the each compound, and the elemental formula of the MS^2 fragments as well as their annotation, intensity of the mass peak, the observed m/z and calculated m/z ratios and the error in ppm.

ID	Monoistopoic mass El. Formula	Elemental Formula Fragments	Annotation	Intensity	Fragment Mass	Calc	Δ ppm
AS 4	608.3044 $C_{28}H_{48}O_{14}$	$C_{28}H_{48}O_{14}Na^+$	$[M+Na]^+$	18,98	631,2962	631,2926	3,6
		$C_{22}H_{38}O_9Na^+$	$[(M -C_6H_{10}O_5)+Na]^+$	100,00	469,2403	469,2408	0,5
		$C_{17}H_{28}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	2,54	367,1625	367,1727	10,2
		$C_{16}H_{26}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	7,66	353,1575	353,157	0,5
		$C_6H_{10}O_5Na^+$	$[(M -C_{22}H_{38}O_9)+Na]^+$	2,71	185,0419	185,042	0,1
AS 10	650.315 $C_{30}H_{50}O_{15}$	$C_{30}H_{50}O_{15}Na^+$	$[M+Na]^+$	100,00	673,305	673,3041	0,9
		$C_{25}H_{40}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	2,72	571,2365	571,2361	0,4
		$C_{24}H_{38}O_{13}Na^+$	$[(M - C_6H_{12}O_2)+Na]^+$	4,80	557,2244	557,2204	4
		$C_{22}H_{38}O_9Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	31,03	469,2377	469,2408	3,1
		$C_{17}H_{28}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	1,95	367,1767	367,1727	4
		$C_{16}H_{26}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	3,46	353,155	353,157	2
		$C_8H_{13}O_6^+$	$[M -C_{22}H_{37}O_9Na]^+$	5,67	205,07	205,0706	0,6
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3,36	97,0276	97,0285	0,9
AS 12	650.2786 $C_{29}H_{46}O_{16}$	$C_{29}H_{46}O_{16}Na^+$	$[M+Na]^+$	100,00	673,2723	673,2678	4,5
		$C_{25}H_{38}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	3,31	585,2072	585,2153	8,1
		$C_{24}H_{36}O_{14}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	4,42	571,1896	571,1997	10,1
		$C_{21}H_{34}O_{10}Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	51,73	469,205	469,2044	0,6
		$C_{17}H_{26}O_8Na^+$	$[(M -C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	8,37	381,1516	381,1519	0,3
		$C_8H_{13}O_6^+$	$[M -C_{21}H_{33}O_{10}Na]^+$	7,53	205,0727	205,0706	2,1
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3,72	97,0263	97,0285	2,2

The last loss of $C_4H_8O_2$ corresponds to either 2-methyl propanoic acid or butanoic acid. To confirm the fatty acid composition, we saponified the *O*-acyl sugars from a crude plant extract and subjected them to GC-FID analysis. Through comparison with the retention indices of authentic standards we could identify the four major fatty acids previously described **(Weinhold, et al. 2011)** (**Fig. 8-3** and **Table 8-2**). In addition, we identified 2-methyl-propanoic acid, which predicted from the neutral losses of m/z 88.0525 ($C_4H_8O_2$) in the MS^2 spectra.

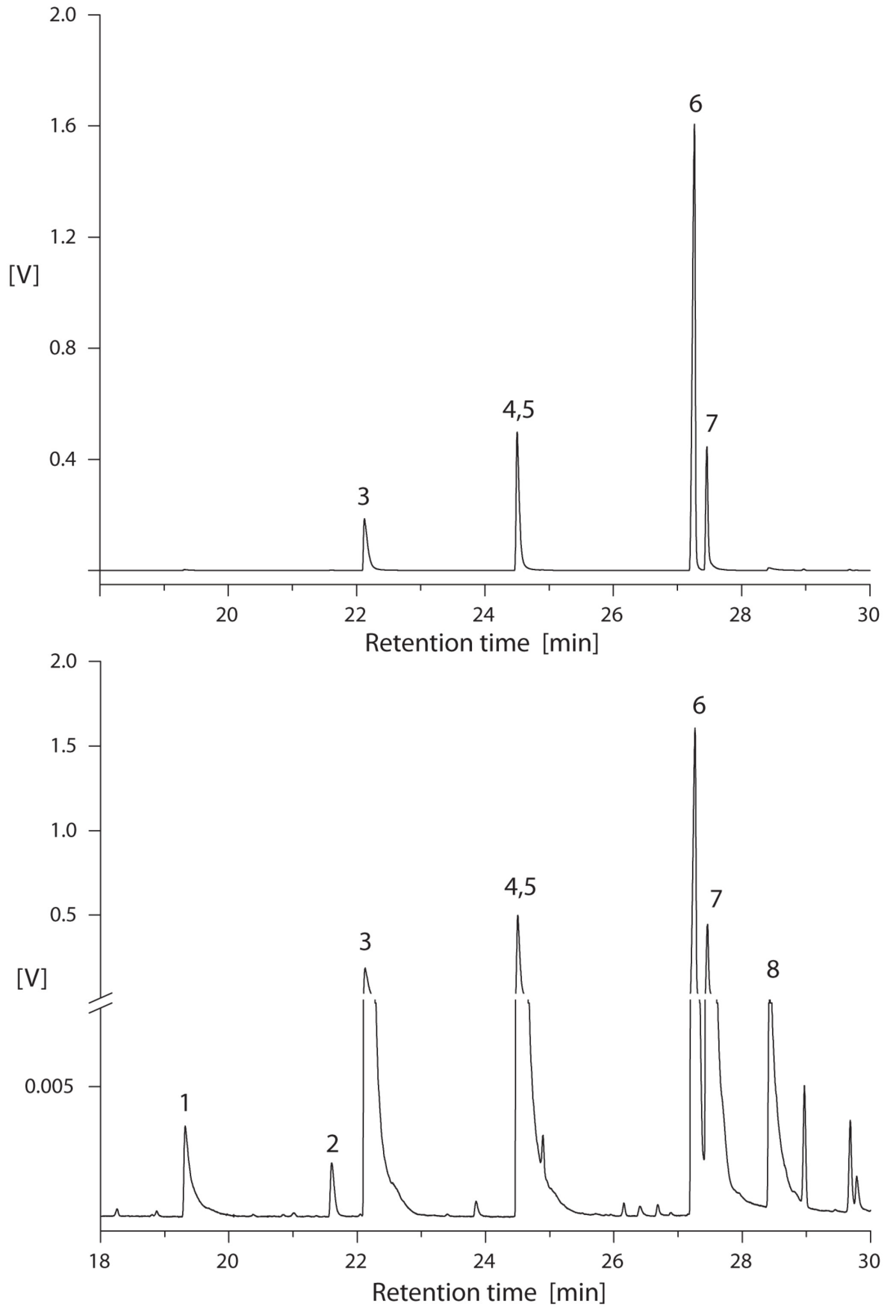


Figure 8-3: GC-FID run from a saponified crude extract of *N.attenuata O*-acyl sugars: Five major acids (upper part) were identified as 2-methyl propanoic acid **(3)**, 2-methyl butanoic acid **(4)**, 3-methyl butanoic acid **(5)**, 3-methyl pentanoic acid **(6)** and 4-methyl pentanoic acid **(7)**. Three less abundant acids (lower part) were identified as acetic acid **(1)**, propionic acid **(2)** and hexanoic acid **(8)**.

Table 8-2: BCAA composition of *N. attenuata O*-acyl sugars. Retention times and indices of authentic standards are compared to those found in the extract of saponified *O*-acyl sugars. The most abundant BCAAs are highlighted (bold).

		RT	RI	RT	RI
	Acid	Single Standards		extract hydrolysis	
1	acetic acid	19,386	1456	19,319	1453
2	propionic acid	21,538	1543	21,603	1546
3	**2-methyl propanoic acid**	22,281	1574	22,124	1567
4	**2-methy butanoic acid**	24,679	1678	24,501	1670
5	**3-methyl butanoic acid**	24,678	1678	24,501	1670
6	**3-methyl pentanoic acid**	27,393	1802	27,265	1796
7	**4-methyl pentanoic acid**	27,601	1813	27,46	1806
8	hexanoic acid	28,536	1859	28,426	1853

8.3.3 N. attenuata *O-acyl sugars belong to three different classes*

MS^2 experiments have shown that *O*-acyl sugars of *N. attenuata* can be classified into two groups according to **(Arrendale, et al. 1990)** (SE-2; SE-3) which were previously reported in *N. glutinosa,* and one group that was reported by Ding et al. in *Nicotiana tabacum* **(Ding, et al. 2006)** (SE-4). The latter contains four compounds (**Table 8-5, Fig. 8-5**) that are characterized by an acetylation on the fructose and glucose moiety and represent (*O*-acetyl-tri-*O*-acyl)-α-D-glucopyranosyl-(-*O*-acetyl)-β-D-fructofuranosides. The other two groups are classified according to Arrendale et al. as SE-2 (**Table 8-3, Fig. 8-3**) and SE-3 (**Table 8-4, Fig. 8-4**) acyl sugars. SE-2 is the biggest class, with six compounds, and not acetylated on the glucose or on the fructose moiety, which makes them (tri-*O*-acyl)-α-D-glucopyranosyl-β-D-fructofuranosides. The class SE -3 contains an acetylated fructose moiety resulting in six (tri-*O*-acyl- α-D-glucopyranosyl-(*O*-acetyl)-β-D-fructofuranosides.

8.3.4 Conclusion

The total number of different *O*-acyl sugars (15) shows how highly diverse this compound class is in *Nicotiana attenuata*. We have shown that *O*-acyl sugars can be divided into three different groups that differ in the acetylation on the glucose or fructose part. Although the ecological function of the different classes remains elusive, one could speculate that a larger number of acid substituents increases the lipophilicity of a particular compound. Since they are important leaf surface chemicals that are exuded by trichomes this might be advantageous in the exudation process. Deployed on the surface or the tips of trichomes *O*-acyl sugars release their defensive potential for the plant by being ingested by herbivores resulting in a specific insect body odor that attracts predators **(Weinhold, et al. 2011)**. Especially in the case of the tobacco hornworm *Manduca sexta* this body odor consists of the C_5 and C_6 acid that are clearly observable as neutral losses in the MS^2 spectra (**Fig. 8-2**). In this study we do report the presence of 2-methyl propanoic acid that was not observed in the insect body odor in our previous report. This discrepancy might be due to the low abundance of *O*-acyl sugars containing this substituent in the plant.

O-acyl sugars have been reported to directly affect attacking herbivores. **(Puterka, et al. 2003)** compared their effects to those of insecticidal soap. They argued that *O*-acyl sugar might destroy the membranes of soft-bodied insects and cause their death by desiccation.

