Probing the herbivore’s responses to plant defenses
using plant-mediated RNAi

DISSERTATION

To fulfil the requirements for the degree of

Doctor of Philosophy (Ph. D.)

Submitted to the Council of Faculty of Biology and Pharmacy,

Friedrich Schiller University Jena

By

Spoorthi Poreddy, M.Sc.

Born on 11.11.1986 in Achampet, India.
Gutachter:

Prof. Dr. Ian Thomas Baldwin, Max-Planck Institute for Chemical Ecology, Jena, Germany

Prof. Dr. Rolf Beutel, Friedrich-Schiller-University, Jena, Germany

Prof. Dr. Phillip Reymond, University of Lausanne, Lausanne, Switzerland

Beginn der Promotion: 13. February 2013

Eingereicht am: 05. February 2016

Tag der Verteidigung: 19. April 2016
# Table of Contents

Chapter 1- Introduction.................................................................1
  1.1. Plant-insect interactions.......................................................1
  1.2. The *Nicotiana attenuata* system and associated arthropods........2
  1.3. *Nicotiana attenuata's* antiherbivore defense compounds..............4
  1.4. RNA interference ......................................................................6
  1.5. Scope of the thesis...............................................................9

Chapter 2- Manuscript overview..................................................11

Chapter 3- Manuscript I...............................................................13

Chapter 4- Manuscript II.............................................................43

Chapter 5- Discussion.....................................................................67

6. References..................................................................................75

7. Summary.....................................................................................83

8. Zusammenfassung.......................................................................85

8. Eigenständigkeitserklärung.........................................................87

9. Erklärung über laufende und frühere Promotionsverfahren.............87

10. Curriculum vitae.........................................................................88

11. List of publications.................................................................91

12. Acknowledgements....................................................................93
Introduction

1.1. Plant-insect interactions

Plants and insects have co-existed for more than 400 million years, evolving a complex array of beneficial and deleterious interactions. For instance, plants provide shelter, food and oviposition sites for insects and in return plants benefit from cross-fertilization that occurs in the process of nectar collection by insects (Panda and Khush 1995). However, insect herbivores damage host plants, and depending on the severity of damage, herbivores might be extremely harmful and even lead to death of the plants. In order to withstand insect attack, plants have developed a plethora of defense mechanisms, including physical barriers such as trichomes (Fordyce and Agrawal 2001) and chemical barriers such as production of constitutive and induced defense metabolites (Baldwin 2001; Kliebenstein et al. 2001), defensive proteins (Haruta et al. 2001) and herbivore-induced plant volatiles that, among other things, attract predators of insects (Birkett et al. 2000). On the other hand, in order to overcome plant defense barriers, insects have developed counter-defense mechanisms such as detoxification (Scott and Wen 2001), rapid excretion (Self et al. 1964) or sequestration (Nishida 2002) of plant allelochemicals and have thus adapted to survive and reproduce on their host plants.

Many important plant defense compounds are lipophilic, including cardenolides, phenylpropanoids, flavonoids, iridoids, and several cyanogenic compounds. Plants often synthesize and store these toxic compounds in an inactive precursor form such as hydrophilic glycosides to avoid self-intoxication and to increase their stability (Jones and Vogt 2001). Tissue damage caused during insect feeding results in the exposure of these inactive compounds to their activating plant- or insect glycosidases to form aglycones. The aglycones severely affect the physiology and fitness of non-adapted herbivores (Boeckler et al. 2011; Dobler et al. 2011). However, lepidopteran herbivores like *Mythimna separate*, *Epirrita autumnata* etc. are able to detoxify these aglycones via a glycosylation process (Pentzold et al. 2014). In insects, deglycosylation of glycosides to form aglycones is considered as toxin activation and glycosylation of aglycones is regarded as a common detoxification process (Lindroth 1988; Winde and Wittstock 2011; Ahn et al. 2012). Most of the research on deglycosylation has been conducted in the compound class of glycosides having single sugar moieties, however
deglycosylation of plant allelochemicals having more than one sugar moiety remained uninvestigated.

1.2. The Nicotiana attenuata system and associated arthropods

I studied tobacco hornworm (Manduca sexta Linnaeus, 1763) and tomato hornworm (M. quinquemaculata Haworth, 1803) (Lepidoptera: Sphingidae) and their host plant wild coyote tobacco (Nicotiana attenuata Torr. ex Wats) as model organisms.

