Isomers of green leaf volatiles in Nicotiana attenuata and their role in plant-insect interactions

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Green leaf volatiles as a ‘cure-all’ – biosynthesis, regulation and function

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CHAPTER 1

General Introduction

The advocate and writer Cheryl Saban once wrote in an online article (The Huffington Post; Monday, 3 March 2008) “Freshly Mown Grass – The Legal Kind”: ‘The smell of freshly mown grass is like a ticket to a time machine. Every Wednesday, when the gardeners mow the lawn, I enjoy a trip to my childhood that leaves me with a satisfied grin on my face. The earthy, fresh scent makes me remember easier days filled with neighborhood kids on bicycles riding freehand down our street, with warm, afternoon air billowing through our shirts like sails.’

Probably everyone is familiar with the characteristic odor of freshly cut grass and has some memories associated with it. Chemically, this ‘earthy, fresh scent’ of freshly mown grass is mainly derived from C6 compounds, also called green leaf volatiles (GLVs) (Matsui, 2006). While we generally associate these GLVs with pleasant memories, for plants and their biotic partners they may not be as pleasant as for us, and may have completely different and very diverse meanings. This thesis aims to shed light on the biological role of plant GLVs.

1. Introduction to herbivore-induced plant defenses

At first glance, plants are easy food for hungry herbivores (Bede et al., 2007; Merkx-Jacques et al., 2008). While carnivores often have to chase their prey and put up a struggle before they can eat, herbivores seem to have it much easier because plants don’t run away. However, herbivores don’t get their meals for free: plants do fight back.

Plants do not have easy lives and this is reflected by their anatomy and physiology. Their cell membranes are shielded by walls and their surface is covered with a protective cuticula and other structures to prevent dehydration as well as penetration by pathogens or feeding by herbivores (Müller and Riederer, 2005; Hématy et al., 2009). Moreover, plants accumulate diverse substances that interfere with herbivore digestive physiology. Some herbivores withstand such constitutive plant defenses. If so, the plant will increase its defenses to hinder the herbivore more while also switching to other measures such as selective tissue-death, i.e. the hypersensitive response (HR), and altered resource allocation (Anten and Pierik, 2010), collectively referred to as “direct defenses” and “tolerance mechanisms”. Plants will also increase their release of volatiles (Kant et al., 2009). Herbivore-induced plant volatiles (HIPVs) can be used by foraging predators or host-searching parasitoids to track down plants with prey, thereby augmenting the plant’s direct defenses. This is referred to as “indirect defense” because it depends on recruiting herbivores’ enemies (Heil, 2008; Dicke, 2009). Direct defenses are costly and require resources otherwise used for
growth and reproduction (Walters and Heil, 2007). Therefore plants have evolved a complex, largely hormonal, signaling network to arrange their physiological priorities during defense and resource allocation (Pieterse et al., 2009).

2. How do plants arrange their defenses? – the role of phytohormones

Whereas plant resistance against immobile (biotrophic) pathogens is often characterized by an HR, defense against herbivores is associated with a decrease in tissue palatability (Anten and Pierik, 2010). Central in the organization of anti-herbivore defenses is the plant hormone jasmonic acid (JA) and its active derivative JA-Isoleucine (JA-Ile) which rapidly accumulate during herbivory (Koo and Howe, 2009). JA and its metabolites are collectively referred to as jasmonates (JAs). The mode of action of JAs has been studied in detail using JA biosynthesis- or perception-impaired mutant plants, which are often preferred by herbivores in choice tests and allow higher herbivore fitness (Kessler, 2004; Howe and Jander, 2008). Accumulation of JAs-dependent defense proteins and metabolites is often co-regulated by ethylene (ET) in a synergistic manner. In contrast, the hormone salicylic acid (SA) antagonizes the action of JAs (Pieterse et al., 2009). SA is well known for its signaling role in defenses induced by biotrophic pathogens, but many stylet-feeding herbivores, like spider mites, whiteflies and aphids, induce a cocktail of JAs- and SA-related responses (Kant et al., 2008). Although it is not clear to which extent this mixed response is required for the plant to establish the appropriate defenses, the “decoy hypothesis” suggests that in some cases the herbivore could benefit from a SA-mediated suppression of JAs-mediated defenses (Zarate et al., 2007). However, phytohormone signaling is much more complicated than that and by now we know that not only the classical defense hormones JA, SA and ET are involved, but that also other hormones like abscisic acid, auxin, cytokinins, brassinosteroids and gibberellin influence the properties of the signaling network (Pieterse et al., 2009; Robert-Seilaniantz et al., 2011). The dynamics of this complex regulatory network, in which hormonal synergisms and antagonisms determine the final output of the defense response, depends largely on the type of herbivore as well as the physiological status of the plant.