According to this hypothesis, a higher lipophilicity might be as well advantageous and higher rate of acid substituents will increase the defensive function of *O*-acyl sugars. This hypothesis might be tested in the future by fraction-guided bioassays. A perquisite for such an approach will be the purification of the single *O*-acyl sugars. Since the reverse phase HPLC method applied in this study did not provide sufficient separation the phase system should be changed. Because *O*-acyl sugars represent a highly lipophilic compound class, normal phase HPLC system may provide sufficient separation to fractionate single compounds for use in bioassays.

8.4 Supporting Information

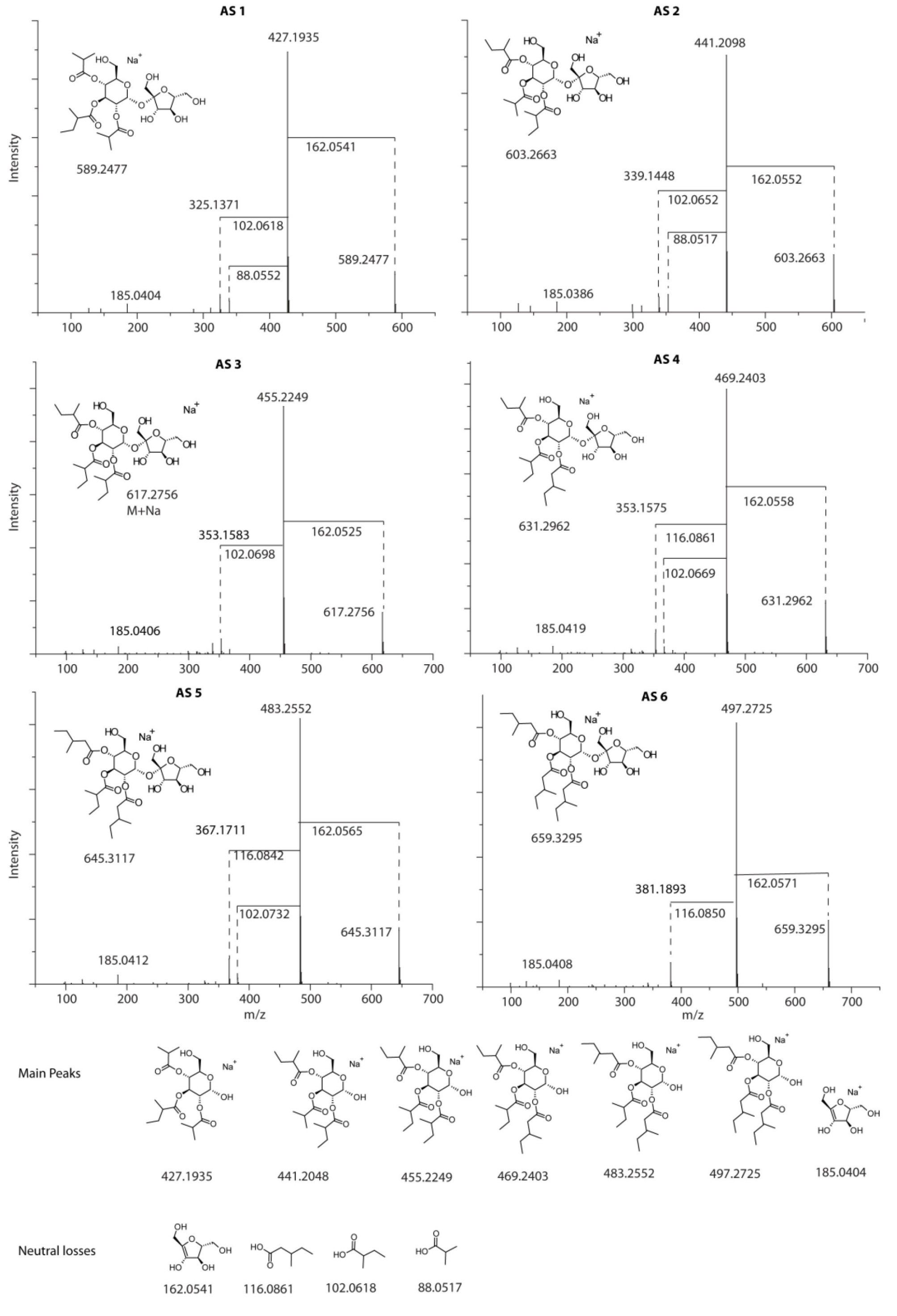


Figure 8-4: MS^2 spectra of class SE-2 O-acyl sugars class and annotation of the main fragment peaks (Main peaks) and neutral losses. The position of the BCAA could not be verified and may vary.

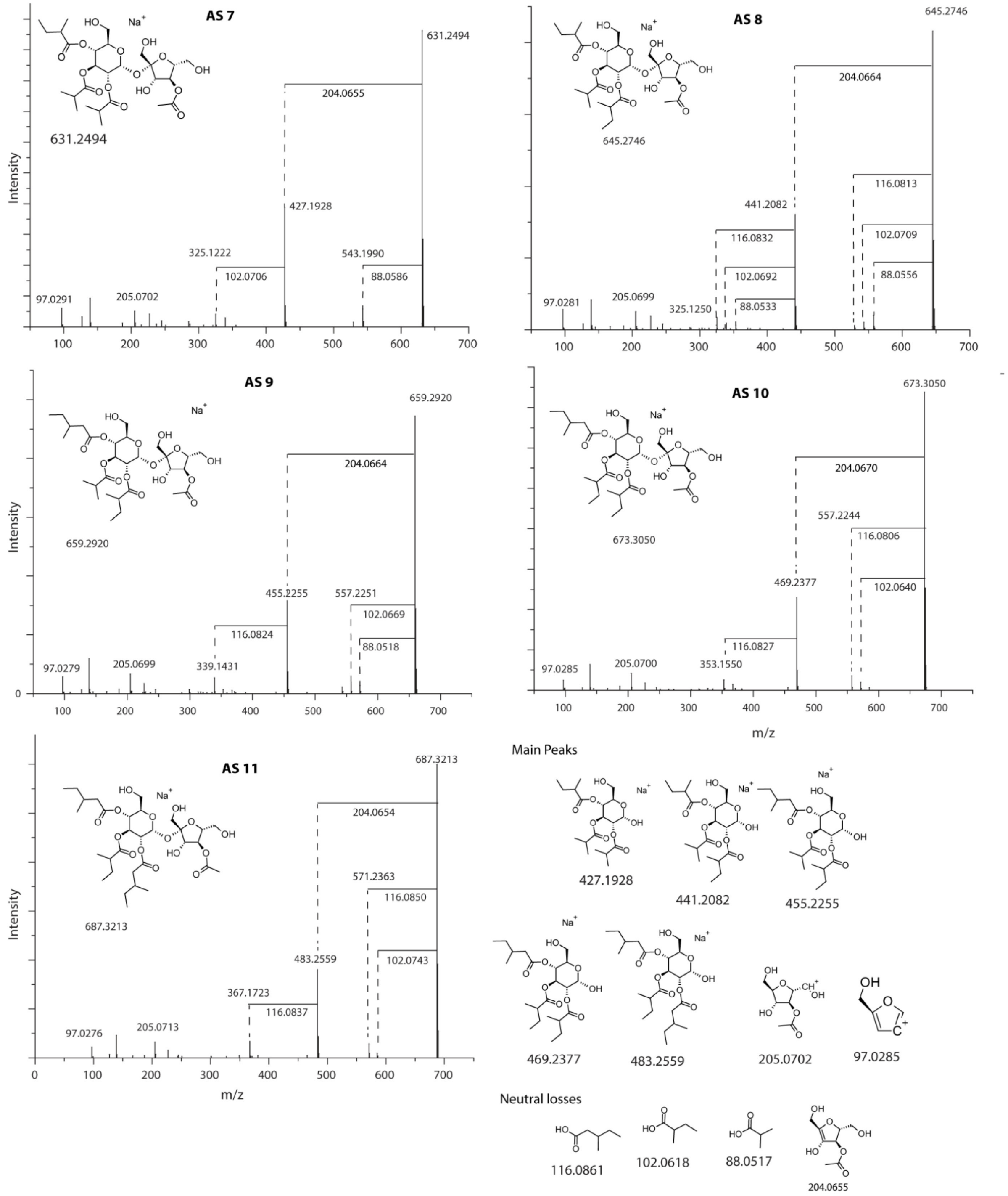


Figure 8-5: MS^2 spectra of class SE-3 O-acyl sugars and annotation of the main fragment peaks (Main peaks) and neutral losses. The position of the fatty acids could not be verified and may vary.

