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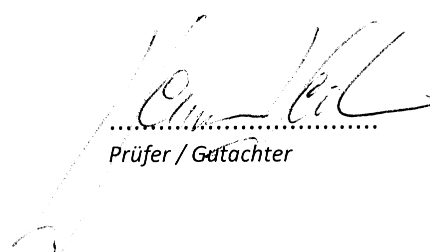

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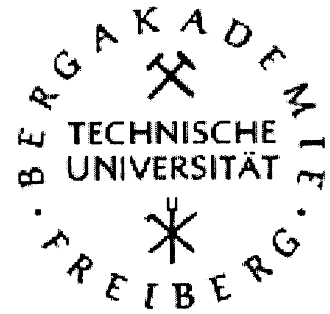

Green leaf volatiles function as an indirect defense and potentially interact with trypsin protease inhibitors in nature

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The enemy of my enemy is my ally.

M. W. Sabelis

This diploma thesis is based on a two-year field project begun in 2010 by Meredith C. Schuman, Ph. D. candidate at the Max Planck Institute for Chemical Ecology, Jena. Experiments were performed at the Lytle Ranch Preserve in southwestern Utah, owned by the Brigham Young University, Provo, Utah. Under the supervision of Ms. Schuman and Professor Dr. Ian T. Baldwin, the results out of this two-year experiment were submitted to *Science* in February 2012. This publication is the basis of the diploma thesis.

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Table of Abbreviations

Bt	*Bacillus thuringiensis*
dNTP	deoxynucleoside triphosphate
FAC	fatty-acid-amino-acid conjugate
FID	flame ionization detector
GLV	green leaf volatile
HIPV	herbivory-induced plant volatile
IS	internal standard
IR	inverted repeat
JA	jasmonic acid
L.O.D.	limit of detection
M	*Manduca* infestations
Ms	*Manduca sexta* (only in figure captions)
MS	mass spectrometry
OS	oral secretion
PCR	polymerase chain reaction
PI	protease inhibitor
PV	plant volatile
R	regurgitant
RNAi	RNA interference
SA	salicylic acid
TPI	trypsin protease inhibitor
VOC	volatile organic compound
WT	wild type

1. Introduction

In the face of herbivory, plants have developed a variety of physical barriers as well as chemical and structural traits which function as defenses against these enemies. Plant defense strategies compromise both constitutive mechanisms, which are always expressed, and inducible mechanisms activated or synthesized in response to herbivore attack, a strategy which avoids incurring the cost of defenses in the absence of enemies (Agrawal, 1998; Agrawal & Karban, 1999; Baldwin, 1998). Both inducible and constitutive plant defensive traits contribute to the effectiveness of resistance (Karban & Baldwin, 1997) and can be divided into two types of defense compounds: direct defenses – toxins, antidigestives or antinutritives that deter or debilitate herbivores directly (Bennett & Wallsgrove, 1994; Preston & Baldwin, 1999) – and indirect defenses such as the emission of herbivore-induced plant volatiles (HIPV) that disable or remove herbivores indirectly by attracting their predators and parasitoids following herbivore attack (Dicke & Sabelis, 1988; Price *et al*., 1980).

Nicotiana attenuata, a wild tobacco species native to Great Basin Desert in western North America, provides well-studied resistance traits in response to a wide array of attacking generalist and specialist herbivores (Baldwin, 1999a, 2001). Although total inducible defenses have been shown to benefit plant fitness under ecological conditions, most individual metabolites have not been tested for their effect on plant fitness. The best-characterized defense in *N. attenuata*’s arsenal is the neurotoxin nicotine, an acetylcholine receptor agonist, which is very effective against non-adapted animals (Baldwin, 1998). Moreover, herbivory on *N. attenuata* causes a decrease in the (*Z*)/(*E*)-isomer ratio of the released green leaf volatiles (GLV) blend, tripling the attraction of predators like *Geocoris* insects (Allmann & Baldwin, 2010). But GLVs also play other roles in plant-herbivore (e.g. feeding stimulants and cues for host location (Halitschke *et al*., 2004; Meldau *et al*., 2009)), also in plant-pollinators interactions (Baldwin *et al*., 2006; Kessler & Baldwin, 2001). Although the variety on interactions between plants and their enemies may have detrimental effects on plants reproduction (see above) and the recruitment of natural predators benefits plants by reducing their herbivore load, no studies have yet determined their net effect on plant fitness (Allison & Hare, 2009; Dicke & Baldwin, 2010). Two laboratory studies have shown that parasitization of herbivores increases plant reproduction, but parasitization was not a result of HIPVs in either of these studies (Fritzsche-Hoballah & Turlings, 2001; van Loon *et al.*, 2000).

In concert with HIPVs, *N. attenuata* expresses direct resistance traits such as trypsin protease inhibitors (TPIs), which inhibit herbivores’ protein digestion and thus reduce their

growth and performance by decreasing their access to essential amino acids (Zavala & Baldwin, 2004; Zavala *et al*., 2008). TPIs effectively reduce performance and survivorship of the natural folivores of *N. attanuata*, *Manduca* and *Spodoptera* larvae (van Dam *et al*., 2000; Glawe *et al*., 2003; Zavala and Baldwin, 2004) and thus increase fitness of *Manduca*-attacked plants under controlled glasshouse conditions (Zavala & Baldwin, 2004, Zavala *et al*., 2004a). But because specialist insects have developed effective strategies to cope with dietary PIs like ingesting more or higher-quality tissue (compensatory feeding), producing insensitive or desensitized proteases, or inactivating or degrading PIs, which could result in a negative fitness impact for the plant (Jongsma *et al*., 1995; Winterer & Bergelson, 2001; Zavala *et al*., 2008), PIs may only function as defenses in concert with other defenses in nature. The antifeedant effect of nicotine functionally complements the defensive function of TPIs against generalist herbivores like *S. exigua* (Steppuhn & Baldwin, 2007). This raises the question of whether a functional synergy exists between TPIs and indirect defenses for the nicotine-adapted specialist *Manduca*. The functional concert between the antinutritive effect of TPIs which reduce the resources available to herbivores for growth and behavior, and the attraction of their predators by the release of GLVs indicate the potential enhancement of each other's effectiveness (e.g. Allmann & Baldwin, 2010; Kessler & Baldwin, 2001; Zavala & Baldwin, 2004). In other words, an increase in herbivores' vulnerability and therefore expansion of the window of their susceptibility to predation due to dietary TPIs can increase the effectiveness of indirect defenses (Williams, 1999). Functional interaction among these two defensive traits might contribute to plant defense by shifting the costs of defense metabolites towards a measurable increase in plant fitness (Steppuhn & Baldwin, 2008).

Finally, whether a resistance trait functions as a defense or the functional complement of resistance trait is a cost-saving mechanism needs to be firmly established in the context of the native community, not only with trained herbivores, predators and parasitoids in controlled laboratory experiments (Baldwin, 2010, Halitschke *et al*., 2003).

2. Background

2.1 Plant defense strategy– response mechanisms, interactions and benefits

Plant defense responses to herbivores are complex as a result of a co-evolutionary history which goes back millions of years (Walters, 2011). In nature over 400.000 herbivorous insects feeding on 300.000 vascular plant species exist (Schoonhoven *et al*., 2005). Plants exposed to herbivores, attacked by them below ground as well as above ground, might lose more than 20% of annual net primary productivity (Agrawal, 2011; Schoonhoven *et al*., 2005). To defend against this loss of reproductive capacity to insect herbivores, the most important herbivore pressure on plants, and, of course, because they are sedentary, plants have evolved various defense strategies (Walters, 2011). The term anti-herbivore defense has been defined as plant traits that increase plant Darwinian fitness under herbivore attack (Karban & Baldwin, 1997) whereas resistance designates any trait that negatively affects herbivore performance or preference, but for which the defensive function is still unproven (Walters, 2011). Plants' survival depends on their ability either to get rid of enemies or tolerate damage, i.e., to regrow and reproduce after herbivory without sustaining a reduction in fitness (Strauss & Agrawal, 1999). In nature, plants' attackers encounter a complex variety of defenses ranging from physical barriers such as waxes and trichomes as first line of defense, to the constitutive and inducible synthesis of chemical compounds, to the induction of structural traits (Baldwin & Preston, 1999; Bennett & Wallsgrove, 1994; Karban & Baldwin, 1997). If herbivores manage to overcome physical barriers they need to arm themselves against an enormous variety of defense compounds, referred to as "secondary plant metabolites" which comprise more than 100.000 different compounds (Walters, 2011). Plants employ different types of defense: constitutive components, that are always expressed in various plant tissues, or inducible compounds synthesized in response to attacking pests or pathogens (Agrawal & Karban, 1999; Walters, 2011; reviewed in Ryan, 1990). Inducible defenses allow the plant to conserve resources in the absence of attack, as well as to tailor its response of different attackers and it may be more difficult for herbivores to develop resistance to inducible than to constitutive defense (Agrawal, 1998; Baldwin, 1998). Inducibility furthermore allows fine tuning of energy and resource allocation to defense according to the prevailing environmental conditions (Baldwin, 1998; Agrawal, 2011; Agrawal & Karban, 1999). Although plants benefit from the ability of induced defense to optimize the allocation of resources to growth, reproduction and defense (Karban & Baldwin, 1997) the time lag between initiation of attack and the complete activation of induced defenses generates a vulnerable state and edacious enables enemies to remove biomass (van Dam *et*

al, 2001a; Karban & Baldwin, 1997). Theory predicts on one hand, a strong but complex effect of induced responses in the face of herbivores and hence a positive selection on defense for the attacked plant (Agrawal, 1998, 2011; Baldwin, 1998; van Dam *et al*., 2001a; Karban *et al*., 1997; fig. 1), on the other hand in the absence of enemies, saved costs of defense elicitation may result in a positive impact on plants fitness and therefore an offset of the time delay disadvantages (Heil & Baldwin, 2002).

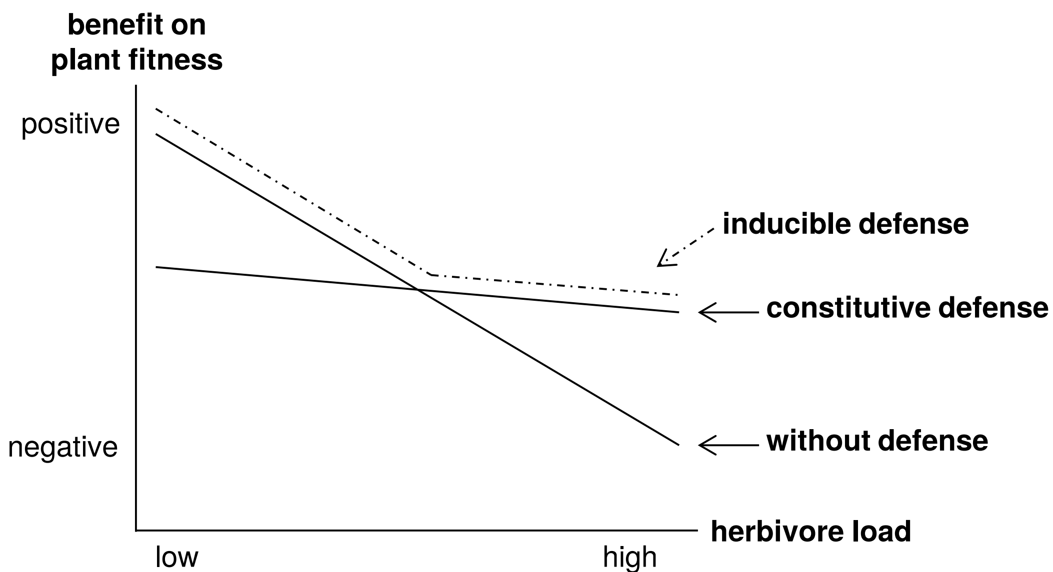


Figure 1: Induced resistance providing increased variability in defense. Plants employing defenses suffer less damage and therefore increase their fitness under herbivore pressure (continuous line). Plants without defense can save the costs of unnecessary defense and remain relatively undefended if future herbivore loads being low allows. Initial attack acts as a cue for the plant to induce defenses. Inducible changes in plant defense incur fewer costs in the absence of attackers. The model of inducible defenses predicts an optimum of employing defenses when needed to react more effectively to incoming risk of future herbivory (dotted line). Modified from Karban *et al*., 1997.

Moreover, both inducible and constitutive plant defensive traits are key mechanisms of plant resistance, acting either alone or in concert to contribute to the effectiveness of resistances (Karban & Baldwin, 1997; Walters, 2011). In response to herbivory, but also constitutively two types of defenses are expressed: direct and indirect defenses, which may functionally interact to shift the costs of defense metabolites towards the fitness benefits for the plant (Steppuhn & Baldwin, 2008). Direct defenses comprise various metabolites, plant volatiles and physical barriers like spines, thorns and trichomes that directly affect the performance, behavior and survival of herbivores (Dicke, 1999a; Karban & Baldwin, 1997; Kessler &

Baldwin, 2001; Steppuhn *et al.*, 2004) and increase plant resistance to further herbivory (Baldwin & Preston, 1999). According to their effects defense metabolites are classified into toxins like nicotine or furanocoumarins, enabling plants to kill their enemies outright, antifeedants and antidigestive proteins, such as protease inhibitors (PIs) or polyphenol oxidases (PPOs), which lead to a reduction of the digestibility of plant tissues and therefore to a decrease of the attackers performance, survival and fertility, or deterrents which make plants tissue more appetizing or palatable (e.g. Baldwin & Preston, 1999; Bennett & Wallsgrove, 1994; Birk, 2003; Broadway, 1996; Green & Ryan, 1972; Ryan, 1990). In contrast plant indirect defense disables or removes herbivores indirectly by attracting and improving foraging efficiency of herbivores natural enemies, such as pathogens, parasitoids or predators (Dicke & Sabelis, 1988; Karban & Baldwin 1997; Price *et al.*, 1980; Turlings & Wäckers, 2004). Plants manipulate tri-trophic plant-herbivore-natural enemy interactions using domatia, food bodies and herbivore-induced extrafloral nectar (EFN), which provide shelter and nutrition for predators and parasitoids, but also via herbivore-induced plant volatiles (HIPVs), sometimes termed the "cry for help", which convey cues for natural enemies about herbivore species and location (De Moraes *et al.*, 1998; Dicke, 2009; Dicke & Baldwin, 2010; Heil, 2008; Heil *et al.*, 2001; Turlings *et al.*, 1995). Although there is evidence that HIPVs enhance the effectiveness of the third trophic level and therefore facilitate top-down control of herbivore populations (Allmann & Baldwin, 2010; Baldwin & Preston, 1999; Halitschke *et al*. 2008; Karban & Baldwin 1997; Kessler & Baldwin, 2001*),* HIPV-mediated positive net effects on plant fitness remain unproven. Only two laboratory studies demonstrated an increase of plant reproduction by parasitization of herbivores (Fritzsche-Hoballah & Turlings, 2001; van Loon *et al.*, 2000), but in neither of these studies was parasitization a result of HIPVs. Thus for three decades HIPVs have been interpreted as being an indirect defense, but their defense function needs to be established by showing a positive impact on plant fitness in nature (Allison & Hare, 2009; Dicke & Baldwin, 2010; Heil, 2008).

2.2 Herbivore-induced direct versus indirect defenses

Nicotinana attenuata has been established as a model organism in plant-insect interactions, and the mechanisms and effects of several of its defense compounds have been well-described in response to generalist and specialist herbivores (Baldwin, 1999a, 1999b, 2001, Schuman & Baldwin 2012). In response to herbivore attack *N. attenuata* produces a wide array of herbivore-induced direct and indirect defense metabolites reducing herbivore load in

nature (Kessler & Baldwin, 2001; 2004b). Although total inducible defenses have been shown to increase plant fitness in nature, most individual metabolites have not been tested for their effect on plant fitness, and therefore it is not known whether the individual components of induced defense all benefit plant fitness (Baldwin, 2001). The best-characterized defense in *N. attenuata*'s arsenal is the neurotoxin nicotine, an acetylcholine receptor agonist, which is very effective against non-adapted animals (Baldwin, 1998). The biosynthesis of a variety of secondary metabolites, elicited by the introduction of elicitors contained in herbivores' saliva into herbivory-caused wounds, is primarily induced via the jasmonic acid (JA) signaling pathway. Therefore the production of both direct defense allelochemicals, such as toxic alkaloids (nicotine) and antidigestive proteins (trypsin protease inhibitors (TPIs)), and indirect defense compounds, such as volatile mono- and sesquiterpenes, is controlled by JA in different plant tissues (roots and shoots, respectively) (Baldwin, 1999b; Halitschke *et al.*, 2001; Kessler & Baldwin, 2002b; for review, see Baldwin & Preston, 1999). In addition to terpenes, plants emit green leaf volatiles (GLVs): C_6 alcohols, aldehydes and esters, immediately after wounding and prior to the JA signaling cascade. GLVs are released independently of JA-signaling (Allmann *et al*., 2010) and enzymes in caterpillar oral secretions (OS) change their isomeric ratio which results in a herbivore-specific signature (Allmann & Baldwin, 2010).

Upon attack by the nicotine-resistant specialist lepidopteran *Manduca sexta*, fatty-acid-amino-acid conjugates (FACs) in the herbivores' saliva are introduced into foliage wounds of *N. attenuata* (Baldwin *et al*., 2001; Halitschke *et al*., 2003). In plant research the first identified FAC was Volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] in the OS of beet armyworms (Alborn *et al*., 1997). *Manduca* larval feeding or application of their OS and regurgitant (R) to mechanical wounds amplifies wound-induced JA levels by twofold or more in a JA "burst" (Halitschke *et al*., 2001; McCloud & Baldwin, 1997). Additionally, during larval feeding a rapid ethylene (ET) burst, another herbivore-specific hormonal response, is emitted (Kahl *et al*., 2000; McCloud & Baldwin, 1997). The ET burst suppresses the induced nicotine response to feeding nicotine-tolerant *Manduca* via a transcriptional down-regulation of nicotine production, without affecting the emission of volatiles and induces accumulation changes of herbivore-responsive genes (Baldwin *et al*., 2001; Halitschke *et al*., 2001; Kahl *et al*., 2000; M. Schuman, unpublished data). Thus, *N. attenuata* employs different defensive strategies for generalists and specialists (Schuman & Baldwin, 2012). The employment of growth-retardant and indirect defenses seams to defend it against this nicotine-resistant specialist *Manduca*.

In addition, the pathogen-induced hormone salicylic acid (SA) negatively regulates the effects of JA and ET (Diezel *et al.*, 2009). Thus the orchestra of JA, SA and ET elicits a suite of specific induced defense responses including herbivore-induced volatile (HIPV) emissions, which can attract predators and parasitoids: in this case *N. attenuata* and the native predator *Geocoris* spp. (DeMoraes *et al.*, 1998; Diezel *et al.*, 2009; Halitschke *et al.*, 2000; Kahl *et al.*, 2000; Kessler & Baldwin, 2001).

2.2.1 GLVs: part of HIPV-mediated indirect defenses?

Herbivory causes a change in the release of plants' volatile bouquets into the air that can affect both direct and indirect plant defense. **Herbivore-induced plant volatiles (HIPVs)** are low molecular weight hydrocarbons compounds (below 300 Da) (Dudareva *et al.*, 2006) that are either only synthesized upon damage or constitutively synthesized by undamaged plants, but only released, or released in greater amounts following damage (Dicke & Baldwin, 2010). The emission of HIPV blends occurs from the epidermal cells of vegetative, reproductive and root tissues, as well as in the secretory structures or glandular trichomes (Dudareva *et al.*, 2004; Schuman & Baldwin, 2012). The first analyses of plant volatile blends revealed on average l0 - 20 compounds (Dicke *et al.*, 1990, Turlings *et al.*, 1990). Recent analyses with more sensitive methods have shown that herbivore-infested plants emit an odor cocktail composed of more than 200 different compounds (Dicke, 2000, Dudareva *et al.*, 2006). The suite of HIPVs comprises three major classes: terpenoids (also known as isoprenoids), phenylpropanoids/benzenoids, and fatty acid (FA) derivatives (Dudareva *et al.*, 2004, 2006) whose composition differs with plant species and genotype. Within a plant species the HIPV composition may change according to endogenous diurnal rhythms, developmental stage of leaves and flowers, pollination status, species or instar of attacking herbivore and abiotic conditions such as light and temperature (Dudareva *et al.*, 2004; Halitschke *et al.*, 2000, Turlings *et al.*, 1995). Furthermore, the herbivore-induced release of volatiles is generally induced by elicitors in the herbivore saliva to the wound sites, for example FACs in the OS of feeding tobacco hornworm larvae (Halitschke *et al.*, 2000) or volicitin of feeding beet armyworms (Alborn *et al.*, 1997). Treatments of herbivores OS and R or methyl JA (MeJA) to mechanical wounds also elicit PV release (Dicke, 1999b; Halitschke *et al.*, 2000; Turlings *et al.* 1995), but mechanical damage can provoke releases itself (Baldwin et al., 2002; Halitschke *et al.*, 2000). Thus, only HIPVs which are reliable indicators of herbivore feeding provide cues for herbivores enemies to hunt their prey.

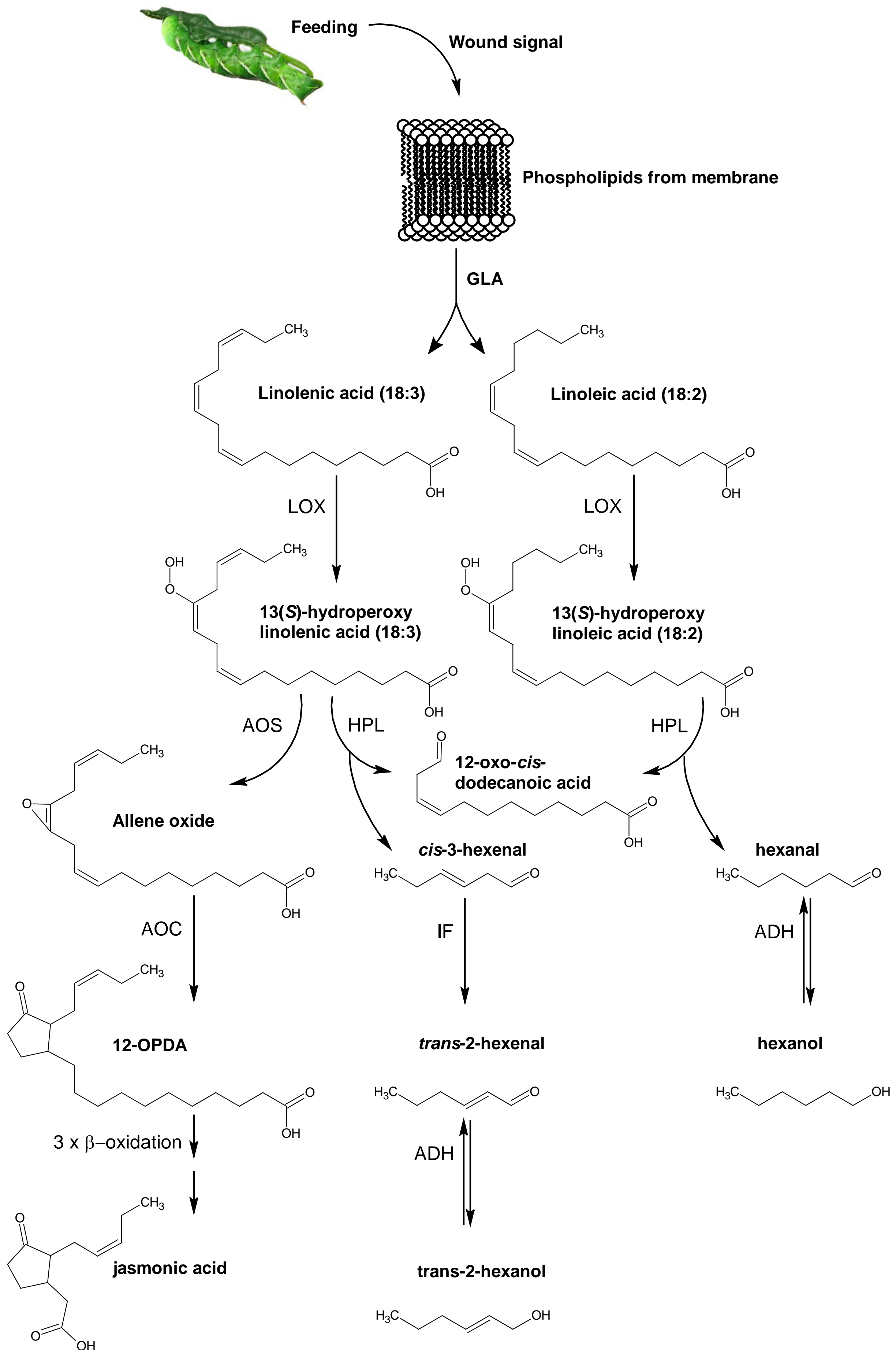
Given the fact that the composition of emitted HIPV bouquets specifies plant species and insect species that attack the plant (De Moraes *et al.*, 1998; Dicke 1999a) volatiles are

thought to mediate specific interactions between plants, their herbivores and the third trophic level (Price *et al*., 1980). The ecology of HIPV-mediated inter- and intra-plant communication is similarly complex and includes not only protection and information – but also potentially misinformation (Baldwin, 2010; Dicke, 1999b). HIPVs may also act as feeding stimulant and host location for herbivores in plant-herbivore interactions (Dicke, 1999b), alert plants to a neighboring plant's presence or herbivore infestation in plant-plant communication (the "talking plants" phenomenon) (Baldwin *et al*., 2006; Heil & Karban, 2010) or alleviate oxidative stress due to heat, drought or high UV exposure (Vickers *et al*., 2009; Loreto & Schnitzler, 2010). Thus, HIPVs can fill a variety of roles; it is unclear whether they really function to recruit natural enemies in nature. Research studies for HIPVs have only shown a fitness benefit for the plant by employing the recruitment of natural enemies under extremely artificial conditions (De Moraes *et al*., 1998; Otte, 1974; Turlings *et al*., 1995). This botanical "cry for help", provides information about identity, location, activity and perhaps even developmental stage of the attacking herbivore and guides insect predators or parasitoids or other members of the third trophic level to their prey (De Moraes *et al*., 1998; Dicke, 1999b; Turlings *et al.*, 1995). In scientific research this attraction phenomenon was first described by Dicke & Sabelis (1989) and Dicke *et al.* (1990) with predatory mites *Phytoseiulus persimilis* and by Turlings *et al*. (1990, 1995) and Turlings & Tumlinson (1992) with the parasitic wasps *Cotesia marginiventris*.

In the research in HIPVs-mediated top-down effects (control through organisms at the top of food chains) **green leaf volatiles (GLVs),** the most commonly released volatiles, play an important role. GLVs represent a suite of C18 fatty-acid derived six-carbon (C_6) aldehydes, alcohols and their esters that were constitutively produced in healthy, undamaged plant tissue in trace amounts, but first emitted following damage, producing the typical freshly cut grass smell (Schuman & Baldwin, 2012; Turlings *et al*., 1995). In contrast to GLVs herbivory releases the second major classes of HIPVs, terpenes, which carry more herbivore specific information (Turlings *et al*., 1998). In plant responses to herbivore attack, for instance caterpillar feeding, the octadecanoid signaling pathway has been shown to play a central role in regulating the release of volatiles in addition to most of inducible defenses (Baldwin, 2010, Kessler & Baldwin, 2002a). The biosynthetic pathway in *Nicotiana attenuata* for GLVs starts with the release of C_{18} polyunsaturated fatty acids, linolenic (LE, $18{:}3^{\Delta 9,12,15}$) or linoleic (LA, $18{:}2^{\Delta 9,12}$) acids, from leaf lipids such as galactolipids out of the plastid membrane by glycolipase activity (GLA) (Schuman & Baldwin, 2012; Heil, 2008). 18:3 and 18:2 free fatty acids are subsequently peroxidated to 13-hydroperoxy linolenic acid and 13-hydroperoxy linoleic acid by lipoxygenase NaLOX2 (Halitschke & Baldwin, 2003; Heil, 2008). LOXs

constitute a large gene family of non-heme-iron containing fatty acid dioxygenases, which are multifunctional enzymes catalyzing at least three different types of reactions: (1) dioxygenase, (2) hydroperoxidase and (3) leukotriene synthase (Feussner & Wasternack, 2002). The ubiquitous variety of biochemical reactions provides their distinct regulatory functions in the production of signaling molecules and defense compounds in plants, fungi, algae and mammals (Brash, 1999; Feussner & Wasternack, 2002). The regio- and stereo-specific dioxygenation of polyenoic fatty acids containing a (1*Z*,4*Z*)-pentadiene system such as LA to their hydroperoxides (HPs) by LOX represents the initial step in the octadecanoid pathway (ketodiene-forming pathway) (Feussner & Wasternack, 2002). According to the position where the oxygen group is introduced in the hydrocarbon backbone either at the C-9 (9-HPs) or C-13 position (13-HPs) the LOX-catalyzed hydroperoxidase reaction yields to the corresponding (9*S*)-hydroperoxy- and (13*S*)-hydroperoxy derivatives (Brash, 1999; Feussner & Wasternack, 2002; Liavonchanka & Feussner, 2006) (fig. 2). Therefore the enzymes are referred to as 9-LOX or 13-LOX, respectively. Both HPs are either subsequently cleaved by the action of hydroperoxide lyases (HPL), leading to GLVs, or, modified by allene oxide synthase (AOS) into a precursor of JA and its derivates (Feussner & Wasternack, 2002; Liavonchanka & Feussner, 2006) (fig. 2). These compounds derived from LOX activity commonly named oxylipins (Liavonchanka & Feussner, 2006). The cleavage of 13-HPs to C_{12} [12-oxo-(*Z*)-9-dodecenoic acid] and C_6 compounds is catalyzied by HPL (Feussner & Wasternack, 2002; Léon *et al.*, 2002; Liavonchanka & Feussner, 2006). Depending on the reaction substrate of HPL different C_6 aldehydes, commonly referred to as GLVs, are produced: *cis*-3-hexenal is formed from linolenic acid 13-HP, *n*-hexanal is formed from linoleic acid 13-HP. Consequent enzymatic or non-enzymatic isomerization of hexenal leads to its isomer (*trans*-2-hexenal), the C_6 corresponding alcohols are produced by the action of alcohol dehydrogenases (e.g. *cis*-hexen-1-ol) and an acyltransferase to form acetates from the alcohols (e.g. *cis*-3-hexen-1-yl acetate) (Feussner & Wasternack, 2002; Kessler & Baldwin, 2002b; Walling, 2000) (fig. 2).

The specific blend of GLVs in plant-herbivore-herbivore and plant-pathogen-interactions and with the third trophic level least in part determines their function. The composition GLVs is unique to the particular plant species provided by distinct balance of linolenic- and linoleic-derived C_6 volatiles (Walling, 2000). Indeed GLVs can have diverse defense-associated roles, for example as effects on insects ranging from host-selections, feeding stimulant of some lepidopteran larvae (Halitschke *et al.*, 2004; Meldau *et al.*, 2009), as antimicrobials or antifungals (Deng *et al.*, 1993; Shiojiri *et al.*, 2006) and as intra- and inter-plant signals (Kost & Heil 2006; Paschold *et al.*, 2006).



Feeding
Wound signal
Phospholipids from membrane
GLA
Linolenic acid (18:3)
Linoleic acid (18:2)
LOX
LOX
13(S)-hydroperoxy linolenic acid (18:3)
13(S)-hydroperoxy linoleic acid (18:2)
AOS
HPL
HPL
12-oxo-cis-dodecanoic acid
Allene oxide
cis-3-hexenal
hexanal
AOC
IF
ADH
12-OPDA
trans-2-hexenal
hexanol
3 x β–oxidation
ADH
jasmonic acid
trans-2-hexanol

Figure 2: Herbivore-induced defense mechanisms in plants: the biosynthetic pathway of GLV and JA production. Wound-induced signals upon herbivory induce the release of C_{18} polyunsaturated fatty acids from leaf lipids that are subsequently peroxidated to the corresponding hydroperoxid derivates by two distinct lipoxygenases. The hydroperoxides are either converted by allene oxide synthase and via a successive cascade of enzymes to JA or cleavage by a hydroperoxide lyase to volatile GLVs and C_{12} oxilipins. Alcohol dehydrogenase (ADH), Allene oxide cyclase (AOC), Allene oxide synthase (AOS), green leaf volatile (GLV), glycolipase (GLA), hydroperoxides (HPs), hydroperoxide lyases (HPL), isomerization factors (IF) jasmonic acid (JA), lipoxygenase (LOX), 12-Oxo-10,15(*Z*)-phytodienoic acid (12-OPDA). Modified according to Heil, 2008; Schuman & Baldwin, 2012; Walling, 2000.

Why may GLVs function as an indirect defense?