3. Elicitors of plant defense responses

Plants can respond very quickly to imminent danger because they have developed strategies to recognize their enemies and boost defense responses even before herbivores take their first bite. Herbivores can betray themselves to plants by the damage they cause with their footsteps when wandering on the leaf surface (Hall et al., 2004; Hilker and Meiners, 2010), or at an
even earlier stage, upon egg deposition. Oviposition often comes along with the wounding of plant tissue (Hilker and Meiners, 2010), but it is not only the damage itself that plants can recognize: to attach their eggs to the leaf surface, herbivorous adult females secrete oviposition fluids which contain elicitor compounds, which in some plants have been shown to induce local plant defense responses and the release of an egg parasitoid-attracting volatile blend (Hilker and Meiners, 2010). So far two of these oviposition-mediated elicitor compounds have been chemically identified: 1) bruchins (mono- and bis-(3-hydroxypropanoate) esters of long chain \(\alpha,\omega\)-diols) are secreted from ovipositing pea weevil females (Bruchus pisorum L.). They stimulate cell division and neoplasm formation in several legumes (Doss et al., 2000; Hilker and Meiners, 2010); 2) benzyl cyanides have been found in oviposition fluids of mated female cabbage white butterflies (Pieris brassicae). They induce transcriptional changes in several defense-related genes and changes in the leaf surface, causing the egg parasitoid Trichogramma brassicae to stay longer at or near the oviposition sites (Fatourou et al., 2008; Hilker and Meiners, 2010).

Plants are able to distinguish real folivory from pure mechanical damage by recognizing the frequency of feeding damage (Mithöfer et al., 2005) or the presence of chemical elicitors introduced into the wound during feeding (Howe and Jander, 2008; Wu and Baldwin, 2010; Bonaventure et al., 2011). These chemical elicitors are in general able to increase the accumulation of phytohormones (Howe and Jander, 2008; Wu and Baldwin, 2009) and to induce the herbivore-specific release of volatiles, thereby attracting predators and parasitoids to the plant’s attacker (Wu and Baldwin, 2010). Herbivores can produce different cocktails of elicitor compounds, and each plant species can in principle respond very specifically to different herbivores (Schmelz et al., 2009; Eichenseer et al., 2010).

Fatty acid-amino acid conjugates (FACs) are a group of compounds formed in insect regurgitant by conjugation of a plant-derived fatty acid to an insect-derived amino acid (Parè et al., 1998). The first chemically described FAC was \(N\)-17-hydroxylinolenoyl-L-glutamine. It was identified from the oral secretions (OS) of Spodoptera exigua larvae, and named volicitin since it induced, like the caterpillar’s OS, the emission of several terpenoids in Zea mays (Alborn et al., 1997). By now FACs have been found in the OS of many lepidopteran species (Yoshinaga et al., 2010) and recently also in the OS of two cricket species (Teleogryllus taiwanemma and T. emma) and fruit flies (Drosophila melanogaster) (Yoshinaga et al., 2007). However, the composition and proportion of FACs depends on the availability of fatty acids in the diet (Parè et al., 1998; De Moraes and Mescher, 2004),
species-specific rates of biosynthesis (Lait et al., 2010) and subsequent modifications catalyzed by the plant (VanDoorn et al., 2010) or the insect itself (Mori et al., 2001). FACs represent a well-studied class of plant elicitors which do not only activate phytohormone-mediated direct defense responses, but also the production and emission of HIPVs (Hilker and Meiners, 2010; Bonaventure et al., 2011).

Caeliferins are a group of sulfated fatty acids and, depending on whether the ω-carbon-chain is decorated with either a sulfated hydroxyl group or a carboxyl group conjugated to glycine, are called caeliferin A or B, respectively. Caeliferins have recently been identified from the OS of the grasshopper Schistocerca americana and they induce, like volicitin, the release of herbivore-specific terpenes from maize seedlings (Alborn et al., 2007; Hilker and Meiners, 2010; Wu and Baldwin, 2010).

A third group of elicitors are proteolytic peptides, called inceptins, which were isolated from the OS of Spodoptera exigua (Schmelz et al., 2006). Inceptins are formed in the insect midgut by degradation of the plant chloroplastic ATP synthase γ-subunit. Even very small amounts (1 fmol Inceptin per leaf) are able to effectively amplify the accumulation of the phytohormones JA, ET and SA and to induce the emission of the homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) from cowpea (Schmelz et al., 2006; Schmelz et al., 2007; Hilker and Meiners, 2010; Wu and Baldwin, 2010).