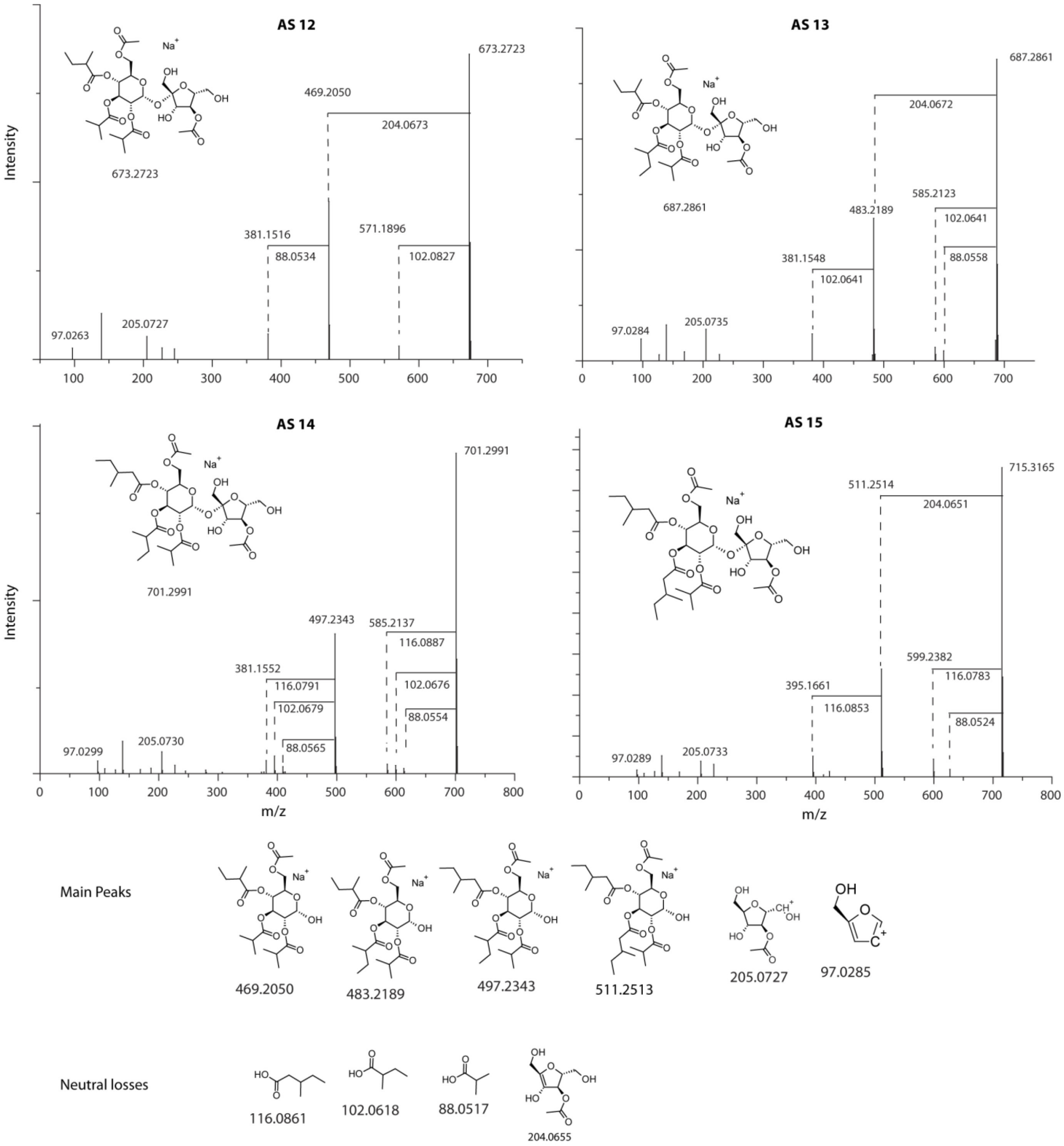


Figure 8-6: MS^2 spectra of class SE-4 O-acyl sugars and annotation of the main fragment peaks (Main peaks) and neutral losses. The position of the fatty acids could not be verified and may vary.

Table 8-3: MS^2- spectral data showing the annotation of class SE-2 *O*-acyl sugars elemental formulas, their calculated monoisotopic masses, the elemental formula of fragment ions, and their annotations, intensity and m/z values compared to the calculated m/z values.

ID	Monoistopoic mass El. Formula	Elemental Formula Fragments	Annotation	Intensity	m/z Fragment	m/z Calc	Δ ppm
AS 1	566.2926 $C_{25}H_{42}O_{14}$	$C_{25}H_{42}O_{14}Na^+$	$[M+Na]^+$	14,68	589,2477	589,2467	1
		$C_{19}H_{32}O_9Na^+$	$[(M - C_6H_{10}O_5)+Na]^+$	100,00	427,1935	427,1938	0,3
		$C_{15}H_{24}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_4H_8O_2)+Na]^+$	4,36	339,1383	339,1414	3,1
		$C_{14}H_{22}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	5,88	325,1317	325,1257	6
		$C_6H_{10}O_5Na^+$	$[(M - C_{19}H_{32}O_9)+Na]^+$	3,33	185,0404	185,042	1,6
AS 2	580.2731 $C_{26}H_{44}O_{14}$	$C_{26}H_{44}O_{14}Na^+$	$[M+Na]^+$	100,00	603,2663	603,2623	4
		$C_{20}H_{34}O_9Na^+$	$[(M - C_6H_{10}O_5)+Na]^+$	33,88	441,2048	441,2095	4,7
		$C_{15}H_{24}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_4H_8O_2)+Na]^+$	7,52	353,1583	353,1571	1,2
		$C_{15}H_{24}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	6,58	339,1448	339,1414	3,4
		$C_6H_{10}O_5Na^+$	$[(M - C_{20}H_{34}O_9)+Na]^+$	4,36	185,0404	185,042	1,6
AS 3	594.2887 $C_{27}H_{46}O_{14}$	$C_{27}H_{46}O_{14}Na^+$	$[M+Na]^+$	16,82	617,2756	617,2779	2,3
		$C_{21}H_{36}O_9Na^+$	$[(M - C_6H_{10}O_5)+Na]^+$	100,00	455,2249	455,2251	0,2
		$C_{17}H_{28}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_4H_8O_2)+Na]^+$	1,83	367,1745	367,1727	1,8
		$C_{16}H_{26}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	6,13	353,1538	353,157	3,2
		$C_{15}H_{24}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	4,30	339,1365	339,1414	4,9
		$C_6H_{10}O_5Na^+$	$[(M - C_{21}H_{36}O_9)+Na]^+$	2,88	185,0406	185,042	1,4
AS 4	608.3044 $C_{28}H_{48}O_{14}$	$C_{28}H_{48}O_{14}Na^+$	$[M+Na]^+$	18,98	631,2962	631,2926	3,6
		$C_{22}H_{38}O_9Na^+$	$[(M - C_6H_{10}O_5)+Na]^+$	100,00	469,2403	469,2408	0,5
		$C_{17}H_{28}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	2,54	367,1625	367,1727	10,2
		$C_{16}H_{26}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	7,66	353,1575	353,157	0,5
		$C_6H_{10}O_5Na^+$	$[(M - C_{22}H_{38}O_9)+Na]^+$	2,71	185,0419	185,042	0,1
AS 5	622.3201 $C_{29}H_{50}O_{14}$	$C_{29}H_{50}O_{14}Na^+$	$[M+Na]^+$	20,23	645,3117	645,3093	2,4
		$C_{23}H_{40}O_9Na^+$	$[(M - C_6H_{10}O_5)+Na]^+$	100,00	483,2553	483,2564	1,1
		$C_{18}H_{30}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	2,33	381,1848	381,1883	3,5
		$C_{17}H_{28}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	9,52	367,1711	367,1727	1,6
		$C_6H_{10}O_5Na^+$	$[(M - C_{23}H_{40}O_9)+Na]^+$	3,42	185,0412	185,042	0,8
AS 6	636.3249 $C_{30}H_{52}O_{14}$	$C_{30}H_{52}O_{14}Na^+$	$[M+Na]^+$	25,23	659,324	659,3249	0,9
		$C_{24}H_{42}O_9Na^+$	$[(M - C_6H_{10}O_5)+Na]^+$	100,00	497,2725	497,2721	0,4
		$C_{18}H_{30}O_7Na^+$	$[(M - C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	9,24	381,1893	381,1883	1
		$C_6H_{10}O_5Na^+$	$[(M - C_{24}H_{42}O_9)+Na]^+$	2,54	185,0408	185,042	1,2

Table 8-4: MS^2- spectral data showing the annotations of class SE-3 *O*-acyl sugars elemental formulas, their calculated monoisotopic masses, the elemental formula of fragment ions, their annotations, intensity and m/z values compared to the calculated m/z values.

ID	Monoistopoic mass El. Formula	Elemental Formula Fragments	Annotation	Intensity	m/z Fragment	m/z Calc	Δ ppm
AS 7	608.2680 $C_{27}H_{44}O_{15}$	$C_{27}H_{44}O_{15}Na^+$	$[M+Na]^+$	100,00	631,2554	631,2572	1,8
		$C_{23}H_{36}O_{13}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	5,99	543,199	543,2048	5,8
		$C_{19}H_{32}O_9Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	40,35	427,1928	427,1939	1,1
		$C_{14}H_{22}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	4,27	325,1222	325,1258	3,6
		$C_8H_{13}O_6^+$	$[M - C_{19}H_{31}O_9Na]^+$	5,32	205,0702	205,0706	0,4
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	6,32	97,0291	97,0285	0,6
AS 8	622.2837 $C_{28}H_{46}O_{15}$	$C_{28}H_{46}O_{15}Na^+$	$[M+Na]^+$	100,00	645,2746	645,2729	1,7
		$C_{24}H_{38}O_{13}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	4,83	557,219	557,2204	1,4
		$C_{23}H_{36}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	2,55	543,2058	543,2048	1
		$C_{22}H_{34}O_{13}Na^+$	$[(M - C_6H_{12}O_2)+Na]^+$	1,48	529,1925	529,1891	3,4
		$C_{20}H_{34}O_9Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	38,54	441,2082	441,2095	1,3
		$C_{16}H_{26}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	1,57	353,1538	353,157	3,2
		$C_{15}H_{24}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	2,22	339,1365	339,1414	4,9
		$C_{14}H_{22}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	4,11	325,125	325,1258	0,8
		$C_8H_{13}O_6^+$	$[M - C_{20}H_{33}O_9Na]^+$	6,09	205,0699	205,0706	0,7
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	6,79	97,0281	97,0285	0,4
AS 9	636.2993 $C_{29}H_{48}O_{15}$	$C_{29}H_{48}O_{15}Na^+$	$[M+Na]^+$	100,00	659,292	659,2885	3,5
		$C_{25}H_{40}O_{13}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	2,92	571,2342	571,2361	1,9
		$C_{24}H_{38}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	3,64	557,2251	557,2204	4,7
		$C_{23}H_{36}O_{13}Na^+$	$[(M - C_6H_{12}O_2)+Na]^+$	2,38	543,2059	543,2048	1,1
		$C_{21}H_{36}O_9Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	33,45	455,2255	455,2251	0,4
		$C_{17}H_{28}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	1,14	367,1748	367,1727	2,1
		$C_{16}H_{26}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	1,41	353,1542	353,157	2,8
		$C_{15}H_{24}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	5,64	339,1431	339,1414	1,7
		$C_8H_{13}O_6^+$	$[M - C_{21}H_{35}O_9Na]^+$	7,08	205,0699	205,0706	0,7
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	6,08	97,0279	97,0285	0,6

Table 8-4 continued:

ID	Monoistopoic mass El. Formula	Elemental Formula Fragments	Annotation	Intensity	m/z Fragment	m/z Calc	Δ ppm
AS 10	650.315 $C_{30}H_{50}O_{15}$	$C_{30}H_{50}O_{15}Na^+$	$[M+Na]^+$	100,00	673,305	673,3041	0,9
		$C_{25}H_{40}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	2,72	571,2365	571,2361	0,4
		$C_{24}H_{38}O_{13}Na^+$	$[(M - C_6H_{12}O_2)+Na]^+$	4,80	557,2244	557,2204	4
		$C_{22}H_{38}O_9Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	31,03	469,2377	469,2408	3,1
		$C_{17}H_{28}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	1,95	367,1767	367,1727	4
		$C_{16}H_{26}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	3,46	353,155	353,157	2
		$C_8H_{13}O_6^+$	$[M - C_{22}H_{37}O_9Na]^+$	5,67	205,07	205,0706	0,6
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3,36	97,0276	97,0285	0,9
AS 11	664.3306 $C_{31}H_{52}O_{15}$	$C_{31}H_{52}O_{15}Na^+$	$[M+Na]^+$	100,00	687,3213	687,3198	1,5
		$C_{26}H_{42}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	1,69	585,2495	585,2517	2,2
		$C_{25}H_{40}O_{13}Na^+$	$[(M - C_6H_{12}O_2)+Na]^+$	4,84	571,2363	571,2361	0,2
		$C_{23}H_{40}O_9Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	30,06	483,2559	483,2565	0,6
		$C_{17}H_{28}O_7Na^+$	$[(M - C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	5,62	367,1723	367,1727	0,4
		$C_8H_{13}O_6^+$	$[M - C_{23}H_{39}O_9Na]^+$	5,41	205,0713	205,0706	0,7
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3,72	97,0285	97,0285	0

Table 8-5: MS^2- spectral data showing the annotations of class SE-4 *O*-acyl sugars elemental formulas, their calculated monoisotopic masses, the elemental formula of fragment ions, and their annotations, intensity and m/z values compared to the calculated m/z values.