The question remains. The potential defensive function of GLVs in *N. attenuata* is based on their attraction of foraging predators such as the big eyed-bug *Geocoris pallens* (Kessler & Baldwin, 2001; Halitschke *et al*. 2008). *Manduca* herbivory of *N. attenuata* induces a decrease in the (*Z*)/(*E*)-isomer ratio of the released GLV blend. The alteration of plant volatile profiles triples the foraging efficiency of predators like *Geocoris* insects in nature (Allmann & Baldwin, 2010.). But the many roles of GLVs as attractants for both predators (Allmann & Baldwin, 2010; Kessler & Baldwin, 2004a) as well as herbivores and also as feeding stimulants (Halitschke *et al*., 2004, 2008) for herbivores may nullify their potential positive impact on plant fitness from enhanced predation. Plant research has shown that although GLV-emitting plants are more attractive to native lepidopteran herbivores like *M. sexta* and stimulate feeding in *M. sexta* larvae (Halitschke *et al*., 2004; Meldau *et al*., 2009), GLV-emitters also attract more predators (Halitschke *et al*., 2008; Kessler & Baldwin, 2001; Kost & Heil, 2006). In summary the release of GLVs influence herbivores both directly and indirectly (Allmann & Baldwin, 2010; Halitschke *et al*., 2004, 2008; Kessler & Baldwin, 2001; Meldau *et al*., 2009). Plants likely benefit from attracting natural enemies like predators, because they enable the plant to get rid of attacking herbivores (Allison & Hare, 2009). But the recruitment of natural enemies as plants “bodyguards” neither guarantees a reduction in herbivory nor an increase on evolutionary fitness for the plant (Allison & Hare, 2009). Hence three decades since the seminal paper by Price *et al*. in 1980 suggesting indirect defense as a plant defense mechanism, HIPVs have been interpreted as indirect defenses, but there is a lack of clear evidence showing any effects of their emissions on plants’ fitness (Allison & Hare, 2009; Dicke & Baldwin, 2010). Given that no studies have yet investigated whether GLVs may function as indirect anti-herbivore defense their beneficial positive net effect on plant

fitness needs to be firmly established in the context of the native community, not only with trained herbivores, predators and parasitoids in controlled laboratory experiments (Baldwin, 2010; Halitschke *et al.*, 2003).

2.2.2 TPIs and the interactions with other defenses

Plants respond to insect attack by producing defensive proteins, such as **protease inhibitors (PIs)**, which are composed of polypeptides or proteins and occur naturally in a wide range of plants like tomato, potato and tobacco (Ryan, 1990). 40 years ago the pioneering study of Green & Ryan (1972) presented the iconic example of PIs as induced plant defense mechanism. Following up on this study, researchers discovered and characterized a class of antidigestive endogenous proteinaceous inhibitors active against herbivorous insects, bacteria and pathogens. The induction of PIs in response to herbivore attack within 12 hours in undamaged younger tomato leaves on damaged plants inspired Green & Ryan (1972) to hypothesize PIs as a potential plant defense. Whereas PIs are developmentally regulated and therefore constitutively expressed, a rapidly induced PI-level increase upon herbivore attack results in a local and systemic accumulation of PIs in plant tissue, in concert with the production of numerous secondary metabolites (Ryan, 1972, 1990). In addition to enzymes such as arginase and threonin desaminase PIs are grouped into the large family of arthropod-inducible proteins (AIPs) that play a critical role in plant defense by targeting the digestive system of insects, widespread in nature (Ryan, 1990; Walters, 2011). The defensive mechanism of PIs is based on the inhibition of major intestinal proteases in the insect gut (Green & Ryan, 1972). In detail, PIs bind irreversibly to the substrate-binding site of cognate proteolytic enzymes of herbivorous insects (Koiwa *et al.*, 1997; Laskowski, 1985). According to the four mechanistic classes of proteolytic enzymes: serine, cysteine, aspartic and metallo-protease, that PIs specifically inhibit, the families are organized into serine PIs (e.g. Soybean trypsin inhibitor (Kunitz) family, Potato Inhibitor I family), cysteine PIs (e.g. Cysteine proteinase inhibitor family (cystatins)), aspartic PIs (e.g. Aspartyl proteinase inhibitor family, cathepsin D inhibitor family) and metalloprotease PIs (e.g. metallo-carboxypeptidase inhibitor family) (Garcia-Olmedo *et al.*, 1987; Ryan 1990). Serine-inhibitor families are universal throughout the plant kingdom and represent with cysteine- inhibitor families the most studied class of inhibitors in plant defense (Garcia-Olmedo *et al.*, 1987; Ryan, 1990; Walters, 2011). The interaction of an inhibitor (*I*) with a cognate serine protease ***(E)*** to its substrate-binding site follows the standard “lock and key” mechanism of inhibition. The formed intermediate inhibitor-enzyme complex dissociate directly in enzyme and a modified inhibitor (*I**) by hydrolysis of its reactive-site peptide bond.

As a result of extremely and the high slow hydrolysis of the inhibitor-enzyme-complex a simple equilibrium between the enzyme and free intact inhibitor on the one hand and bond modified inhibitor is reached (Garcia-Olmedo *et al*., 1987; Laskowski & Kato, 1980). The efficiency of a specific protease inhibitor is determined not exclusively by the structural compatibility of all amino acid residues of the reactive site of the protease inhibitor and those of the substrate-binding site of the proteases in the target organism, but also by the physiological conditions within the herbivore midgut (e.g. pH) and the plant quality (e.g. polyphenyloxidase activity, protein quality and quantity) (Laskowski, 1985; Broadway & Duffey, 1986, for review see Broadway, 1995).

Inhibiting the proteolysis in the gut of the insect decreases its access to essential amino acids, thus the absorption or digestion of plant protein is impaired immensely (Green & Ryan, 1972; Ryan, 1972; Ryan, 1990; Walters, 2011; Zhu-Salzman *et al*., 2008). The amino acid deficiency for protein synthesis causes several physiological effects in insects: decreased performance, survival and fertility (Birk, 2003; Broadway & Duffy, 1986; Broadway, 1996). But the employment of non-toxic digestibility-reducers can elicit behavioral and physiological counter-responses in insects that increase enemy efficacy for plants. Hence, insects may adapt to high TPI levels by (1) production of proteases that are less susceptible to inhibitors (Broadway 1995; Jongsma *et al*., 1995), (2) using other classes of proteases (Jongsma *et al*. 1995; Broadway 1996), (3) inactivating or degrading the PIs to prevent binding to sensitive proteases (Gruden *et al*., 1998; Ishimoto & Chrispeels 1996) or (4) compensatory feeding responses to increase their total protein intake (Winterer & Bergelson, 2001). If herbivores respond to PIs by compensatory feeding, the greater removal of photosynthetic tissue can have a negative impact on plant fitness (Steppuhn & Baldwin, 2007; Price *et al*., 1980; Walters, 2011). One way for plants to avoid the “proteolytic breakdown” of PIs is by producing multidomain inhibitors of several PIs, which collectively target a broad range of insect gut proteases (for review see Jongsma & Bolter, 1997).

In response to herbivore attack *N. attenuata* produces a combination of toxins and antidigestive proteins like nicotine and TPIs. The elicitation of these defense compounds is mediated by JA signaling (Kessler & Baldwin, 2002b; van Dam *et al*., 2000, 2001b; Steppuhn *et al*., 2004), which varies with ontogeny and leaf age (Ohnmeiss & Baldwin, 2000; van Dam *et al*., 2001b) and JA certainly transcriptional down-regulates and posttranscriptional increased many defense genes, respectively (Horn *et al*., 2005; Kahl *et al*., 2000). Endogenous TPIs belong to the potato PI-II family have a 7-repeat TPI domain (Zavala & Baldwin, 2004), and effectively reduce the performance of folivores such as *Manduca* and *Spodoptera* larvae and mirids (*Tupiocoris notatus*) (van Dam *et al*., 2000; Glawe *et al*., 2003;

Zavala and Baldwin, 2004; Horn *et al*., 2005; Zavala *et al*., 2008). Under glasshouse conditions, TPI-mediated decreases in *M. sexta* growth and survivorship translates into a fitness benefit for *N. attenuata* plants. Although TPI production reduces fitness in the absence of herbivory, TPI-producing plants produce more seeds than TPI-deficient plants when they were attacked by *Manduca sexta* under controlled glasshouse conditions (Zavala & Baldwin, 2004, Zavala *et al*., 2004a). But this has not been demonstrated under natural conditions.

Although fitness benefits of TPI production outweigh their large costs for attacked *N. attenuata* plants, insects have developed effective strategies to cope with dietary protease inhibitors which could result in a negative fitness impact for the plant (see above) (Zavala & Baldwin, 2004, Zavala *et al.*, 2004b). The functional complement of TPIs and nicotine, a neurotoxin, is one strategy to deter generalist herbivores like *Spodoptera exigua.* The compensatory feeding response and therefore the enhanced performance of *S. exigua* to TPIs alone are prevented by the antifeedant effect of nicotine (Steppuhn & Baldwin, 2007). However, the inhibition of both nicotine and TPI individually increased the performance of the nicotine-tolerant specialist *M. sexta* (Glawe *et al.*, 2003; Voelckel *et al*., 2001). In summary the presence of antifeedants like nicotine complements the defense function of PIs synergetically to reduce herbivores' ability to overcompensate and thus provides a defensive synergism for generalist herbivores, but not for the nicotine-adapted specialist *M. sexta* (Steppuhn & Baldwin, 2007).

2.2.3 The potential functional mechanisms of TPI-GLV interaction

In nature plants have to cope with a large diversity of enemies and stressors, sometimes simultaneously. Due to the pressure from resource limitation or pleiotropic effects plants developed both individual defense compounds with additional functions in growth and development and "cross-talk" among different defensive traits (Ballhorn *et al*., 2008; Walters, 2011). The mechanisms linking the expression of one defense trait to another trait include genomic, metabolic or functional linkages that bring with them not only benefits, but also constraints due to high costs of co-expression and evolutionarily generated "defense syndromes" (for review see Bergelson & Purington, 1996; Steppuhn & Baldwin, 2008). Thus depending on the type of interaction, synergetic or mutually exclusive, positive or negative outcomes are possible (Walters, 2011).

Functional interactions among defenses can increase plant fitness if they shift the costs of defense metabolites towards the fitness benefits (Steppuhn & Baldwin, 2008). Therefore to

the extent that these interactions have evolved under selective pressure, they should function to increase plant fitness and decrease overall costs of defense. As discussed above, particularly those direct defenses that decrease insect herbivore growth and performance by reducing nutrient intake (such as PIs), may only function as defenses in concert with other antifeedant or deadly defense responses in nature. An antidigestive defense may not function for specialists in nature without their removal by the third trophic level, because specialists are especially likely to have adapted to antinutritive defenses and particularly to tolerate the larger dose of other defense metabolites ingested through compensatory feeding (Baldwin & Preston, 1999; Bergelson & Purington, 1996). However, what are the pivotal keys for an existence of a functional linkage between TPI production and indirect defense for *N. attenuata*? Firstly both are induced upon herbivore attack or mechanical wounding and OS application (e. g. Steppuhn *et al*., 2004; van Dam *et al*., 2000; Zavala & Baldwin, 2004). Secondly TPIs and VOCs are coordinately expressed because they share the same elicitor (Halitschke *et al*., 2000, 2001; van Dam *et al*., 2001b). Thirdly the costs of TPI production which divert nitrogen-containing resources and essential amino acids (Zavala *et al*., 2004a) versus the minor carbon resource requirements for the volatile release (Halitschke *et al*., 2000) are not too high to provide a net fitness benefit of employing both defenses (Baldwin, 1999b). Finally, the functional concert between reducing the nutrition available to herbivores for growth and health (via TPIs) and increasing their mortality by attracting the generalist predator *G. pallens* (via GLVs) indicate that these two defense compounds would enhance each other's effectiveness (e.g. Allmann & Baldwin, 2010; Birk, 2003; Kessler & Baldwin, 2001; Zavala & Baldwin, 2004). Because the antinutritive effect of TPIs potentially increases the vulnerability of herbivore to their predators without poisoning them (Koiwa *et al*., 1997), they may expand the window of susceptibility to predation, increasing the effectiveness of indirect defense, which must be measurable as an increase in plant fitness (Williams, 1999). But a specialist herbivore like *Manduca* resistant to plant toxins has many means of adapting to TPIs for instance with compensatory feeding or moving to non-induced, more protein-rich plant parts (Zavala & Baldwin, 2004). Moreover GLV-emitting plants are more attractive to native herbivores and stimulate feeding in *M. sexta* larvae (Halitschke *et al*., 2004; Meldau *et al*., 2009), which may outweigh the benefit of increased predation by *Geocoris*. However, if PIs weaken or slow the growth of *Manduca* larvae before they have fed enough to overcome the antinutritive effect, the herbivores might be more vulnerable to indirect defense.

Furthermore a functional linkage between nicotine and the release of VOCs seems to be possible, because the prediction of inducibility and coordinately activating by the JA signaling cascade following insect attack are fulfilled (Baldwin, 1999a). Although the potentially

synergy of VOCs and nicotine may result in a positive net fitness benefit for the plant in consideration to the large investment of fitness-limiting resources for nicotine production (Halischke *et al.*, 2000) the transcriptional suppression of nicotine may reflect the incompatibility of its defensive use against nicotine-tolerant specialists whose parasitoids are sensitive to the toxin (Baldwin, 1999b; Kahl *et al.*, 2000). In addition toxin-tolerant herbivores are able to sequester plant toxins for their own defense (Steppuhn & Baldwin, 2008).

Although production of TPIs and GLVs is certainly costly to the plant (Halitschke *et al.*, 2000; Steppuhn & Baldwin, 2008; Zavala *et al.*, 2004a, 2004b) the synergistic linked interaction of both defenses is supposed to provide an "increase of the resistance above the sum of the resistance provided by each defense alone" (Steppuhn & Baldwin, 2008). But this has not been demonstrated in nature. In summary, the functional complement of indirect and direct defense responses is predicted to be a cost-saving mechanism.

2.3 *Nicotiana attenuata*, *Manduca* and *Geocoris* – an ecological model system

The wild tobacco ***Nicotiana attenuata*** Torr. ex Watts (Solanaceae; synonymous to *N. torreyana* Nelson & Macbr.), a post-fire annual plant, is native to the Great Basin Desert in western North America (Baldwin, 2001). This native tobacco, also referred to as coyote tobacco, "chases" fires by germinating synchronously into post-fire habitats, stimulated by the presence of chemical cues in smoke and the absence of inhibitors from competing vegetation (Baldwin *et al.*, 1994; Preston and Baldwin, 1999). Its seeds can lay dormant up to 150 years in seed banks, waiting for new fires, and germinate the first and second years after a fire (fig. 3) (Baldwin, 2001; Preston and Baldwin 1999, Steppuhn, 2007). In the ephemeral post-fire environment, which features nitrogen-rich soils and a very small number of herbivorous insects and few intra-specific competitors, *N. attenuata* is a primary settler selected for rapid growth in monocultures during times of high water availability (Baldwin *et al.*, 1994; Lynds & Baldwin, 1998; Whelan, 1995). A diploid set of chromosomes, self-compatibility and the above-mentioned specific properties make *N. attenuata* capable of populating this primordial agricultural niche, but expose plants to high intra-specific competition (Baldwin, 2001; Steppuhn, 2007).

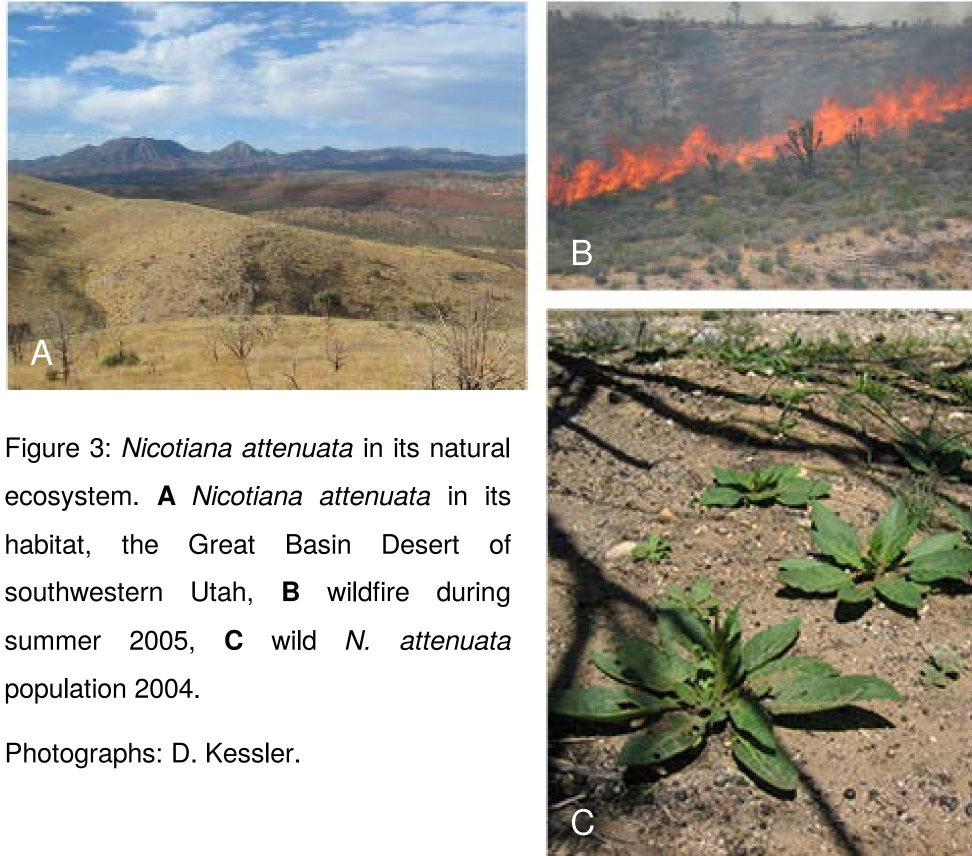


Figure 3: *Nicotiana attenuata* in its natural ecosystem. **A** *Nicotiana attenuata* in its habitat, the Great Basin Desert of southwestern Utah, **B** wildfire during summer 2005, **C** wild *N. attenuata* population 2004.

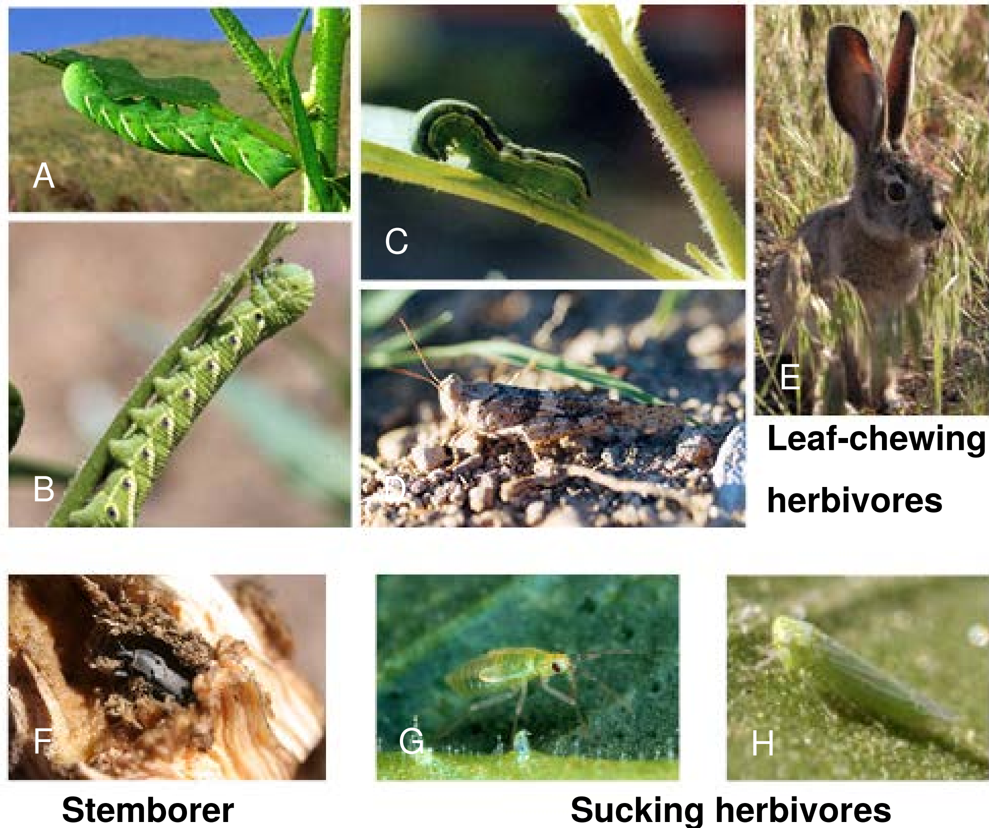
Photographs: D. Kessler.

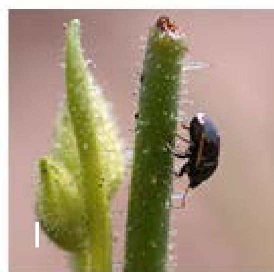
Herbivores from more than 20 different phylogenetic taxa recolonize new populations quite quickly the burned habitats "closing" the fugacious niche of exclusive growth for *N. attenuata*, whereas the most dominant and therefore damaging herbivores differ from year to year (Baldwin, 2001). In the Great Basin Desert native populations of *N. attenuata* are usually first colonized by mirids (*Tubiocoris notatus*) (Glawe *et al*., 2003). Furthermore mirids present with caterpillars of *Manduca quinquemaculata* (Lepidoptera, Sphingidae) and the flea beetle *Epitrix* spp. (Coleoptera, Chrysomelidae) are the dominant folivores of these wild tobacco populations (Kessler & Baldwin, 2001). Additionally, there is a diverse community of natural opportunistic generalist herbivores which attack *N. attenuata* including grasshoppers, (*Acrididae* spp. and *Trimerotropis* spp., Acrididae) and noctuids (*Spodoptera exigua* Hübner, Noctuidae) and mammal species, like black-tailed jack rabbits (*Lepus californicus,* Leporidae) (fig. 4) (Baldwin & Ohnmeiss, 1993; Kessler & Baldwin, 2004a; personal experience).

In the following, the naturally occurring sphingid hornworm *M. sexta* Linnaeus and the closely related species *M. quinquemaculata* Haworth will be considered in more detail. These two specialist hawkmoth species, commonly named tobacco hornworm and tomato hornworm, are native in a geographic range from Canada to Argentina and specialized on nightshades including *Nicotiana*, *Lycopersicon*, *Solanum*, and *Datura* species (Steppuhn, 2007).

Manduca larvae are often responsible for the greatest defoliation of *N. attenuata* plants in the great basin desert in Utah (Kessler & Baldwin, 2001). They are specialized to their host plant *N. attenuata* by adaptation to one of the most efficient defense against herbivores, the potent alkaloid toxin nicotine, through specific detoxification (Kessler & Baldwin, 2004a). The nocturnal activity of adult hawk moths allows protected oviposition, because predators are day-active, and simultaneous pollination of flowers during nectar feeding. Single eggs are preferentially deposited lower surfaced of mid-stem leaves of plants in the flowering stage (70 % of eggs) (Kessler & Baldwin, 2002a). Larvae hatch ca. five days after oviposition and undergo five, rarely six, instars over ten to fourteen days before entering the pupal stage. Because of the protracted and variable duration of the pupal stage, the life cycle of *Manduca* spp. spans 30 to 50 days, or longer for overwintering pupae (Steppuhn, 2007; Villanueva, 2009). Consuming more than 98 % of the lifetime leaf mass during the fourth and fifth instars (Baldwin, 2001) or in other words three to five plants up to the pupal stage, *Manduca* larvae are the major defoliators of the wild tobacco (Kessler & Baldwin, 2001, 2002a).

Decreases in plant fitness by *Manduca* larvae are effectuated by both intra-plant movement from highly elicited to non-induced, younger leaves upward the plant and inter-plant movement onto neighboring, potential non-induced plants (van Dam *et al.,* 2000, 2001a; Zavala & Baldwin, 2006). The behavioral decisions are driven by the quality of host plant and the heterogeneity of defensive secondary metabolites within the plant such as higher protein content and lower levels of TPI activity in younger leaves corresponding to the optimal defense hypothesis (Kessler & Baldwin, 2002a, van Dam *et al.,* 2001a, Horn *et al.* 2005). As a result of *Manduca* larval intra-plant movement the changed feeding foliage increases not only larval mass for 6.3 fold by reducing plant fitness but also a decrease of predation risk for 40% (Kessler & Baldwin, 2002a; van Dam *et al.*, 2000, 2001a; Zavala & Baldwin, 2004). Nonetheless moving has been shown to be costly during the first three instars, whereas since the third instar *M. sexta* larval development is immune to either induced defenses or starvation. Hence this instar is also referred to as “window of sensitivity” (van Dam *et al.*, 2000, 2001a). Additionally, inter-plant movement of larvae onto neighboring competitors imply both large costs as a result of defenses activating to the new colonized plant and therefore a fitness benefit to the abandoned plant. For a plant, Lepidoptera caused damage in the early larval ontogeny only represents a fraction of the foliage damage caused in latter instars (van Dam *et al.*, 2000, 2001a).





Seed feeder

Photographs:
A: D. Kessler;
B, G: A. Kessler;
C-I: A. Steppuhn.

Figure 4: *Nicotiana attenuata* is attacked by a wide range of herbivores of different feeding guilds with varying host ranges: present herbivory insects during the field season in 2011. Leaf-chewing herbivores: **A** *Manduca sexta* (tobacco horn worm), **B** *Manduca quinquemaculata* (tomato horn worm), **C** *Spodoptera exigua* (beet army worm), **D** *Trimerotropis* spp. (band-winged grasshoppers) and **E** *Lepus californicus* (black-tailed jack rabbit). Stemborer: **F** adult *Trichobaris mucorea* (tobacco stalk borer). Sucking herbivores: **G** *Tupiocoris notatus* nymph (suckfly), **H** *Empoasca* spp. (leaf hoppers) and seed feeder: **I** *Thyreocoridae* (negro bugs).

Hornworm mortality is due to a complex interaction between behavioral and physiological ability of the present ontogenetic stage, on-plant position, host plant quality and age, weather, micro-environmental effects and interactions with the next trophic level (Kessler & Baldwin, 2002a; van Dam *et al.*, 2000, 2001a; Zalucki *et al.*, 2002; Zavala & Baldwin, 2004). Although high but variable rates of neonate mortality could be attributed to challenges like leaf structure such as trichomes and surface waxes, laticifers, glands or tissues filled with allelochemicals, locally induced plant changes, pathogens and parasitoids, predation has been identified as main cause (41 studies) (Zalucki *et al.*, 2002). In the narrow window of

time for larval vulnerability neonates defend themselves by aposematic, cryptic, hiding, flight and distributional behavior (Zalucki *et al*., 2002).

The small invertebrate predatory bug *Geocoris pallens*, a ground-dwelling, invertebrate generalist hemipteran predator, attacks herbivory insects like *Tupiocoris notatus*, *Epitrix hirtipennis and M. quinquemaculata* as well as *M. sexta* hornworms (Kessler & Baldwin, 2002a, 2004b). Geocoris *pallens*, commonly called big-eyed bug, is the most abundant predator in *N. attenuata*'s *habitat*, however other predators, such as crap spiders (*Thomisidae),* assassin bugs (*Redividae)* and dessert lizards frequently occur (fig. 5). Due to the determination of egg and larval predatebility to leaf position and larval age *Geocoris* insects preferentially feeds on eggs, followed by first up to second instar hornworm larvae, decreasing with ascending leaf positions. Increasing size and therefore the effectiveness of defensive behavior of *Manduca* larvae reduces predation risk by *Geocoris* (Kessler & Baldwin, 2002a; van Dam *et al*., 2001a; Walters *et al.,* 2001). Nonetheless late-instar *Manduca* larvae are large, vigorous moving and regurgitating, Stork *et al.* (2011) provides evidence for lizard predation of these Lepidoptera (Dyer, 1995; own experience). Additionally, inter-plant moving increases the predation risk by arthropod predators, such as spiders and ants (van Dam *et al*., 2001a).

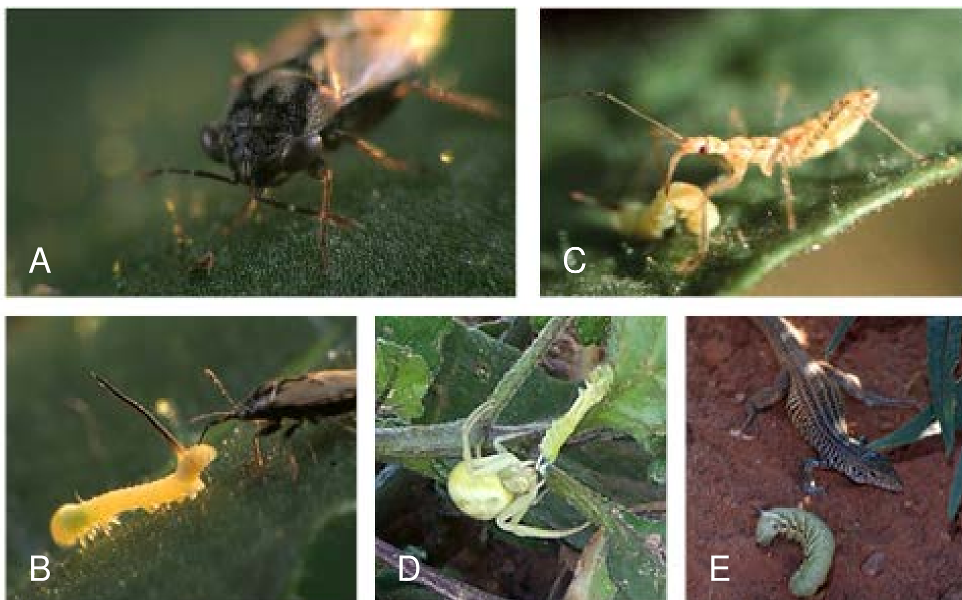


Figure 5: Predators present on *N. attenuata* attacking herbivory insects during the field season in 2011. **A** *Geocoris pallens* (big-eyed bug), **B** *Geocoris pallens* preying on a *Manduca sexta* larvae, **C** nymph of a *Redividae* (assassin bugs) preying on a *M. sexta* larva, **D** *Thomisidae* (crab spider) and **E** desert lizard predating a *M. sexta* larva.

Photographs A-B: Massih media; C: A. Steppuhn; D: K. Barthel; E: A. Kessler.

Why choosing N. attenuata as ecological model system?

Baldwin (2001) answered this question by indicating two crucial aspects: 1) a highly adaption via “a large amount of morphological and chemical phenotypic plasticity”, 2) the growth in a “primordial agricultural niche: the immediate post-fire environment”. Indeed, numerous studies, few accomplished in the Department for Molecular Ecology at the MPI-CE, established a fundamental knowledge of *N. attenuata*: a complete genome sequence, several identified genes related defenses and a variety of natural plant-insect interactions (e.g. Hermsmeier *et al.*, 2001; Halitschke *et al.*, 2003; Kessler & Baldwin, 2001, 2002a, 2004b; van Dam *et al.*, 2001a; Voelckel & Baldwin, 2004; Zavala & Baldwin, 2004). Furthermore, according to Hermsmeier *et al.* (2001) the interaction between *M. sexta* and its natural host plant *N. attenuata* fits to the demand of an ecological model system for plant-herbivore interactions. But for deterring the question more specifically both the interacting between *Manduca* and *N. attenuata* and the interaction between *Manduca* and the next trophic level, its native predator *Geocoris* are essential. Therefore the following literature provides supporting arguments: e.g. Allmann & Baldwin, 2010; Kessler & Baldwin, 2001, 2004a; Halitschke *et al*., 2008; Heil, 2008.

Finally, once it has been shown in nature that a plant compound defends against adapted herbivores, this compound becomes a better candidate for use in agricultural ecosystems. In this case, experimental results from *N. attenuata* afford the practice in similar agricultural plants like cultivated solanaceous species (*Lycopersicon esculentum, Solanum tuberosum*, *N. tabacum*).

2.4 Gene silencing to study plant defense

Silencing single defense genes is a valuable tool to address the functions of putative defenses as well as their fitness costs for the plant. The manipulation of individual genes enables the specific manipulation of resistance traits, the disentanglement of their interactions with other defensive traits, and their effects on individual plant-insect interactions or community dynamics (Bergelson & Purrington, 1996). Molecular biologists have developed tools including RNA interference (RNAi), virus-induced gene silencing (VIGS) and T-DNA knockouts with maximum genetic control (see Wesley *et al*. 2001; Zheng & Dicke, 2008 for review). Post-transcriptional gene silencing (PTGS) in plants, also known as RNAi, provides a specific mechanism of RNA degradation and gene silencing to regulate the activity of genes and developmental processes within eukaryotic cells. But it also functions as a defense to protect eukaryotic organisms against foreign or aberrant endogenous RNAs,

like viruses and retrotransposons (Mülhardt, 2009; reviewed in Bazzini *et al*., 2007; Hamilton *et al.*, 2002).

How does RNAi work?