Not only relatively small molecular weight compounds but also bigger molecules, like enzymes, have been shown to serve as elicitors of plant defenses: β-glucosidase is a digestive enzyme which catalyzes the hydrolysis of glycosidic linkages in glycosides. Its activity was detected in the OS of Pieris brassicae. Application of the pure enzyme to the wounds of Brussels sprouts resulted in a volatile release very similar to that of OS-treated plants, and this release attracted the egg parasitoid Cotesia glomerata (Mattiacci et al., 1995).

In this thesis I describe an enzyme that directly affects induced changes in plant volatile emission without eliciting the plant. The enzyme activity has been found in the OS of Manduca sexta. When M. sexta caterpillars fed on Nicotiana attenuata the (Z)/(E)-ratio of green leaf volatiles (GLVs) dramatically changed, and this change in the volatile bouquet tripled the foraging efficiency of the generalist predators Geocoris spp. (Chapter 3) and decreased the oviposition frequency of gravid hawkmoths (Chapter 4). Interestingly the rearrangement from (Z)-isomers to (E)-isomers is not made by the plant but by the insect’s OS itself (Allmann and Baldwin, 2010). In Chapter 5 the initial characterization of the responsible enzyme is described.
The fact that the oral secretions of herbivores contain specific elicitors and other compounds that trigger e.g. the emission of HIPVs indicates that plants can evolve to recognize a pest and to initiate direct and indirect defenses. It is thus not surprising that herbivores can evolve to suppress these defenses. Suppression of direct and indirect defenses has been shown for chewing (Musser et al., 2002; Bede et al., 2006; Gaquerel et al., 2009), and sucking insects (Zarate et al., 2007; Kant et al., 2008; Bos et al., 2010; Alba et al., 2011; Sarmento et al., 2011). The mechanisms by which herbivores suppress plant defenses are not well understood, but they can often be explained by antagonizing effects of phytohormones, as suggested for glucose oxidase, which was the first identified salivary enzyme that was able to suppress herbivore-induced defenses in plants (Musser et al., 2005; Diezel et al., 2009; Eichenseer et al., 2010).

4. Herbivore-induced plant volatiles (HIPV)
More than 1700 volatile compounds have been isolated from over 90 plant families (Maffei 2010), and these plant volatiles, derived from reproductive or vegetative tissues, establish interactions with the biotic environment of the plant. Despite the complex interactions in which these volatiles play a role, they are derived from only a handful of biochemical pathways (Dudareva et al., 2006). The majority of organic plant volatiles are either mono- or sesquiterpenes, derived from the isoprenoid pathways (in plants there are two); aromatic metabolites, such as methyl salicylate, indole and benzenoids from the shikimate/ tryptophan pathway; or green leaf volatiles, derived from the oxylipin pathway (Maffei, 2010).

4.1 Biosynthesis of GLVs
GLVs form an important group of HIPVs. While emitted only in trace amounts from healthy, undamaged plant tissue, they are rapidly released upon herbivory (Allmann and Baldwin, 2010). The increase in emission is transient but can be sustained over days by repetitive wounding from feeding herbivores (Turlings et al., 1995). GLVs are generated from the C18 fatty acids α-linolenic acid (18:3) and linoleic acid (18:2) which are liberated from membrane lipids, probably enzymatically by (a) yet unknown lipase(s) (Fig. 1-1). These fatty acids are subsequently dioxygenated by a 13-lipoxygenase (LOX). LOXs are non-heme, iron-containing enzymes catalyzing the incorporation of molecular oxygen at the C-9 and/or C-13 position (Liavonchanka and Feussner, 2006) of polyunsaturated fatty acids. The 13-hydroperoxy fatty acids (13-HPs) are then cleaved by the action of the cytochrome P450 hydroperoxide lyase (HPL; Pinot and Beisson, 2010) leading to the formation of the C12
compound (9Z)-traumatin and a C6 compound. Depending on whether α-linolenic acid or linoleic acid was the starting material, the resulting C6 compound is either the unsaturated (Z)-3-hexenal or the saturated n-hexanal. (Z)-3-hexenal is relatively unstable and thus easily re-arranges either non-enzymatically or through catalysis by a (3Z):(2E)-enal isomerase to (E)-2-hexenal (Takamura and Gardner, 1996; Noordermeer et al., 1999). All aldehydes can be further metabolized by the activity of alcohol dehydrogenase (ADH) and alcohol acyltransferase (AAT; D’Auria et al., 2007) to the corresponding alcohols and their esters (Matsui, 2006).