ID	Monoistopoic mass El. Formula	Elemental Formula Fragments	Annotation	Intensity	Fragment Mass	Calc	Δ ppm
AS 12	650.2786 $C_{29}H_{46}O_{16}$	$C_{29}H_{46}O_{16}Na^+$	$[M+Na]^+$	100,00	673,2723	673,2678	4,5
		$C_{25}H_{38}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	3,31	585,2072	585,2153	8,1
		$C_{24}H_{36}O_{14}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	4,42	571,1896	571,1997	10,1
		$C_{21}H_{34}O_{10}Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	51,73	469,205	469,2044	0,6
		$C_{17}H_{26}O_8Na^+$	$[(M - C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	8,37	381,1516	381,1519	0,3
		$C_8H_{13}O_6^+$	$[M - C_{21}H_{33}O_{10}Na]^+$	7,53	205,0727	205,0706	2,1
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3,72	97,0263	97,0285	2,2
AS 13	664.2942 $C_{30}H_{48}O_{16}$	$C_{30}H_{48}O_{16}Na^+$	$[M+Na]^+$	100,00	687,2861	687,2835	2,6
		$C_{26}H_{40}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	3,36	599,2303	599,231	0,7
		$C_{25}H_{38}O_{14}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	4,52	585,2123	585,2153	3
		$C_{22}H_{36}O_{10}Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	47,27	483,2189	483,2201	1,2
		$C_{17}H_{26}O_8Na^+$	$[(M - C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	8,99	381,1548	381,1519	2,9
		$C_8H_{13}O_6^+$	$[M - C_{22}H_{35}O_{10}Na]^+$	10,54	205,0735	205,0706	2,9
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	7,30	97,0284	97,0285	0,1
AS 14	678.3098 $C_{31}H_{50}O_{16}$	$C_{31}H_{50}O_{16}Na^+$	$[M+Na]^+$	100,00	701,2973	701,2991	1,8
		$C_{27}H_{42}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	1,58	613,2455	613,2467	1,2
		$C_{26}H_{40}O_{14}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	2,58	599,2348	599,231	3,8
		$C_{25}H_{38}O_{14}Na^+$	$[(M - C_6H_{12}O_2)+Na]^+$	2,90	585,2137	585,2153	1,6
		$C_{23}H_{38}O_{10}Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	43,62	497,2343	497,2357	1,4
		$C_{19}H_{30}O_8Na^+$	$[(M - C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	2,15	409,1778	409,1832	5,4
		$C_{18}H_{28}O_8Na^+$	$[(M - C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	5,48	395,1664	395,1676	1,2
		$C_{17}H_{26}O_8Na^+$	$[(M - C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	4,11	381,1552	381,152	3,2
		$C_8H_{13}O_6^+$	$[M - C_{23}H_{37}O_{10}Na]^+$	6,83	205,0703	205,0706	0,3
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3,96	97,0299	97,0285	1,4
AS 15	692.3225 $C_{32}H_{52}O_{16}$	$C_{32}H_{52}O_{16}Na^+$	$[M+Na]^+$	100,00	715,3165	715,3148	1,7
		$C_{28}H_{44}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	2,46	627,2641	627,2623	1,8
		$C_{26}H_{40}O_{14}Na^+$	$[(M - C_6H_{12}O_2)+Na]^+$	5,74	599,2382	599,231	7,2
		$C_{24}H_{40}O_{10}Na^+$	$[(M - C_8H_{12}O_6)+Na]^+$	34,86	511,2514	511,2513	0,1
		$C_{18}H_{28}O_8Na^+$	$[(M - C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	6,68	395,1661	395,1676	1,5
		$C_8H_{13}O_6^+$	$[M - C_{24}H_{39}O_{10}Na]^+$	5,07	205,0733	205,0706	2,7
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	2,24	97,0289	97,0285	0,4

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Chapter 9: Summary and discussion

Trichomes have long been considered to be the first line of defense for many plant species. But only a more rigorously insight into the true chemical nature of their metabolites reveals the mechanisms behind trichome-based plant defense. In this chapter I will summarize the results of the previous chapters and discuss their impact on our understanding of the role of trichomes in the defense of the wild tobacco *Nicotiana attenuata* against the tobacco hornworm *Manduca sexta*.

9.1 Trichome exudates in plant defense (Manuscript I, II)

The trichomes of *N. attenuata* secrete viscous liquids to the plant surface. As we have shown, these droplets and liquids primarily consist of *O*-acyl sugars. These compounds are composed of a sucrose core molecule, which is esterified on the free hydroxyl groups by branched chain aliphatic acids (BCAAs). Through the HPLC-ToFMS analysis of methanolic leaf washes from *N. attenuata,* we showed that *O*-acyl sugars are the dominant leaf surface chemical in this plant species (**Manuscript I, Fig. 1**). We confirmed previously described molecular weights of these molecules **(Ding, et al. 2007),** taking advantage of the high mass accuracy ToFMS provides. Furthermore, we identified the BCAA residues linked with an ester bond to the sucrose core molecule. This was achieved using two-dimensional comprehensive gas chromatography coupled to ToF-MS (GCxGC-ToFMS). Crude extracts of *O*-acyl sugars were purified and then subjected to alkaline hydrolysis. Analysis of the hydrolysis products revealed the presence of four major compounds. These compounds were identified as 2-methyl and 3-methyl butanoic acid and 3-methyl and 4-methyl pentanoic acid. The identities were verified by the injection of authentic standards and the comparison of retention times as well as mass spectra.

These four BCAAs are characterized by a distinct smell. During their analysis we realized that this specific odor is similar to the body odor that larvae of the tobacco hornworm *Manduca sexta* emit when feeding on *N. attenuata*.

To demonstrate that the insect body odor could be due to BCAA residues hydrolyzed from *O*-acyl sugars during the feeding process, we trapped the headspace of larvae feeding on *N. attenuata* and showed that the compounds emitted from the larval frass, as well as from the larvae itself, were identical to those found in the *O*-acyl sugar hydrolysis products.

In addition, we collected the headspace of larvae feeding on an *O*-acyl sugar free diet and found that, in the absence of *O*-acyl sugars, the body odor lacks its BCAA components. Since these BCAAs are released from the sucrose via alkaline hydrolysis, we propose that *O*-acyl sugars are cleaved in the alkaline milieu (pH 8) of the caterpillar gut **(Martin, et al. 1987b)**. This process is occurs quickly, and the release of BCAAs from the caterpillars increases about 2 hours after they started feeding (**Manuscript I, Fig. 2 E**). Because insect predators and parasitoids use volatile cues to locate their prey or host **(Herard, et al. 1988)**, the caterpillar's body odor might be a fast and specific signal to attract them. Hence, we hypothesized that this particular body odor is used by predators of *M. sexta* as well. We quantified the amounts of BCAAs in the insect body and frass odor and used synthetic standards in these concentrations for a series of experiments in *N. attenuata's* native habitat, where we demonstrated that omnivorous ants of the species *Pogonomyrmex rugosus* follow BCAA cues to prey on *M.sexta* larvae.

During our field studies, we frequently found that large numbers of late-instar *M. sexta* caterpillars disappeared from the field sites (**Manuscript II, Fig. 1**). Since larvae of this stage are too big to be predated by either ants or other known insect predators, like the big-eyed bug *Geocoris spp.* **(Allmann, et al. 2010)**, we hypothesized that lizards, native to the field site, are also active predators of *M. sexta*. We demonstrated in a series of bioassays, that ground-foraging lizards are attracted by BCAA cues. In small field assays, we observed that the lizards respond to these BCAAs cues (**Manuscript II, Fig 2**) and we videotaped predation of *M. sexta* larvae by the lizards. These results indicate the importance of *O*-acyl sugars in the defensive arsenal of *N. attenuata*, because they affect the mortality of *M.sexta* larvae in every developmental stage.

9.2. Trichome constituents in plant defense (Manuscript III):

O-acyl sugars are secreted to the leaf surface, but other trichome constituents are not exuded and instead stored within the leaf hairs. The literature about trichomes provides various examples for chemicals that are produced and stored within these special structures. The chemical diversity of these metabolites is enormous and ranges from sesquiterpenes and monoterpenes to alkaloids of different kinds **(Kennedy 2003, Laue, et al. 2000, van Schie, et al. 2007)**. The composition of these components within *N. attenuata* trichomes is rarely known; therefore it is of interest to elucidate their entire chemical profile. Since the harvesting of trichomes in considerable amounts for extraction and analysis is a difficult task, there is a need for an analytical method that can deliver sufficient resolution with low amounts of tissue.

Nuclear magnetic resonance (NMR) spectroscopy allows the non-destructive analysis of small amounts of samples and robust quantification of chemical constituents. Especially in the case of trichomes, NMR holds the advantage of introducing unprocessed tissue samples to the measurement. Hence, one can avoid extraction procedures that will come along with a potential loss of substance and low-abundance metabolites. In order to characterize the chemical composition of trichomes, we distinguished between the two different trichome types present on *N. attenuata,* the large type C and the smaller type D trichomes (**Manuscript III, Fig. S1**). When subjected to ^{1}H-NMR, the signals of nicotine were identified in the spectra and used for quantification (**Manuscript III Fig.2**). We could also detect large signals in the aliphatic region (**Manuscript III Fig.S3**), most likely caused by the BCAAs residues of *O*-acyl sugars. Moreover, we observed proton signals in the range of 6.5 – 7.0 ppm that could be attributed to homogentisic acid (2,5–Dihydroxyphenylacetic acid) glucoside (phaseoloidin). Furthermore, we showed that there were differences in the abundance of nicotine and phaseoloidin in the different trichome types analyzed. This was the first time that this compound was reported in a Solaneceous plant, since it was described before only in the seeds of *Entada phaseoloides* **(Barua, et al. 1988)**. We confirmed the identity via NMR and LC-HRToFMS by comparison with phaseoloidin extracted from *E. phaseoloides* seeds.

To assess the ecological role of the trichome constituents, we conducted caterpillar performance assays with the specialist *M. sexta* and the generalist *Spodoptera littoralis*.