The first evidence of RNAi emerged out of the pioneering study of Fire *et al*. (1998) in *Caenorhabditis elegans* (Rhabditidae). They showed RNAi is efficiently triggered by double stranded RNA (dsRNA), which sequence substantially determines the degeneration of messenger RNAs (mRNA) (Mülhardt, 2009). dsRNA intermediates are formed due to homologous base pairing in the presence of sense and antisense mRNA strands with complimentary sequences for a certain gene (Waterhouse *et al*., 1998). dsRNA are generated by transcription of single inverted repeat (IR) or hairpin sequences, transcription and pairing of antisense with sense sequences or by converging promoters (for review see Hamilton *et al.*, 2002) or virus-encoded RNA-dependent RNA polymerases (Dalmay *et al*., 2000). In the first catalytic step of RNAi a specific endonuclease, an RNAse III-like enzyme referred to as Dicer, cleaves dsRNA into RNA duplexes of 21-23 nucleotides with overhangs of 2 nucleotides at both 3'-ends. (Bernstein *et al.,* 2001; Fire *et al.*, 1998; Mülhardt, 2009; Wesley *et al*., 2001; Zamore *et al*., 2000). Once produced, these small "guide RNA" molecules or short interfering RNAs (siRNAs) will be subsequently incorporated into a ribonucleoprotein complex called the RNA-induced silencing complex (RISC) (Mülhardt, 2009; Zamore, 2001). After removing one of the double strands the activated RISC complex can bind via its integrated antisense or guide strand of the siRNA to the complementary sense or passenger RNA strand and then cleave this target mRNA (Mülhardt, 2009; Zamore, 2001). Thus, the base pairing between siRNA and the target gene generates a specific degradation of siRNA homologous mRNAs (Hamilton *et al.*, 2002; Zamore *et al.*, 2000). The cleaved target mRNAs lose their function (aberrant RNA) and are further degraded; RNA-dependent RNA polymerases (RdRPs) amplify the guide RNA and lead to a recycling of the RISC complex several cycles (Dalmay *et al*., 2000; Steppuhn, 2007). RNAi is used in plant genetics by integrating a gene sequence in anti-sense (AS) orientation or inverted-repeat (IR) constructs, which contain both the sense and antisense sequences and thereby enhance the efficiency of RNAi (Waterhouse *et al*., 1998; Wesley *et al*. 2001). Furthermore, RNA silencing in plants can be achieved by DNA methylation and transcriptional suppression, pre-mRNA processing and inhibition of translation (reviewed in Hamilton *et al.*, 2002).

Before use in functional studies, the success of RNAi must be verified for every transformant. Side effects, such as mutation of inserts and variation in transcript levels with plants

development and generation, and effects due to the site of construct insertion into the genome, can be minimized by using transformants with a single, homozygous insertion, measuring transcript levels of the targeted gene and screening for off-target effects on plant growth, development and metabolism (Strauss *et al*., 2002; Steppuhn, 2007; Steppuhn & Baldwin, 2008). Using multiple lines of independent transformation events are common methods to correlate the observed resistance effects with the inserted transgene with the greatest probability (Steppuhn & Baldwin, 2008). The constructs in the single lines used in this research study were previously characterized with multiple lines.

2.5 The key questions

In this diploma thesis I ask the plant for the evolutionary function of GLV emission, TPI production and their potential functional interaction for *N. attenuata* in its natural habitat the Great Basin Desert in western North America. To understand the function of GLVs a transgenic line silenced via a single upstream 13-lipoxygenase, *LOX2*, which specifically supplies the HP substrates for their production, was created (Allmann *et al*., 2010; Halitschke & Baldwin, 2003). Different levels of GLV silencing were provided by generating both homozygous (Allmann *et al*., 2010) and hemizygous IR*lox2* plants. This ensures that GLV-silenced plants would still suffer decreased fitness from herbivore feeding, because GLVs act as feeding stimulants (Meldau *et al.*, 2009). In addition homozygous IR*pi* plants, transformed to silence the TPI biosynthetic gene, were used to suppress the expression of TPIs (Steppuhn & Baldwin, 2007). In field studies with these transgenic lines compared with wild type (WT) *N. attenuata*'s resistance, development and fitness are investigated in its natural ecosystem. By manipulating both the emission of GLVs and the expression of TPIs individually two hypotheses could be addressed: (1) herbivore-infested GLV-emitting plants are fitter than non-emitters, but only in the presence of active predators; and (2) TPIs weaken herbivores' response to attempted predation. These hypotheses comprise the following question:

- Do GLVs effectively increase *Manduca* larval and egg predation in the presence of *Geocoris* predators?
- Does the GLV-mediated increase in predation increase plant fitness?
- Do *Manduca* larvae feeding on TPI-producing plants grow more slowly and suffer higher mortality, or are they otherwise weakened in response to predator attack?
- Does TPI production increase plant fitness?

In addition to plants silenced for *LOX2* and *PI* separately, a genotype was generated that is deficient for both TPIs and GLVs created by crossing IR*lox2* and IR*pi* homozygous lines. Because interactions among different defensive traits might contribute to plant defense, this transgenic plant was designed to determine whether and how *N. attenuata*'s endogenous TPI production and GLV emission influence each other and work together synergistically. By considering the dilemma why plants produce TPIs as antinutritive defenses as well as toxins like nicotine, their synergy is only efficient against generalists (Steppuhn & Baldwin, 2007), the question of a functionally work of TPIs and indirect defenses for specialists was raised to test a third hypothesis: (3) if there is a synergy between TPIs and GLVs, their defensive effects interact in a non-additive way resulting in a fitness benefit for the plant.

$z > x+y$

z - relative fitness of plants with both TPIs and GLVs
x - relative fitness of plants with GLVs and no TPIs
y - relative fitness of plants with TPIs and no GLVs

relative fitness - fitness compared to plants which have neither TPIs nor GLVs

Following questions are part of this hypothesis:

- Are plants producing neither GLVs nor TPIs less or more fit than plants which only produce TPIs or GLVs?
- Does synchronized production of GLVs and TPIs affect each other's function against the specialist *Manduca*?
- Do TPIs function as a defense or only as an enhancement of indirect defenses in nature?

Because of the IR*pi* construct was not successfully silenced in the cross line, the synergy question between GLVs and TPIs could not be addressed directly. However, employing both homozygous and hemizygous IR*lox*2 plants provided different levels of GLV silencing and a vector control. Therefore, particular attention was paid to the question of fitness consequences for both defense compounds, TPIs and GLVs, individually.

Finally, whether a resistance trait functions as a defense needs to be established under natural conditions.

3. Materials and Methods

3.1 Plant transformation and growth conditions

Plant material and transformation

Seeds of the 31st generation of an inbred line *Nicotiana attenuata* Torr. ex Wats (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae), originated from seeds collected at the Desert Inn ranch (Utah) in 1988 (Baldwin, 1998), were used as the wild-type (WT) in all experiments. Germination of WT and transgenic seeds was conducted according to the protocol described in Krügel *et al*. (2002). Seeds were first sterilized in 5 ml aqueous solution of 0.1 M dichloroisocyanuric acid (Sigma-Aldrich, Taufkirchen, Germany) with 0.005% (v/v) Tween-20 (Merck, Darmstadt, Germany), for 5 min and subsequently incubated in 5 mL 1:50 (v/v) diluted liquid smoke (House of Herbs, Passaic, NJ) with 50 µL of 0.1 M gibberellic acid (GA_3, Roth, Karlsruhe, Germany) for 1 h. Afterwards, seeds were transferred individually onto the germination media containing Gamborg's B5 medium (c = 3.16 g/L, pH 6.8; Duchefa, Haarlem, The Netherlands) and 0.6% (w/v) plant agar (Sigma-Aldrich, Taufkirchen, Germany) and maintained in Percival chambers (Perry, IA). The incubation conditions were set to 27°C/16 h 100% light, 24°C/8 h dark cycles for ten days. For the field experiment, seedlings were transferred into 50 mm peat pellets (Jiffy) 15 days after germination as above and gradually adapted to the environmental conditions of high sun exposure and low relative humidity of the Great Basin Desert habitat over ten days. Adapted, size-matched seedlings were transplanted into a field plot in a native habitat at the Lytle Ranch Preserve (Utah). Planted seedlings were watered over the first two weeks in field until the roots were established. The release of field plantations with transgenic lines was carried out under Animal and Plant Health Inspection Service (APHIS) permission number 10-349-102r. For glasshouse experiments, seedlings were transferred into individual 1 L pots and grown under day/night cycles of 16 h (26-28°C)/8 h (22-24°C) under daylight supplement with Master Sun-T PIA Agro 400 W or Master Sun-T PIA Plus 600 W lights (Philips, Turnhout, Belgium) as described by Krügel *et al*. (2002).

In order to silence the expression of *LOX2* and *PI* genes in *N. attenuata* previously characterized, homozygous, IR-RNAi transformants of the second transformed generation (T_2) were used. Transformation of IR constructs into the genome was done using the *Agrobacterium tumefaciens* (strain LBA 4404)–mediated transformation procedure in Krügel *et al*. (2002). Plant transformation was performed by technicians (W. Kröber, S. Kutschbach or A. Wissgott). Vector constructs contained either a fragment of the *PI* gene or *LOX2* gene twice in inverted orientation and linked by an artificial intron to facilitate hairpin formation

(Steppuhn *et al*., 2004) Transformed homozygous IR*lox2* lines (number A-04-52-2; Allmann *et al*., 2010) were silenced in GLV biosynthesis and emission and homozygous IR*pi* lines (number A-04-186-1; Steppuhn & Baldwin, 2007) were silenced in TPI activity; whereas hemi-IR*lox2* plants, created in an IR*pi* background by crossing IR*lox2* and IR*pi* homozygous lines, were hemizygous for both silencing constructs (M.C. Schuman, in review). The transformation process itself does not affect plant fitness or competitive ability (Schwachtje *et al*., 2008), TPI production and GLV emission (figs. 8B-D, 9), however, TPI activity or transcripts were not silenced in the hemizygous state (fig. 8A). Therefore these plants were phenotypically intermediate between WT and IR*lox2* with regard to GLV emission and served only as vector controls for comparison with IR*pi*.

Plant treatments, sample harvest and handling

In field experiments plants were either infested with *Manduca* larvae (described in chapter 3.6) or served as control plants. For glasshouse assays to measure *Manduca* larval performance and consumption of plant tissue, all plants were infested in early elongation stage (pre-flowering). To analyze differences in GLV emission in the plant headspace, field- and glasshouse-grown plants were treated with a standardized method of mimicking *Manduca* larvae feeding by wounding and applying of *M. sexta* OS (W+OS) (fig. 7). A similar, mature, non-senescent leaf was chosen for each field-grown plant. For glasshouse-grown plants at early elongation-stage, second fully elongated leaf at node +2 was used. Leaves at node +1 (one younger than +2) and +2 of a separate set of rosette-stage plants were used to determine *PI* and *LOX2* transcripts, respectively. Wounding was performed by running a fabric pattern wheel six times over the adaxial surface, thrice on either side of the midvein, for all chosen leaves. Twenty µL of OS diluted 1:5 (v/v, with deionized water) was immediately added to wounds; pure OS was collected from fourth- to fifth-instar *M. sexta* larvae from the colony in Jena, fed on WT plants. Control plants were left untreated.

Leaf tissue was sampled by excising the leaf at the petiole and immediately wrapping the excised leaf tightly in aluminum foil and freezing. In the field, harvested leaves were frozen at -20°C using dry ice and ice packs. Until the transport to Jena on dry ice field samples were at kept -20°C and in Jena stored at -80°C until analysis. For glasshouse sampling leaves were immediately flash frozen in liquid nitrogen and kept at -80°C until analysis.

Before analysis, frozen leaf material was homogenized with mortar and pestle over liquid nitrogen and subsequently transferred into 2 mL microcentrifuge tubes. Prior to extraction aliquots weighed from homogenized samples were finely ground in microcentrifuge tubes containing two steel balls (ASK, Korntal-Muenchingen, Germany) with a GenoGrinder® 2000

(SPEX Certi Prep, Metuchen, NJ): 30 s, 350 strokes min^{-1} prior to TPI activity assays and 30 s, 250 strokes min^{-1} prior to RNA extraction (dependent on size of microcentrifuge tubes used for aliquots). Prior to analysis homogenized leaf material was stored at -80°C or immediately processed over liquid nitrogen until adding extraction solvents.

3.2 Phenotypical characterization of the transformed lines

To confirm that the transformed lines used had the expected phenotypes, *PI* and *LOX2* transcripts, GLV emission and TPI activity was analyzed from each line.

3.2.1 RNA extraction and quantification of *PI* and *LOX2* transcripts

To ascertain the silencing efficiency of the transformed lines, *PI* and *LOX2* transcript levels were determined in unelicited and W+OS-elicited leaves of glasshouse-grown WT *N. attenuata* plants, the homozygous lines of each genotype, IR*pi* and IR*lox2,* and a hemizygous line hemi-IR*lox2* (five plants per genotype), respectively. According to the peak of transcript accumulation for *PI* (Wu *et al*., 2006) and *LOX2* (Allmann *et al*., 2010) leaves at node +2 were harvested 12 h after W+OS treatment for *PI* transcripts and after 14 h at node +1 for *LOX2* transcripts. Total RNA was extracted using the acid guanidin thiocyanate-phenol-chloroform method from Chomezynski & Sacchi (1987) from 150 mg of fresh frozen, well-ground leaf tissue with 1 mL TRIzol® reagent (0.4 M ammonium thiocyanate, 0.8 M guanidinium thiocyanate, 0.1 M sodium acetate trihydrate, 5% (v/v) glycerol, 0.73% (v/v) acetic acid, 38% (v/v) phenol; Chomezynski & Sacchi ,1987; Invitrogen, Karlsruhe, Germany) into 2 mL microcentrifuge tubes on ice. After 5 min shaking at RT aliquots were centrifuged for 10 min at 12 000 g and 4°C to pellet extracted tissue and separate the supernatant containing RNA, DNA and protein. The supernatants were transferred to 1.5 mL microcentrifuge tubes and 200 µL chloroform was added to separate DNA, protein and RNA into three phases. The chloroform-supernatant mixture was shaken vigorously for 15 s, incubated for 2 to 3 min at RT and then centrifuged for 15 min at 10 000 g and 4°C. After collecting the ~0.6 mL of the upper (aqueous) phase which contains RNA, RNA was precipitated with 480 µL isopropanol (2-propanol) during a 10 min incubation at RT. Precipitated RNA was pelleted by centrifugation 10 min at 10 000 g, washed twice with 300 µL 75% ethanol (in DEPC water, ice cold) and finally resuspended in 20 to 50 µL DEPC-H_2O.

Before proceeding to cDNA synthesis, RNA quality was checked in ten randomly chosen samples using gel electrophoresis. For this, 1 µL of each RNA sample was aliquoted with

5 µL of 1x RNA loading dye (95% formamide, 0.025% SDS, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF, 0.025% ethidium bromide, 0.5 mM ethylenediaminetetra-acetic acid (EDTA); MBI Fermentas, St. Leon-Rot, Germany) in a 1.5 mL microcentrifuge tube. After denaturing by incubating 5 min at 65°C with the Termomixer comfort and transferring immediately to an ice/water mixture to prevent renaturation, RNA was loaded into a 1% agarose gel: agarose (Sigma-Aldirch, Taufkirchen, Germany) was diluted in 1 x TAE-(Tris-Acetat-EDTA)-buffer (Sambrook & Russel, 2001). Electrophoresis ran 30 min at 100 V. The visualization and determination of quality of the separated RNA molecules can be assessed by using ultraviolet (UV) spectrophotometry following ethidium bromide staining. For this, ethidium bromide, contained in the RNA loading dye, intercalates between bases in nucleic acids of the RNA molecule and its UV fluorescence was measured at 300 to 360 nm. Intact total RNA could be visibly identified according to two distinct ribosomal bands corresponding the 18S and 28SrRNA, while highly degraded RNA produces a smear of indistinct 18S and 28S rRNA bands shifted to lower molecular weight (Chen *et al*., 2010). RNA visualization and quality was done using the ChemiGenius BioImaging System (Syngene, Cambridge, U.K.) and the software program Genesnap (Syngene, Cambridge, U.K.). RNA concentration was determined using the NanoDrop® ND-1000 spectro-photometer (peqlab Biotechnologie GmbH, Erlangen, Germany) an aliquot of 500 ng/µL was made for each sample by mixing 5 µL of sample with the appropriate amount of with DEPC-H_2O.

cDNA was generated from 100 ng of total RNA by reverse transcription. For this, 2 µL of each total RNA 500 ng/µL aliquot was pipetted into a 96-well plate with 3 µL denature master mix (Σ 5 µL) (tab. 1A) and covered with a SealMat (Biozym). For RNA denaturation the mix was heated to 65°C for 5 min on a thermocycler (Mastercycle gradient, Eppendorf, Hamburg, Germany) and immediately transferred to a pre-cooled Alu-Rack (Carl-Roth, Karlsruhe, Germany) to prevent renaturation. After 1 min 5 µL of reaction master mix (Σ 10 µL) (tab. 1B) was added into each well.

Subsequently, the reverse transcription reaction carried out on a thermocycler (Mastercycle gradient, Eppendorf, Hamburg, Germany) using the following program: annealing step at 42°C for 60 min and elongation step at 70°C for 15 min. After the reaction finished, 15 µL of mili q-water were added to each sample for a total volume of 25 µL and the covered plate was stored at -20°C until use for quantitative real-time PCR (qPCR).

Table 1: Denature master mix and reaction master mix for cDNA synthesis to quantify *PI* and *LOX2* transcripts.

A: Denature master mix

reagent	stock solution	final concentration	producer
Oligo (dT)$_{18}$	0.5 µg µL^{-1}	0.025 µg µL^{-1}	MBI Fermentas
dNTP Mix	∑ 10 mM	0.5 mM	MBI Fermentas
RNA template	500 ng µL^{-1}	100 ng µL^{-1}	
DEPC-H_2O		ad. 5 µL	

B: Reaction mix

reagent	stock solution	final concentration	producer
5x RT Buffer	5x	1x	MBI Fermentas
RiboLock	40 u µL^{-1}	1 u µL^{-1}	MBI Fermentas
RevertAid	200 u µL^{-1}	1.5 u µL^{-1}	MBI Fermentas
DEPC-H_2O		ad. 10 µL	

RiboLock - ribonuclease inhibitor, RevertAid - H Minus Reverse Transcriptase

Finally, *PI* and *LOX2* transcripts were quantified by performing a qPCR with a Mx3005P Multiplex qPCR system (Stratagene, Waldbronn, Germany) In the qPCR reaction cDNA was amplified using gene-specific primer pairs, binding to a region not used for IR construct generation in either *PI* or *LOX2* (tab. 2), and the qPCR Core kit for SYBR Green I (Eurogentec, Köln, Germany) (tab. 3). *ELONGATION FACTOR 1A* (*EF1A*) was used to control for differences in cDNA quantity or quality among samples The qPCR program for *PI* and *EF1A* transcripts consisted of a two-step amplification program: initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 s denaturation at 95°C, 1 min annealing at 60°C (amplification and reaction monitoring) and a final cycle of 15 s denaturation at 95°C, 30 s annealing at 60°C and 15 s elongation at 95°C (melting curve generation to confirm product specificity). In contrast the qPCR program for *LOX2* transcripts consisted of a 3-step program: initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 s denaturation at 95°C, 1 min annealing at 65°C and 1 min elongation at 72°C and a final cycle of 15 s denaturation at 95°C, 30 s annealing at 65°C and 15 s elongation at 95°C. T The 3-step program was necessary for reliable amplification of the *LOX2* template.

Transcripts were quantified using external standard curves for each gene. Transcript abundance for each sample was normalized to the abundance of *EF1A* in that sample.

Table 2: Primers used for qPCR (SYBR Green) to quantify *PI* and *LOX2* transcripts.

gene	primer	sequence (5' → 3')	citation
PI	forward	TCA GGA GAT AGT AAA TAT GG	Fragoso *et al*., 2011
	reverse	ATC TGC ATG TTC CAC ATT GC	
LOX2	forward	TTG CAC TTG GTG TTT GAG ATG GT	Kallenbach *et al*., 2010
	reverse	TTA GTA GAA AAT GAG CAC CAC AA	
EF1A	forward	CCACACTTCCCACATTGCTGTCA	I. Galis
	reverse	CGCATGTCCCTCACAGCAAAAC	

Table 3: SYBR analysis master mix for qPCR to quantify *PI* and *LOX2* transcripts.

reagent	stock solution	final concentration	producer
Master mix	10x	1x	Eurogentec
$MgCl_2$	50 mM	3.5 mM	Eurogentec
dNTP Mix	∑ 5 mM	0.2 mM	Eurogentec
Forward primer	5 µM	0.3 µM	Sigma
Reverse primer	5 µM	0.3 µM	Sigma
SYBR diluted	100%	3% (v/v)	Eurogentec
ROX (1:10)	100%	4% (v/v)	Eurogentec
Hotgoldstar enzyme	5 u μL^{-1}	2.5 u μL^{-1}	Eurogentec
cDNA	10 ng	1 ng	
milli q-H_2O		ad. 20 µL	

3.2.2 Green leaf volatile collection and quantification

To qualitatively asses GLV pools in leaf tissue from *M. sexta*-infested field-grown plants (28th May, M2; chapter 3.6.2) and to determine appropriate amounts of leaf tissue and internal standard (IS) for GLV extraction, aliquots of leaf material from individual samples were pooled into single samples for each genotype. Prior to extraction, aliquots of 150 mg were spiked with 3 µg of tetralin (Sigma-Aldirch, Taufkirchen, Germany) as an IS over liquid nitrogen and immediately extracted with 300 µL *n*-hexane (Sigma-Aldirch) in 1.5 mL gas chromatography (GC)-vials. Samples were incubated by rotating at 25 rpm and RT overnight and 100 µL water- and tissue-free hexane supernatant was transferred to a new GC vial containing a 250 µL microinsert. Individual analytes were analyzed by Varian CP-3800 GC-Saturn 4000 ion trap Mass Spectrometer (MS) connected to a ZB5 column (30 m×0.25 mm i.d., 0.25 µm film thickness, Phenomenex, Aschaffenburg, Germany). For analysis, 1 µL extract was injected by a CP-8400 autoinjector (Varian) onto the column with a 1/10 split ratio. Two minutes after injection the injector was returned to a 1/70 split ratio to avoid external contamination. The GC program was as follows: injector temperature 250°C, initial column temperature at 40°C for the first 5 min, then ramped at 5°C min^{-1} to 185°C and finally at 30°C min^{-1} to 300°C, held for 0.17 min. Helium was used as the carrier gas with a flow rate of 1 mL min^{-1}. After elution from the column the volatile compounds were analyzed via MS. The MS program was as follows transfer line temperature 250°C, trap temperature 110°C, manifold temperature 50°C, source heater 200°C and scan range from 40 to 399 m/z at 1.33 spectra per second as described (Schuman *et al*., 2009). GLVs were identified according to their GC retention time and the recorded mass spectra compared to mass spectra databases, Wiley version 6 (Wiley) and NIST (National Institute of Standards and Technology) spectra libraries.

Quantitative GLV pools were analyzed from individual leaves of matched WT, IR*pi* and hemi-IR*lox2* field-grown plants collected at the beginning (6th June) and in the middle of the wild *Manduca* infestation M3 (22nd June) (ten randomly chosen triplets) (fig. 6B). Aliquots of 50 mg frozen, well-ground leaf tissue were extracted with 300 µL *n*-hexane (Sigma-Aldirch) and spiked with 15 µg of *(Z)*-hex-3-enyl acetate (Sigma-Aldirch) as an IS according to the protocol described above. Analytes were separated by a Varian CP-3800 GC-FID (flame ionization detector) connected to a ZB-Wax column (30 m×0.25 mm i.d., 0.25 µm film thickness, Phenomenex). For analysis, 1 µL extract was injected by a CP-8400 autoinjector (Varian) onto the column in a splitless mode, whereas the injector returned to a 1/70 split ratio 2 min after injection. The GC program was as follows: injector held at 230°C, initial column temperature at 40°C held for 7 min, then ramped at 5°C min^{-1} to 115°C and finally at

30°C min^{-1} to 250°C, held for 0.5 min, with helium as the carrier gas, flow rate 1 mL min^{-1}. After elution from the GC column the volatile compounds were transferred to a connected Varian FID (flame ionization detector). The FID detection conditions were set up to: temperature 250°C, airflow 300 mL min^{-1}, hydrogen 30 mL min^{-1}, nitrogen (make-up gas) 5 mL min^{-1}. For identification of individual volatile compounds peaks were compared to spectra of (*Z*)-hex-3-en-1-ol (Sigma-Aldirch), (*E*)-hex-2-enal (Sigma-Aldirch) and *(Z)*-hex-3-enyl acetate (IS) using the MS Work Station Method Builder and Batch Report software (Varian). Moreover, for quantification peak areas of volatile compounds were normalized with them of *(Z)*-hex-3-enyl acetate (IS) in each sample and compared to standard curves of (*Z*)-hex-3-en-1-ol, (*E*)-hex-2-enal and *(Z)*-hex-3-enyl acetate, diluted in *n*-hexane.

To measure GLVs in the headspace of field-grown plants intact leaves of randomly chosen plants of each genotype (three replicates) were harvested on May 21st (M2) (fig. 6B) and subsequently placed in microcentrifuge tubes filled with water for transport. Immediately after W+OS elicitation a 1 cm^2 disc was stamped out and enclosed in a 4 mL GC vial for 15 min incubation. GLV emissions were analyzed with a zNose® 4200 portable gas chromatograph with a 1 m DB5 column (Electronic Sensor Technology, Newbury Park, CA, USA) by inserting the zNose inlet needle through the septum of the GC vial into the headspace. The measurement was conducted under the following conditions: valve set at 165°C, inlet at 200°C, trap at 250°C; 30 s sampling time, column ramped from 30 to 190°C at 4°C s^{-1}, data collection for 20 s. To avoid artifacts from detached leaves affecting one genotype more than others, genotypes were analyzed in an alternating order within each replicate. For peak identification retention times of the most abundant GLVs, the aldehydes and alcohols, were determined using pure standards.

3.2.3 Quantification of trypsin protease inhibitor activity

TPI activity induced by *Manduca* feeding was measured in systemic leaves of field-grown plants two times each at the beginning and end of Manduca infestation M2 (16th May WT and IR*pi*, 23rd May IR*lox2* and hemi-IR*lox2*, 28th May all genotypes) and M3 (14th and 22nd June), as well as in glasshouse-grown plants after nine days *Manduca* feeding (fig. 6B). TPI activity was quantified in 100 mg of fresh frozen, well-ground leaf tissue using a radial diffusion assay as described in van Dam *et al.* (2001b). PIs were extracted with 0.3 mL of ice-cold extraction buffer (0.1 M Tris-HCl (pH 7.6), 5% (w/w) polyvinylpolypyrrolidone, 2 mg mL^{-1} phenylthiourea, 5 mg mL^{-1} diethyldithiocarbamate, 0.05 M Na_2EDTA (Sigma-Aldrich, Taufkirchen, Germany); Jongsma *et al.*, 1994) on ice and completely suspended by vortexing 10-15 min at RT. After centrifugation to pellet out tissue at 4°C for 20 min at 12 000 g,

0.1-0.2 mL supernatant was transferred to a fresh 1.5 mL microcentrifuge tube and kept on ice for protein quantification and PI analysis. Protein concentration was measured for three technical replicates of each sample using the method of Bradford (1976): 10 µL of diluted protein extracts (1:20 or 1:30) and 200 µL 1:5 diluted Bio-Rad protein assay (Dye Reagent for Bradford, Bio-Rad, München, Germany) were pipetted in triplets in ELSA plates (BD Biosciences, Heidelberg, Germany) and incubated for 10 min at RT. Protein determination was carried out in the TECAN ELISA reader (TECAN, Männedorf, Switzerland) and results were analyzed with the Magellan™ 3 software (TECAN). A standard series of bovine serum albumina (BSA; Sigma-Aldrich) solution, ranging from 0.5 mg mL^{-1} to 0.0313 mg mL^{-1} (with 0.1 M Tris, 5 solutions), was incubated with 1:5 diluted Bradford reagent on plates with samples and used to determine relative protein concentration in samples.

PI activity was analyzed via a radial diffusion assay according to the protocol from van Dam *et al.* (2001b) for *N. attenuata* samples, based on a method first described by Jongsma *et al.* (1993). To determine PI activities a series of soybean Tryp inhibitor (STI) (15.3 µM, 4.59 µM, 2.3 µM, 1.15 µM, 0.57 µM and 0.29 µM; with 0.1 M Tris; Sigma-Aldrich) solutions were used to obtain a reference curve. For the radial diffusion 42 nM bovine trypsin (Tryp; type III) (Sigma-Aldrich) was dissolved in plant agar (Sigma-Aldrich), which function as substrate for TPIs to be detected. Protein extracts and standard solutions were filled in wells until the liquid surface was completely level with the agar. PIs were allowed to diffuse at 4°C for 16-18 h overnight and subsequently dyed with 25 mL staining solution (1 mM Fast Blue B Salt, 80% (v/v) 0.1 M Tris, 0.7 mM APNE 5% (v/v) Dimethylformamide (DMF; Sigma-Aldrich). The plates were incubated at 37°C for 55 min and after poured off the staining solution, diameters of colorless PI inhibition zones were measured using a caliper over an illuminator. Visualization of these clear zones is achieved by using agar gels containing a trypsin protease, which conducts esterolytic hydrolysis of APNE. After diffusion of variable amounts of PI from a well, colorless zones developed, their size is directly proportional to the amount of PI (Jongsma *et al*., 1993). Therefore by using APNE as substrate for the protease the activity of PIs could be determined. The detection limit of this method is at PI concentrations lower than 0.2 mM (Jongsma *et al*., 1993).

3.3 Experimental design of Utah field experiments

Field experiments were performed during the field season 2011 from 28th April to 30th June at Lytle Ranch Preserve (Utah, USA). Seedlings of WT and IR*lox2*, hemi-*IRlox2* and IR*pi* transgenic lines of *N. attenuata* were matched for size and planted into the experimental field plot over one week (see plant material and growth conditions in chapter 3.1). WT and IRpi seedlings were initially bigger; in order to plant out all seedlings at the right stage of growth, which is important for their successful establishment in the soil, WT plants were planted pairwise with a size-matched IR*pi* plant, IR*lox2* with a size-matched hemi-*IRlox2* over the course of one week, with WT and IR*pi* planted at the beginning of the week and IR*lox2* and hemi-*IRlox2* at the end. For genotype identification, each plant was labeled with a 10 cm bamboo stick near the plant bearing 0, 1, 2, or 3 marks and a plastic label buried under the roots bearing the APHIS identification code. The experimental field consisted of 160 plants, 40 for each genotype, in a quadruplet design in which plants of each genotype were placed 0.5 m apart and arranged such that no two neighbors were of the same genotype. Between quadruplets plants were also planted in a distance of 0.5 m. This distance allows predators and herbivores to distinguish volatiles from neighboring plants (Kessler & Baldwin, 2001; Halischke *et al*., 2008). The planting design of the field plot is illustrated in figure 6A, C-D.

The experimental design consisted of three stages of *Manduca* infestation: M1 (5th-15th May), M2 (16th May-1st June) and wild *Manduca* assay (14th-29th June) and two *M. sexta* egg predation assays (2nd-4th and 5th-7th June) (chapter 3.6; fig. 6B). Due to the variation in time and extent of *Manduca* oviposition from year to year, which can be influenced by GLVs (Kessler & Baldwin, 2001; De Moraes *et al*., 2001), synchronous oviposition events were experimentally created by distributing *Manduca* neonates and eggs, either from a lab-reared culture of *M. sexta* (*Manduca* infestations M1 and M2) or from wild collections (wild *Manduca* infestations M3, fig. 6B), simultaneously and evenly across genotypes. Additionally naturally oviposited wild hornworm eggs were removed. All plants were monitored daily for canopy damage due to experimentally infested *Manduca* and naturally occurring herbivores (chapter 3.5), plant growth and reproduction (chapter 3.4) and the predation and mortality of infested *Manduca* larvae (chapter 3.6.2). During M2 and the egg predation assay the predator community of *Geocoris* insects was recorded (21st-22nd May, 3rd-7th June) (chapter 3.6.3). Because of larvae suffer a reduction in growth and are less responsive due to TPI-mediated amino acid starvation of larvae the question of whether TPIs change larval defense behavior in response to predation was tested. For this, on-plant and as well as off-plant mock-predation assays with first- and second-instar *Manduca* larvae were performed during wild *Manduca* infestation M3 with either wild collected or lab-reared larvae.