N. attenuata is a solanaceous annual plant native to the Great Basin Desert in the southwestern USA. N. attenuata's germination is triggered in response to smoke cues in post-fire environment (Baldwin and Morse 1994). Over the years, N. attenuata has been well studied for its defense mechanisms and its interactions with several native herbivores belonging to different phylogenetic taxa, and occupying different feeding guilds with diverse host ranges (Halitschke et al. 2001; Kessler and Baldwin 2004). Herbivores that attack N. attenuata in its native habitat comprise different insect orders including Lepidoptera, Coleoptera, Orthoptera and Hemiptera.

The leaf-chewing lepidopteran herbivores M. sexta and M. quinquemaculata solely feed on solanaceous plants and are specialist herbivores of N. attenuata while noctuid lepidopteran generalist herbivores Spodoptera exigua, Heliothis virescens and cutworms of the genus Agrotis, often hide in the soil during the day and feed on a broad range of host plants including N. attenuata during the night. The leaf-chewing coleopteran flea beetles Epitrix subcrinita and E. hirtipennis bore small holes into the leaves and stems of N. attenuata, and larvae of other two coleopteran insects Trichobaris mucorea and Trichobaris compacta, live inside the stems of N. attenuata and feed on the pith (Diezel et al. 2011). Leaf-chewing orthopteran grasshoppers of the genus Trimerotropis occur occasionally in high abundance and destroy plants over wide areas. The sap-sucking hemipteran specialist herbivores Tuniocors notatus, adults and nymphs, are the most abundant sucking insects of N. attenuata (Kessler and Baldwin 2004), and generalist Empoasca leaf hoppers preferentially attack wild N. attenuata jasmonate mutants in natural populations (Kallenbach et al. 2012). The seed feeding hemipteran specialist Corimelaena extensa feeds on N. attenuata seeds and decreases seed mass and viability of N. attenuata.
Manduca sexta and M. quinquemaculata

The two sphingid moths, *M. sexta* and *M. quinquemaculata* are congeneric closely related (Halitschke et al. 2001; Reisenman et al. 2010; Yoshinaga et al. 2010; Kawahara et al. 2013), or sister (Kessler and Baldwin 2002; Nihout and Suzuki 2008), or sympatric sibling species (Kessler and Baldwin 2004). They are specialized to feed on nightshades such as *Nicotiana, Lycopersicon, Solanum*, and *Datura* species and are major defoliators of *N. attenuata* in its native habitat. *M. sexta* and *M. quinquemaculata* have similar morphology (Sannino et al. 1995), behavior (Peterson et al. 1993), and ecology (Bossart and Gage 1990; Kester and Barbosa 1994) and are common study subjects for plant-herbivore interactions (Raguso and Willis 1997; Schittko et al. 2000; Kessler and Baldwin 2002; Kessler and Baldwin 2004; Schuman et al. 2012). Their geographic distribution, ranging from Canada to Argentina, matches that of *N. attenuata*.

![Image](A) Ecological model plant, the wild tobacco *N. attenuata* germinates in post fire environment. **(B) N. attenuata's** leaf chewing specialist herbivores *M. sexta* (left) and *M. quinquemaculata* (right) **(C) Transgenic N. attenuata** plants (rosette stage) growing in a field plot located in its native habitat, the Great Basin desert, Utah, USA. (Photos S. Poreddy).

Adult moths are nocturnal, feed on the nectar of flowers, and lay eggs on the underside of their host plants' leaves. Eggs hatch after on average of 3-4 days and neonates (about 1 mg body mass) start to feed on leaves. Usually, larvae pass through five instars within nearly twenty days with a molting period between each instar, during which their body mass increases by a thousand fold, and later they enter pupation. *M. sexta* and *M. quinquemaculata* eggs and larvae are
morphologically similar and hard to distinguish until the second-instar. In the third-instar, larvae of both the species can clearly be distinguished based on their horn color and line pattern on their skin; *M. sexta* larvae have seven white straight lines and a red colored horn whereas *M. quinquemaculata* larvae have 'V'-shaped yellow color line patterns and a black horn.

**Predator community in *N. attenuata*'s native habitat**

*N. attenuata*’s native habitat is also home to various predators that feed on the abovementioned herbivores, causing a constant threat to them. The major predators include big-eyed bugs (*Geocoris pallens* and *G. punctipes*), wolf spiders (*Camptocosa parallela*) and ants; in addition, some antlions and lizards also appeared to feed on these herbivores. *Geocoris spp* are the most abundant predators that mainly feed on eggs and first-instar larvae of *Manduca spp* (Schuman et al. 2013). *C. parallela* is a nocturnal predator and is deterred by nicotine exhaled from spiracles of *M. sexta* larvae (Kumar et al. 2014).