The bioassays were conducted on an artificial diet spiked with nicotine or phaseoloidin concentrations similar to those found in trichomes. Both caterpillar species showed a significant decrease in growth that was more pronounced for the generalist *S. littoralis*. Furthermore, we fed diet containing the aglycone of phaseoloidin, homogentisic acid, to *M. sexta* larvae and observed a clear reduction in growth. This suggests that the aglycone is the active component, even though the mode of action remains elusive and will be discussed in a following paragraph (9.4).

9.3 The defensive function of trichomes in N. attenuata

I would argue that the trichomes of *N. attenuata* can honestly be called the plant's first line of defense. In this work I have shown that trichome exudates (*O*-acyl sugars) as well as trichome contents (phaseoloidin) have a profound impact on the resistance of *N. attenuata* against attacking insects. Moreover I showed that the trichome-based defense can be attributed to different defensive mechanisms.

O-acyl sugars and phaseoloidin are constitutively present in the trichomes of *N. attenuata*, but they function in different ways. Phaseoloidin and nicotine act as direct defenses. These two metabolites are stored in the trichomes, and consumed by the insect. Nicotine is a strong agonist of nicotinic acetylcholine receptors and **(Wink, et al. 2002)** therefore a strong neurotoxin that is effectively used as an insecticide **(Jackson, et al. 2002, Schmeltz 1971)**.

In contrast, the mode of action of phaseoloidin against insects is still elusive. However the metabolism of the aglycone (homogentisic acid) is known in humans. It is derived from the proteinogenic amino acids phenylalanine or tyrosine (**Fig 9-1, A**) and further catabolized to fumaric and acetoacetic acid (**Fig.9-1, C**). Studies in the *Arabidopsis thaliana* indicate that plants utilize the same metabolic pathway as mammals **(Dixon, et al. 2006)**. If this pathway is blocked in humans, for instance due to a genetic deficiency and a lack of homogenitisate dioxygenase, patients will suffer from the accumulation of homogentisic acid (alkaptonuria) **(Williams, et al. 2012)**. Studies have shown that homogentisic acid can undergo spontaneous or enzymatic oxidation, resulting in the formation of quinone-like structures **(Wolff, et al. 1989, Zannoni, et al. 1969)** (**Fig. 9-1 D, E**). One possible product of homogentisic acid oxidation might be benzoquinone acetic acid, which has been reported to react quickly with proteins **(Stoner, et al. 1967)** and to promote the formation of reactive oxygen species and free radicals **(Martin, et al. 1987a).**

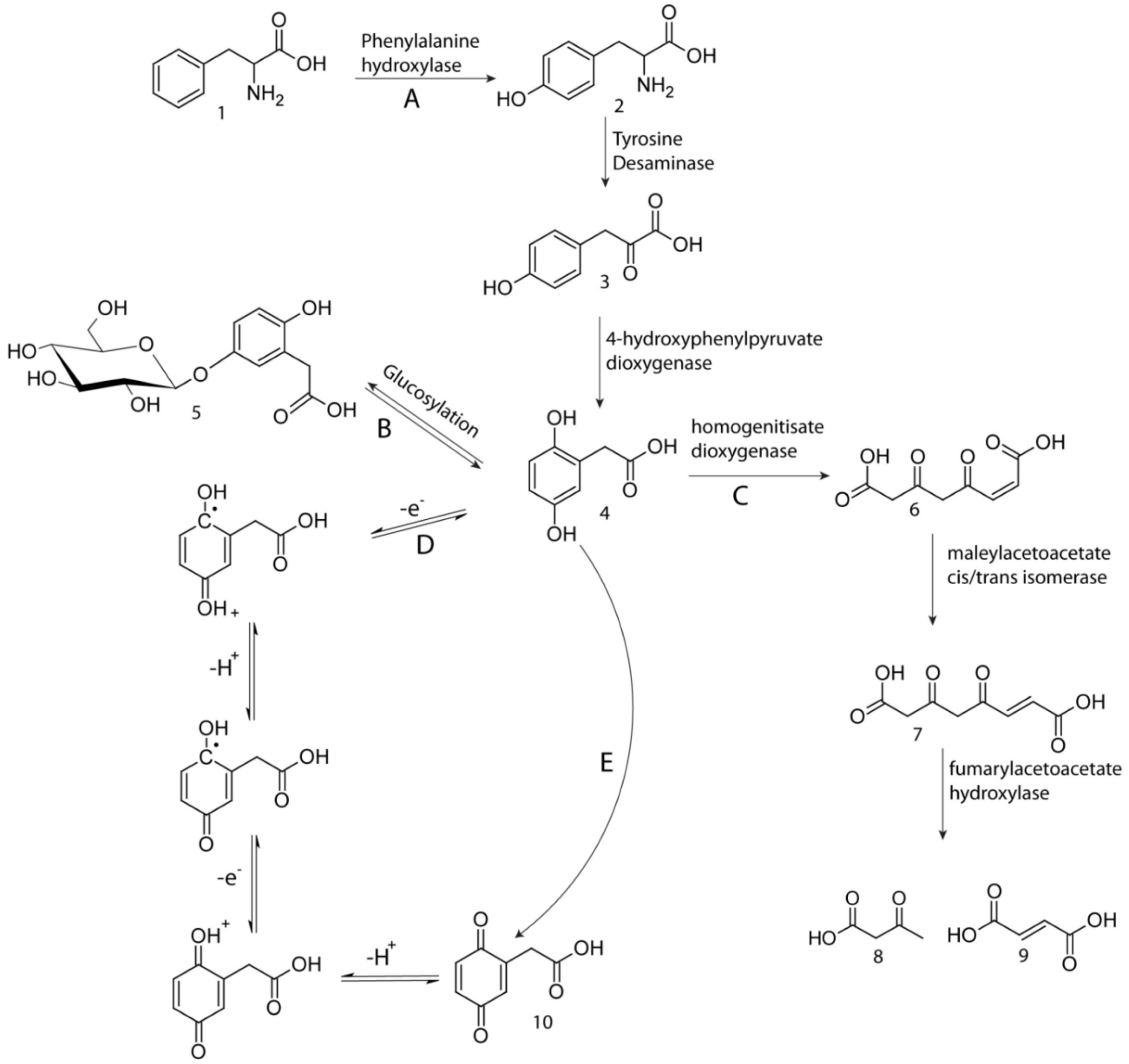

Figure 9-1: Origin and fate of homogentisic acid: **A** Biosynthesis of homogentisic acid **(4)** via phenylalanine **(1)**, tyrosine **(2)**, and 4-hydroxyphenylpyruvate **(3)**; **B** Storage or detoxification via glucosylation to phaseoloidin **(5)**; **C** Catabolism of homogentisic acid **(Arias-Barrau, et al. 2004, Dixon, et al. 2006)** via maleylacetoacetic acid **(6)**, and fumarylacetoacetic acid **(7)** to 3-oxo-acetic acid **(8)** and fumaric acid **(9)**; **D** Possible oxidation steps for the formation of benzoquinone acetic acid **(10)**; **E** enzymatic oxidation of homogentisic acid to benzoquinone acetic acid

Benzoquinone acetic acid or the semiquinone intermediates can also cause oxidative DNA damage **(Hiraku, et al. 1998)**. Hence, homogentisic acid could easily cause severe damage to the consuming animals. Since we have shown that the aglycone is the active part of the molecule, one might ask why it is found bound to a glucose moiety. There are two possible explanations for these findings.

One could be the avoidance of autotoxicity for the plant. Indeed, the quinine-like structures originating from homogentisic acid may not only react with the biomolecules of the targeted insect but also with those of the plant. Glucosylation stabilizes the aglycone and blocks the further catabolism. Therefore, the glycoside form of this defensive metabolite might only be a storage form of homogentisic acid.

This concept is not unique for phaseoloidin. Other plant toxins, e.g. glucosinolates, are stored as glycosides and activated by de-glycosylation in response to insect attack **(Fahey, et al. 2001)**. Another explanation for the glucosylation of homogentisic acid might be the transport of this compound. So far, it is not known how phaseoloidin is synthesized and in which tissue. It is possible that site of biosynthesis is not within the trichomes. Glycosylation may help to transport phaseoloidin into the trichomes. Additionally, it is known that trichomes are also used by plants to excrete phytotoxins or heavy metals **(Choi, et al. 2001)**. Hence, the compartmentalization of phaseoloidin into trichomes may just be the route via which the plant is excreting autotoxic compounds like phaseoloidin.

In contrast to the direct mechanism of action of other trichome constituents, *O*-acyl sugars seem to have no deleterious effects on caterpillars. At least for neonates of *M. sexta,* we could show that feeding on *O*-acyl sugars did not have any negative consequences (Manuscript I, Fig.S2), although there are reports indicating a possible toxicity for *M. sexta* **(Puterka, et al. 2003, Van Dam, et al. 1998)**. *O*-Acyl sugars act indirectly, since they rely on a third party to release their defensive potential. The *O*-acyl sugar-dependent body odor that is utilized by predatory ants is a classic example of an indirect defense, similar to the release of GLVs **(Allmann, et al. 2010)** or terpenoids **(Unsicker, et al. 2009)** from wounded plant tissues. Indirect defenses based on volatiles emitted by attacked plants are widely spread throughout the plant kingdom and are often referred to as a “cry for help” **(Dicke 2009)**. The novelty of the *O*-acyl sugar-based defense in *Nicotiana attenuata* lies in how the defensive signals are generated. In contrast to volatile compounds, e.g GLVs, the branched chain aliphatic acids (BCAAs) are not directly emitted, but instead must be post-ingestively hydrolyzed by the attacking insect. Furthermore, the exudation of *O*-acyl sugars combines nutritional- and information-based resistance traits. They share similarities with the provision of extrafloral nectars or food bodies by various plant species **(Heil 2008, Heil, et al. 2001)** that attract predators and are nutritional based defensive traits.

O-acyl sugars provide a first food source for caterpillars, but then develop into a defense trait upon digestion and the release of the BCAAs from the caterpillars. Trichome derived *O*-acyl sugars can therefore be regarded as an intermediate between those kinds of defenses and will be a highly interesting target for future research.

The substitution pattern of BCAAs in *O*-acyl sugars may influence their defensive function. We could identify three different classes of *O*-acyl sugars, of which two were already described in *Nicotiana glutinosa* (SE-2, SE-3) **(Arrendale, et al. 1990)** and one in *Nicotiana tabacum* **(Ding, et al. 2006)** (SE-4 **Fig. 9-2**). The number of BCAA substituents determines the lipophilicity of the compounds. Therefore, a higher substitution rate could enhance their predicted detergent-like function against soft bodied insects **(McKenzie, et al. 2004, Puterka, et al. 2003)**. Since it is not clear how *O*-acyl sugars are exuded, an increase in lipophilicity might also facilitate anexudation process through the plant cuticle **(Shepherd, et al. 2007)**.