The GLV-pathway represents only one branch of oxylipin biosynthesis, which comprises multiple biosynthetic branches that all start with dioxygenated fatty acid substrates (hydroperoxides, HP). Other branches lead, for example, to the formation of JA and divinyl ether fatty acids. However, not all HPs necessarily come from the same pool, nor are all necessarily provided by the same enzymes, i.e., the same lipases and LOXs. While no GLV-specific lipase has been identified so far, several lipases have been found that are involved in the fatty acid supply for JA biosynthesis: In *Arabidopsis*, the activation of the JA pathway is dependent on several partly redundant lipases, since knocking out one of them did not completely abolish the wound-induced JA-burst (Ellinger et al., 2010). *N. attenuata* plants with reduced expression of the plastidial lipase GLA1 showed a decrease in wound-induced JA levels of approx. 75% (Kallenbach et al., 2010; Bonaventure et al., 2011). However, these plants were unaffected in their GLV levels, indicating that this lipase does not supply fatty acids for the GLV pathway (Bonaventure et al., 2011). Such pathway specificity has also been reported for LOXs in several plant species. In potato, two distinct wound-inducible LOX genes (*LOX-H1* and *LOX-H3*) have been identified. While transgenic plants with reduced expression levels of *LOX-H1* exhibited a clear decrease in their GLV emission, the release was normal in plants with reduced *LOX-H3* expression (Leon et al., 2002). In tomato, silencing *TomLOXC* expression led to a significant reduction of GLVs in fruits and leaves (Chen et al., 2004) while silencing *TomLOXA* and *TomLOXB*, which are expressed in seeds or roots (Ferrie et al., 1994), had no effect on the production of GLVs (Griffiths et al., 1999). Rice possesses at least five different LOXs: while OsHI-LOX channels 13-HPs specifically to the JA-branch (Zhou et al., 2009), another 9/13-LOX, OsLOX1, has been shown to supply both the JA as well as the GLV branch (Wang et al., 2008). *N. attenuata* possesses two 13-LOXs, of which one (NaLOX3) specifically feeds into the JA-pathway, while the second LOX (NaLOX2) provides HPs to synthesize GLVs (Allmann et al., 2010 and Chapter 2).
The C18 fatty acids α-linolenic acid (18:3) and linoleic acid (18:2) are liberated from membrane lipids either enzymatically or non-enzymatically, and subsequent incorporation of molecular oxygen is catalyzed by a 13-lipoxygenase (LOX). The resulting hydroperoxides are cleaved by a hydroperoxide lyase (HPL) into (9Z)-traumatina and (Z)-3-hexenal or n-hexanal. (Z)-3-hexenal partly isomerizes to (E)-2-hexenal. For few plant species, (3Z):(2E)-enal isomerase activity has been documented. All aldehydes can be further metabolized by the activity of an alcohol dehydrogenase (ADH) and an alcohol acyltransferase (AAT) to the corresponding alcohols and their esters.

Figure 1-1. Biosynthetic pathway of green leaf volatiles

The C18 fatty acids α-linolenic acid (18:3) and linoleic acid (18:2) are liberated from membrane lipids either enzymatically or non-enzymatically, and subsequent incorporation of molecular oxygen is catalyzed by a 13-lipoxygenase (LOX). The resulting hydroperoxides are cleaved by a hydroperoxide lyase (HPL) into (9Z)-traumatina and (Z)-3-hexenal or n-hexanal. (Z)-3-hexenal partly isomerizes to (E)-2-hexenal. For few plant species, (3Z):(2E)-enal isomerase activity has been documented. All aldehydes can be further metabolized by the activity of an alcohol dehydrogenase (ADH) and an alcohol acyltransferase (AAT) to the corresponding alcohols and their esters.
4.2 Versatile roles of GLVs

GLVs are ubiquitous compounds as almost every green plant releases them. The release of GLVs is initiated by cell disruption caused by mechanical damage (Halitschke et al., 2004) or herbivory (De Moraes et al., 2001; Allmann and Baldwin, 2010), by pathogen attack (Croft et al., 1993; Shiojiri et al., 2006), and also as a consequence of abiotic stress (Gouinguene and Turlings, 2002; Heiden et al., 2003) or a sudden switch from light to dark (Brilli et al., 2011). Their functions are as broad as their occurrence:

Effects on microorganisms

GLVs can have a direct repellent or toxic effect on microbes or fungi (Hamiltonkemp et al., 1992; Nakamura and Hatanaka, 2002; Prost et al., 2005) and might thus be produced and released by plants to protect themselves from infection. In 1960, Major et al. reported that (E)-2-hexenal extracted from ginkgo leaves functioned as a fungal growth inhibitor. Especially this aldehyde, with its α,β-unsaturated-carbonyl group, has potent antimicrobial activity (Croft et al., 1993; Prost et al., 2005; Menniti et al., 2009) and it has already been tested and successfully used for improving the shelf life and the hygienic safety of processed food (Lanciotti et al., 2003; Belletti et al., 2007). While most studies used plate-assays to determine the antimicrobial activity of GLVs, only few studies have tested their properties directly in the plant: Plants inoculated with Botrytis cinerea increased their HPL transcript levels and concomitantly their GLV release (Shiojiri et al., 2006; Kishimoto et al., 2008). Furthermore, overexpression of HPL in Arabidopsis resulted in greater resistance of the transgenic plants against B. cinerea, while suppression of HPL caused greater susceptibility to the pathogen (Shiojiri et al., 2006). Pre-infestations of rice plants with the white-backed planthopper Sogatella furcifera induced the expression of HPL2 and subsequently increased the release of (E)-2-hexenal, finally leading to an increased resistance to bacterial blight (Gomi et al., 2010).

Effects on insects

HIPVs can mediate indirect defenses, i.e. by attracting foraging carnivorous predators and parasitoids that kill herbivores. Many studies have shown that attraction of predators and parasitoids is largely depending on HIPVs (Dicke, 2009; Mumm and Dicke, 2010). Dicke & Sabelis (1988) were the first to show that HIPVs indeed can be key foraging cues for natural enemies of herbivores. By means of a Y-tube olfactometer assay, they showed that the blind predatory mite Phytoseiulus persimilis depends on HIPVs for finding plants infested with its
prey, the spider mite *Tetranychus urticae*. Since then, many other studies have followed and they showed a similar attractive behavior of several parasitoids and predators to single volatile compounds or complex mixtures (for reviews see Heil, 2008; Dicke, 2009; Mumm and Dicke, 2010). Most of these studies were performed under laboratory conditions (Allison and Hare, 2009; Mumm and Dicke, 2010) and only few have challenged the indirect plant defense paradigm under field conditions (Allison and Hare, 2009).

As GLVs are immediately released from the wounded leaf of a plant, this group of HIPVs can provide rapid and reliable information about the exact location of the attacking herbivore. However, because GLVs are released from almost every plant and under various stress conditions, they might not provide reliable information to the prey-searching carnivore. Nonetheless, many studies showed that predators and parasitoids are indeed attracted to single GLVs or to a set of GLVs (Kessler and Baldwin, 2001; James, 2005; Shiojiri et al., 2006; Shimoda, 2010). It has been hypothesized that GLVs, due to their ubiquity and nonspecific information, should be mainly attractive to naïve natural enemies (Hoballah et al., 2002). Indeed, Hoballah et al (2005) showed that the parasitoid *Cotesia marginiventris* shifted its odor preference from a mainly GLV-containing mixture, when inexperienced, to a mixture that contained GLVs and terpenoids after it had experienced the latter blend in association with hosts (Hoballah and Turlings, 2005). However, we recently showed that the rapid release of GLVs can in fact contain herbivore-specific information: the (Z)/(E)-ratio of GLVs released from *N. attenuata* plants changed when plants were attacked by its specialist herbivore, the tobacco hornworm (*M. sexta*). This herbivore-induced change in the (Z)/(E)-ratio tripled the foraging efficiency of the generalist predators *Geocoris* spp. in nature (Allmann and Baldwin, 2010 and Chapter 3).

Unfortunately, at least from the plant’s perspective, the volatile information that plants release into the air upon herbivory is (probably) not encrypted and can thus be used and abused by non-beneficial insects for e.g. host plant recognition. Interestingly, the responses of herbivores to HIPVs can vary tremendously depending on the volatile composition, the insect gender or species; while some herbivores are repelled by GLVs, others are clearly attracted (Dicke and van Loon, 2000; Dicke and Baldwin, 2009).

Early results from Visser and Ave (1978) showed that the odor of potato plants was attractive to the Colorado potato beetle, *Leptinotarsa decemlineata*, but that individual components ((E)-3-hexenol, (Z)-2-hexenol, (E)-2-hexenol or (E)-2-hexenal) were not. Interestingly the addition of single GLVs to the potato odor disrupted the attraction of the potato beetle, suggesting that certain GLV ratios are important for host odor recognition.
Field experiments with tobacco (*N. attenuata*) revealed that flea beetles (*Epitrix hirtipennis*) were more abundant on GLV-producing wild type plants compared to plants with reduced HPL expression (*as-hpl*; Halitschke et al., 2008). Interestingly, for the tobacco hornworm, *M. sexta*, GLVs seem to serve as feeding stimulant (Halitschke et al., 2004; Meldau et al., 2009).