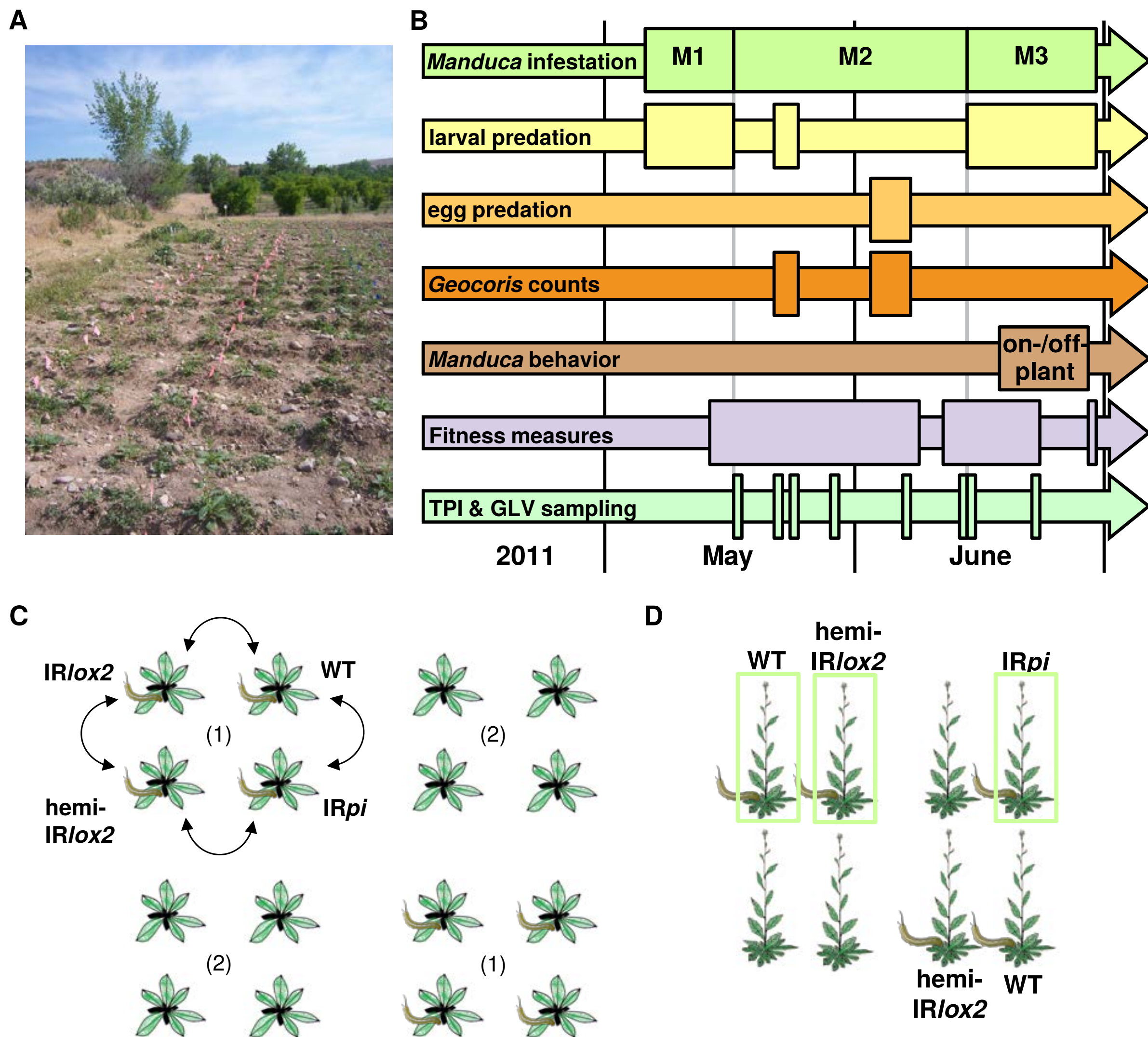


Figure 6: Experimental design during the field season in 2011 at Lytle Ranch Preserve in southwestern Utah. **A** Experimental field plot at Lytle Ranch Preserve. **B** Timeline for experimental approach. Different assays and measurements are represented by individual arrows, and rectangles span the time frame of each assay or measurement; narrow rectangles represent single days. Three experimental *Manduca* infestations (M1-M3) structure the overall experimental design: M1-M2, with laboratory *M. sexta*, and M3, with wild *Manduca* larvae. **C** Depiction of plot design during infestation M1 and M2: two with *Manduca* larvae induced (1) and two non-induced (2) quadruplets including one plant per genotype (WT, IR*pi*, IR*lox2* and hemi-IR*lox2*). Quadruplets were infested in a checkerboard design. Whole field plot included 20 induced and 20 non-induced quadruplets, ten of each planted at upper row and ten at lower (not shown). **D** Depiction of plot design during infestation M3. Triplets of matched WT, IR*pi* and hemi-IR*lox2* plants. Plants were not adjacent but rather distributed throughout the experimental plot. Only one out of 21 triplets is shown, corresponding plants are distributed throughout two quadruplets (see design in (C)) and marked with green square frames. Photograph: K. Barthel.

3.4 Plant growth and reproduction in the field experiment

Plant growth and reproduction were quantified correlates. Because by law, the flowering of transgenic plants must be controlled in the field, and because transgenics must not produce ripe seed, we counted numbers of buds, flowers and unripe seed capsules and did not quantify viable seed production. It must be acknowledged that lifetime viable seed production is a more accurate correlate of a plant's fitness (female fitness, Baldwin *et al.*, 1990).

Plant growth and reproduction during Manduca infestation M1 and M2

Because size-matched plants had been planted over one week (see plant transformation and growth conditions in chapter 3.1), growth and reproduction data from M1 and M2 were organized by the number of days since planting for comparison for between-genotype comparisons. Plant size (rosette diameter, stem length and number of side branches) were measured from the beginning of *M. sexta* infestations M1 (5th and 13th May) and M2 (days 33-47 in field for all plants: WT and IR*pi*, 16th-31st May every third day; IR*lox2* and hemi-IR*lox2*, 23rd-26th May and 28th May-7th June every third day) and reproductive output (time to first flower, buds and flowers) was recorded every morning from the time that plants produced buds during M2 from 16th May to 1st June (days 33-47) (fig. 6B; chapter 3.6.1, 3.6.2). Rosette diameter and stalk elongation were measured by gently laying a ruler over the rosette or by placing a ruler beside the stem and measuring from the base of the stem to the tip of the apical inflorescence, respectively, and all side branches 50 mm or longer and buds 2 mm or longer were counted. Flowers were removed upon counting during M2, and flower counts during M2 represent newly produced flowers.

Plant growth and reproduction during wild Manduca infestation M3

To determine the fitness consequences for GLV-mediated predation of hornworm larvae for the plant, 21 triplicates of WT, IR*pi* and hemi-IR*lox2* plants similar in size, previous reproducing output, apparent health, damage caused by naturally occurring herbivores and prior *Manduca* damage stage were chosen (M3; chapter 3.6.2). The choice of hemi-IR*lox2* in contrast to homozygous IR*lox2* was due to the reduction of growth as well as reproduction by *Manduca* infestation. For a clear between-genotype comparison, plants were first matched prior to M3 to exclude differences in growth, fitness and damage arising during M1 and M2 and second suffer the loss of all reproductive meristem to set initial reproductive units to zero. Thereafter new reproductive output of equal infested plants with one wild *Manduca* larvae was quantified by recording flower buds, closed flowers, 2 mm or larger, and unripe seed capsules on 17th, 18th, 20th, 22nd and 29th June (day 3, 4, 6, 8 and 15).The time frame of

the M3 was restricted such that plants could not produce ripe seed and thus flowers were not removed during this assay.

3.5 Quantification of herbivore damage and plant health

To determine whether genotypes suffered different levels of herbivore damage, which could influence the plant fitness, damage from naturally occurring herbivores as well as experimentally infested *Manduca* was monitored.

Prior to M1 (5th May) and twice during M2 (16th May WT and IR*pi*, 23rd May IR*lox2* and hemi-IR*lox2*, 27th May all genotypes; fig. 6B) each plant was examined four times for herbivore attack, to determine whether GLV-silenced plants suffered different amounts of herbivore damage, which could influence the fitness measurements. During field experiments the most abundant herbivores observed in the field plantation were *Tupiocoris notatus* (mirids), noctuid larvae; *Trimerotropis* spp. (grasshoppers), *Epitrix* spp (flea beetles) and *Trichobaris mucorea* (tobacco stalk borer). Leaf canopy damage due to different types of herbivores was identified by their characteristic feeding patterns and estimated as a percentage of the total leaf area in categories of starting with 1%, 5%, and then in 5% steps. For these estimates the number of leaves per plant (small leaves were counted as 1/5 to 1/2 of a leaf based on leaf area and large leaves were counted as one leaf) was counted, and the proportional damage area from each leaf type was summed and divided by the total number of leaves.

Characteristic damage caused by *Manduca* larvae during M1 and M2 was recorded for separately by photographs of *Manduca*-damaged leaves and entire plants. Plant damage was rated by an independent observer with no knowledge of plant identity. Four groups of damage were identified: 1: ≤5%, 2: >5-15%, 3: >15-35% and 4: 35-50% (fig. 14).

Furthermore, plant health was rated on a scale ranging from 1 (dead) to 5 (healthy) using the index in figure 17 as part of matching plants in triplets.

3.6 Field bioassays

In field bioassays *Manduca* behavior, predation, growth and movement assays were conducted with first- and second-instar *Manduca* larvae, except the final infestation with wild *Manduca*, in which larvae were reared from the first through fifth instars on plants (fig. 6B). The nicotine-tolerant specialist *Manduca* was chosen because it is the most damaging lepidopteran folivore of *N. attenuata* in nature (Kessler & Baldwin, 2001). *Manduca* larvae

and eggs used for predation assays were either from laboratory-reared *M. sexta* kindly provided by Dr. Carol Miles at SUNY Binghampton (North Carolina, USA) or collected when available from natural ovipositions. Until hatching eggs were placed in aerated plastic boxes over moist paper towels. For each plant infestation *Manduca* larvae were placed as neonates at a standardized position, on a rosette or lower stem leaf (fig. 7), and equally distributed among plants and monitored daily mornings and evenings, during times outside of the main period of *Geocoris* activity that occurs at midday. Thus, disappearance and appearance of eggs and larvae, larval movement, within-plant position were used to determine larval mortality and predation. A larva was considered predated if (1) its dead, sucked out body was found on the plant, characteristic for *Geocoris* spp. attacking larvae or (2) it disappeared leaving behind fresh characteristic feeding damage without any sign of moving to another adjacent plant and (3) its disappearance could be verified over several days. *Manduca* eggs were considered depredated if they were pierced and emptied, which is also characteristic for *Geocoris* insects (Kessler & Baldwin, 2001). Hence, disappeared larvae and eggs without predation or feeding characteristics as well as eggs occasionally collapse were excluded from the predation tabulation. Mortality was defined as the total number of missing larvae.

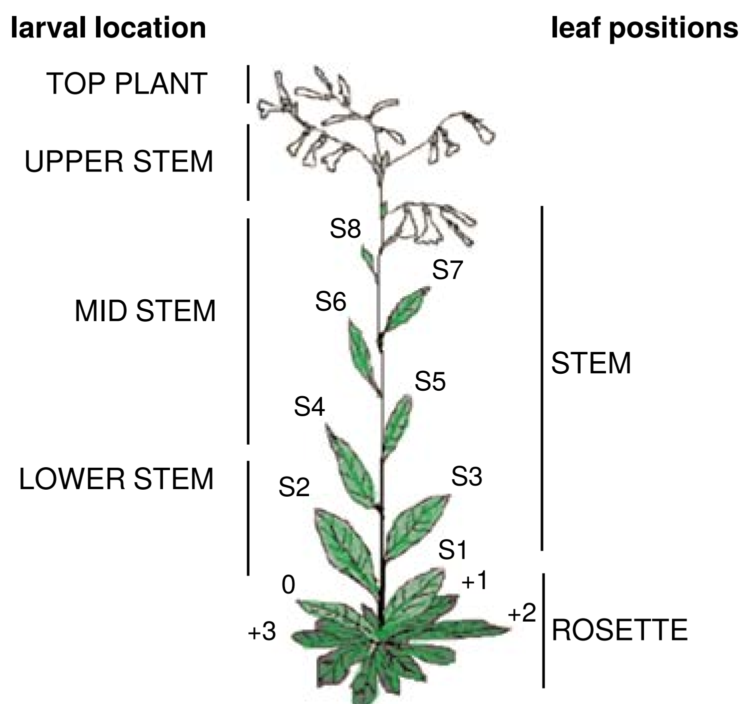


Figure 7: Sketch of full-grown *Nicotiana* plant showing different leaf positions for predation assays and larval locations for intra-plant movement. Modified from Zavala & Baldwin, 2004.

3.6.1 Initial predation assay and GLV fumigation

The time and extent of the predator community varies from year to year (M.C. Schuman, personal communication) and GLVs have been shown to attract predatory *Geocoris* insects (Allmann & Baldwin, 2010; Halitschke *et al*., 2008; Kessler & Baldwin, 2001). *Geocoris* was allowed to associate all four genotypes with the presence of prey during the first *M. sexta* infestation M1 (5th-15th May). In order to do so, all plants in half of the quadruplets (every second) were initially infested with one neonate *M. sexta* (lab-reared) at node +1 (figs. 6B-C; 7) and GLV emission was externally supplemented to the headspace of GLV-deficient plants. A cotton swab with ca. 20 µL of lanolin paste containing pure GLVs dissolved in hexane (tab. 4), representative of the *M. sexta*-fed *N. attenuata* headspace (Allman & Baldwin, 2010), was placed adjacent to the *Manduca*-infested leaf of IR*lox2* and hemi-IR*lox2* plants. Next to *Manduca*-infested leaves of WT and IR*pi* plants cotton swabs with 20 µL lanolin paste with hexane was used as control. During two subsequent predation assays, each three days, lanolin pastes were refreshed in the early afternoon and in the morning. Placing GLVs adjacent to, rather than on the infested leaves ensured both GLV supplementation to the plant's headspace without altering its metabolism, and a clear termination of the perfuming by removing the cotton swabs.

Table 4: GLV mix used to externally supplement plant GLV emission in M1.

20 µL GLV mix contains pure GLVs diluted in 1 mL hexane and mixed into 14 mL lanolin and represents the emission per g leaf tissue within the first 20 min of W+OS elicitation (Allman & Baldwin, 2010).

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

3.6.2 *Manduca* larval and egg predation assays

To determine whether GLV emission increases larval and egg predation all cotton swabs and remaining larvae were removed after termination of the GLV perfuming experiment and predation of newly-infested *M. sexta* larvae and eggs without GLV complementation was monitored. Due to the difference in planting 15 pre-induced WT and IR*pi* plants were immediately infested at 16th May with two first- and second-instar (reared on these plants since 13th May; M2) *M. sexta* larvae on equal lower stem leaves (figs. 6B-C, 7). Predation rates were recorded daily for five days. Because of high predation rates and to create comparability to IR*lox2* and hemi-IR*lox2* plants two additional neonates were placed on these plants after this period. 15 pre-induced IR*lox2* and hemi-IR*lox2* plants were infested with two first- and second-instar larvae at 23rd May, as for WT and IR*pi* plants.

Additionally, during 2nd-4th and 5th-7th of June two *M. sexta* egg predation assays were conducted (fig. 6B-C). For this, two frozen eggs per plant were glued with droplets of α-cellulose glue, which does not affect plants' VOC emissions (Kessler & Baldwin, 2001), to the underside of a lower stem leaf (fig. 7) at a standardized position for 12 quadruplets, six pre-induced and six un-induced. The use of frozen eggs is to prevent plants from hatching and therefore uncontrolled *Manduca* damage, and, in this respect, to be still palatable for *Geocoris.* For continuous induction one wild *Manduca* (first instar) per plant was clip-caged on a comparable leaf in upper stem section. Predation rates were recorded daily for three days after the experimental oviposition and predated eggs were replaced daily.

After the collection of a sufficient number of naturally oviposited eggs a wild *Manduca* assay M3 was conducted during the 14th-29th of June in which larvae were reared from the first through fifth instars on plants (fig. 6B, D). To mimic natural oviposition rates (Kessler & Baldwin, 2001) one neonate per plant was applied to a lower stem at a standardized position within 21 matched triplicates (see chapter 3.4). Cumulative mortality of these larvae was recorded until plants began to set seed. Because fifth-instar *Manduca* can move to new plants (Kessler & Baldwin, 2002a), abandoned plants without recent *Manduca* feeding were excluded from the mortality calculation.

3.6.3 Monitoring of *Geocoris pallens* population density near plants

The present predator population of *Geocoris* insects in the immediate vicinity of experimental plants was monitored during M2 and egg predation assays every second or third day (fig. 6B). Based on predator observations during M1 *Geocoris* counts were conducted in the early afternoon, the main period of activity. Following a standardized procedure, two to four observers counted *Geocoris* adults and nymphs by looking within a 5 cm radius of vicinity focal plant for 15 s and then quickly inspecting the leaves. All observers moved synchronously with each other from one end of the field plot to the other in order to prevent disturbance of not-yet-counted predators. Counts were completed within 20 to 30 min of *Manduca*-infested plants, control plants and also dead plants as negative controls.

3.6.4 *M. sexta* larval performance and plant tissue consumption

To examine the TPI effect on larval growth, development and the change in consumption for *M. sexta* larval predation assays were combined with performance assays M2 and M3. In the field during M2 growth of surviving hornworm larvae starting with two fist- and second-instar *M. sexta* larvae (lab-reared) was measured on each plant (18th, 21th and 23rd May for WT and IR*pi*, 23rd May for IR*lox2* and hemi-IR*lox2,* see chapter 3.6.2) (fig. 12). Because of the spread of planting and high predation rates on WT and IR*pi* two additional neonates were put on WT and IR*pi* plants at the begin of M2 for comparison with those on IR*lox2* and hemi-IR*lox2* plants. Additionally during M3 growth and instar developmental changes were monitored daily on wild *Manduca* larvae, starting with the fourth day feeding on plants (18th June, 4 days after hatching). Length measurements were made by gently placing a ruler beside the larva's body without disturbing it. As a result of the high predation rates on WT and IR*pi* plants and therefore the low numbers of replicates in this matched triplet design progressing of larval instars over time is presented for all genotypes, without comparing them.

To increase replicate numbers, comparability as well as reproducibility, *M. sexta* larvae growth was also monitored on glasshouse-grown plants. *M. sexta* neonates (in-house colony at the Max Planck Institute for Chemical Ecology, Jena) were placed at node +1 and their mass was determined after nine days (fourth instar) and 12 days (fifth instar). To determine whether GLV- and TPI-silenced plants suffer reduced *Manduca* damage, which could influence plant fitness under *Manduca* attack, larval consumption was recorded via photographs two days after neonates were placed. Consumption was quantified as number of pixels in comparison to a 1 cm^2 standard for conversion of pixels to cm^2 leaf area, and

normalized using a size standard included in each photograph, using SigmaScan 5.0 (Systat Software Inc.).

3.6.5 Within-plant movement of wild *Manduca* larvae

In order to determine the effect of TPI activity and GLV emission on larval movement, within-plant movement of each wild *Manduca* larva was monitored during infestation M3 (14th-29th June; fig. 6B). All larvae were allowed to move freely on the plants for 15 days during which larval movement was monitored daily. To monitor within-plant movement larval position was classified according to following categories: ROSETTE (only rosette leaves, level 0), LOWER STEM (where larvae started to feed, from leaf S1 to S3, level 1), MID STEM (form S4 to S8 leaf position, valuation 2), UPPER STEM (from leaf S9 to S14, level 3) and TOP PLANT (upper part of stem, level 4) (fig. 7). Caterpillar movement was observed throughout development (chapter 3.6.4).

3.7 Mock predation: on- & off-plant behavior assays with *Manduca* larvae

In order to evaluate the potential of TPIs to weaken the response behavior of *Manduca* larvae to predators, behavior assays mimicking *Geocoris* predation were conducted with 2nd-instar *Manduca* on plants (18thJune, wild *Manduca* infestation M3) and first- and second-instar *M. sexta* of the laboratory strain off plants (first instar 24th June, 2nd instar 27th June) (fig. 6B). *Geocoris* predators first pierce their prey with their sharp beaks and then often lift and carry it away; *Manduca* larvae were often pierced in the back below the horn, or in the flank. Because larvae in the on-plant assay were needed for monitoring their natural mortality (chapter 3.6.2), within-plant movement (chapter 3.6.5) and their effect on plant reproduction (chapter 3.4), the on-plant assay consisted of a very mild imitation of *Geocoris*. Larvae in each of five replicates were matched for size and instar stage (beginning, middle, or end of the instar). Larvae fed on WT or IR*pi* plants were first poked with the end of a toothpick below the horn three times, 3 s apart, and then lifted from the plant using a featherweight forceps and held for 15 s (fig. 13A). The duration of each poke was approximately 0.5 s. An attack of the toothpick was defined as the larva whipping its head around toward the toothpick and making contact; a successful attack to lifting counted when the larva moved from hanging at a 180° angle below the forceps toward the forceps and made contact with its front end before returning to its original position. In contrast, in an attempted attack of the forceps the larva only moved vertically toward the forceps without touching it. Responses to

mock *Geocoris* predation were recorded as videos and counted in slow motion. Molting larvae were excluded.

In order to increase replicate numbers, reproduce the experiment and imitate Geocoris predation more realistically off-plant mock predation assays with first- and second-instar *M. sexta* (20 and 12 replicates, size-matched) were developed. After hatching neonates were raised for 48 h (first instar) or five days (second instar) on either WT or IR*pi* leaf tissue from field-grown plants in aerated plastic boxes over moist paper towels. Leaves were refreshed twice daily and kept fresh by placing the petioles in water in 1.5 mL microcentrifuge tubes which were closed around the petiole with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA). The assay was similar to the on-plant assay (described above) except that it was conducted with an insect pin to mimic the Geocoris beak (fig. 13B). Because of the reduced number of replicates due to high mortality rates for second-instar hornworm larvae fed on IR*pi* plants, a size-matched, quantitative representative analysis for second-instar larvae was not possible. Recovery following mock attack was monitored by placing each larva in a cup with a moist paper towel round and the leaf tissue on which they had been previously fed (WT or IRpi) and allowing them to feed for 24 h, after which mortality and length were recorded.

3.8 Data analysis & statistics

All statistical analyses were conducted with SPSS 17.0 (IBM), besides Fisher's exact tests, which were conducted using a macro (J. H. Macdonald, http://udel.edu/~mcdonald/ statfishers.html) for Excel (Microsoft). Prior to all statistical analysis all data were inspected for normality and variance homogeneity using Levene's test; when necessary data were $\log_2$ transformed (volatile and transcript data) or square root transformed (count data) to meet requirements for homogeneity of variance. Parametric data were compared using ANOVAs, multivariate ANOVAs or repeated-measure ANOVAs followed by Scheffe *post-hoc* tests. If variance was not homogeneous following transformation, data were compared using Kruskal-Wallis tests (for multiple comparisons) followed by Bonferroni P-value corrections or Mann-Whitney U-tests (for two-way comparisons). For Kruskal-Wallis tests and Mann-Whitney U-tests, a Monte Carlo algorithm was used with 10000 permutations and a 95% confidence level.

4. Results

4.1 Characterization of WT and transgenic *Nicotiana attenuata* plants

To evaluate the potential of HIPVs and TPIs to defend plants in nature by increasing herbivore predation and thereby plant fitness, plants were transformed to silence GLV biosynthesis (IR*lox2*) or TPI biosynthesis (IR*pi*) by RNAi, and a hemizygous cross of these two constructs was created (hemi-IR*lox2*). GLV emission and TPI production of WT *N. attenuata* in response to *Manduca* larvae attack were characterized and compared to that of RNAi lines. The transformation process itself did not affect plant fitness or competitive ability (Zavala & Baldwin, 2004) (figs. 15, 16), TPI production or volatile emission (fig. 8). GLV tissue pools and emission was reduced by 80-100% in IR*lox2* and hemi-IR*lox2* lines but equivalent to WT in IR*pi* lines; whereas TPI activity was reduced to below the limit of detection in IR*pi*, but equivalent to WT in IR*lox2* and hemi-IR*lox2* (fig. 8A). Nevertheless the cross line provided an intermediate GLV phenotype, a vector control and an alternative to IR*lox2* plants during *Manduca* infestation M3 (chapter 3.6.2). Non-target metabolites are not affected in these lines (Allman *et al*., 2010; Steppuhn & Baldwin, 2007; M. Schuman, in press) and thus manipulation of GLV emission or TPI production represents an ideal way to test their defense function in nature.

Silencing of GLV and TPI production in transgenic lines was quantified by measuring the transcript accumulation of the target genes in elicited leaf tissue of glasshouse-grown plants using qPCR (chapter 3.2.1). qPCR permits real-time detection of PCR products measuring the accumulation of fluorescently labeled product. An amplification plot is created of the fluorescence signal (in this case SYBER Green) recorded at the end of each cycle versus cycle number. Because each PCR product has a characteristic melting temperature dependent on its length and nucleotide composition; the melting curve is produced by monitoring fluorescence as the final qPCR product is heated from the annealing temperature to the melting temperature of the reaction, if the reaction is specific, then all product will dissociate at one temperature (Mülhardt, 2009). Successful amplification was determined qualitatively by the slope of the standard curve, using cDNA standards with known concentration, and the presence of only one peak in dissociation curves (shown in figures A1-6 in chapter appendices). For quantification of transcript levels, a logarithmic regression based on the number of cycles it takes for the reaction to reach the exponential phase was generated from the standard curve.

Differences in transcript accumulation between genotypes were analyzed by two-way ANOVAs on log_2-transformed data with factors treatment and genotype (*PI*: genotype

$F_{3,29}$=174.077, P<0.001, treatment $F_{1,29}$=75.909, P<0.001, genotype*treatment $F_{3,29}$=0.727, P=0.544; *LOX2*: genotype $F_{3,32}$=635.477, P<0.001, treatment $F_{1,32}$=0.021, P=0.887, genotype*treatment $F_{3,32}$=1.261, P=0.304; fig. 8A). Elicitation via W+OS treatment significantly affect *PI* (P<0.001) but not *LOX2* (P=0.887) transcript accumulation. Transcript accumulation of *PI* was 0.134% of WT levels in IR*pi*, 78% of WT in hemi-IR*lox2* plants and similar to WT plants in IR*lox2* plants. Transcript accumulation of *LOX2* was 1.19% of WT levels in IR*lox2*, 1%in hemi-IR*lox2* and unaffected in IR*pi* plants.

TPI activity was measured via dial diffusion assay (van Dam *et al.*, 2001a) in similar systemic (undamaged) leaves of *Manduca*-infested glasshouse as well as field-grown plants (chapter 3.2.3). In the glasshouse and throughout the field experiment IR*pi* plants had no detectable TPI activity; in contrast, TPI activity was similar to WT plants in IR*lox2* and hemi-IR*lox2* (fig. 8B). Thus the IR*pi* construct was not active in the cross line (hemi-IR*lox2*).

To ensure that IR*lox2* and hemi-IR*lox2* lines produce and release strongly reduced amounts of GLVs compared to WT or IR*pi*, GLV emission was assessed by GC analyses of leaf headspace with a zNose™ 4200 and GLV production by hexane extracts (chapter 3.2.2). Headspace measurements provided a similar 80-100% reduction in GLV emissions from field-grown IR*lox2* and hemi-IR*lox2* plants compared to WT and IR*pi* (fig. 8C). Differences between genotypes were analyzed by one-way ANOVA ($F_{3,8}$=7.346, P=0.011). GC-FID analysis of hexane extracts from pooled leaf samples of field-grown plants on May 28th showed (*E*)-hex-2-enal as only detectable GLV compound in WT and IR*pi* lines, in IR*lox2* and hemi-IR*lox2* GLV amounts were below quantifiable levels (figs. 8D, 9A). However, (*E*)-hex-2-enal was detectable by GC-MS in pooled leaf samples of hemi-IR*lox2* , but not IR*lox2*. In hexane extracts from samples taken during M3 (6th and 22nd June) (*E*)-hex-2-enal remained the main component but (*Z*)-hex-3-en-1-ol was also quantifiable in much smaller amounts (figs. 8D, 9B). Differences between genotypes were analyzed by one-way-ANOVAs for (*Z*)-hex-3-en-1-ol (6th June: $F_{2,26}$=9.556, P=0.001; 22nd June: $F_{2,26}$=12.196, P<0.001). Hemi-IR*lox2* leaf extracts contained up to 50% as much (*Z*)-hex-3-en-1-ol as WT and IR*pi* extracts. Together, these results indicate that hemi-IR*lox2* are intermediate between WT and IR*lox2* plants for GLV production.

In summary, the silencing of GLV emission from transgenic IR*lox2* and hemi-IR*lox2* lines or TPI production in IR*pi* plants was verified in measurements of transcript levels of target genes, GLV emission from plants and GLV content and TPI activity in leaf tissue. Because *PI* transcripts as well as activity levels were detected in the cross, these hemizygous plants were only silenced in GLV, but not in TPI production.

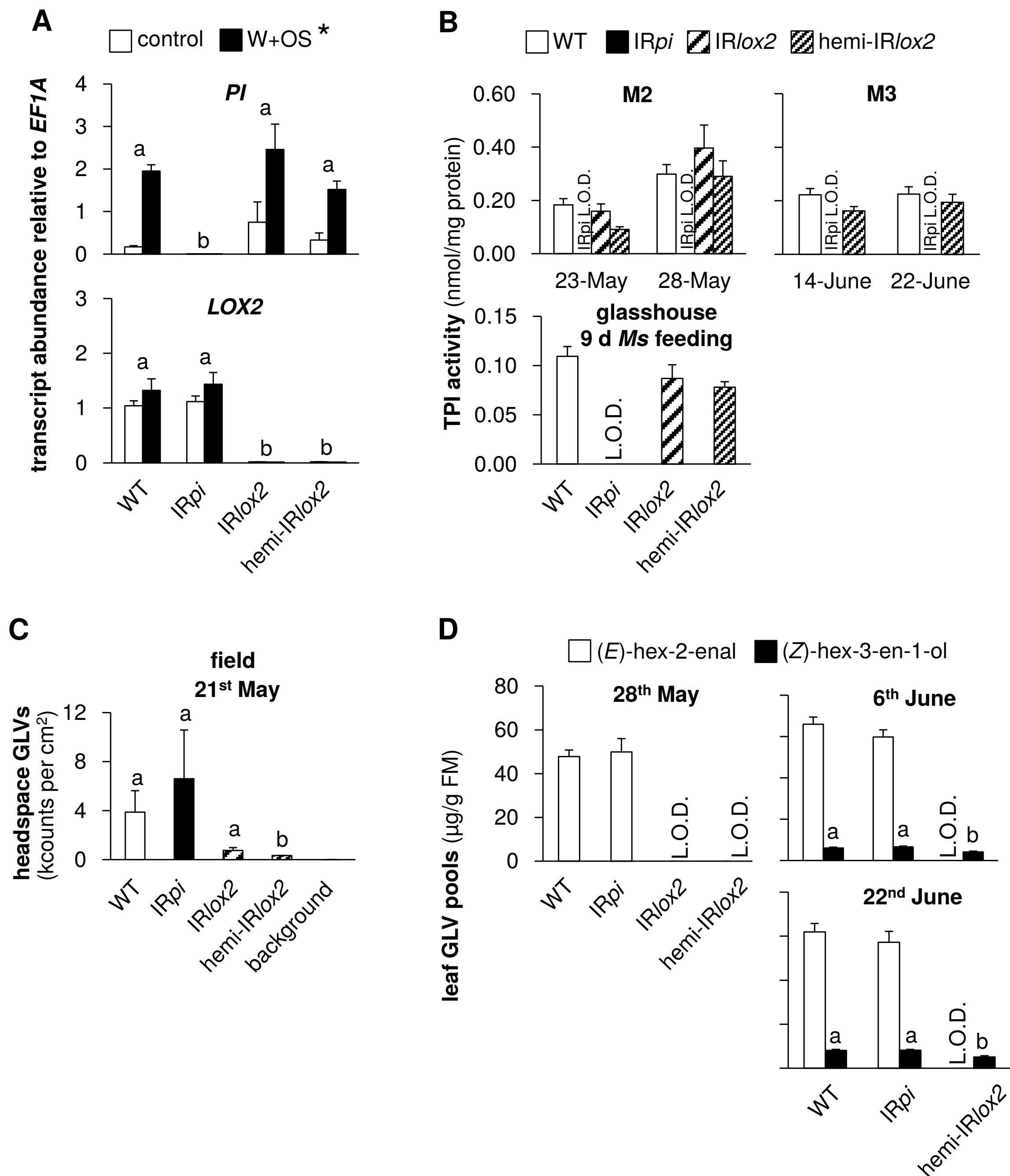


Figure 8: Genetic manipulation of GLV and TPI production in transgenic lines of *N. attenuata*. Graphs show means+SEM. **A** Levels of *PI* and *LOX2* transcripts in unelicited leaf tissue (control) and at the point of maximum accumulation in W+OS-treated leaf tissue in glasshouse-grown plants (N=5). Silencing efficiency was verified using qPCR. *W+OS treatment significantly affected *PI* (P<0.001) transcript accumulation. Different letters indicate significant differences between genotypes (P<0.001) in Scheffe *post-hoc* tests following two-way ANOVAs on log_2-transformed data with factors treatment and genotype (*PI*: genotype $F_{3,29}$=174.077, P<0.001, treatment $F_{1,29}$=75.909, P<0.001, genotype*treatment $F_{3,29}$=0.727; *LOX2*: genotype $F_{3,32}$=635.477, P<0.001, treatment $F_{1,32}$=0.021, P=0.887, genotype*treatment $F_{3,32}$=1.261, P=0.304). **B** TPI activity measured in systemic leaves of *Manduca*-infested field-grown (top two

panels, N=11-14 for *Manduca* infestation M2 and N=21 for M3; chapter 3.6.2) or glasshouse-grown plants (bottom panel, N=10). **C** GLVs in headspace samples of leaves from field-grown plants with a zNose™ 4200 (N=3, just before M2). Different letters indicate significant differences ($P \leq 0.05$) in Scheffe *post-hoc* tests following one-way ANOVA with genotype as the factor ($F_{3,8}=7.346$, $P=0.011$). **D** GLVs extracted with hexane from leaf tissue of field-grown WT, IR*pi*, and hemi-IR*lox2* plants in triplets (N=4 pooled samples, M2; N=10, M3). Different letters indicate significant differences ($P \leq 0.05$) in Scheffe *post-hoc* tests following one-way ANOVAs with genotype as the factor for (*Z*)-hex-3-en-1-ol (top panel, $F_{2,26}=9.556$, $P=0.001$; bottom panel, $F_{2,26}=12.196$, $P<0.001$). L.O.D., below limit of detection for measurement.