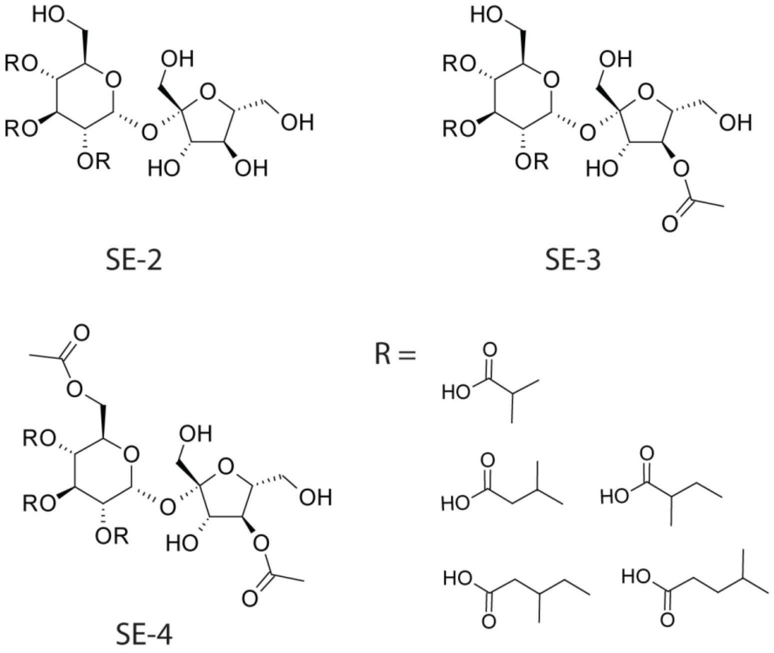

Figure 9-2: Examples of the three different classes of O-acyl sugars present in *N. attenuata*, SE-2 tri-*O*-acyl-α-glucopyranosyl-β-fructofuranosides; SE-3 tri-*O*-acyl-α-glucopyranosyl-*O* -acetyl-β -fructofuranosides, SE-4 *O*-acetyl-tri-*O*-acyl-α-glucopyranosyl-*O*-acetyl-β -fructofuranosides

To summarize, we have identified two different defensive mechanisms deployed in the trichomes of *N. attenuata*: direct defense via nicotine and phaseoloidin and both the putative direct and demonstrated indirect defense mediated by *O*-acyl sugars. Interestingly, we found that the trichomes of type D, which exude most of the *O*-acyl sugars, have lower amounts of phaseoloidin, while type C trichomes have high amounts of phaseoloidin and do not exude *O*-acyl sugars (Manuscript III, Fig S3). This indicates that defenses mediated by phaseoloidin and *O*-acyl sugars might act synergistically: high phaseoloidin concentrations would deter larvae from the *O*-acyl sugars and therefore undermine their defensive potential in attracting predators. Trichome types instead seem to specialize in either perfuming or poisoning larvae.

9.4 Timing of trichome-based defenses:

The timing of the plants' defense response to herbivory is of the essence, as a plant has to repel the attacker as fast as possible for several reasons. The loss of foliage is accompanied by the loss of photosynthetic capacity and metabolic building blocks like sugars or proteins. Plants can mitigate this loss by the allocation of these metabolites to other tissues, like roots **(Schwachtje, et al. 2006)**. Additionally, insects are vectors for pathogens like phytoplasmas **(Weintraub, et al. 2006)**, and the wounding sites are the infection sites for fungi and bacteria. Therefore a fast defense response is necessary to counteract possible infections, but an immediate defense response is only possible if the defensive chemicals are produced *a priori* and stored in the plant tissue. For example, glucosinolates *in Brassicae* **(Chen, et al. 2001)** are constitutively synthesized and after disruption of plant tissues come into contact with glucosidase enzymes to release the effective chemical species.

In contrast, *de novo* synthesis after attack saves resources for the plant. For instance, the nicotine that is produced in *N. attenuata* roots after herbivory **(Wink, et al. 1998)** consumes important nitrogen resources. Therefore, *N.attenuata* maintains basal levels of nicotine but accumulates this alkaloid in response to herbivory. The herbivore-induced synthesis of defense chemicals delays plant defense. It depends on signaling cascades that involve recognition of the attack and transmission of the information to effect phenotypic changes. Several kinases and phytohormones are involved in this process that leads to the expression of the biosynthetic genes for defensive metabolites (for an overview see **(Howe, et al. 2008)**). This gives the attacking insect the advantage by allowing it to begin feeding on undefended tissue. However, there is a great diversity of induced defenses in *N. attenuata* (**Fig. 9-3**). Phenolic compounds that are deleterious for *M. sexta* are induced within 24 hours after wounding **(Kaur, et al. 2010, Kim, et al. 2011)**. Diterpene glycosides are constitutively present in *N. attenuata* but do also increase in concentration after attack **(Heiling, et al. 2010)**. The timing of these defense metabolites illustrates the delayed response to insect attack.

In contrast trichome-based defenses like *O*-acyl sugars or phaseoloidin are constitutive, and represent a permanent protection against herbivores. Phaseoloidin is consumed by larvae and affects their growth. On the contrary the *O*-acyl sugar mediated defense is delayed, because a prerequisite is the ingestion and digestion of *O*-acyl sugars by the larvae. Nevertheless it is still faster than the other plant defenses and acts in a time span like the emission of GLVs (**Fig. 9-3**).

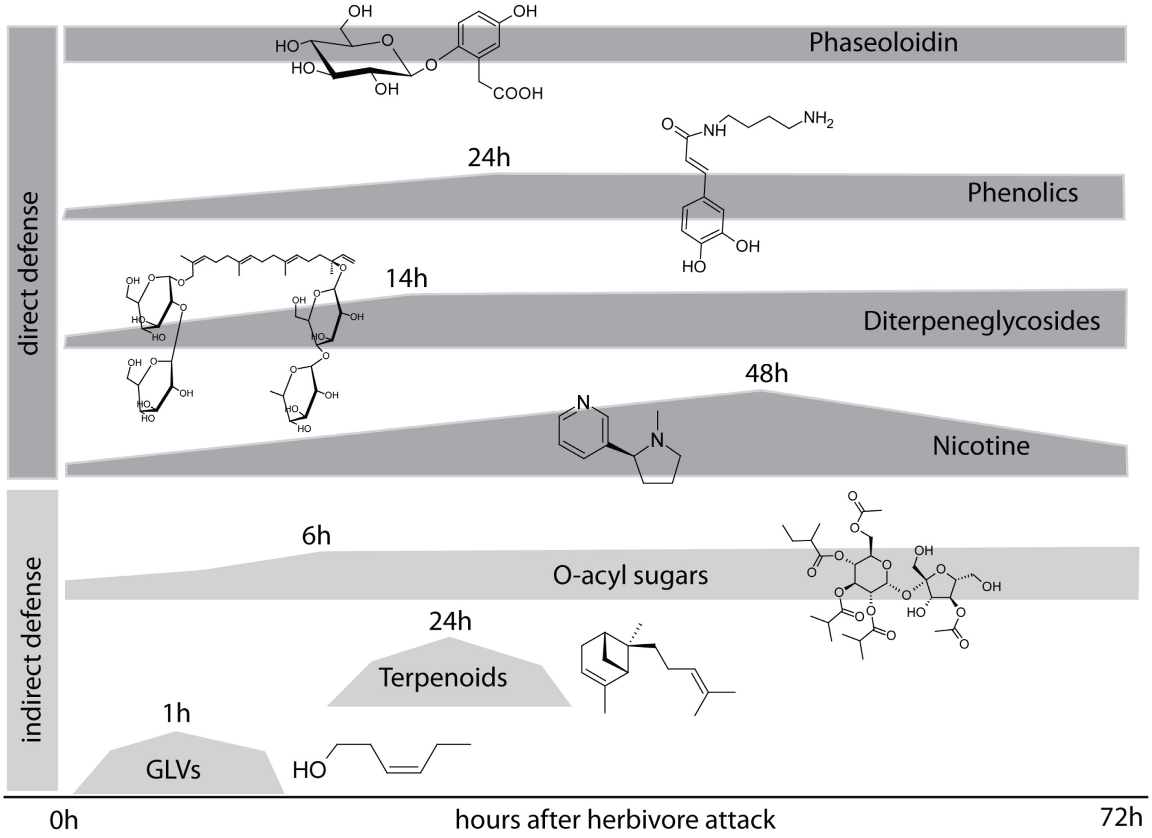

Figure 9-3: Overview of the different defense strategies of *Nicotiana attenuata* and their timing after herbivore attack: Times are indicating the time points when the “highest” defensive potential is reached. The bars show the peaks of either the highest accumulation (phaseoloidin,nicotine, diterpene glycoside, phenolics) or the highest emission timepoint (GLVs, terpenoids, *O*-acyl sugars and their BCAAs respectively).

To conclude, trichomes of *N. attenuata* are indeed the first line of defense against attacking insect herbivores. Their location on the leaf surface makes them the first plant parts that come into contact with attackers, and insects are forced to feed on these literally evil lollipops. This holds as well true for the timing of the different plant defenses mediated by trichomes. Phaseoloidin acts immediately against insects upon trichome consumption and *O*-acyl sugars are causing the specific body odor within hours after ingestions that attracts predators.

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Chapter 10: Zusammenfassung

10.1 Pflanzliche Verteidigungsstrategien

In einer Welt voller pflanzenfressender Insekten ist es durchaus überraschend, wie viele Pflanzenarten es vollbracht haben, zu überleben. Da Pflanzen im Gegensatz zu Tieren nicht vor ihren Fraßfeinden flüchten können, haben sie im Laufe der Evolution verschiedene Verteidigungsstrategien entwickelt. Diese können grob sowohl in direkte und indirekte als auch in konstitutive und induzierte Pflanzenverteidigung eingeteilt werden. Direkte Verteidigungen richten sich direkt gegen das angreifende Insekt. Beispiele hierfür sind die Produktion von Alkaloiden in verschiedenen Nachtschattengewächsen (Solanaceae). Indirekte Verteidigungen hingegen richten sich nicht direkt gegen Angreifer, sondern beinhalten die Interaktion mit einem anderen Trophieniveau. Beispielsweise geben die Wurzeln von Maispflanzen nach dem Befall durch dem Maiswurzelbohrer (*Diabrotica virgifera*) das Sesquiterpen (E)-β-Caryophyllen ab **(Rasmann, et al. 2005)**. Durch dessen Emission werden Fadenwürmer aus dem Boden angelockt, die ihrerseits den Maiswurzelbohrer befallen und so die Pflanze schützen. Dies ist auch ein Beispiel für eine induzierte Verteidigung, da die Emission von (E)-β-Caryophyllen erst nach dem Insektenbefall und nicht kontinuierlich geschieht. Im Gegensatz dazu können konstitutive Verteidigungen zum Beispiel die Herausbildung von Dornen und Stacheln sein, welche Pflanzen gegen pflanzenfressende Tiere schützen. Trichome (Blatthaare) werden von einer Vielzahl von Pflanzenspezies gebildet. Diesen Blattausstülpungen wurden in der Literatur wichtige ökologische Funktionen, im Speziellen in Bezug auf pflanzliche Verteidigungsstrategien gegen Insekten, zugeschrieben. Doch erst genauere Untersuchungen der chemischen Natur der Sekundärmetaboliten in den Trichomen des wilden Tabaks *Nicotiana attenuata* haben gezeigt, welche Rolle diese Blatthaare in der Pflanzenverteidigung spielen.