GLVs are not only used to find appropriate host plants, but also to find mating partners; cockchafer males (*Melolontha* sp.) swarming at dusk use plant-derived GLVs as primary attractants for mate finding (Reinecke et al., 2002; Ruther et al., 2002). Similar results have been shown for the garden chafer, *Phyllopertha horticola*, as exclusively male, but not female garden chafers were attracted to (Z)-3-hexenol (Ruther, 2004).

Furthermore, GLVs enhanced the attraction of both male and female diamondback moths (*Plutella xylostella*) when added to a pheromone blend in the field (Reddy and Guerrero, 2000).

As the increased release of plant volatiles may indicate to the herbivore that defensive compounds in the volatile-emitting plant have been induced, or that conspecific competitors and/or natural enemies are already present (Dicke and van Loon, 2000), the release of GLVs can also have a repellant effect on herbivores. Both C6 aldehydes and especially their alcohols were effective in reducing tobacco aphid (*Myzus nicotinae*) fecundity (Hildebrand et al., 1993), and the closely related green peach aphid *Myzus persicae*, fed on HPL-depleted potato plants (*Solanum tuberosum*) showed an almost two-fold increase in fecundity compared to wild type-fed insects (Vancanneyt et al., 2001). However, it is not clear whether GLVs have a direct toxic or repellent effect on aphids, or rather an indirect effect by inducing changes in the plant’s leaf chemistry. HIPVs, including GLVs, are also used by gravid moths to choose appropriate host plants for their offspring. In field experiments, undamaged control plants of *N. attenuata* received more eggs from the moth of the tomato hornworm *M. quinquemaculata* than plants that were already damaged by conspecific caterpillars (Kessler and Baldwin, 2001). Recently, we showed that female *Manduca* moths can even distinguish between (Z)- and (E)-isomers of GLVs. In field experiments they laid fewer eggs on *Datura wrightii* plants that were perfumed with (E)-isomers or low (Z)/(E)-ratios, resembling the GLV bouquet of *Manduca*-attacked plants (Chapter 4). Additionally, *spr2* mutants of tomato plants, which release lower amounts of unsaturated GLVs and some terpenoids, were preferred over wild type plants by ovipositing *Manduca* moths (Sánchez-Hernández et al., 2006).
CHAPTER 1

Figure 1-2. The versatile roles of GLVs
Green leaf volatiles are released by plants and their emission increases upon exposure to various biotic and abiotic factors. GLVs can affect plants’ interactions with many organisms, including microorganisms (1), insects (2-3, 6) and plants (4-5).

Effects on plants
Neighboring plants can also receive HIPVs and consecutively induce their own defenses (Heil and Karban, 2010). The idea that signaling between plants might be mediated by volatiles to activate defense responses emerged in the early eighties (Baldwin and Schultz, 1983; Rhoades David, 1983). Although controversial at that time, many studies have followed since, confirming that volatiles can initiate plant defense responses (Baldwin et al., 2002; Heil and
Karban, 2010). Treating plants with GLVs has shown to induce the expression of several defense-related genes and downstream metabolites (Zeringue Jr, 1992; Bate and Rothstein, 1998; Arimura et al., 2001; Farag et al., 2005; Engelberth et al., 2007) including a subsequent release of HIPVs (Farag and Parè, 2002; Ruther and Furstenau, 2005; Ruther and Kleier, 2005) or the secretion of extrafloral nectar (Kost and Heil, 2006). Strikingly, Paschold et al. (2006) found that the absence of GLVs from a WT wounding-induced volatile blend caused the transcriptional up-regulation of several defense related genes in *N. attenuata*. This demonstrates that GLV signaling and the subsequent processing of the information might not always be straightforward and it indicates that plants may ‘expect’ to perceive a wild type GLV blend from a neighbor, and are ‘confused’ when they do not; but perhaps this is not the same as adding extra GLVs to the neighbor blend, either for the receiver’s physiology, or for the ecological relevance of the information.

However, plants do not always directly up-regulate their defenses when exposed to plant volatiles. They can also be alerted by these volatiles, enabling them to induce their defenses more rapidly and/or more effectively at the actual time of herbivore attack. This form of “alertness” is also called priming and it has the advantage that it does not involve a significant fitness penalty for the plant (Heil and Baldwin, 2002; van Hulten et al., 2006). One of the best-described forms of priming is systemic acquired resistance (SAR), i.e., when a local leaf becomes infected by a pathogen, the systemic leaves can develop resistance to a broad spectrum of pathogens (Vlot et al., 2008; Shah, 2009). A comparable mechanism for priming against herbivore attack by GLV exposure was reported by Engelberth et al. (2004). Since then, several other studies have followed and confirmed the priming effect of GLVs on plants (Kessler et al., 2006; Frost et al., 2007; Heil and Bueno, 2007; Frost et al., 2008).