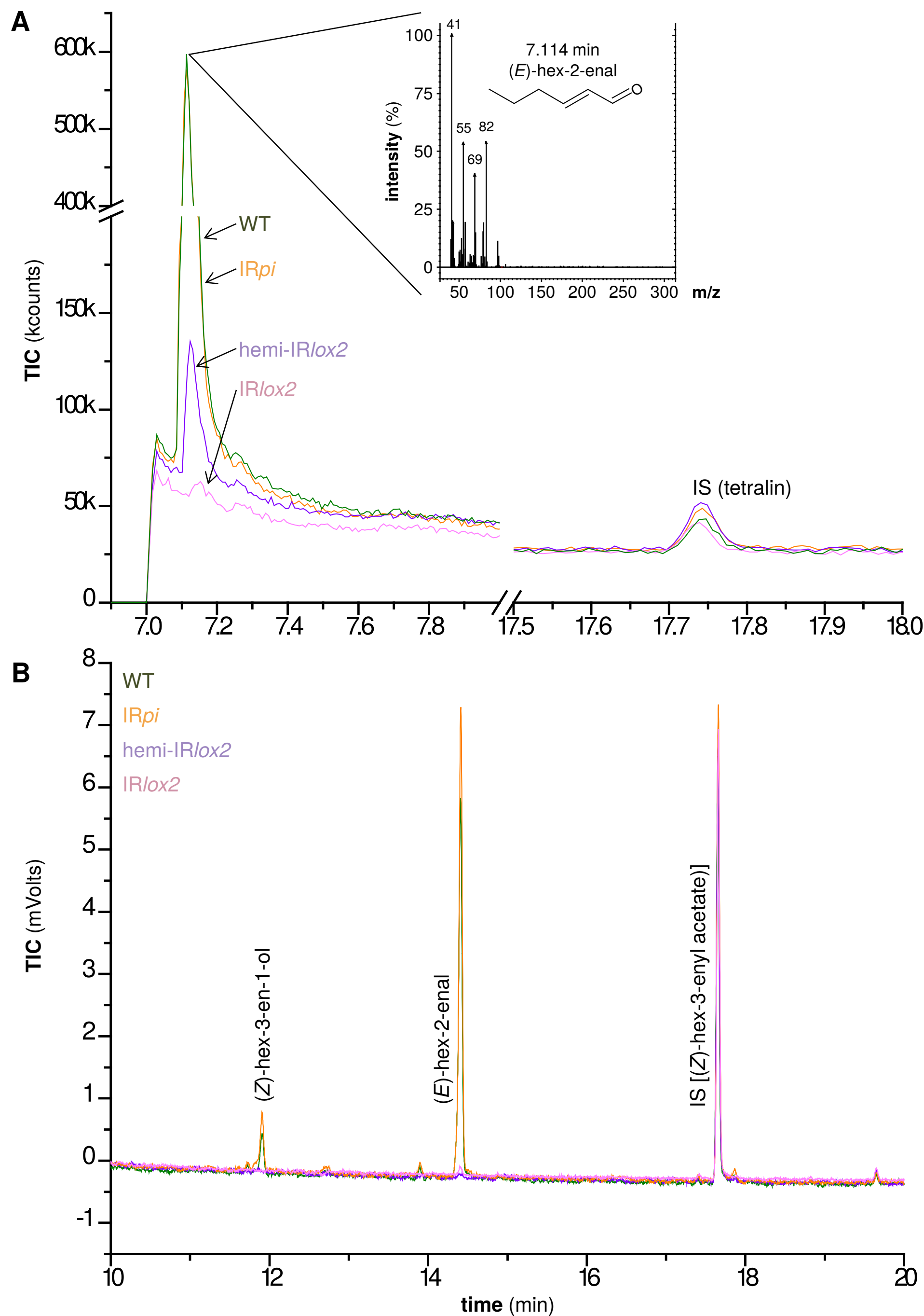


Figure 9: Qualitative and quantitative analysis of GLVs in hexane extracts from field-grown plants. **A** Qualitative measurement of GLV pools from pooled leaf samples by GC-MS with tetralin as IS (internal standard) (May 28th, M2); only (*E*)-hex-2-enal was detected **B** Example chromatograms from hexane extracts of individual leaf samples for quantitative GLV analysis, analyzed by GC-FID (6th and 22nd June before and in the middle of the wild *Manduca* infestation M3; chapter 3.6.2). The dominant compound was (*E*)-hex-2-enal; (*Z*)-hex-3-en-1-ol was also present in quantifiable amounts.

4.2 GLVs effectively increase *Manduca* larval and egg predation in the presence of *Geocoris* predators

To test whether GLVs attracted native *Geocoris* predators, resulting in increased predation pressure on *Manduca* larvae and whether TPIs reduce larval growth or strength and increase mortality in nature, two different *Manduca* infestations (M1 and M2, see fig. 6B, see chapter 3.6.1, 3.6.2) were conducted during the field season in 2011. For all assays, predation of *Manduca* larvae and eggs by *Geocoris* was monitored daily and *Geocoris* individuals (adults and nymphs) around plants were counted every second or third day (*Geocoris* counts, see chapter 3.6.3). GLVs mediate interactions between herbivores, plants and the third trophic level such as attractions for *Geocoris* predators (Allmann & Baldwin, 2010; Halitschke *et al*., 2008), which likely benefit plant fitness. Thus, by supplementing GLVs to GLV-deficient plants during the first two predation assays (5th-6th and 13th-15th May, M1; see chapter 3.6.1) *Geocoris* was allowed to associate all four plant genotypes with the presence of prey. As a result, synchronously placed first-instar larvae of lab-reared *M. sexta* suffered similar predation pressure due to *Geocoris* insects across all genotypes. *Manduca* larvae were predated at a rate of 22-38% over 2 d and respectively 12-21% over 3 d trials. There was a tendency for *Geocoris* to predate more larvae from GLV-supplemented plants which was significant overall, but not in each trial (Fisher's exact tests; 35-37% versus 22-27% in trial 1, P=0.066, 17-21% versus 12% in trial 2, P=0.069, combined trials, Bonferroni-corrected P=0.0063) (fig. 11A).

After removal of cotton swabs, plants were infested with new *Manduca* larvae, without GLV supplementation for GLV-deficient plants (see chapter 3.6.2). Because WT and IR*pi* were planted three up to five days earlier than IR*lox2* and hemi-IR*lox2* plants, WT and IR*pi* plants were larger and had more leaf area. Therefore, these genotypes were equally re-infested with *Manduca* neonates immediately after removal of cotton swabs, and this infestation confirmed that there was no difference in predation between these genotypes (71.7% predation of *Manduca* larvae from WT versus 81.7% from IR*pi*, P=0.28 in Fisher's exact test). Once IR*lox2* and hemi-IR*lox2* had caught up in size and recovered sufficient leaf area following M2 to support new *Manduca* larvae (one week later), all four genotypes were equally infested with new *Manduca* neonates. Predation of *Manduca* larvae from all four genotypes was simultaneously monitored over one week starting at May 21st, during which IR*lox2* and hemi-IR*lox2* were infested with more larvae than WT and IR*pi* plants. Although *Geocoris* had time to associate IR*lox2* and hemi-IR*lox2* plants with prey during M1 and predator counts during May 21st-28th indicated that *Geocoris* continued to explore IR*lox2* and

hemi-IR*lox2,* higher predation rates sustained on GLV-emitters compared GLV-deficient plants (80% from WT and IR*pi* versus 47% from IR*lox2* and 67% from hemi-IR*lox2*) (see chapter 3.6.2). During simultaneous predation assays of 1st-instar *Manduca* larvae (21st-28th May) and eggs (2nd-4th and 5th-7th June) without GLV supplementation predation rates on WT and IR*pi* plants reached a maximum of 60% over two days versus 17-33% for IR*lox2* and hemi-IR*lox2*, associated with a steady *Geocoris* population of 16-23 individuals/day within a 5 cm radius around plants (fig. 10). The differences in predation on GLV-emitters versus non-GLV-emitters were significant in Fischer's exact tests against WT (IR*lox2* larvae: P=0.047, eggs: P=0.0002) or IR*pi* (hemi-IR*lox2* eggs: P=0.025). However, there was no difference among plant genotypes in the number of *Geocoris* in a Fischer's exact test, indicating that *Geocoris* regularly survey all plants and use GLVs as a short-distance cue to determine which plants harbor prey. Thus, different *Manduca* loads during predation assays did not bias *Manduca* predation and therefore plant reproduction.

It is known that *Geocoris* can predate *Manduca* larvae during the first- to third-instar (Kessler & Baldwin, 2001) but it is not known whether *Geocoris* prefers large or small larvae. In a separate predation trial during M2 to compare total predation rates for first-(small) and second-(large)-instar *Manduca* larvae between WT and IR*pi* (16th-21st May) as well as IR*lox2* and hemi-IR*lox2* (21st-28th May, there was no difference as determined by Fisher's exact tests (fig. 11B). Predation of small hornworm larvae reach a maximum of 80% compared to 63.3% for large caterpillars on WT plants (P=0.252) and 83.3% compared to 80% on IR*pi* plants (P=1.000). For IR*lox2* and hemi-IR*lox2* plants predation rates for small *Manduca* larvae were 1-1.4 fold higher than for large ones, but not significantly different within genotypes in Fischer's exact tests (IR*lox2*: P=0.3, hemi-IR*lox2*: P=1). Total predation rate of small versus large *Manduca* larvae across genotypes was not significant for both WT-IR*pi* as well as IR*lox2*-hemi-IR*lox2* plants (WT-IRpi: P=0.28, IR*lox2* and hemi-IR*lox2*: P=0.46).

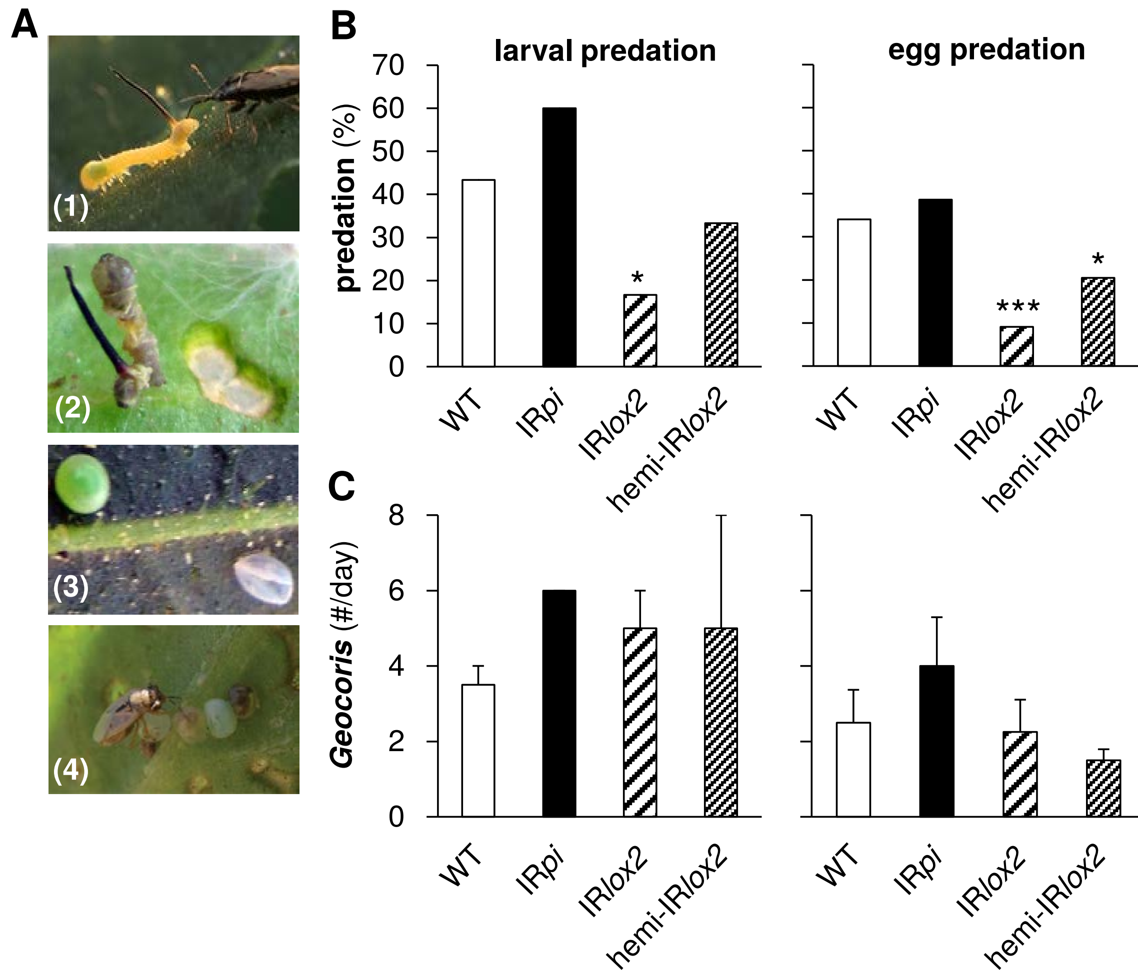


Figure 10: Predation of *Manduca* larvae and eggs by *Geocoris* for GLV-emitting versus non-GLV-emitting field-grown plants. **A** Pictures of a *Geocoris* adult predating a first-instar *Manduca* larva (1), the residual carcass of a predated first-instar *Manduca* larva and their characteristic feeding damage (2), a predated (right) and intact (left) *Manduca* egg (3) and a *Geocoris* adult predating a *Manduca* egg (4). **B** Total percentage of *Manduca* larvae (left panel, N=30 larvae) and eggs (right panel, N=88 eggs) predated during two separate trials during M2 (chapter 3.6.2). Differences between genotypes were significant in a Bonferroni-corrected Fisher's exact tests against WT (IR*lox2*) or IR*pi* (hemi-IR*lox2*); *P<0.05, ***P<0.001. Graphs show total percent. **C** Numbers of *Geocoris* (adults and nymphs) near plants during *Manduca* larval and egg predation trials in M2. Differences were not significant in Fisher's exact tests. Graphs show means+SEM per day.

Photographs: 1: Massih media, 2-3: M. Schuman, 4: M. Stitz (previously published in Allmann & Baldwin, 2010)

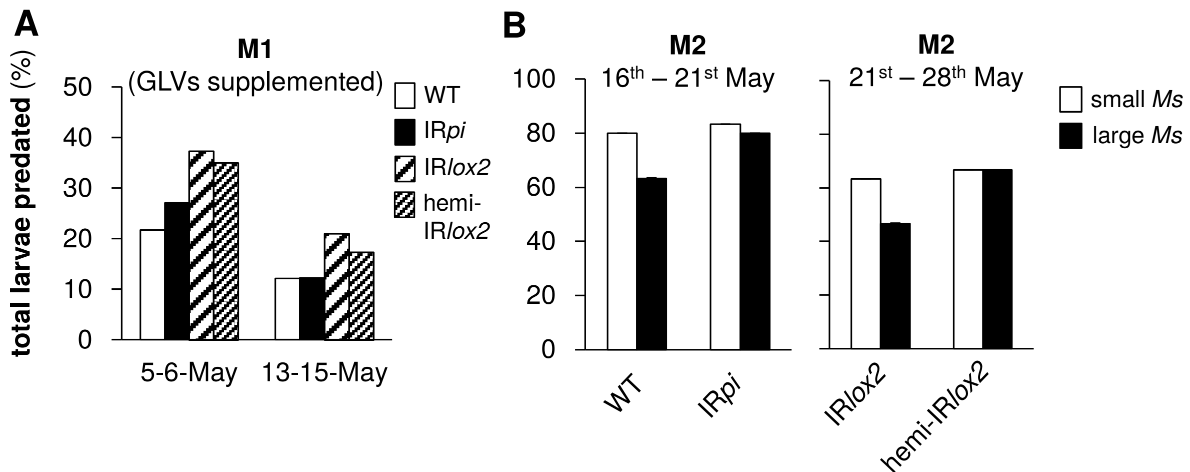


Figure 11: Total predation rates of *Manduca* larvae by *Geocoris* during GLV supplementation and infestation with different-sized larvae. Graphs show total percent. **A** Two larval predation trials during GLV fumigation of IR*lox2* and hemi-IR*lox2* plants (59-60 larvae on May 5th-6th, 92-100 larvae on May 13th-15th; N=20 plants per genotype; chapter 3.6.1) **B** Two infestation trials with two first-(small) and two second-(large)-instar larvae each on GLV-emitting (WT, IR*pi*) and GLV-deficient plants, respectively (N=15 plants per genotype; chapter 3.6.2). Differences between and within genotypes were not significant in Fisher's exact tests.

4.3 TPIs make *Manduca* larvae more sluggish in response to mock predation

To test for a function of TPIs in reducing larval performance and in increasing their susceptibility to predators, *Manduca* larval growth and resistance to mock predator attack were monitored in field and laboratory trials. Results of *Manduca* performance assays of 1st-instar larvae feeding two days on plants during M2 (21st-23rd May, see chapter 3.6.2) on equal infested field-grown plants showed no significant difference between genotypes (one-way ANOVA with genotype as the factor $F_{3.77}$=2.792, P>0.074; fig. 12A). Furthermore, *M. sexta* larvae grow equal well on all genotypes in an additional laboratory performance experiment. Among all genotypes larvae gain weight in the range of 137 to 189 mg in the 4th and 280 to 560 mg in the fifth instar and consumed on average 0.57 to 0.60 cm^2 leaf area over two days during the first instar. The progression of larval instars, weight gain during these instars as well as larval consumption after two days feeding on plants did not significantly differ in one-way ANOVA with genotype as the factor (consumption $F_{3.75}$=0.067, P>0.985; weight $F_{3.6}$=2.084, P>0.12; figs. 12B-C). The instar development over all larvae is shown in figure 12D. These results demonstrate that *Manduca* larvae on TPI-producing plants can overcome the inhibiting effect in photolytic digestion by production of proteases that are less susceptible to offensive inhibitors (Broadway 1995; Jongsma *et al.*, 1995) or

using other classes of proteases (Jongsma *et al*. 1995; Broadway 1996), what was not tested, but not by higher leaf consumption. Although GLVs can stimulate *Manduca* feeding (Eubanks & Denno, 1999; Meldau *et al*., 2009), GLV-deficient plants suffer same loss on leaf tissue to *Manduca* larvae.

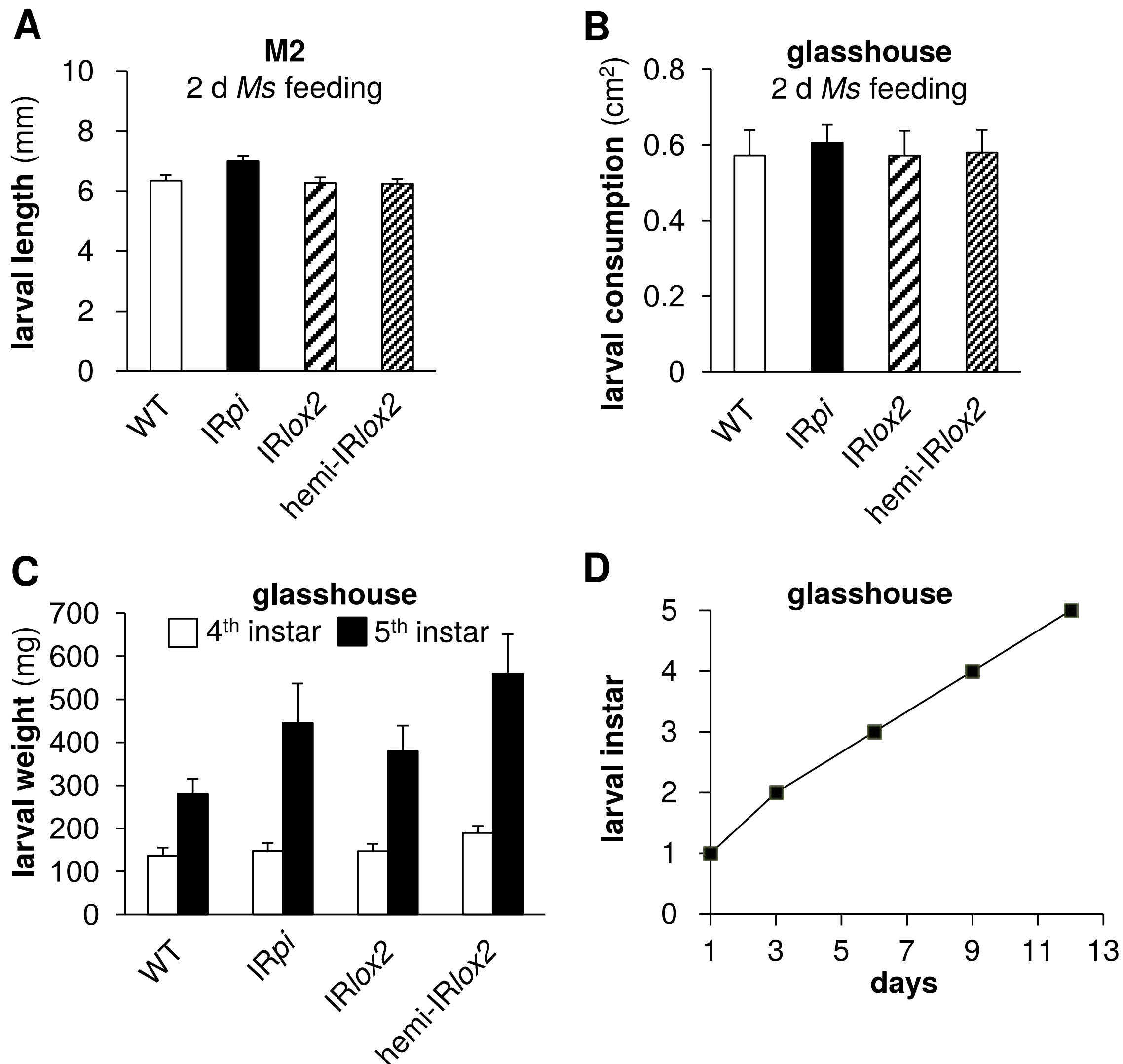


Figure 12: TPI-mediated effects on *Manduca* development and consumption on TPI-producing versus TPI-deficient field- and glasshouse-grown plants. **A** Larval length in the 1st instar after 2 d feeding on field-grown plants. Larval length was measured for surviving larvae (N=13-26) in a predation assay during *Manduca* infestation M2 (fig. 5, see fig. timeline in chapter 3). **B** Leaf tissue consumption of 1st-instar *M. sexta* larvae after 2 d feeding on glasshouse-grown plants in a 12 d performance assay (N=20; fig. 12C). **C** Weight gain of 4th- and 5th-instar *M. sexta* larvae after 9 d and 12 d feeding on glasshouse-grown plants (N=14-17). **D** Progression of larval instars over time for lab-reared *M. sexta* across all genotypes in the 12 d glasshouse performance assay (C). Differences between genotypes were not significant in Scheffe *post-hoc*

tests following a one-way ANOVA (length $F_{3.77}$=2.792, P=0.074; consumption $F_{3.75}$=0.067, P=0.985; weight $F_{3.6}$=2.084, P=0.12). Graphs show means+SEM.

However, after testing the hypothesis that the lower nutritional value of TPI-producing plants might nevertheless affect *Manduca* larvae behavior the data of wild second-instar *Manduca* larvae feeding on WT plants show a decrease in resistance to mimicked *Geocoris* attack in a mock predation assay (18th June, M3, see chapter 3.7). Indeed, wild hornworm larvae fed on WT plants were 75% less likely to attack when lifted off of the leaf (fig. 13A). In more accurate off-plants assays (24th-25th June) lab-reared first-instar *M. sexta* larvae fed on IR*pi* leaves from field-grown plants reacted two times more tackling to the insect pin either when initial poking or piercing and lifting them than WT-fed larvae (fig. 13B). Post trial monitoring of recovery over 24 h showed significant differences (P=0.016, F=-2.656; tab. 5B) between *Manduca* larvae feeding on IR*pi* leaf material, which continued to grow (0.22 mm on average), and WT-fed larvae, which ceased to grow (-0.92 mm on average) (fig. 13B). In contrast, mortality did not differ between genotypes in a Fisher's exact test. Behavioral responses were similarly in both assays and tested in paired t-tests (see tab. 5).

In conclusion, TPIs did not reduce *Manduca* larvae growth, but affect *Manduca* behavior. Thus larvae feeding on WT plants reacted more sluggishly to experimental provocation than larvae on IR*pi* plants.

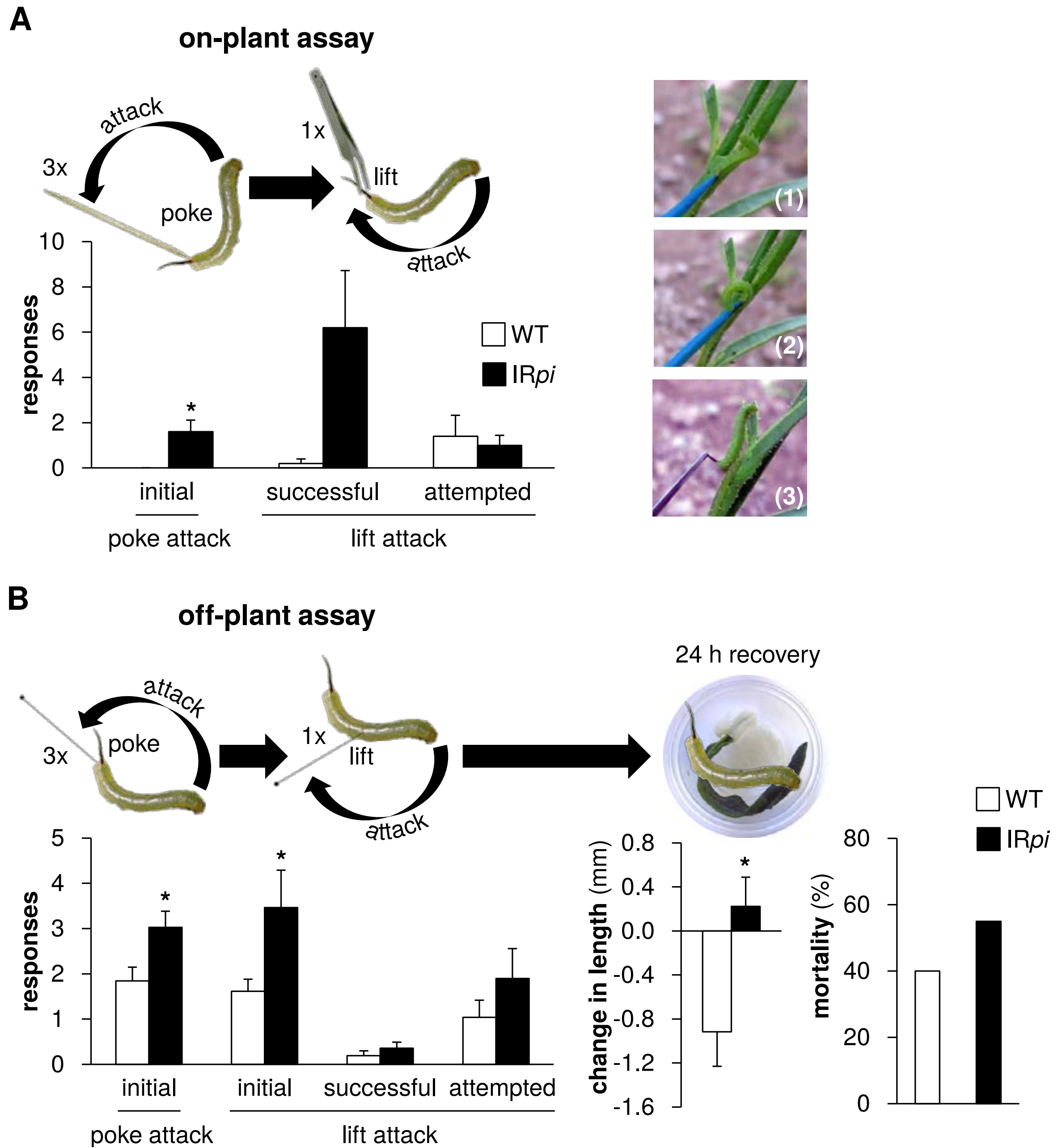


Figure 13: TPI-mediated effects on *Manduca* behavior and recovery in mock *Geocoris* predation assays on TPI-producing versus TPI-deficient field-grown plants Graphs show means+SEM. **A** Response of wild second-instar *Manduca* larvae in an on-plant assay in the field (18th June, M3). Larvae were poked three times, 3 s apart, with the end of a toothpick and thereafter lifted with a forceps for 15 s (N=5 larvae matched for size; chapter 3.7). Right, pictures of larval responses to poking (1 and 2) and lifting (3) during on-plant assays, exported out of video clips. **B** Left, response of lab-reared first-instar *M. sexta* larvae in off-plant assays, to being poked, pierced and lifted with an insect pin (N=20 larvae matched for size; 24th-25th June; chapter 3.7). Right, growth of survival first-instar *M. sexta* larvae in the following 24 h (N=9-12 larvae) and larval mortality after mock *Geocoris* attack. Response and growth differences between genotypes were significantly different in paired t-tests, *P<0.05 (tab. 1). Mortality was not significantly different between genotypes in a Fisher's exact test. *Manduca* icons, M. Schuman.

Table 5: Behavioral differences between WT- and IR*pi*-fed *Manduca* larvae in mock *Geocoris* predation assays. Results show between-genotype differences in behavioral responses of second-instar *Manduca* larvae in on-plants and first-instar larvae in off-plant assays, tested in paired t-tests. Significant P values are **bold**.

A: on-plant assay

Response	df	t	P
attack to poking	4	-3.138	**0.035**
successful attack to lifting	4	-2.268	0.86
attempted attack to lifting	4	0.389	0.717

B: off-plant assay

Response	df	t	P
attack to poking	12	-2.472	**0.029**
initial attack to lifting	12	-2.408	**0.033**
successful attack to lifting	12	-1.363	0.198
attempted attack to lifting	12	-1.172	0.264
growth difference	19	-2.656	**0.016**

4.4 GLVs increase plant fitness in the presence of predation

4.4.1 GLV emission do not promote an increase in herbivory

Because GLVs can stimulate *Manduca* feeding, and silencing plant GLV production results in reduced herbivore damage (Halitschke *et al*., 2004; Meldau *et al*., 2009) it raised the need to determine whether GLV-silenced plants suffered different amounts of herbivore damage which might reduce the impact of herbivore damage on the fitness of GLV-silenced plants. Total canopy damage to field-grown WT *N. attenuata* and transgenic lines (IR*pi*, IR*lox2* and hemi-IR*lox2*) due to naturally occurring herbivores was monitored both before the first infestation M1 (May 5th) and near the end of M2 (May 27th; fig. 14A, see chapter 3.5, 3.6.2). There were significant differences in noctuid and mirid damage between genotypes (one-way ANOVAs: mirids May 27th $F_{3,103}$=5.291, P=0.002; noctuids May 27th $F_{3,103}$=3.503, P=0.018; Bonferroni-corrected Kruskal-Wallis test: noctoids May 5th χ^2 = 11.239, P=0.027). All plants suffered damage of up to 15 % and 3 % leaf area to mirids and nuctuids and less than 2% to grasshoppers and flea beetles. Damage due to grasshoppers and flea beetles was not significantly different between genotypes in one-way ANOVAs on arcsine-transformed data.

Additionally, total canopy damage due to *M. sexta* larvae during the predation assays M1 and M2 (15th May and May 28th; fig. 14B) was recorded and analyzed using a relative damage index (see fig. 10B right panel). As for consumption data from *M. sexta* larvae fed on glasshouse plants (shown in fig. 12B), all plants suffered, in total, a similar amount of *Manduca* damage, corresponding to 1.6 to 2.6 on the damage index; only IR*pi* plants suffered significantly less Manduca damage compared to WT plants during M2 as determined by a Mann-Whitney U-test (U= 54, P=0.046).

Thus because herbivore damage was either not different, or lower on *LOX2*-silenced plants, these data cannot explain differences in plant fitness (see chapter 4.4.2). Of all parameters measured, only differences in the predation of *Manduca* larvae were positively correlated to differences in plant growth and reproduction. This is supported by the fact that these genotypes do not differ in growth and fitness in the absence of *Geocoris* predators (Schuman *et al*., in review).