10.2 Zuckerester und ihre Wirkungsweise auf den Tabakschwärmer Manduca sexta (Manuskript I,II)

Trichome des wilden Tabaks *N. attenuata* sekretieren an ihren Spitzen zähflüssige Tröpfchen. Von dort gelangen diese auch zur Trichom-Basis und verteilen sich auf der Blattoberfläche. In unseren Studien konnten wir zeigen, daß diese Ausscheidungen zum größten Teil aus Zuckerestern bestehen. Eine Saccharose-Einheit ist dabei an den freien Hydroxylgruppen mit kurzkettigen verzweigten Carboxylsäuren verestert. Durch die Anwendung verschiedener analytischer Verfahren gelang es uns, diese Trichom-Metaboliten zu identifizieren.

Die hohe Massenauflösung eines ToF – Massenspektrometers erlaubte uns, anhand der Analyse von Blattextrakten die vorgeschlagenen Summenformeln zu bestätigen. Dies führte zur Identifizierung von acht verschiedener Zuckerestern in den Trichomen von *N. attenuata* (**Manuskipt I Tabelle S2**). Des weiteren identifizierten wir ebenfalls die einzelnen Carboxylsäurereste der verschiedenen Zuckerester. Eine aus Pflanzenextrakten gewonnene Mischung der verschieden Zuckerester wurde im Alkalischen hydrolysiert und die Hydrolyseprodukte anschließend mit einem GCxGC-ToFMS System analysiert. Die Chromatogramme zeigten vier Hauptkomponeten. Diese konnten durch ihre Massenspektren und durch den Abgleich mit kommerziellen Standards folgenden Carboxylsäuren zugeordnet werden: 2-Methyl-Butansäure, 3-Methyl-Butansäure, 3-Methyl-Pentansäure und 4-Methyl-Pentansäure. (**Manuskipt I Fig. S4**).

Diese vier Säuren zeichnen sich durch ihren unverwechselbaren Geruch aus. Während dieser Experimente wurde offensichtlich, daß das spezielle Bouquet dieser Säuremischung ähnlich dem ist, das die auf *N. attenuata* fressenden Raupen des Tabakschwärmers *Manduca sexta* emittieren (**Manuskript I Fig. 1**). Um die Hypothese zu bestätigen, daß der charakteristische Geruch von *M. sexta* durch die vier verschieden Säurereste der Zuckerester verursacht wird, analysierten wir den Headspace der Raupen mit Hilfe von GCxGC-ToFMS. Wir konnten zeigen, daß es sich hierbei um dieselben Säuren handelt, die sowohl von der Raupe als auch von deren Kot emittiert werden. Zusätzlich konnten wir demonstrieren, daß *M. sexta* auf zuckeresterfreien Blättern keine dieser Säuren von sich gibt (**Manuskript I Fig. 2**).

Da die Säurereste durch alkalische Hydrolyse von der Saccharose gespalten werden, nehmen wir an, daß das alkalische Milieu im Raupendarm die geeigneten Bedingungen für eine solche Reaktion bietet. Interessanterweise ist der spezifische Geruch der Raupen bereits kurz nach dem Beginn des Tabakfraß wahrzunehmen. Wir konnten beobachten, daß bereits 2 Stunden nachdem die Raupen Blattmaterial aufgenommen hatten, die Emission an Säuren drastisch anstieg (**Manuskript I Fig. 2**). Daraus läßt sich schließen, daß der Raupengeruch ein sehr schnelles und spezifischen Signal darstellt, das von verschieden Prädatoren oder Parasiten zum Auffinden ihrer Beute genutzt werden könnte. Da dieses Phänomen in verschieden Pflanzenfresser-Parasiten

Systemen beschrieben wurde **(Herard, et al. 1988)** testeten wir im Feldversuch die Wirkung der verschiedenen Komponenten des Raupengeruchs. Zu diesem Zweck quantifizierten wir die abgegebenen Mengen an flüchtigen Säuren und waren so in der Lage einen künstlichen Raupengeruch für unsere Feldversuche nach zu empfinden. Interessanterweise wurden weder parasitäre Wespen noch Raubwanzen durch den Raupengeruch angelockt.

Allerdings konnten wir beobachten, daß Ameisen der allesfressenden Art *Pogonomyrmex rugosus* das Raupenbouquet als Orientierungshilfe benutzten, um *M. sexta* Raupen aufzuspüren und zu fressen (**Manuskript I Fig. 3**).

Während unserer Feldversuche mußten wir feststellen, daß mit großer Regelmäßigkeit Raupen älterer Larvenstadien von unseren Versuchspflanzen verschwanden (**Manuskript II Fig. 1**). Da Raupen in diesen Entwicklungsstadien mit großer Sicherheit weder in das Beuteschema von Raubwanzen noch von Ameisen passen, vermuteten wir, daß in der Great Basin Desert heimische Eidechsen der Grund für den Verlust an Raupen sein müssen. Wir konnten zeigen, daß zwei verschiedene bodenjagende Eidechsenarten die Säuren aus dem Raupen- und Kotbouquet zum Lokalisieren ihrer Beute benutzten (**Manuskript II Fig. 2**). Diese Ergebnisse unterstreichen die wichtige Rolle von Zuckerestern in der Pflanzenverteidigung, da sie Raupen in allen Larvenstadien beeinflussen. Darüber hinaus zeigen beide Studien die Wichtigkeit der zugrundeliegenden chemischen Verfahren, da sie uns die Identifizierungen der Bestandteile des Raupenbouquets und deren Quantifizierung erst ermöglichten.

10.3 Trichom-Inhaltstoffe und ihre Wirkung auf Spezialisten und Generalisten (Manuskript III)

Neben von Trichomen abgegebenen Zuckerestern existieren noch weitere Sekundärmetbolite, die in Trichomen gespeichert werden. Beispiele dafür finden sich an verschiedenen Stellen in der Literatur und reichen von Mono- und Sesquiterpenen bis hin zu den verschiedensten Alkaloiden **(Kennedy 2003, Laue, et al. 2000, van Schie, et al. 2007)**. Daher war es für uns von großem Interesse, auch die Trichominhalte zu untersuchen. Da das Sammeln von Trichomen in ausreichenden Mengen einige Schwierigkeiten bereitet, besteht die Notwendigkeit, eine analytische Methode mit einer ausreichenden Auflösung auch für kleine Substanzmengen zur

Anwendung zu bringen. Die Kernspinspektroskopie (NMR) erlaubt uns, selbst kleine Mengen an Trichommaterial direkt und ohne vorherige Extraktion zu analysieren und hat dabei den Vorteil, nicht destruktiv zu sein. Dadurch können Extraktionsverluste vermieden werden. Des weiteren erlaubt die NMR-Spektroskopie auch eine robuste Quantifizierung der Trichominhalte. Die Trichome von *N. attenuata* haben zwei unterschiedliche Erscheinungsformen, die längeren Typ C- und die kürzeren Typ D-Trichome (**Manuskript III, Fig S1**). Es wurden Proben von beiden Typen gesammelt und mit Hilfe von ^{1}H-NMR analysiert. Die ^{1}H-Spektren waren weit weniger komplex als erwartet. Signale im aromatischen Bereich konnten den Kohlenstoffatomen an Position 2, 4, 5 und 6 des Pyridinrings von Nikotin zugeordnet werden (**Manuskript III, Fig. 2**).

Des weiteren waren Signale im aliphatischen Bereich sichtbar, die höchstwahrscheinlich durch die Carboxylsäurereste der Zuckerester verursacht wurden. Interessanterweise waren auch Signale im Bereich von 6.5 – 7.0 ppm detektierbar, die wir einem Glucosid der Homogenitisinsäure (2,5 – Dihydroxyphenylessigsäure) zuordnen konnten (**Manuskript III Fig. 2**). Dieses Glucosid, Phaseoloidin, wurde bisher nur in den Samen von *Entada phaseoloides* beschrieben **(Barua, et al. 1988)** und war bis dato in Solanaceaen unbekannt. Aus den Samen von *E. phaseoloides* konnten wir ausreichende Mengen an reinem Phaseoloidin extrahieren und damit die Identität dieser Substanz in *N. attenuata* durch Vergleich der NMR und MS Spektren bestätigen (**Manuskript III Fig. 3**).

Um den Effekt von Phaseoloidin auf verschiedene pflanzenfressende Insekten zu untersuchen, erlaubten wir Raupen des Spezialisten (*Manduca sexta*) und Generalisten (*Spodoptera littoralis*) auf mit Phaseoloidin versetztem artifiziellem Futter zu fressen.

Sowohl *M. sexta* als auch *S. littoralis* zeigten nach dem Verzehr des Phaseolioidin-haltigen Futters eine 50%ige Reduktion ihres Wachstums, wobei der Effekt für den Generalisten größer war. Des weiteren konnten wir zeigen, daß auch das Aglykon des Phaseoloidins, Homogenitisinsäure, das Wachstum von *M. sexta* reduziert (**Manuskript III Fig. 4**). Daraus läßt sich schließen, daß Homogenitisinsäure die aktive Komponente ist. Die genaue Wirkungsweise bedarf allerdings weiterführender Untersuchungen.

10.4 Trichome in der direkten und indirekten Pflanzenverteidigung

Die Blatthaare *Nicotiana attenuata's* können in der Pflanzenabwehr als erste Verteidigungslinie gegen pflanzenfressende Insekten verstanden werden. Diese Arbeit verdeutlicht, daß sowohl die Absonderungen der Trichome (Zuckerester) als auch deren Inhaltsstoffe (Phaseoloidin) einen erheblichen Einfluß auf die Widerstandsfähigkeit von *N.attenuata* gegenüber Insekten haben. Darüber hinaus zeigte sich, daß die Trichom-basierte Pflanzenverteidigung auf zwei verschieden Mechanismen beruht. Beide Klassen von Verteidigungmetaboliten, sowohl Zuckerester als auch Phaseoloidin und Nikotin, liegen konstitutiv in den Trichomen vor. Ihr Wirkungsmechanismus auf die Insekten unterscheidet sich jedoch erheblich. Phaseoloidin und Nikotin sind ein sehr gutes Beispiel für eine direkte Verteidigung. Beide Substanzen sind in den Blatthaaren gespeichert, welche dann von den Raupen gefressen werden. Nikotin ist bekannt als starkes Neurotoxin und wird aufgrund seiner Wirkung als Agonist des Nikotin-Acetylcholin Rezeptors **(Wink, et al. 2002)** auch als Insektizid eingesetzt **(Jackson, et al. 2002, Schmeltz 1971)**. Im Gegensatz dazu ist das genaue Wirkungsprinzip von Phaseoloidin noch unbekannt.