Volatile signaling between plants seems to be counterproductive for the emitting plant, as the volatile signals might benefit neighboring and probably genetically unrelated receivers, which may compete with the emitter for resources and fitness. Recently it has been shown that volatile signaling also takes place within a plant (Heil and Bueno, 2007). Intra-plant signaling by volatiles can overcome vascular constraints (Frost et al., 2007; Heil and Bueno, 2007) or might augment vascular systemic signals. GLVs might be of special importance for within-plant signaling events, as they are rapidly released after herbivory and highly volatile and can thus supply information to adjacent leaves within seconds.
The observation that plants can use volatile information coming from neighboring plants or adjacent leaves raises questions about the perception and transduction of green leaf volatiles and the mechanism behind priming. It has been suggested that GLVs with an $\alpha,\beta$-unsaturated carbonyl group, like (E)-2-hexenal, are potent inducers of plant defenses due to their chemical reactivity (Alméras et al., 2003). To elucidate such mechanisms Mirabella et al. (2008) isolated A. thaliana mutants in which phenotypic responses to (E)-2-hexenal were altered. These studies revealed a possible role of $\gamma$-amino butyric acid in mediating (E)-2-hexenal responses. However, GLVs that lack an $\alpha,\beta$-unsaturated carbonyl group and which are unable to yield an electrophile such as (E)-2-hexenal also have been shown to induce defense responses in a plant (Heil et al., 2008). Thus, it has been hypothesized that GLVs interact with membrane proteins of plants, like odorant binding proteins of insects, leading to changes in transmembrane potentials and subsequent induction of gene activity (Heil et al., 2008; Arimura et al., 2011).

5. Model system

For my studies I used two solanaceous plant species: wild coyote tobacco (N. attenuata, Chapters 2 and 3) and jimsonweed (Datura wrightii, Chapter 4). The model plant N. attenuata is a well-studied annual plant which has its native habitat in western North America (Baldwin, 2001). N. attenuata seeds germinate after fire from long-lived seed banks (Baldwin et al., 1994; Preston and Baldwin, 1999). While this germination behavior provides plants with nitrogen-rich soil and a habitat that lacks interspecific competition, N. attenuata is confronted with intense intraspecific competition and variable herbivore communities (Baldwin, 2001).

Among its most devastating attackers in nature are the tobacco and the tomato hornworms M. sexta and M. quinquemaculata (Lepidoptera: Sphingidae) which are also often associated with D. wrightii as a host plant, forming an ambivalent relationship as nectar-feeding pollinators as ovipositing adults, but folivorous larvae after oviposition (Mechaber et al., 2002; Mira and Bernays, 2002). The defense responses of N. attenuata upon M. sexta attack are well described, and a large portion of these defenses can be attributed to the plant’s perception of FACs present in the OS of Manduca caterpillars (Halitschke et al., 2001). During herbivory, OS are introduced into plant wounds, causing not only major transcriptional changes in the plant (Halitschke et al., 2003), but also the elicitation of a transient JA and ET burst (Kahl et al., 2000; Wang et al., 2007; Wang et al., 2008) which exceeds those bursts elicited by simple mechanical damage (Kahl et al., 2000). JA and its
derivatives play an essential role in mediating direct and indirect defenses as they are responsible for the accumulation of several defensive secondary metabolites, including trypsin proteinase inhibitors (Zavala et al., 2004) and diterpene glycosides (Jassbi et al., 2008; Heiling et al., 2010; Jassbi et al., 2010) and the release of the sesquiterpene (E)-α-bergamotene (Halitschke et al., 2000; Schuman et al., 2009). Accordingly, *N. attenuata* plants with a deficiency in JA production (as-lox3; Kessler, 2004) or JA perception (ir-coi1; Paschold et al., 2007) have been shown to be more vulnerable to *N. attenuata’s* adapted herbivore community when transplanted into their native habitat. Furthermore these plants also attracted novel herbivore species which were not present on normally defended wild type plants (Kessler, 2004).

Both *N. attenuata* and *D. wrightii* respond to *M. sexta* attack by emitting HIPVs (Gaquerel et al., 2009; Hare and Sun, 2011). However, *M. sexta* feeding does not only increase the emission of HIPVs; it also suppresses the release of certain volatiles, suggesting that plants have the tools to fine-tune their herbivore-induced volatile emissions (Gaquerel et al., 2009). Interestingly, although FACs played a central role in this pattern of regulation, as they inhibited the production of almost all OS-suppressed volatiles and accounted for half of the OS-elicited volatiles, they cannot explain all OS-induced regulatory changes in the volatile emissions. For example, the *M. sexta* OS-induced isomeric changes in the GLV profile of *N. attenuata* (Chapter 3) and *D. wrightii* (Chapter 4) are clearly not dependent on FAC-perception, but occur by the action of a yet unknown enzyme (Allmann and Baldwin, 2010 and Chapter 3).