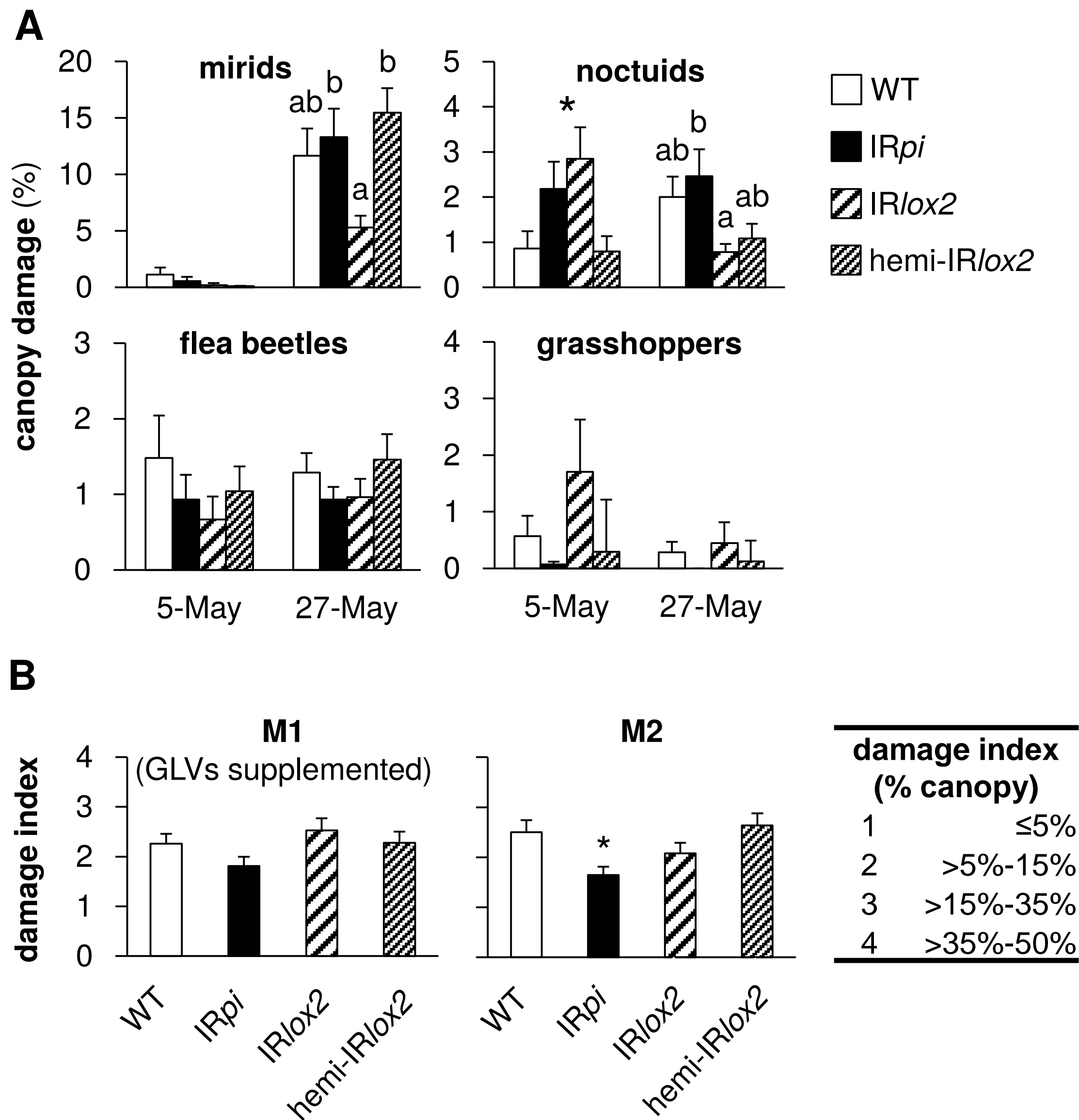


Figure 14: Herbivore damage to plants during the field season 2011. Graphs show means+SEM. **A** Total canopy damage due to naturally occurring herbivores before the first infestation *Manduca* M1 (May 5th) and near the end of M2 (May 27th) (chapter 3.6.1, 3.6.2). Differences denote significant differences between genotypes ($P<0.05$) in Scheffe *post-hoc* tests following one-way ANOVAs of arcsine-transformed data at each timepoint (mirids May 27th $F_{3,103}=5.291$, $P=0.002$; noctuids May 27th $F_{3,103}=3.503$, $P=0.018$); *$P<0.05$ for the main effect of genotype on noctuid damage in a Bonferroni-corrected Kruskal-Wallis test, May 5th ($\chi^2 = 11.239$, $P=0.027$). **B** Total damage from *M. sexta* larvae (N=11-17) used in the predation assays in M1 (May 15th, left panel), in which GLVs were externally supplemented to plants, and M2 (May 28th, middle panel). Total canopy damage was estimated according to the index (right panel). *$P<0.05$ in a Mann-Whitney U-test between IR*pi* and WT in M2 ($U= 54$, $P=0.046$); the difference on May 15th was not significant ($P>0.1$).

4.4.2 *Manduca* feeding reduces plant fitness

To evaluate the effect of Manduca feeding on plant fitness, fitness correlates including rosette diameter, stem length, numbers of branches, buds and flowers were measured or counted every third day during M2 (see chapter 3.4). All plants suffered, in total, a similar amount on *Manduca* damage and loss of leaf tissue to *Manduca* attack, and G*eocoris* had the same opportunity to locate *Manduca* larvae and eggs on all genotypes (fig. 10C; chapter 4.2). Final growth measurements showed a significant difference overall between *Manduca*-infested versus uninfested control plants of each genotype (44-45 d after planting) in two-way multivariate ANOVAs with factors genotype and treatment (treatment: $F_{6,52}$=2.287, P=0.049; genotype*treatment $F_{18,147}$=0.771, P=0.732) or a separate Mann-Whitney U-test for number of branches (figs. 15 A-B; stem $F_{1,57}$=9.155, side branches U = 270, buds $F_{1,57}$=4.572, first flower $F_{1,1}$=5.802). (Comparison according the number of “days in field” controls for the different dates on which plants were placed into the field over a total of one week). Individual measurements of growth and reproduction differed significantly among genotypes: rosette diameter ($F_{3,57}$=8.791, P<0.001), stem length ($F_{3,57}$=4.192, P=0.009), number of branches (χ^2 = 10.958), number of buds ($F_{3,57}$=9.876, P<0.001) and days to first flower ($F_{3,1}$=3.617, P<0.016) (figs. 15A-B). Furthermore, within genotypes, the difference between *Manduca*-infested and control plants was significant for WT and hemi-IR*lox2* (stem length and numbers of buds), but not IR*lox2* or IR*pi*. *Manduca* feeding significantly reduced growth and reproductive output of WT and hemi-IR*lox2* plants of by 30% to 40% (for stem length, number of bud and flowers) but not of IR*pi* and IR*lox2* plants (figs. 15, 16, tab. 6; WT stem: $F_{5,22}$=3.871, P=0.011, buds: $F_{5,22}$=3. 188, P=0.026; hemi-IR*lox2* buds: $F_{5,18}$=3.001, P=0.038). The lack of *Manduca*’s negative effect in growth and reproduction on IR*pi* and IR*lox2* plants is likely due to reduced feeding damage resulting from a lack of GLV feeding stimulants in IR*lox2* (Halitschke *et al*., 2004) and TPI-induced compensatory feeding in IR*pi* (Steppuhn & Baldwin, 2007). But *Manduca* feeding reduced growth and reproduction in hemi-IR*lox2* plants, despite strongly reduced GLVs, and reduced herbivore damage on IR*lox2* cannot explain why these plants were less fit than WT (figs. 14, 15, 16, tab. 6). Finally, results showed that lower predation of *Manduca* from IR*lox2* and hemi-IR*lox2* is correlated with a reduction in growth as well as reproduction of both genotypes, by 30-50% for IR*lox2* and 20-30% for hemi-IR*lox2* versus WT during infestations M2 (figs. 10B, 15, 16).

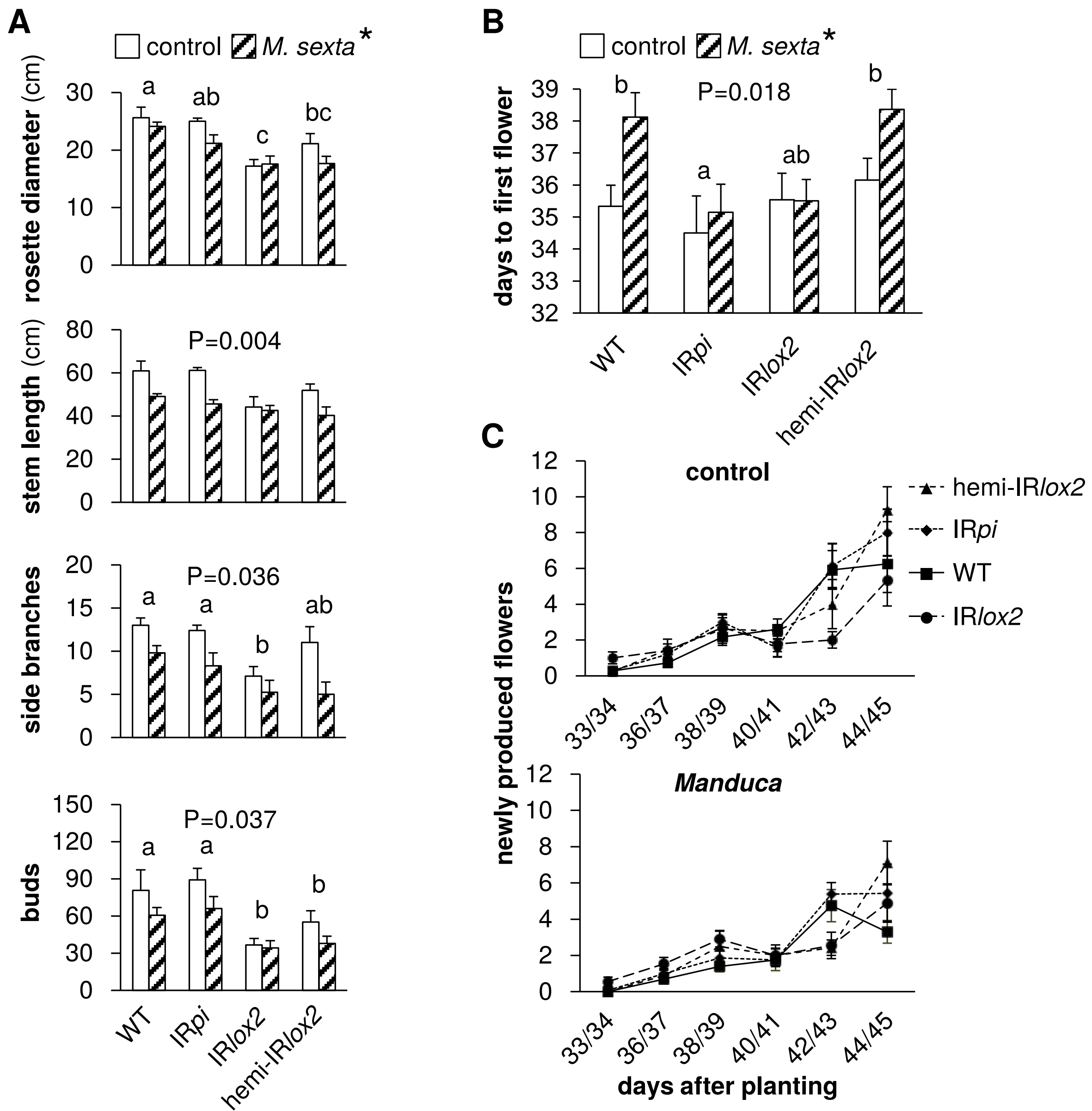


Figure 15: Growth and reproduction of *Manduca*-infested versus uninfested field-grown plants measured at the end of *Manduca* infestation M2. Graphs show means+SEM. **A** Final growth measurements for *Manduca*-infested versus uninfested control plants of each genotype (44-45 d after planting, N=11-17, fig. timeline in chapter 3). *Manduca* feeding had a significant effect on growth and reproduction as determined by a Wilks' Lambda test in a two-way multivariate ANOVA for rosette diameter, stem length and number of buds, with factors genotype and treatment (treatment $F_{6,52}$=2.287, P=0.049; genotype*treatment $F_{18,147}$=0.771, P=0.732), or a separate Mann-Whitney U-test for number of branches. **B** Days to first flower for *Manduca*-infested versus control plants. P-values above individual graphs denote the significance of *Manduca* feeding over all genotypes (stem $F_{1,57}$=9.155, side branches U = 270, buds $F_{1,57}$=4.572, days to first flower $F_{1,1}$=5.802). Different letters denote significant (P<0.05) differences between genotypes in Scheffe *post-hoc* tests (rosette diameter $F_{3,57}$=8.791,

P<0.001, stem length $F_{3,57}$=4.192, P=0.009, number of buds $F_{3,57}$=9.876, P<0.001, first flower $F_{3,1}$=3.617, P<0.016) or Bonferroni-corrected P-values for Mann-Whitney U-tests following a Kruskal-Wallis test (number of branches χ^2 = 10.958). **C** Flower production for *Manduca*-infested and uninfested control plants from the beginning of flowering until the end of M2 (May 28th). Flowers were counted and removed after each measurement, each time point represents new flower production.

Table 6: Growth and reproduction for *Manduca*-infested versus uninfested field-grown plants of each genotype during the field season in 2011.Results are shown from Student's t-test for the final measurement of rosette diameter and number of branches and from Wilks' Lambda tests in repeated-measures ANOVAs across all measurements of stem length, numbers of buds and flowers during M2 (fig. 16). Significant P values are **bold**.

genotype	**WT**			**IR*pi***			**IR*lox2***			**hemi-IR*lox2***		
growth & fitness	df	t	P	df	t	P	df	t	P	df	t	P
branches	26	1.696	0.102	26	1.024	0.315	25	1.112	0.277	22	1.753	0.094
rosette	26	-0.870	0.932	26	-0.161	0.873	25	-0.058	0.954	22	1.140	0.267
	df	F	P	df	F	P	df	F	P	df	F	P
stem	5,22	3.871	**0.011**	5,22	0.991	0.446	5,21	0.606	0.696	5,18	1.118	0.386
buds	5,22	3.188	**0.026**	5,22	0.656	0.660	5,21	0.535	0.748	5,18	3.001	**0.038**
flowers	3,24	1.213	0.326	5,22	0.525	0.755	5,21	0.540	0.744	4,19	0.723	0.587

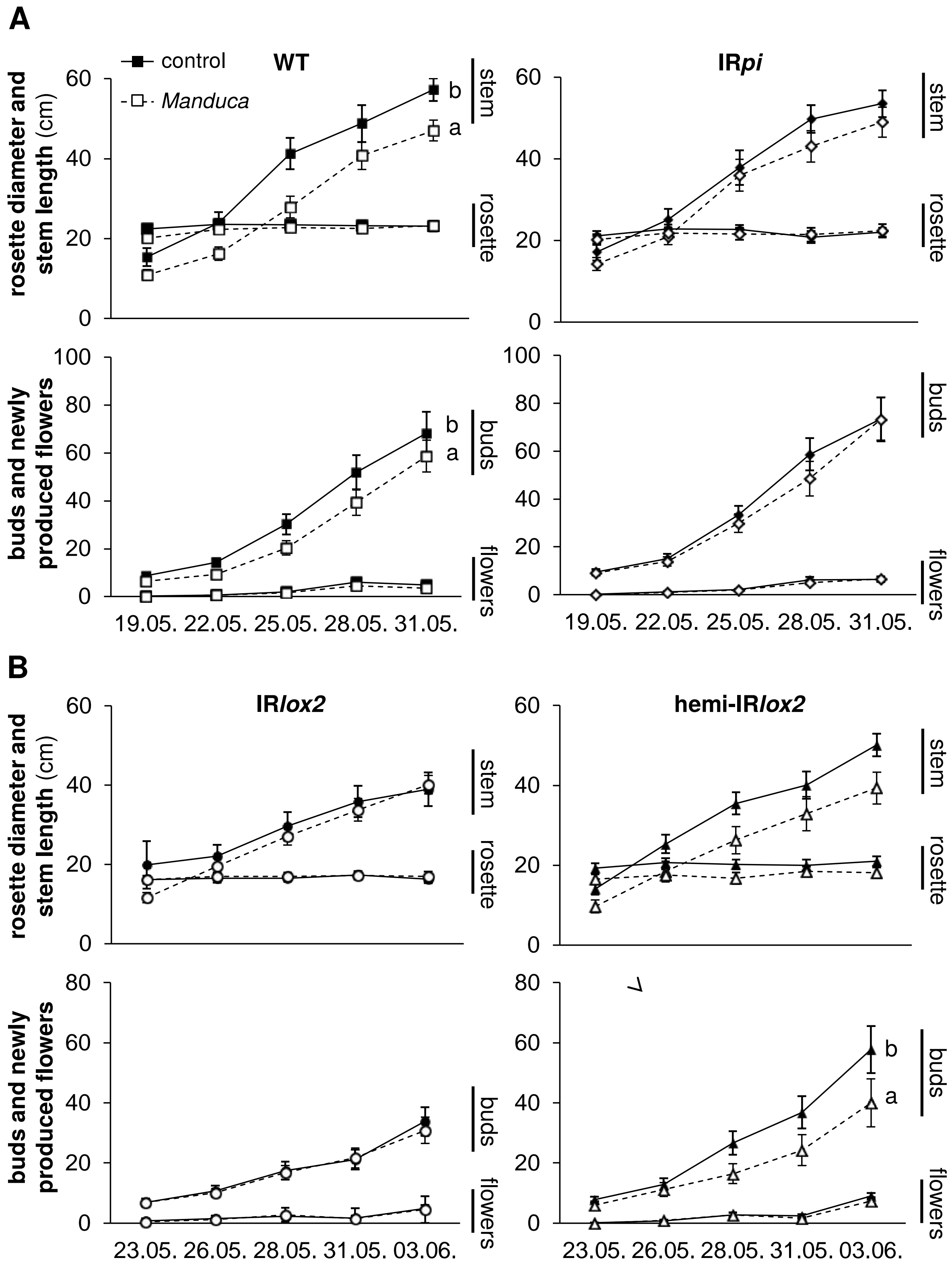


Figure 16: Growth and reproduction of *Manduca*-infested versus uninfested plants of each genotype grown in the field in 2011. Graphs show means+SEM. **A** Growth measurements for WT and IR*pi* plants during M2 (16th-21st May, N=15; chapter 3.6.2). **B** Growth measurements for IR*lox2* and hemi-IR*lox2* plants during M2 (21st-28th May, N=15). The significant effect (*P<0.05) of *Manduca* feeding on growth and reproduction was tested with Wilks' Lambda tests in

repeated-measures ANOVAs with treatment as the factor or in separate Student's t-tests of the final measurement of rosette diameter and number of branches, because plants had few or no side branches before the final measurement and rosette diameters did not change during M2. Different letters denote significant ($P<0.05$) differences within genotypes in Scheffe *post-hoc* tests (values for individual genotypes are in table 6).

4.4.3 Fitness advantage of GLV emission is correlated to *Manduca* predation in a matched design

Finally, to determine whether TPIs or GLVs increase *N. attenuata*'s fitness when plants are attacked by *Manduca* larvae a *Manduca* predation and plant performance assay was conducted during infestation M3 (14th-29th June; see chapters 3.6.2, 3.6.4, 3.6.5). The hypothesis was that 50% lower predation rates of *Manduca* from hemi-IR*lox2* plants (fig. 10B), combined with the decrease in the growth and fitness of these plants due to M*anduca* feeding (fig. 15), would result in reduced reproduction for hemi-IR*lox2* versus matched WT and IR*pi* plants if *Geocoris* was present. Twenty-one WT, IR*pi* and hemi-IR*lox2* plants were matched for size as well as reproductive output, apparent health and prior *Manduca* damage to exclude differences in growth, fitness and damage arising during infestations M1 and M2 (fig. 17). Damage from naturally occurring herbivores did not differ among these genotypes (fig. 14A). Because *Manduca* feeding did not significantly affect growth and reproduction for homozygous IR*lox2* plants (fig. 15), they were excluded from this experiment. Hemi-IR*lox2* plants used for M3 had produced significantly more flowers (but not buds) than WT prior to infestation M3 and this difference was by removing all reproductive meristems from matched plants ($F_{2,60}=8.668$, $P<0.001$; fig. 17B).

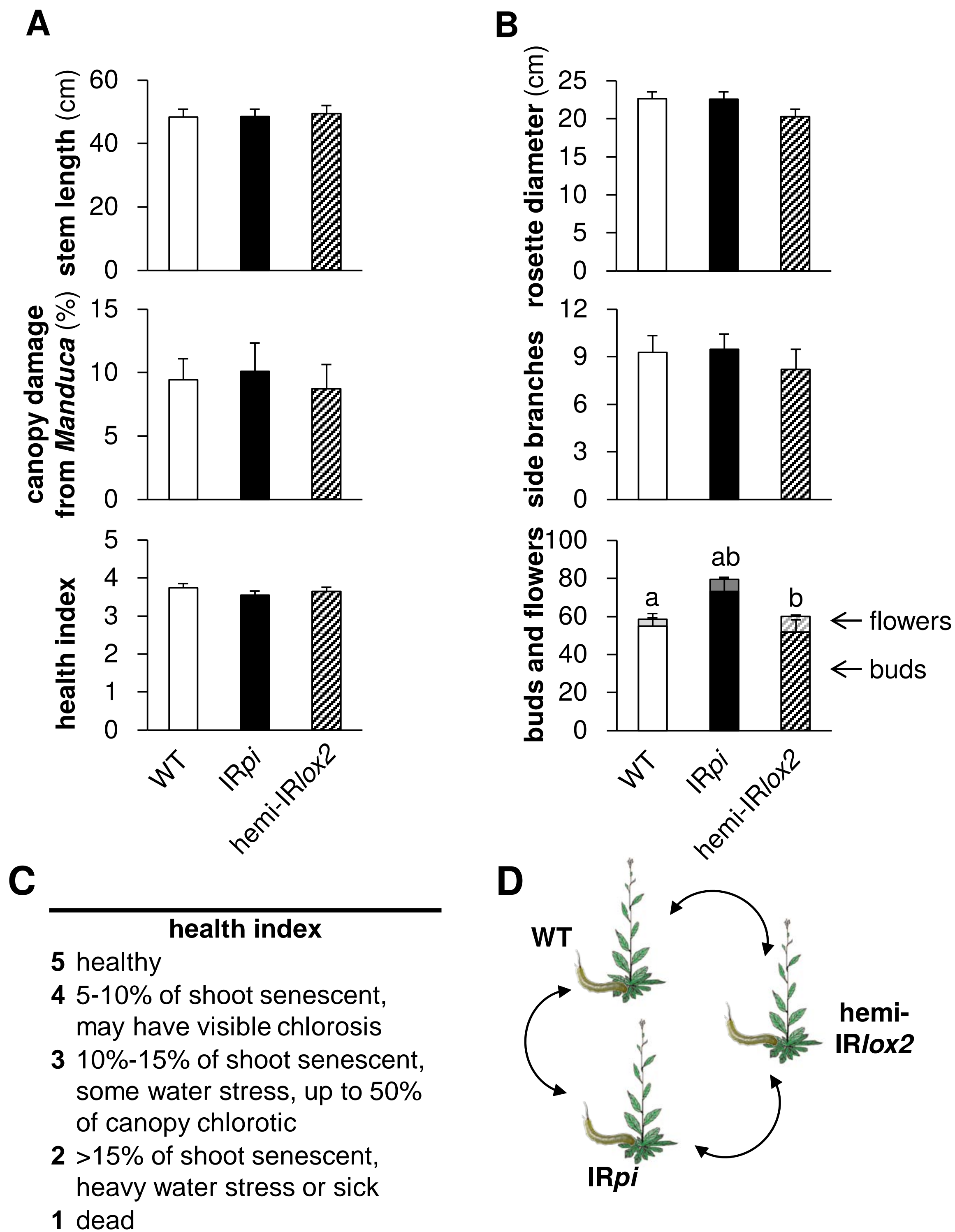


Figure 17: Comparison of plants used in triplets for wild *Manduca* infestation during the field season in 2011. Graphs show mean+SEM. **A** Parameters used to match WT, IR*pi* and hemi-IR*lox2* plants in triplets (N=21, M3; chapter 3.4, 3.5). Measurements and counts are from the first day of the infestation assay (14th June). **B** Final measurement of prior growth and reproduction for plants used for triplets; data are from the final two measurements during infestation M2 (28th May for WT and IR*pi*, 1st June for hemi-IR*lox2*). Different letters denote

significant differences between genotypes ($P<0.001$) for flower number in Scheffe *post-hoc* tests following a multivariate ANOVA with all measurements ($F_{2,60}=8.668$). **C** Health index used in A. **D** Layout of matched triplet design of WT, IR*pi* and hemi-IR*lox2* plants during M3. Plants were not adjacent but rather distributed throughout the experimental plot. Each plant was infested with one wild *Manduca* neonate. The interactions to be measured between genotypes in one out of 21 triplets are illustrated (see plot design in fig. 6D).

All genotypes were infested simultaneously with one wild *Manduca* larva and caterpillar mass, instar progression, larval intra-plant movement and cumulative predation/mortality as well as newly produced flowers, buds and unripe seed capsules were measured until plants began to set seed and *Manduca* larvae reached the fifth and final instar (fig. 18). Indeed, wild *Manduca* larvae suffered a lower mortality rate of 38% on hemi-IR*lox2* versus 62-76% on WT and IR*pi* during the first three instars, in which they are vulnerable to *Geocoris* attack (Kessler &Baldwin, 2001). Differences in larval mortality between IR*pi* and hemi-IR*lox2* were significant on days nine and ten (Fisher's exact tests, $P=0.028$), after *Manduca* larvae reached the third instar; after this time, larvae are no longer predated by *Geocoris*. Furthermore, the effect of TPIs in decreasing *Manduca* growth and instar progression as well as reducing within-plant movement over time (Zavala & Baldwin, 2004a; Zavala *et al*., 2008) were not observed in this experiment (figs. 18A, C-D).

In the presence of *Geocoris*, *Manduca* mortality rates on GLV-emitters were twice as great as those for on non-GLV-emitters and resulted in a twofold greater production of buds, flowers and unripe seed capsules on emitting plants (results of Greenhouse-Geisser-corrected univariate tests for the interaction of line and day: buds, $F_{4.988}=5.297$, $P<0.001$; flowers, $F_{3.722}=4.403$, $P=0.003$, fig. 18B; one-way ANOVA with genotype as the factor unripe seed capsules $F_{2,60}=4.142$, $P=0.021$; fig. 18E). In summary, these data demonstrate that the GLV-mediated twofold increase of *Manduca* larvae predation by *Geocoris* is correlated with a twofold increase in plant fitness.

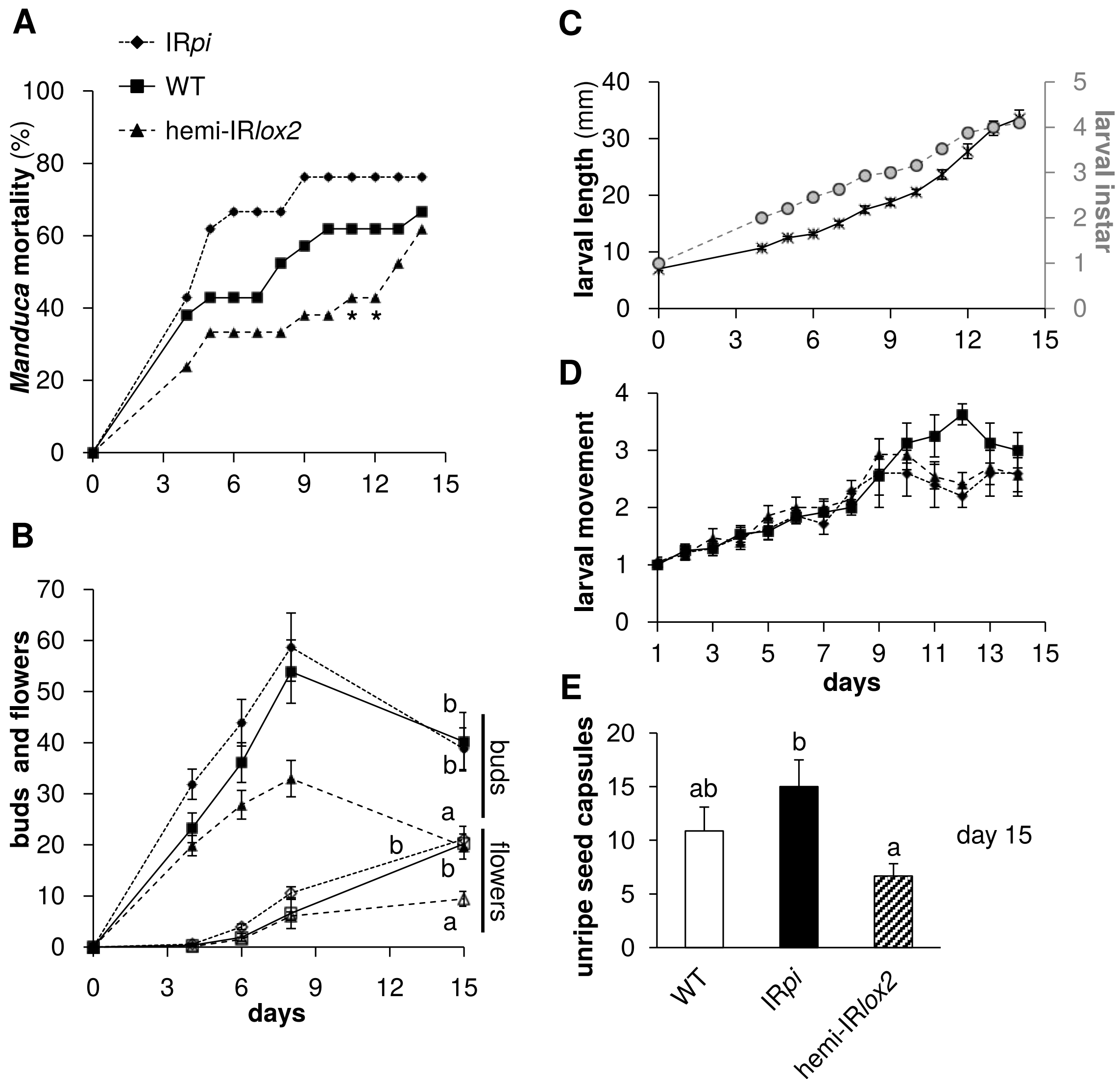


Figure 18: GLV-mediated increase of *Manduca* larval predation by *Geocoris* is correlated to plant fitness. Graphs show percent or mean+SEM. **A**. Mortality of wild *Manduca* larvae over 15 days on infested field-grown plants (M3, N=21). One neonate was placed on each plant. Through day eight, larvae (third instar, (C)) were susceptible to predation by *Geocoris*. Differences in larval mortality between genotypes were significant ($P \leq 0.05$) in Fisher's exact tests comparing hemi-IR*lox2* and IR*pi*; *$P<0.05$. **B** Numbers of reproductive units, flowers and buds, produced by infested matched WT, IR*pi* and hemi-IR*lox2* plants in triplets after cutting their reproductive meristems. Different letters indicate significant differences ($P \leq 0.05$) in Scheffe *post-hoc* tests of hemi-IR*lox2* versus WT and IR*pi* flowers and buds, following a repeated-measures multivariate ANOVA with genotype as factor over all measurements (results of Greenhouse-Geisser-corrected univariate tests for the interaction of line and day: buds, $F_{4.988}=5.297$, $P<0.001$, closed symbols; flowers, $F_{3.722}=4.403$, $P=0.003$, open symbols). **C** Progression of larval instars and larval performance over time for wild *Manduca* larvae across all genotypes during M3 (A). **D** Intra-plant movement over time for wild *Manduca* larvae during M3 (A), according categories in chapter 3.6.5. **E** Unripe seed capsules at the final measurement

(day 15) of plant reproduction. Different letters denote significant differences ($P<0.05$) for in Scheffe *post-hoc* tests following an one-way ANOVA with genotype as the factor ($F_{2,60}=4.142$, $P=0.021$).

4.5 Functional interaction between TPIs and GLVs

Because the IR*pi* construct was inactive in the hemi-IR*lox2* line (fig. 8), the question of synergy between GLVs and TPIs could not be addressed directly by measuring the comparative growth and reproduction of these plants. However, the behavioral results demonstrate that dietary TPIs weakened *Manduca*'s behavioral evasive responses to simulated *Geocoris* attack (see chapter 4.3; fig. 13). Thus reducing herbivores growth and prolonged vulnerability enhances enemy attack by expanding the window of susceptibility to predation (Williams, 1999), postulated by the slow growth-high mortality hypothesis (Benrey & Denno, 1997; Kaplan & Thaler, 2011).

In conclusion, results indicate that TPIs function against specialists by enhancing indirect defense.