Das Aglykon, Homogenitisinsäure, kann sowohl spontan als auch mit Hilfe von Enzymen oxidiert werden und bildet Chinone **(Martin, et al. 1987, Stoner, et al. 1967)**. Diese sind bekannt für ihre schnelle Interaktion mit essentiellen Biomolekülen wie DNA oder Proteinen und den daraus resultierenden Schäden für die fressenden Insekten. **(Martin, et al. 1987)**

Im Gegensatz zu Phaseoloidin und Nikotin haben die konsumierten Zuckerester keinen nachteiligen Effekt auf die sie konsumierenden Raupen. Zumindest für *M. sexta* im ersten Larvenstadium konnten wir zeigen, daß Zuckerester sogar regelmäßig von den Raupen konsumiert wurden. Wir konnten keine Unterschiede in den Mortalitätsraten von Raupen, die auf Zuckerester-freiem und Zuckerester-enthaltendem Futter fraßen, feststellen (Manuskript I Fig. S2). Dies steht im Gegensatz zu Studien, die zeigten, daß eine gewisse Toxizität für *M. sexta* existieren könnte **(Puterka, et al. 2003, Van Dam, et al. 1998)**.

Zuckerester wirken nicht direkt auf das Insekt ein, sondern indirekt nach ihrer Hydrolyse und der Freisetzung ihrer Säurereste im Raupendarm. Der durch die Zuckerester bedingte Raupengeruch ist das eigentliche Verteidigungssignal, das von Ameisen und Eidechsen genutzt wird, um ihre Beute zu lokalisieren. Dies macht Zuckerester zu einem klassisches Beispiel einer indirekten Verteidigungsstrategie, ähnlich der Emission von GLVs (green leaf volatiles). Solche indirekten

Verteidigungen, die auf der Freisetzung von Signalstoffen beruhen, sind weitverbreitet im Pflanzenreich und werden auch gelegentlich als „S.O.S.“-Signal der Pflanze bezeichnet **(Dicke 2009)**. Die Neuartigkeit der Zuckerester-basierten Verteidigung liegt in der Art und Weise, in der das eigentliche „S.O.S“-Signal generiert wird. Im Gegensatz zu GLVs bedarf es der Raupen, die erst durch den Verzehr der Zuckerester das eigentliche Signal, die Säuren, freisetzen. Auch zeigt die Abgabe von Zuckerestern durch die Blatthaare Ähnlichkeit mit der Abgabe extrafloraler Nektare (EFN) **(Heil 2008, Heil, et al. 2001)**, wie sie beispielsweise in Akazien beschrieben wurden. Ameisen werden durch diese EFNs angelockt und verteidigen die Pflanze, ihre Nahrungsquelle, gegen andere Insekten. Während bei EFNs der „Hilferuf“ an die Beschützer der Pflanze durch das Angebot von Nahrung verbreitet wird, geschieht dies bei Zuckerestern erst indirekt über das angreifende Insekt selbst. Daher kann diese Verteidigungsstrategie als intermediär zwischen direkter und indirekter Verteidigung angesehen werden und stellt damit ein ausgezeichnetes Forschungsobjekt für weitere Studien dar.

Zusammenfassend läßt sich sagen, daß in den Blatthaaren von *N. attenuata* zwei verschiedene Verteidigungsmechanismen existieren. Zum einen die direkte Verteidigung durch Nikotin und Phaseoloidin, zum anderen die indirekt/direkte Verteidigung durch Zuckerester.

Interessanterweise konnten wir zeigen, daß der Trichome des Typs D mit niedrigen Mengen an Phaseoloidin mehr Zuckerester abgeben als Trichome mit einer hohen Phaseoloidinkonzentration (Typ C), die kaum Zuckerester absondern (**Manuskript III Fig S3**).

Das zeigt, daß beide Strategien aufeinander abgestimmt sind, denn eine hohe Konzentration an Phaseoloidin würde Raupen vom Fressen der Zuckerester abhalten und dadurch deren Verteidigungspotential erheblich abschwächen.

10. 5 Die zeitliche Regulierung der Trichom-basierten Pflanzenverteidigung

Im Falle eines Insektenbefalles ist eine schnelle Verteidigungsantwort der Pflanze von zentraler Bedeutung. Die befallene Pflanze muß aus den verschiedensten Gründen versuchen, den Angreifer schnellstmöglich zu vertreiben. Zum einen geht der Verlust von Blattgewebe oder gar ganzen Blättern einher mit dem Verlust an Photosynthesekapazität. Des weiteren verliert die Pflanze auch gespeicherte Ressourcen wie Proteine und Kohlenhydrate. Daher ist es im Interesse der Pflanze beides zu vermeiden. Des weiteren können Insekten auch Überträger von Pathogenen **(Weintraub, et al. 2006)** sein, und eine schnelle Antwort der Pflanze ist daher notwendig, um Infektionen vorzubeugen oder diesen angemessen zu begegnen. Eine rasche Reaktion der Pflanze kann dabei nur gewährleistet werden, wenn die Verteidigungsmetaboliten *a priori* zum Befall durch ein Insekt synthetisiert und gespeichert wurden. Beispielhaft dafür stehen Senfölglycoside im Genus *Brassicae*. Diese werden kontinuierlich von den Pflanzen produziert, jedoch erst eine Zerstörung des Pflanzengewebes bringt sie in Kontakt mit bestimmten Enzymen (z.B. Glucosidasen), die die eigentlichen aktiven chemischen Spezies freisetzten **(Wittstock, et al. 2002)**. Die Synthese von Senfölglycoside zeigt aber das Dilemma der Pflanze. Sie muß Ressourcen in die Verteidigung investieren - unabhängig davon, ob ein Insekt die Pflanze befällt oder nicht. Im Gegensatz dazu werden durch *de novo* Synthese kostbare Ressourcen eingespart und erst im Fall einer Insektenattacke umgesetzt.

In den Wurzeln von *N. attenuata* wird nach Insektenbefall verstärkt Nikotin produziert und dann in die Blätter transportiert. Da zur Biosynthese von Nikotin Stickstoff benötigt wird, ist es für eine Pflanze von Vorteil, dieses Toxin nicht in vollem Umfang *a priori* zu produzieren. Nachteilig ist dabei, dßs durch eine solche induzierte Verteidigung die Pflanzenantwort auf den Insektenbefall verzögert wird. Eine solche Verteidigungsstrategie setzt außerdem voraus, daß der Insektenbefall von der Pflanze erkannt wird. Dies geschieht meistens durch verschiedene insektenspezifische Elicitoren in den oralen Sekreten der Insekten, die von der Pflanze erkannt werden können.

Erst danach wird eine Signalkaskade in Gang gesetzt, die eine Vielzahl von Enzymen und Pflanzenhormonen umfaßt und an deren Ende die Biosynthese der Verteidigungsmetaboliten steht **(Howe, et al. 2008)**.

Diese gibt den Insekten den Vorteil, an nicht verteidigten Blättern zu fressen. Im Fall von Nikotin dauert es nach der Induzierung beispielsweise drei Tage, bis die höchsten Konzentrationen in den Blättern gemessen werden können.

Im Gegensatz zu den meisten in *N. attenuata* beschriebenen Verteidigungsmechanismen sind die Abgabe von Zuckerestern und die Einlagerung von Phaseoloidin in den Trichomen kontinuierliche Prozesse. Sie stellen damit einen permanenten Schutz gegen potentielle Angreifer dar. Phaseoloidin im Speziellen wirkt sich sofort auf das Wachstum der angreifenden Insekten aus. Zuckerester wirken erst mit einer Verzögerung, da sie zuvor durch das Insekt in den Zuckerrest und seine Säurereste gespalten werden müssen. Dennoch ist auch diese Verteidigungsantwort der Pflanze deutlich schneller als übliche Verteidigungen und liegt im Bereich der Abgabe von GLVs (1-3 Stunden). Zusammenfassend läßt sich sagen, daß die Trichome von *N. attenuata* mit Recht als erste Verteidigungslinie der Pflanzen bezeichnet werden können. Sie sind das erste Pflanzengewebe, das mit potentiellen Angreifern in Kontakt kommt und können sofort (Phaseoloidin und Nikotin) oder mit leichter Verzögerung (Zuckerester) ihr Verteidigungspotential entfalten.

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Curriculum Vitae

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List of publications:

Publication List:

Gaquerel, E., Weinhold, A., Baldwin, I. T. (2009). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphigidae) and its natural host *Nicotiana attenuata*. VIII. An unbiased GCxGC-ToFMS analysis of the plant´s elicited volatile emissions. **Plant Physiology**, 149(3), 1408-1423. doi:10.1104/pp.108.130799

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Talk Presentations:

Weinhold, A., Shaker, K., Wenzler, M., Schneider, B., Baldwin, I.T. Studying the plant's first line of defense - How analytics reveal new defensive functions of trichomes. 27th ISCE Meeting, International Society of Chemical Ecology, Simon Fraser University, Burnaby, British Columbia, Canada, July 2011

Weinhold, A. The scent of a caterpillar: How trichomes influence M.sexta's body odor. 9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, February 2010

Weinhold, A. Der Hilferuf der Pflanze - Bestimmung von Pflanzen VOC's mit Hilfe von GCxGC-ToFMS, LECO Praxis Workshop, LECO Instrumente GmbH, Mönchengladbach, Germany, September 2009, (invited speaker)

Weinhold, A. GCxGC-TOFMS for Analysis of Changes in Plant Volatile Emissions during herbivory, 1st European Comprehensive GCxGC Workshop & Networking Meeting, LECO Corp., Amsterdam, The Netherlands, June 2009, (invited speaker)

Poster Presentations:	**Weinhold, A.** Trichomes as evil lollipops – a first meal that tags caterpillars for predation. 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, February 2011
	Bonaventure, G., Gaquerel, E., Gilardoni, P.,Kallenbach, M., Schuman, M., Weinhold, A., Wissgott, A. Signaling in the real world: aposomatic JA signaling, evil lollipop, SAB Meeting 2010, MPI for Chemical Ecology, Jena, Germany, October 2010
	Weinhold, A. Sweets are no good, are they? Studying the effects of acylsugars on the herbivore community of *N. attenuata*. 8th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, February 2009
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Erklärung an Eides Statt

Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung, der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

Jena, den 3.10.2012

Alexander Weinhold