The emission of HIPVs attracts predatory big-eyed bugs (*Geocoris* spp.) (Hemiptera: Geocoridae; Kessler and Baldwin, 2001; James, 2005). *Geocoris* spp. are generalist predators which feed amongst others on *Manduca* eggs and early larval instars. These predators have been shown to account for a vast majority of *M. quinquemaculata* mortality in nature (Kessler and Baldwin, 2002).

6. Outline of the thesis

In this thesis, I describe the biosynthesis of GLVs in the wild tobacco *N. attenuata*, how herbivory by the tobacco hornworm *M. sexta* affects the plant’s GLV composition and what the consequences of these herbivore-induced changes are for plants, herbivores and predators.
**Chapter 2** - *Oxylipin channeling in Nicotiana attenuata: Lipoxygenase 2 supplies substrates for green leaf volatile production*

In Chapter 2, we compare isogenic lines of the wild tobacco *N. attenuata* with reduced expression of *NaLOX2* (irlox2) or *NaLOX3* (irlox3) to determine the role of these different LOX isoforms in supplying substrates for two different pathways leading to the formation of either GLVs or JA. We show that reduced *NaLOX2* expression strongly decreases the production of GLVs without influencing the formation of JA and downstream metabolites. Conversely, reduced *NaLOX3* expression strongly decreases JA biosynthesis without influencing GLV production. These results demonstrate that *N. attenuata* channels the flux of hydroperoxides through the activities of different LOXs, leading to different direct and indirect defense responses mediating the plant’s herbivore resistance.

**Chapter 3** – *Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles*

In Chapter 3 we show that the rapid release of GLVs profoundly impacts tritrophic interactions under natural conditions. First, we show that mechanically damaged leaves of the wild tobacco *N. attenuata* release large amounts of (Z)-GLVs and low amounts of (E)-GLVs, but that feeding by the specialist herbivore, the tobacco hornworm (*M. sexta*), dramatically shifts this isomer ratio from (Z)- to (E)-GLVs. Subsequently we show that the herbivore-induced change in the (Z)/(E)-ratio triples the foraging activity of the generalist predators *Geocoris* spp. on caterpillar eggs. Finally we demonstrate, much to our own surprise, that the distinct change in the (Z)/(E)-ratio is not caused by the plant, but by the insect itself: *in vitro* assays show that enzymatic activity of the oral secretions (OS) of *M. sexta* caterpillars isomerize (Z)-3-hexenal to (E)-2-hexenal independently from the plant. Hence we are left with the puzzling observation that *M. sexta* caterpillars are betraying themselves by making the plant’s volatile bouquet more attractive to their natural enemy and, to explain this, we hypothesize that the enzyme may play a role in the insect’s disease immunity.

**Chapter 4** - *Isomerization of green leaf volatiles alters the behavioral responses of female Manduca moths*

In Chapter 4 we show that the herbivore-induced shift in the (Z)/(E)-ratio of GLVs released from *N. attenuata* plants is reproducible in *Datura wrightii*, another plant species of the solanaceous family. In this study we test whether the application of *M. sexta*’s oral secretions causes a change in the GLV profile, not only during the day but also and especially during
sunset and night – the active times of the hawkmoths *M. sexta* and *M. quinquemaculata*. With functional calcium imaging we demonstrate four different regions of interest (ROIs) in the antennal lobe of *M. sexta* females which are activated upon stimulation with several GLVs. Interestingly, two of these ROIs show high isomer specificity for hexenyl acetates as they are either activated by \((Z)-3\)-hexenyl acetate or its \((E)-2\)-isomer. Furthermore, stimulation with different ratios of both acetate isomers results in distinguishable activation patterns. With field experiments, in the native habitat of the moth, we demonstrate that the oviposition behavior of female *Manduca* moths differs between \((Z)\)- and \((E)\)-GLVs, between different \((Z)/(E)\)-ratios and between the two hexenyl acetates.

**Chapter 5 - Purification and properties of a \((3Z):(2E)\)-enal isomerase from *M. sexta’s* oral secretions**

In Chapter 5 we describe our first results on the characterization and purification of a \((3Z):(2E)\)-enal isomerase from *M. sexta’s* oral secretions by biochemical fractionation.

**Chapter 6 – General Discussion**

In Chapter 6 I discuss the results presented in this thesis and I summarize the possible functions that \((Z)/(E)\)-isomerization of GLVs might have, and who might benefit from such isomerizations.

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