5. Discussion

Three decades since HIPVs have been interpreted as indirect defenses (Price *et al.*, 1980), no studies have yet investigated whether HIPVs positively benefit plants' Darwinian fitness in an ecological context (Allison & Hare, 2009). In cooperation with the two-year field project begun in 2010 by Meredith C. Schuman the results of this diploma thesis finally can demonstrate their function as defenses in nature (Schuman *et al.*, in review). GLV-emitting *N. attenuata* plants produced twice as many buds in flowers as GLV-silenced plants in correlation with a twofold increase of *Manduca* larval predation by *Geocoris* from GLV-emitters (fig. 18). The results showed that lower predation of *Manduca* from GLV-silenced plants is correlated with a reduction in growth and reproduction by 20-50% versus WT plants (figs. 10B, 15, 16). Although TPIs neither reduced *Manduca* larvae growth, nor increased plant fitness (figs. 12, 15, 16), results demonstrated the possibility of a functional concert between TPIs and GLVs in *N. attenuata*: TPIs weakened specialist *Manduca* larvae's behavioral defense response to mock *Geocoris* predation, likely via nitrogen starvation, while GLVs increased larval mortality by attracting *Geocoris* (figs. 10, 13).

5.1 Experimental design - benefits and drawbacks

To test the hypotheses that GLVs and TPIs defend plants in nature by increasing *Manduca* predation and thereby plant fitness, experiments were performed with the *N. attenuata* in its natural habitat. Fundamental knowledge about a variety of natural plant-insect interactions and several defenses-related genes (e.g. Halitschke *et al.*, 2003; Kessler & Baldwin, 2001, 2002a, 2004b; van Dam *et al.*, 2001a; Voelckel & Baldwin, 2004; Zavala & Baldwin, 2004) as well as *N. attenuata*'s adaption and growth in an immediate post-fire environment, which features nitrogen-rich soils, a very small number of herbivorous insects and few intra-specific competitors (Baldwin, 2001; Baldwin *et al.*, 1994; Lynds & Baldwin, 1998; Whelan, 1995), provides the crucial aspects for using this model plant. Furthermore, the variety of time and extent of the predator and herbivore communities between years has an impact on the adaptive value of defense traits, also this experiment. To address the putative defensive function and their realistic estimates of their fitness costs for the plant, manipulation of individual genes provides a clean approach to examine the adaptive significance of defense traits with maximum genetic control versus natural variation (Bergelson & Purrington, 1996; Wesley *et al*. 2001; Zheng & Dicke, 2008). Single gene silencing using RNAi, T-DNA knockouts or VIGS enables the specific manipulation of single resistance traits and their effects on individual plant-insect interactions or community dynamics (Bergelson &

Purrington, 1996). The extreme variance of herbivore and predator communities, which can be influenced by effects which are poorly understood, underlines the importance of placing genetically controlled plants in their native environment, coping with competitors, herbivores and their predators, to realistically evaluate the role of resistance traits (van Dam & Baldwin, 1998, Kessler & Baldwin, 2001, 2004a, b; Preston & Baldwin, 1999). Thus, novel approaches to address the ecological function of genes compare molecular genetics and community ecology (Zheng & Dicke, 2008). The use of transgenic plants silenced with high precision by RNAi for the biosynthesis either of GLVs (IR*lox2*), TPIs (IR*pi*) or in a hemizygous cross of these two constructs (hemi-IR*lox2*) permitted the determination of the role of TPIs and GLVs as well as their synergetic interaction in an ecological model system: the interaction between *Manduca,* its natural host plant *N. attenuata,* and the third trophic level, the native generalist predator *Geocoris (*Hermsmeier *et al*., 2001; Kessler &Baldwin, 2001). Reproductive output of wild-type *N. attenuata* plants was compared to that of the transgenic lines in nature. The advantage of genetically silencing inducible GLV emission, rather than creating constitutive emission of these compounds, is that herbivores and predators do not learn to ignore the usually herbivore-induced cues from plants: naturally inducible WT plants serve as HIPV-emitters permitting the comparison with transformants lacking specific volatile components (Halitschke *et al*., 2008; Skibbe *et al*., 2008). The engineering of transformants that constitutively emit HIPVs may prevent predators from learning the association of these cues with prey, if plants "crying wolf" all the time. Although the transformation of an induced resistance provides more control for genetic background than constitutive traits, is difficult to get inducible constructs to work (Schuman & Baldwin, 2012; Turlings *et al*., 1995). Thus comparing silenced to WT plants provides a simple and natural alternative.

The transformation process itself with IR constructs of *PI* and *LOX2* genes successfully silenced the production of TPIs in IR*pi* plants and the emission of *GLVs* from IR*lox2* and hemi-IR*lox2*; the transformation process itself does not affect plant fitness or competitive ability (Zavala & Baldwin, 2004; Zavala *et al*., 2004a; figs. 15, 16), TPI production or volatile emission (fig. 8, chapter 4.1) and for these lines did not affect non-target metabolites (Allmann *et al*., 2010; Steppuhn & Baldwin, 2007; M. C. Schuman, in press). Especially the strong reduction of GLVs in IR*lox2* and hemi-IR*lox2* plants without influencing the production of JA and JA-related secondary metabolites (Allmann *et al*., 2010), justify the use of this lines. To avoid unsuccessful silencing of the target gene or contingent silencing loss with development and generations, the success of RNAi must be verified for every transformant using a genotype screen such as qPCRs to confirm reduced transcript accumulation of the target gene in comparison to WT plants or vector controls using plants transformed with

empty vectors (Strauss *et al*., 2002; Steppuhn & Baldwin, 2008). Phenotype screenings including the measurement of volatile emission in plant's headspace, concentration of defense metabolites, plant size and reproduction are furthermore necessary to confirm the desired phenotype. The production of sufficient amounts of transgenic seeds uses to avoid time-consuming recrossing of plants of the parental generation. Transcript levels, measurements of TPI activity and GLV emission confirmed that the transgenic lines used, except for hemi-IR*lox2*, had the expected phenotypes. Although hemi-IR*lox2* plants were silenced in only GLV and not in TPI production, this line provided an intermediate GLV phenotype, a vector control and an alternative to IR*lox2* plants during *Manduca* infestation M3 (chapter 4.4.3). However, if the *PI* construct would be intact in the cross, hemizygous silencing of *PI* genes may be flawed with the accumulation TPIs in the cross over time and therefore with phenotypical changes in plant fitness or herbivores performance.

Difficulty of demonstrating metabolite defense functions in nature

If plant traits really function as defenses, they should increase Darwinian fitness, defined as successful reproduction, under herbivore attack (Karban & Baldwin, 1997). Because transgenic plants are not allowed to produce ripe seed, correlates of Darwinian fitness including days to first flower, number of buds and flowers and seed production were measured in this research study (figs. 15, 16, 18, chapter 4.4.2, 4.4.3), not Darwinian fitness itself (number of viable offspring). In fact, net effects on fitness of different phenotypes need to be established ideally in the subsequent plant generation under natural conditions (Karban & Baldwin, 1997). By supplementing GLVs for GLV-deficient plants, *Geocoris* was initially allowed to associate all induced and uninduced plants of the four genotypes with the presence of prey in order not to avoid non-GLV-emitters during the first *Manduca* infestation M1 (fig 6B, chapter 3.6.1). Counts of the predator population verified that *Geocoris* were equally associated with GLV-emitting and non-GLV-emitting plants, although the latter maintained a higher *Manduca* load (fig. 10C, chapter 4.2). To ensure that specifically larval predation and not only mortality was monitored, *Geocoris* presence and fresh *Manduca* feeding damage were used as criteria to determine predation; predated carcasses were also occasionally found on plants.

The estimation of the plant fitness effect of employing induced defenses was accomplished by including induced and control plants (fig. 15, chapter 4.4.2; Karban & Baldwin, 1997). To accommodate differences in plant growth and reproduction initial *Manduca* infestations M1 and M2 (chapter 3.6.2) were staggered and several control measurements of plant size and reproduction were conducted. Within genotypes, the difference between *Manduca*-infested

and control plants was significant for WT and hemi-IR*lox2*, but not IR*lox2* or IR*pi*. In IR*pi* plants, a lack of TPI-induced compensatory feeding (Steppuhn & Baldwin, 2007) can cause a reduction in *Manduca* feeding damage (see fig. 14). Similarly, that *Manduca* did not negatively affect growth and reproduction of IR*lox2* plants is likely due to reduced feeding damage resulting from a lack of GLV feeding stimulants in this line (Halitschke *et al*., 2004), but reduced herbivore damage on these plants (figs. 14, 15, 16, tab. 6) cannot explain why there were less fit than WT. Experiments with both WT and homozygous IR*lox2* plants in 2010 and 2011 showed no constitutive differences between these two genotypes (M. Schuman *et al*., in review), which indicates either contracts with a pathogen (mild infection) or stress-related health problems due to problems in seedling handling.

Predation rates of *Manduca* from WT and IR*pi* plants of up to 80% (see chapter 4.2) were significantly greater than those from IR*lox2* (47%) and hemi-IR*lox2* (67%) plants, but prevented the conduction of a simultaneous *Manduca* performance assay allowing larvae to progress through all larval instars until pupation during *Manduca* infestation M2 (chapter 3.6.2). The repetition of the *Manduca* larval performance assay under glasshouse conditions is not entirely comparable to the performance results during the field assay. A possibility to enable performance experiments in field are clip-caging of *Manduca* larvae at high stem positions to protect them from predators, or the use of wild *Manduca* larvae, which were up to 20% less predated than lab-reached larvae in the same time ratio (figs. 10, 18). But on one hand *Geocoris* predators may crawl in holes of these cages, on the other hand cages change photosynthetic rate, increase local temperature and are limited in space, which could affect plant and larval health as well as larval mortality. For behavior assays of wild larvae feeding on plants, larvae were treated gently because they were needed for the final plant fitness assay. Replicate numbers were limited due to available plants for matching in triples, wild *Manduca* eggs and placement of one larva per plant to mimic natural oviposition rates of one egg per plant (Kessler & Baldwin, 2001). In off-plant assays, larvae were attacked with an insect pin to mimic the *Geocoris* beak. Due to not comparable results of the second-instar behavior test in off-plant assays, a repeat with higher replicate numbers could be necessary.

Because GLVs do not only function as attractants for predators (Allmann & Baldwin, 2010; Kessler & Baldwin, 200), but also for herbivores and as feeding stimulants (Halitschke et al., 2004; Meldau *et al*., 2009), measurements of herbivore load in addition to the quantification of damage due to different herbivore groups would be more accurate to determine whether differences in damage are due to differences in attraction or feeding damage. Only differences in the predation of *Manduca* larvae were positively correlated to differences in plant growth and reproduction, which is supported by the lack of growth and fitness

differences between these genotypes in the absence of *Geocoris* predators (Schuman *et al*., in review). During the final *Manduca* predation and plant performance assay (M3), WT, IR*pi* and hemi-IR*lox2* plants were matched in triplets to exclude differences in growth, fitness and damage arising during infestations M1 and M2. Thus it was possible to accurately determine whether TPIs or GLVs increased plants fitness when plants are attacked (fig. 18, chapter 4.4.3, and see discussion in chapter 5.2).

The question of whether GLVs and TPIs act synergistically in the resistance they potentially provide to the specialist *Manduca* and benefit fitness in a non-additive way, could not be addressed directly due to the inactive *PI* construct in the cross. This raises the need for stable transgenic plants with reduced expression levels of both *LOX2* and *PI* genes. Suitable transgenic plants would be: a stable transformed line homozygous silenced in both GLV and TPI expression or plants of the natural *N. attenuata* genotype collected from Arizona (Steppuhn et al., 2008) either homozygous silenced in *LOX*2 or with a sense construct of the full-length cDNA of the 7-domain *PI*-gene using a constitutive promoter, which provides 74% of the WT PI activity level in stem leaves after 11 d of *Manduca* attack (Zavala & Baldwin, 2004). Arizona lines lack PI production due to a mutation in the endogenous 7-domain *PI*-gene activity, have reduced jasmonate accumulation after herbivory, and lack emission of a sesquiterpene HIPV, *trans*-α-bergamotene (Glawe *et al*., 2003; Wu *et al*., 2006; Schuman *et al*., 2009), which functions as an efficient indirect defense in plants from Utah (Kessler & Baldwin 2001). Hence, two different experimental set-ups, one with WT and transgenic lines of the Utah genotype and the other one with Arizona genotype could be designed to test the functional synergy between GLVs and TPIs.

In conclusion, field experiments raise a number of challenges for scientists: changing environmental conditions, the difficulty of planting simultaneously size-matched plants into the field plot, higher mortality of plants as well as *Manduca* larvae, unexpected damage to the plants due to mammals like rabbits and cows and humans and the limited time of one growing season. Many of these problems could be alleviated by increasing the number of plants, but each plant is much more difficult to handle in the field than in the glasshouse. In contrast, laboratory experiments are more controllable, permanent, all-season engineerable and reproducible. Thus, improving the experiment would require not only stable transformed lines homozygous for both GLV and TPI silencing constructs as well as measurement of silencing efficiency before the experimental beginning, but also a greater number of replicates as well as simultaneous control (predator-free) plants for *Manduca* infestation M3 (chapter 3.6.2), and the fully simultaneous conduction of *Manduca* infestations and predation assays among GLV-emitters and non-GLV-emitters.

5.2 The ecological context of indirect defense

Plants indirect defenses are traits that disable or remove herbivores by manipulating tritrophic interactions to increase plant's Darwinian fitness (Karban & Baldwin, 1997). To determine whether a trait functions as indirect defense, three hypothesis need to be addressed: the determination of (1) the presence of natural enemies on WT plants compared to transgenic plants silenced in the defense trait, (2) the enemy predation or parasitism activity, whose increase is correlated with reduction in herbivore performance and survival and (3) the increase of herbivore abundance, which might increase the impact of herbivore damage on plant fitness (Kessler & Heil, 2011). It is likely that plants benefit from the HIPV-mediated attraction of predators (Allmann & Baldwin, 2010; Halitschke *et al*., 2008), but HIPVs have multiple functions others than just as predator attractants. GLVs mediate a variety of interactions between plants, plants and herbivores and herbivores and predators, for example as feeding stimulants and host location or oviposition cues (Baldwin *et al*., 2006; Halitschke *et al*., 2004, 2008; Meldau *et al*., 2009; Kessler & Baldwin, 2001, 2004a), which may nullify their potential positive impact on plant fitness from enhanced predation. For the evaluation of GLVs as an indirect defense, the context of a natural community including competitors, the food web and enemies, is necessary because the recruitment of natural enemies as plants "bodyguards" neither guarantees a reduction in herbivory nor an increase on evolutionary fitness for the plant (Allison & Hare, 2009; Dicke & Baldwin, 2010). Thus, indirect defense requires the presence of predators or parasitoids, which respond innately to HIPVs or preferentially learn the alteration of HIPV blends after herbivory with the presence of prey (Allison & Hare, 2009; Baldwin, 2010). It is reasonable to expect that the experience of predators is positively correlated with the predation rate (Allison & Hare, 2009). Thus, equal *Manduca* larval predation rates of 22-38% over a two-days and respectively 12-21% over a three-day trial (fig. 11A) during GLV supplementation of GLV-silenced plants during the first *Manduca* infestation M1 (chapter 3.6.1) is a result of *Geocoris* ability to associatively learn and incorporate volatile cues with prey. Counts of the predator community confirmed that *Geocoris* continued to explore similarly all GLV-emitters and GLV-silenced plants, but not to predate from the latter during equal *Manduca* infestations during M2 and the egg predation assay (fig. 10B-C, chapter 4.2). The tendency of higher predation on IRpi versus WT plant could be explainable by the fact that *Geocoris* may prefer plants with higher bud and flower numbers, because it was shown that they also feed on seeds and pollen of full-grown *N. attenuata* plants in the field (M. Schuman, personal communication).

The positive correlation of two- or one -quarter lower predation of *Manduca* larvae by *Geocoris* from IR*lox2* and hemi-IR*lox2* plants with a reduction in growth as well as

reproduction of both genotypes, by 30-50% for IR*lox2* and 20-30% for hemi-IR*lox2* versus WT during infestation M2 (figs. 10B, 15, 16, chapter 4.4.2), and also the correlation between the GLV-mediated twofold increase of *Manduca* larvae predation by *Geocoris* with a twofold increase in plant fitness in M3 (fig. 18, chapter 4.4.3), is evidence that associative learning of *Geocoris* predators is linked with an increase in plant fitness (Allison & Hare, 2009). This is supported by the fact that these differences in *Manduca* larval mortality occurred while larvae were vulnerable to *Geocoris* predation (through the end of the second instar) (Kessler & Baldwin, 2001), and the four genotypes do not differ in overall *Manduca* mortality nor in growth and fitness in the absence of *Geocoris* predators (Schuman *et al*., in review). Only few systems provide evidence for the existence of a correlation between associative learning and increased fitness of natural enemies, for example for the associative learning in a parasitoid wasp (Dukas & Duan, 2000), and this is the first such data for a native plant in its natural environment.

In conclusion, this study demonstrates the first clear evidence that GLV emission functions as an indirect defense by increasing predation of a specialist herbivore twofold, resulting in a twofold increase in bud and flower production for a wild plant in its native habitat. Former studies lack molecular and chemical tools or spanned too short a time to reveal Darwinian fitness benefits, or have not reported fitness data at all to investigate whether GLVs or other HIPVs may function as an indirect anti-herbivore defense (e. g. Allmann & Baldwin, 2010; Halitschke *et al.*, 2008; Heil, 2008; Kessler & Baldwin, 2001; Rassmann *et al*., 2005). Moreover, only two laboratory studies showed that parasitization of herbivores can increase plant reproduction, but in neither of these studies was parasitization a result of HIPVs (Fritzsche-Hoballah & Turlings, 2001; van Loon *et al.*, 2000). Only long-term field studies comparing plants emitting endogenously produced HIPVs versus HIPV-silenced plants ensure specific, lasting and consistent differences under field conditions for the demonstration of a defensive function for HIPVs. Furthermore, the manipulation of GLVs influences not only third trophic interactions, but also interaction with the second trophic level, for example feeding stimulants and host location cues (Halitschke *et al*., 2004; Meldau *et al*., 2009). Thus, additional experimental manipulations are required to target only tritrophic interactions and maintain plant-herbivore interactions.

5.3 Potential interaction: the additional impact of TPIs on predatability

In concert with HIPVs, plants also employ antidigestive TPIs, which reduce herbivores' access to essential amino acids and therefore decrease their growth and performance (e.g. (Birk, 2003; Broadway & Duffy, 1986; Green & Ryan, 1972; Ryan, 1990). But herbivores may adapt to high PI levels by producing proteases less susceptible to inhibitors (Broadway 1995; Jongsma *et al*., 1995), using other classes of proteases (Jongsma *et al*. 1995; Broadway 1996) or compensatory feeding responses to increase their total protein intake (Winterer & Bergelson, 2001) which may result in a negative fitness impact for the plant. Because PIs accumulate locally due to wounding throughout the plant's lifespan (Jongsma et al., 1994), movement of *Manduca* larvae to younger, unelicited leaf tissue with higher protein percentage and lower PI levels is an additional possibility to minimize PI inhibitory effects correlated with a 40% lower predation risk and a 6.3-fold greater mass gain for larvae (Kessler & Baldwin, 2002a; Zavala & Baldwin, 2004; Zavala *et al.*, 2008). The lack of *Manduca*'s negative effect on IR*pi* plants is likely due to reduced *Manduca* damage resulting from a lack of TPI-induced compensatory feeding (Steppuhn & Baldwin, 2007). Similar feeding damage, instar progression and intra-plant movement data of *Manduca* larvae among all genotypes corresponded to no significant fitness differences between TPI-producing and -deficient plants. Intra-plant movement of *Manduca* larvae over time to leaves with a higher fitness value (at higher stem positions) was likely due to the within-plant heterogeneity of defensive secondary metabolites (van Dam et al., 2001a; Zavala & Baldwin, 2004). *Manduca* feeding becomes more detrimental to the plant as larvae grow, and move up the plant to younger and reproductive tissues. The motivation deterred by induced defenses of the larvae to move to an unelicited neighboring plant, when they are the most voracious and insensitive to predation (Kessler & Baldwin, 2001, Madden & Chamberlin, 1945; Walters et al., 2001), may benefit both the fitness of the former host plants and larval development (van Dam *et al*., 2000, 2001a).

Although TPI-mediated decreases in *M. sexta* growth and survivorship translated into a fitness benefit for *N. attenuata* plants under glasshouse conditions (Zavala & Baldwin, 2004), results showed neither a significant effect of TPIs on *Manduca* growth nor on plant growth (fig. 12) or reproduction under natural conditions (figs. 15, 16). For the lack of effect of TPIs and plant's Darwinian fitness and because non-toxic digestibility-reducers can elicit behavioral and physiological counter-responses in insects that increase enemy efficacy for plants, TPIs defenses may only function as defenses in concert with other antifeedant or deadly defense responses in nature. The synergy between TPIs and nicotine is only efficient for generalists (Steppuhn & Baldwin, 2007). But the employment of non-toxic digestibility-

reducers may not function for specialists in nature without their removal by the third trophic level, because specialists like nicotine-tolerant *Manduca* larvae are especially likely to have adapted to antinutritive defenses and particularly to tolerate the larger dose of other defense metabolites ingested through compensatory feeding (Baldwin & Preston, 1999; Bergelson & Purington, 1996). Nevertheless, the sensitivity of *Manduca* larvae to TPI-mediated decrease of the nutritional value of plant tissue (Zavala & Baldwin, 2004) affect their behavior and indicates the function of TPIs and indirect defenses for specialists. Indeed, larvae fed on WT plants reacted more sluggishly to experimental provocation than larvae on IR*pi* plants. Wild *Manduca* larvae were 75% less likely to attack when lifted off of the leaf (fig. 13A) in on-plant assays and 50% less likely to successfully attack the insect pin, either when initially poked, or poked and lifted with an pin, in off-plant assays. Furthermore, in post-recovery trails, *Manduca* larvae feeding on IR*pi* leaf material continued to grow (0.22 mm on average), while WT-fed larvae ceased to grow (-0.92 mm on average) (fig. 13B). The reduction of herbivore's growth and the prolonged vulnerability due to the antinutritive effect of TPIs is postulated by the slow growth-high mortality hypothesis, first described by Feeny (1976) and Clancy & Price (1987) (Benrey & Denno, 1997; Kaplan & Thaler, 2011; Kessler & Baldwin, 2001; Wiliams, 1999). According to this hypothesis, slowing an herbivore's growth enhances enemy attack by expanding the window of susceptibility to predation (Williams, 1999). Indeed, neonate lepidopterans defend themselves against enemies through the use of aposematic, cryptic, hiding, flight, and distributional defenses (Zalucki *et al*., 2002) and during the first three instars, *Manduca* larvae are vulnerable to *Geocoris* predation (Kessler & Baldwin, 2001). Increasing size and therefore the effectiveness of defensive behavior of Manduca larvae, employing vigorous movement and regurgitation, reduces predation risk (Dyer, 1995; Kessler & Baldwin, 2002a; Walters *et al.*, 2001). Therefore, the risk of predation is negatively correlated with herbivore size (Dyer, 1995). The increase in herbivores' mortality due to natural enemies via expansion of the vulnerable time period to predation may increase plant fitness (Benrey & Denno, 1997).

Finally, TPIs did not increase plant Darwinian fitness under attack from a specialist, but weakened the behavioral evasive responses of the specialist to simulated *Geocoris* attack, indicating that TPIs function by enhancing indirect defense, not as a defense themselves against a specialist in nature.

5.4 How to integrate TPIs and indirect defenses into integrated pest management?

Understanding ecological resistance mechanisms and demonstrating their defensive function in nature allows the integration of this defense strategy into integrative crop-protection programs with reliable risk assessments (Heil & Baldwin, 2002). This research study demonstrates the long-sought defense function of HIPVs, which could be engineered in pest management (IPM) and push-pull strategies to sustainably mediate top-down control of pest insects (Halitschke *et al.*, 2008; Cook *et al.*, 2007). The integration of indirect defenses into push-pull pest management provides an option for natural, environmentally friendly, non-toxic and behaviorally manipulative pest control by making the protected resource plant unattractive or unsuitable to the pests via integrating of stimuli (pull) while actively repelling herbivores away from HIPV-emitting crop plants (push) by the recruitment of biological control agents such as native predators and parasitoids (Allison & Hare, 2009; Cook *et al.*, 2007; Eubanks & Denno, 1999, 2000) and will contribute to future yield increases in agriculture (Heil & Baldwin, 2002).

Due to the world's growing population and therefore the increased demands for food production, novel strategies for improved crop resistance are required to prevent serious yield and quality losses of 15% of global crop production to insects and diseases (Gatehouse, 2011; Hedin, 1991; Walters, 2011). For example selective breeding or engineering of HIPV emission (Kos *et al.*, 2009) in contrast to controlled release dispensers, which have mixed success and require large amounts of synthetic HIPVs (Kaplan, 2012), or use of PIs to enhance to indirect defense promise success for increasing cop plant resistance, especially for specialist herbivores, and may support the reduction of agricultural pesticide and herbicides to which plenty of insects can adapt (Gatehouse, 2011; Haq *et al.*, 2004). PIs are ubiquitous in plant species and are potential candidates for the development of insect-resistant transgenic crops by inhibiting herbivores' protein digestion and thus reducing their growth and performance (Schlüter *et al.*, 2010; Zavala & Baldwin, 2004; Zavala *et al.*, 2008), and the combination with *Bacillus thuringiensis* (Bt) toxins is likely to expand the impact of Bt. But results with transgenic lines such as the *Bt/CpTI* transgenic cotton led to suggest that PIs either enhance the activity of Bt toxins (Zhang *et al.*, 2000) or counteract the effectiveness of Bt toxins at protecting plant tissue due to the adaption of insect to dietary PIs (Gatehouse, 2011; Winterer & Bergelson, 2001; see Haq *et al.*, 2004 for review). Thus, herbivores tolerance to PIs rarely results in a sufficient reduction in their growth and survivor to commercially acceptable level (Gatehouse, 2011).

Finally, crop protection combining direct and indirect defenses requires both the comparison of the relative effectiveness of these two defense strategies in the same plant species (Dicke, 1999a) and the engineering of plants, whose defense metabolites and volatile emission are effective against host-plant relating herbivores as well as cues for their predators in the present ecosystem (Dudareva & Pichersky, 2008).

5.5 Conclusion

Finally, three decades since indirect defense were described as a plant defense mechanism (Price *et al*. in 1980), this research study demonstrates the first evidence that HIPVs function as an indirect defense in plants. Although the effect of a functional interaction between TPIs and GLVs for plants reproduction could not be tested in this field study, it characterizes that TPIs only enhance GLVs indirect defense function, but do not function as defense themselves against specialists in nature. Therefore, the integration of TPIs and HIPVs in crop pest management presents a novel strategy in agriculture.

To attribute HIPV function in plants, not only are molecular and chemical tools including mutants of native plants deficient or enhanced in the biosynthesis of specific metabolites (Schuman & Baldwin, 2012) necessary, but also long-term studies in the context of a native community, not with trained predators and parasitoids in controlled laboratory experiments (Baldwin, 2010, Halitschke *et al*., 2003). Future research should include examination of more controlled performance assays of *Manduca* larvae and a repetition of the experimental approach to address the synergy question between GLVs and TPIs with regard to plant fitness.

In summary, the "value" of a defense in plants depends on its demonstrated fitness effect in their native environmental background.

Abstract

Plants employ a variety of defense strategies against attacking enemies. To measure the evolutionary benefit of direct and indirect defense traits in plants, different transgenic lines of the native tobacco *Nicotiana attenuata* were planted in its natural ecosystem, the Great Basin Desert, Utah. A toolbox of wild-type (WT) and RNAi lines, silenced for the production either of green leaf volatiles (GLVs; IR*lox2*) or of trypsin protease inhibitors (TPIs; IR*pi*), was assembled. Because the nicotine-tolerant specialist *Manduca* can cope with dietary PIs by compensatory feeding, and GLVs are not only cues for the attraction of *Manduca*'s predator *Geocoris* but also attract herbivores and stimulate *Manduca* larvae to feed, manipulation of GLV emission or TPI production represents an ideal way to test two hypotheses: (1) herbivore-infested GLV-emitting plants are fitter than non-emitters, but only in the presence of active predators; and (2) TPIs weaken herbivores' response to attempted predation. In addition, the generation of a genotype deficient for both TPIs and GLVs (hemi-IR*lox2*), enabled the test of a third hypothesis: (3) if there is a synergy between TPIs and GLVs, their defensive effects interact in a non-additive way resulting in a fitness benefit for the plant.

The results of this diploma thesis support the knowledge that GLVs play a crucial role in mediating the interactions of *Manduca* larvae, *N. attenuata,* its natural host plant, and their predator *Geocoris*. Supplementing GLVs for GLV-deficient *N. attenuata* plants allowed *Geocoris* to associate all four plant genotypes with the presence of prey and resulted in a similar predation pressure on *Manduca* due to *Geocoris* insects across all genotypes: 22-38% and 12-21% over a two-day and respectively a three-day trial. Without GLV supplementation, 50% lower predation rates of *Manduca* larvae and eggs from GLV-deficient plants (17-33%) were correlated with a reduction in growth and reproduction by 20-50% versus WT plants (60% predation). Furthermore, in a triplet design of WT, IR*pi* and hemi-IR*lox2* plants matched for size, reproductive output, apparent health and prior *Manduca* damage, GLV-emitting plants produced twice as many buds in flowers as GLV-deficient plants in correlation with a twofold increase of *Manduca* larval predation by *Geocoris* on GLV-emitters. Although TPI did not significantly affect *Manduca* larval growth, mortality or plant fitness, TPIs weakened *Manduca*'s behavioral evasive responses to simulated *Geocoris* attack. Wild *Manduca* larvae fed on WT plants were 75% less likely to attack when lifted off of the leaf in on-plant assays as well as lab-reared larvae, which were 50% less likely to successfully attack the insect pin, either when initially poked, or poked and lifted with an pin, in off-plant assays. Thus, results demonstrated TPIs function as enhancement of indirect defense against specialists in nature, not as a defense themselves.

Zusammenfassung

Pflanzen verwenden eine Vielzahl von Verteidigungsstrategien, um vielzählige Fraßfeinde abwehren zu können. Die Rolle direkter und indirekter Verteidigungsmechanismen in Pflanzen konnte durch Verwendung verschiedener transgener Linien des Wildtabaks *Nicotiana attenuata* in seinem natürlichen Ökosystem, der Great Basin Desert in Utah, bestimmt werden. Dabei wurden der Utah-Genotyp als Wildtyp sowie mit RNAi-transformierte Pflanzen eingesetzt, in denen entweder die Biosynthese von grünen Blattduftstoffen (GLVs; IR*lox2*) oder von Trypsin Protease-inhibitoren (TPIs; IR*pi*) mittels Gen silencing gehemmt wurde. Da der Nikotin-tolerante Spezialist *Manduca* sich an die diätische Wirkung von Pis über kompensatorisches Fraßverhalten anpassen kann und GLVs neben der Anziehung des Prädators *Geocoris*, auch anderer Herbivoren und zusätzlich stimulierend auf *Manducas* Fressverhalten wirken, ist es nicht klar, ob GLVs und TPIs nützlich für die Pflanzen sind. Durch gezielte Manipulierung der GLV-Emission sowie TPI-Produktion konnten folgende Hypothesen in dieser Arbeit gestellt: (1) In der Gegenwart von Räubern sind mit Herbivoren befallene Pflanzen, die GLVs emittieren, fitter als nicht emittierende; und (2) TPIs schwächen die Reaktion von Herbivoren gegen versuchte Prädation. Weiterhin ermöglichte die Generierung eines Genotyps, dessen Biosynthese von TPIs und GLVs unterbunden wurde, eine dritte Hypothese zu stellen: (3) Wenn es eine synergetische Beziehung zwischen TPIs und GLVs gibt, dann interagieren ihre defensiven Wirkungen nicht-additiv zu einem Fitnessnutzen der Pflanze.

Die Ergebnisse dieser Diplomarbeit unterstützen die Annahme, dass GLVs eine übermittelnde Kernrolle in den Interaktionen zwischen *Manduca* Larven, ihrer natürlichen Wirtspflanze *N. attenuata* und ihrem Raubfeind *Geocoris* einnehmen. Durch den Zusatz von GLVs zu Pflanzen mit mangelnder GLV-Emission konnte *Geocoris* alle Genotypen gleichermaßen mit einem Beutevorkommen verbinden. Durch diese aktiven Prädatoren wurden gleiche Prädationsraten an *Manduca*-Larven von 22-33% bzw. 12-21% in zwei- bis dreitägigen Experimenten an allen Genotypen generiert. Aber ohne GLV-Zusatz korrelierten die 50% niedrigeren Prädationsraten von *Manduca*-Larven und -Eiern an Pflanzen, die keine GLVs emittieren, mit einer 20-50%-igen Reduktion in Größe und Reproduktion gegenüber Wildtyppflanzen (60% Prädationsrate). In einem Triplettdesign aus WT, IR*pi* und hemi-IR*lox2*, die nach ihrer Größe und Reproduktion, ihrem vorherrschenden Gesundheitszustand und ihrem vorherigen *Manduca*-Schaden gepaart wurden, produzierten GLV-Emitter doppelt so viele Knospen und Blüten wie Pflanzen, denen es an GLVs mangelte. Dieser

Fitnessunterschied korrelierte mit einem zweifachen Anstieg in der Prädation von *Manduca*-Larven von *Geocoris* an GLV-emittierenden Pflanzen.

Obwohl TPIs weder einen negativen Einfluss auf die Größe oder Mortalität von *Manduca* Larven noch auf die Pflanzenfitness hatten, konnten sie dennoch *Manducas* ausweichendes Verhalten auf simulierte Angriffe von *Geocoris* schwächen. In nachgeahmten Prädationsversuchen konnten 75% der *Manduca*-Larven, die an Wildtyppflanzen fraßen, schlechter angreifen, wenn sie von der Pflanze gehoben wurden. Auch im Labor aufgezogene *Manudca*-Larven konnten in Laborversuchen nur halb so viele erfolgreichen Angriffe gegen eine Insektennadel vollführen, wenn sie erst damit gestochen oder gestochen und gleichzeitig hochgehoben wurden.

Demzufolge lassen die Ergebnisse den Rückschluss zu, dass TPIs nur als Ergänzung der indirekten Verteidigung und nicht als eigenständige Verteidigungsstrategie gegen Spezialisten wirken.

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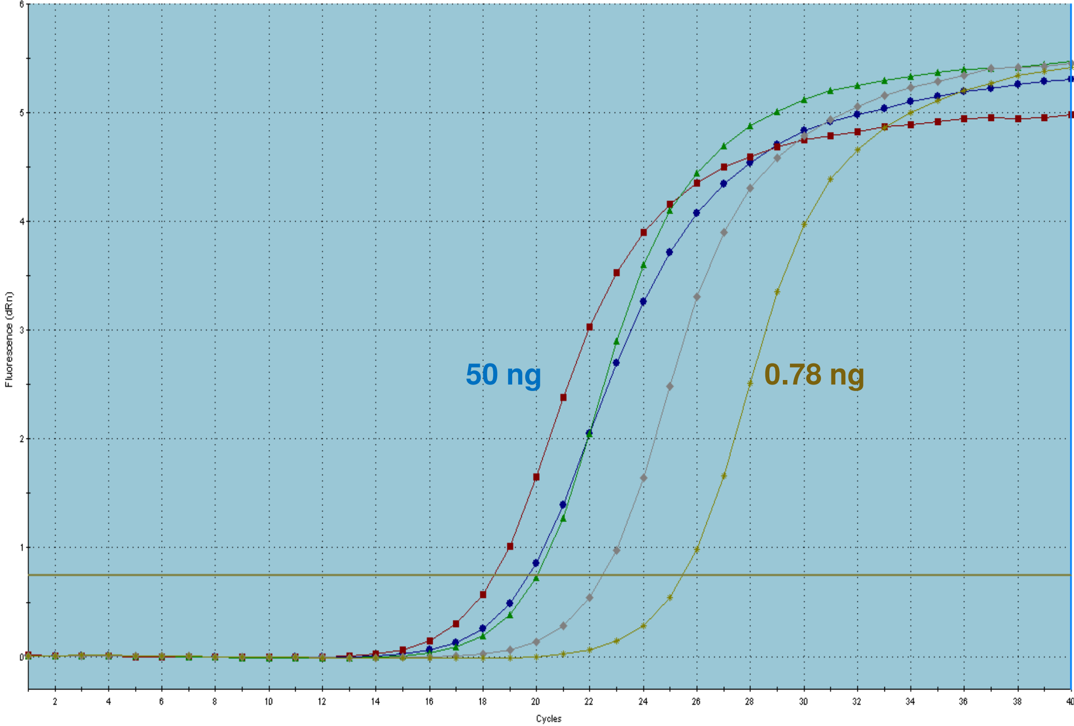
Appendices

A Amplification plot *EF1A* transcripts: standards



B Dissociation curve *EF1A* transcripts: standards

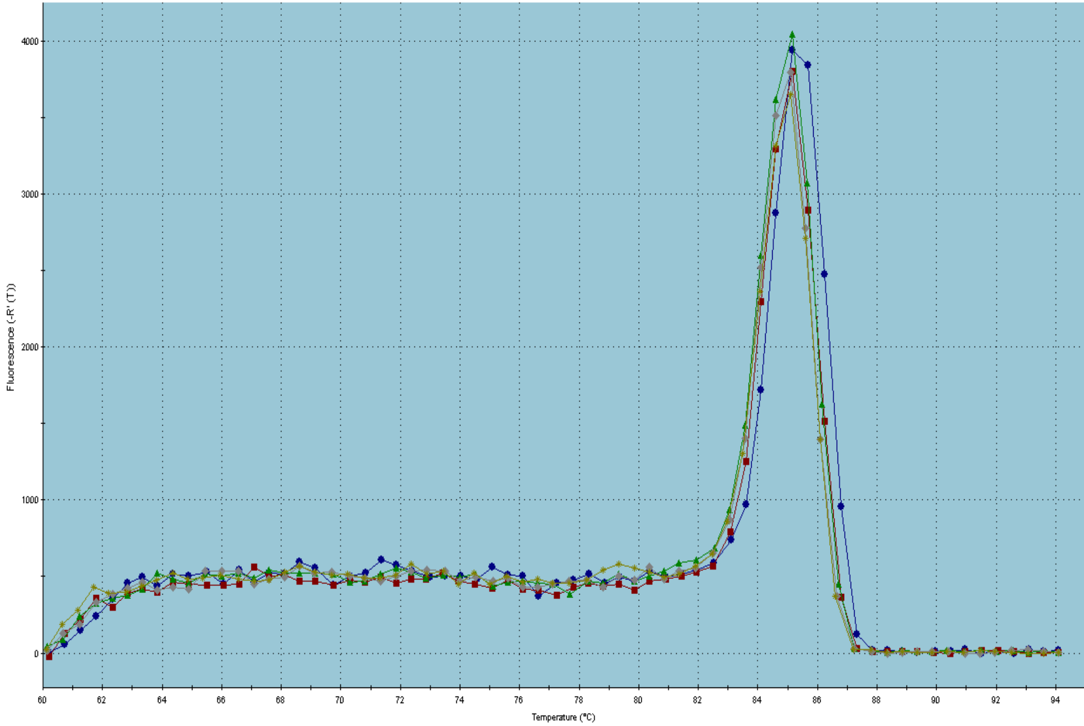
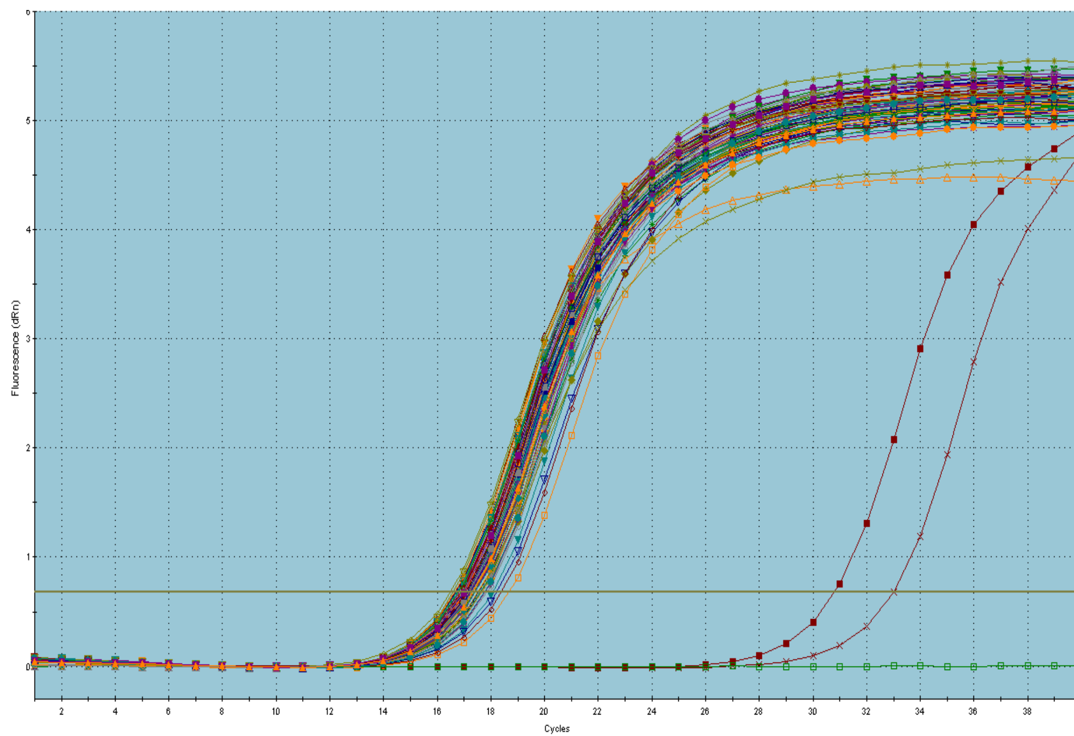


Figure A1: qPCR of for *EF1A* transcripts of cDNA standards to quantify *PI* and *LOX2* transcripts.**A** Amplification plot of standards (N=5), concentration ranging from 0.78 to 50 ng. **B** Dissociation curve of standards to confirm product specificity.

A Amplification plot *EF1A* transcripts: samples



B Dissociation curve *EF1A* transcripts: samples

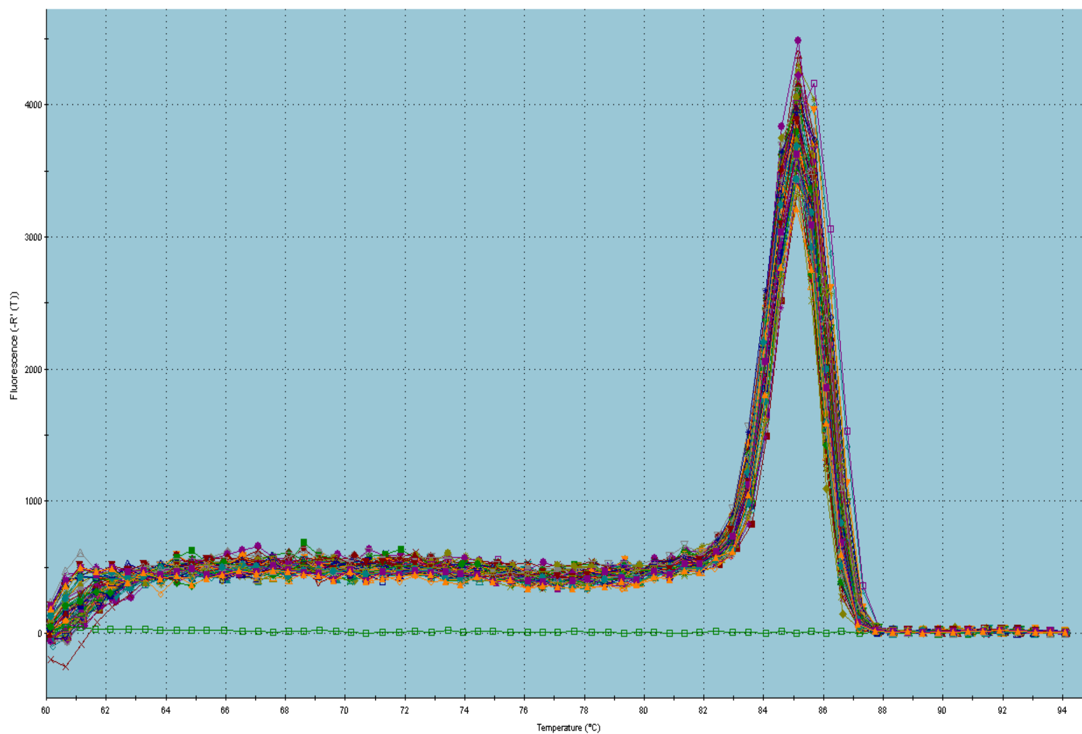
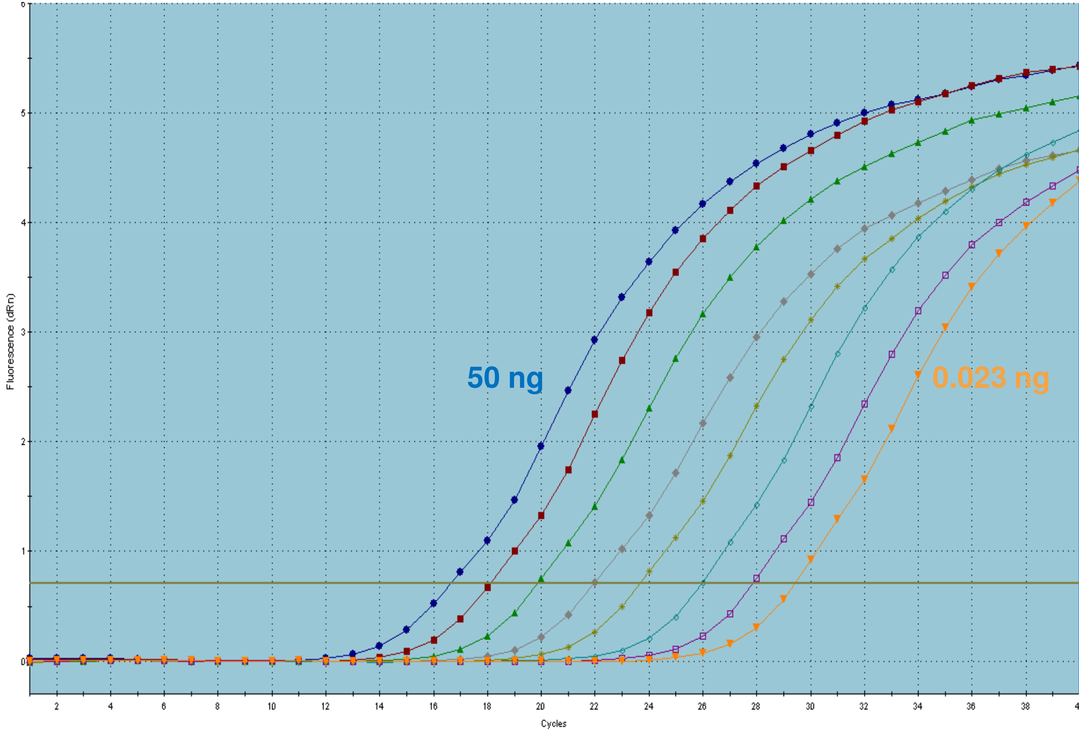


Figure A2: qPCR of for *EF1A* transcripts of cDNA extracted from leaf samples to quantify *PI* and *LOX2* transcripts. **A** Amplification plot of samples (N=40) **B** Dissociation curve of samples to confirm product specificity. Two samples without positive amplification and dissociation were excluded from transcript concentration analyses.

A **Amplification plot *LOX2* transcripts: standards**



B **Dissociation curve *LOX2* transcripts: standards**

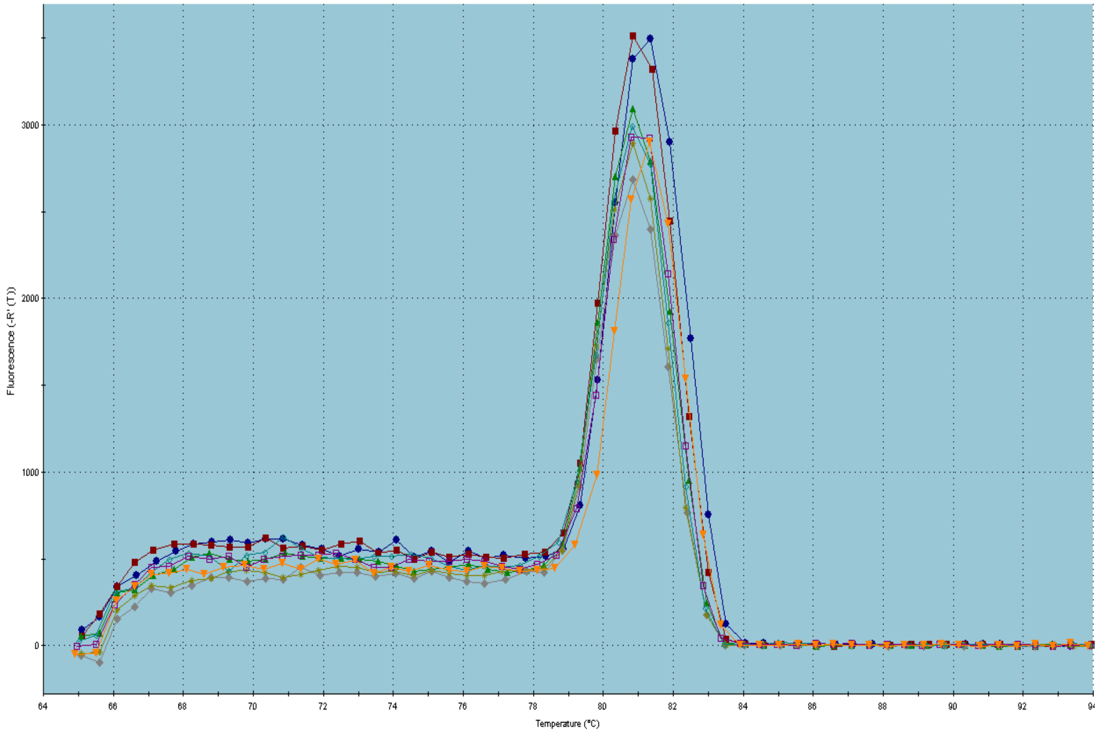
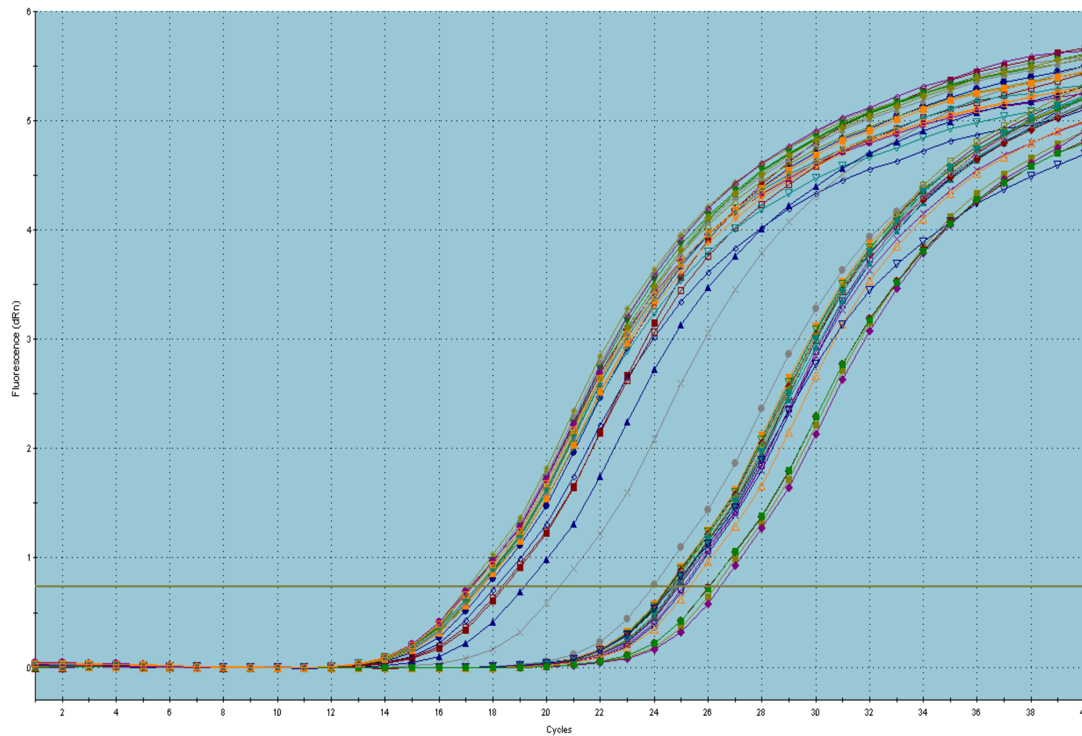


Figure A3: qPCR of for *LOX2* transcripts of cDNA standards to quantify *LOX2* transcripts. **A** Amplification plot of standards (N=8), concentration ranging from 0.023 to 50 ng. **B** Dissociation curve of standards to confirm product specificity.

A Amplification plot *LOX2* transcripts: samples



B Dissociation curve *LOX2* transcripts: samples

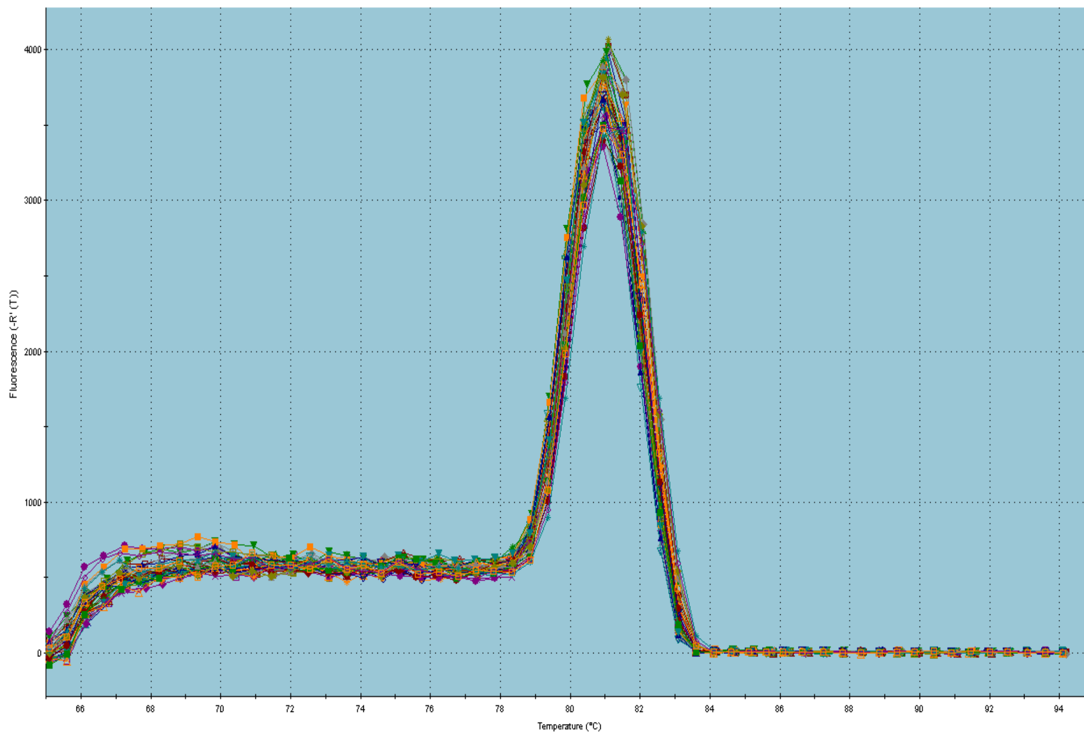
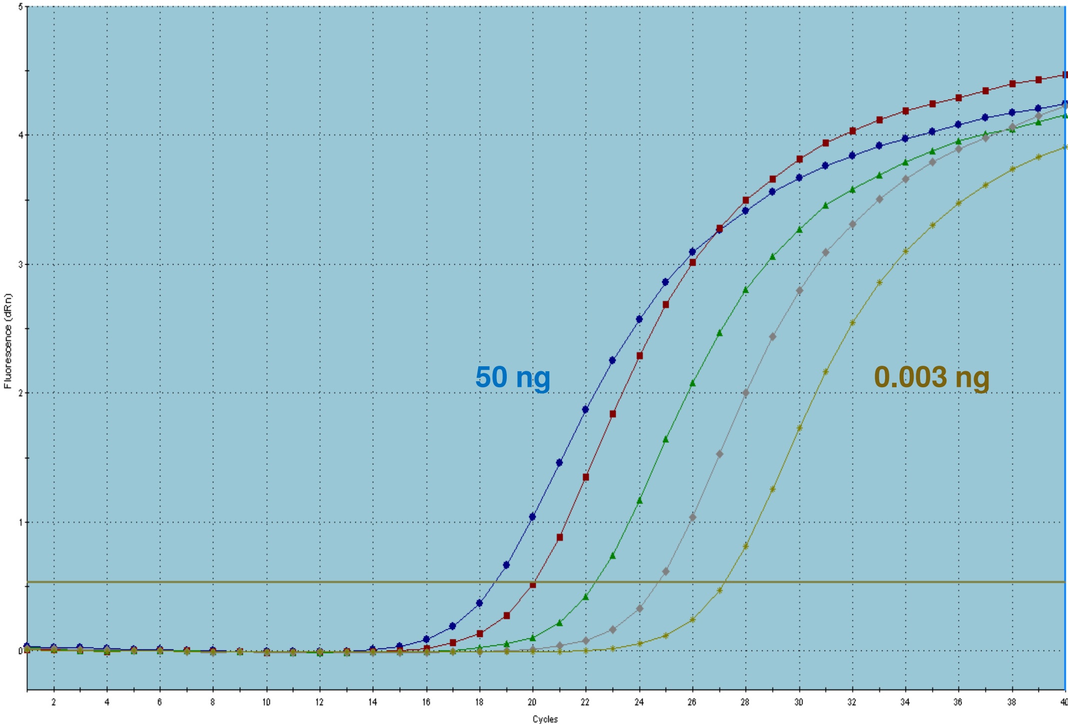


Figure A4: qPCR of for *LOX2* transcripts of cDNA extracted from leaf samples to quantify *LOX2* transcripts. **A** Amplification plot of samples (N=40) **B** Dissociation curve of samples to confirm product specificity.

Amplification plot *PI* transcripts: standards



B Dissociation curve *PI* transcripts: standards

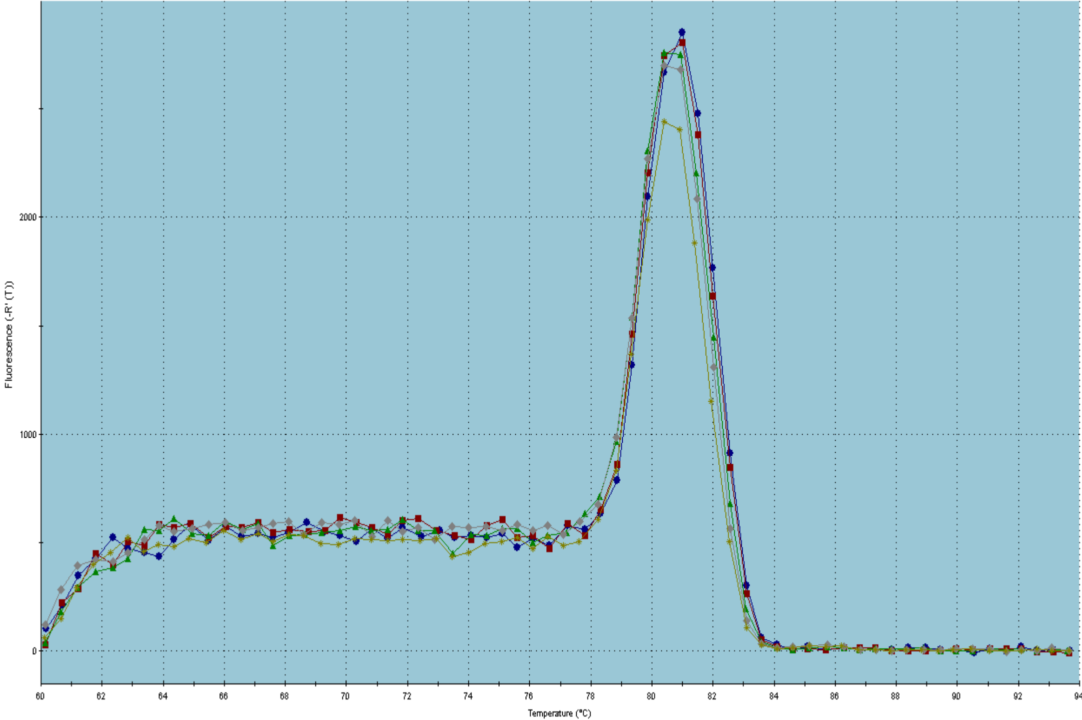
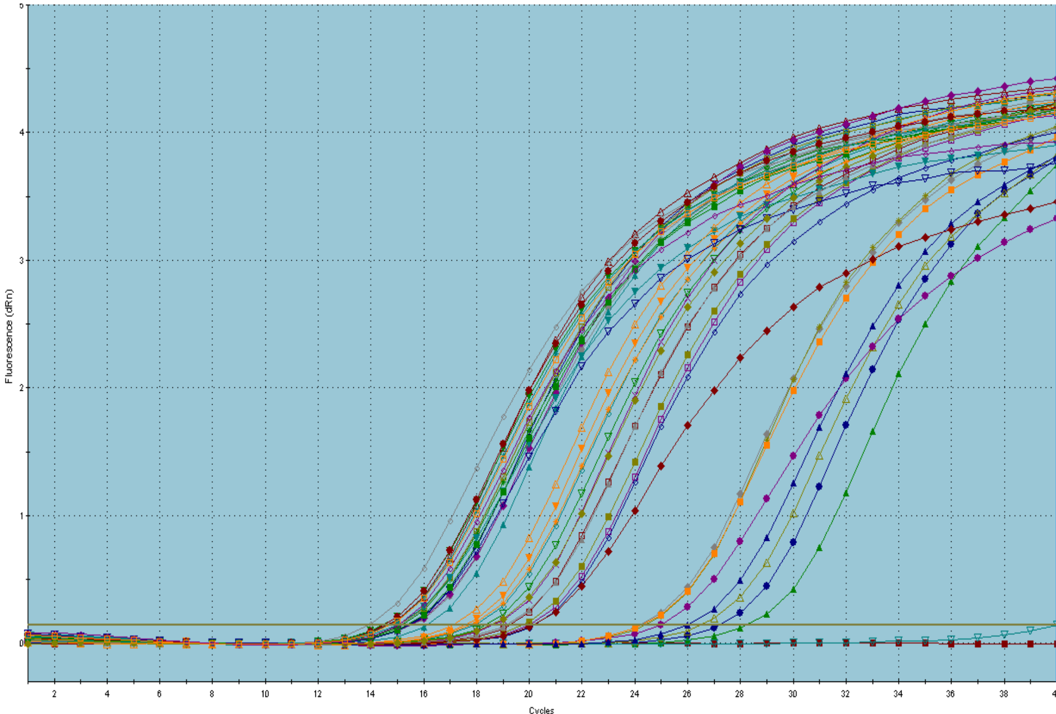


Figure A5: qPCR of for *PI* transcripts of cDNA standards to quantify *PI* transcripts. **A** Amplification plot of standards (N=5), concentration ranging from 0.003 to 50 ng. **B** Dissociation curve of standards to confirm product specificity.

A Amplification plot *PI* transcripts: samples



B Dissociation curve *PI* transcripts: samples

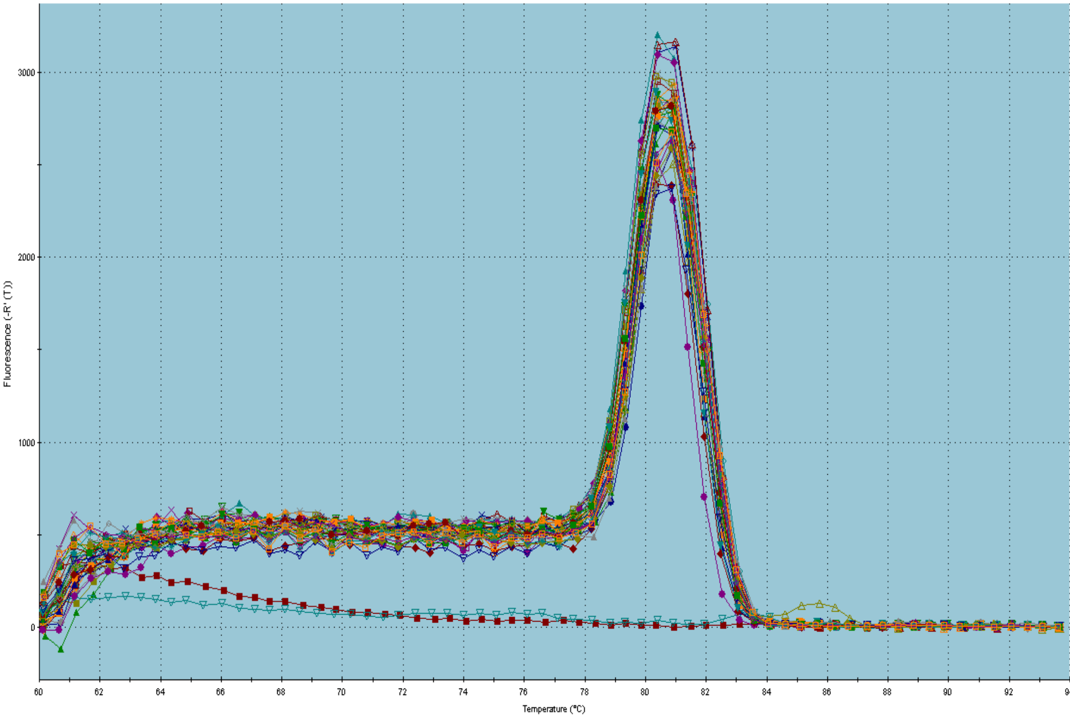


Figure A6: qPCR of for *PI* transcripts of cDNA extracted from leaf samples to quantify *PI* transcripts. **A** Amplification plot of samples (N=40) **B** Dissociation curve of samples to confirm product specificity. Two samples without positive amplification and dissociation were excluded from transcript concentration analyses.

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Kathleen Barthel

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