### 1.3 *Nicotiana attenuata*’s antiherbivore defense compounds

*N. attenuata* produces a plethora of direct and indirect chemical defenses in order to thwart herbivory. *N. attenuata*’s defense responses against *M. sexta* attack have been extensively studied (Baldwin 1999b; Schittko et al. 2000; Halitschke et al. 2001; Voelckel et al. 2001; Diezel et al. 2009; Schuman et al. 2012). After real or simulated herbivory, *N. attenuata* plants rapidly accumulate jasmonic acid (JA) and its bioactive form (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) in amounts two- to tenfold to that of a standardized mechanical wounding and subsequently, activate direct and indirect defense responses (Schittko et al. 2000; Halitschke et al. 2001; Halitschke et al. 2003). As a result of JA-Ile accumulation, herbivore associated elicitors elicits *N. attenuata*’s direct defenses by promoting the production and mobilization of compounds which directly affect larval growth, such as trypsin protease inhibitors (Zavala and Baldwin 2004), 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) (Jassbi et al. 2008; Heiling et al. 2010), caffeoylputrescine, dicaffeoylspermidine and nicotine (Baldwin 1999a). Simulated herbivory with *M. sexta*’s oral secretion or regurgitant (OS) is enough to induce *N. attenuata*’s defense responses. Fatty acid-amino acid conjugates and 2-hydroxylinolenic acid present in larval OS is responsible for this induction (Halitschke et al. 2001; Gaquerel et al. 2012). In addition to the direct defenses, *N. attenuata* releases volatile organic compounds, sesquiterpenes
(Halitschke et al. 2000) and green leaf volatiles (Schuman et al. 2012) that function as an indirect defense by attracting predators of herbivores and thereby reducing herbivore load.

**17-hydroxygeranyllinalool diterpene glycosides**

![Figure 2: Schematic of biosynthesis and structures of lyciumoside IV and its malonylated forms.](image)

HGL-DTGs are produced by several solanaceous species. HGL-DTGs have been reported to exhibit antibiosis activity and reduce larval growth in the tobacco budworm (*Heliothis virescens*) when larvae fed on artificial diets containing HGL-DTGs of *N. tabacum* (Snook et al. 1997). Negative effect of HGL-DTGs on the growth of *M. sexta* larvae have been
reported in several studies using different *Nicotiana* species with different HGL-DTG profiles, such as *N. attenuata*, *N. bigelovii*, *N. clevelandii* and transgenic isogenic lines of *N. attenuata* as a food source (Lou and Baldwin 2003; Jassbi et al. 2006; Jassbi et al. 2008). HGL-DTGs have a larger effect on the growth of *M. sexta* larvae than do nicotine and trypsin proteinase inhibitors (Jassbi et al. 2008). *M. sexta* larvae grew three times larger than controls when fed on transgenic *N. attenuata* plants depleted in their HGL-DTG content by silencing *geranylgeranyl pyrophosphate synthase* (*GGPPS*), the key enzyme involved in the synthesis of the HGL-DTG precursor, geranylgeranyl pyrophosphate (Jassbi et al. 2008; Heiling et al. 2010).

*N. attenuata* contains more than 12 HGL-DTGs with a little diversity in their structures. *N. attenuata's* HGL-DTGs contain multiple sugar moieties attached to an acyclic C\textsubscript{20} HGL backbone. Hydroxyl groups on the third and seventeenth carbons (C-3 and C-17) of C\textsubscript{20} HGL are always attached to glucose moieties, which are in turn attached to either glucose or rhamnose moieties at C'-2, C'-4, or C'-6 hydroxyl groups. Malonyl groups are additionally attached to glucoses at their C'-6 hydroxyl groups, resulting in malonylated HGL-DTGs. Based on the number of malonyl groups, these are classified as singly- or di-malonylated HGL-DTGs. Lyciumoside IV and its malonylated forms are the major HGL-DTGs that constitute more than 80\% of total HGL-DTGs. (Heiling et al. 2010).

### 1.4. RNA interference

RNA interference (RNAi) has emerged as a potential molecular tool to study plant or insect gene functions in ecological context. RNAi is a sequence-specific gene silencing mechanism that operates endogenously in all eukaryotes to control gene expression both at transcriptional or post-transcriptional levels (Fire et al. 1998). The initial idea of gene silencing phenomenon was originated from attempts to alter visual characteristics of petunia plants. The over-expression of the chalcone synthase (*CHS*) gene in an attempt to enrich flower pigmentation unexpectedly resulted in an albino phenotype in some of the petunia plants due to silencing of both the transgene and endogenous *CHS* gene (Napoli et al. 1990), this phenomenon is called as “co-suppression”. The RNAi mechanism is first discovered in *Caenorhabditis elegans* (Fire et al. 1998). The RNAi mechanism starts with either endo- or exogenous double stranded RNA (dsRNA) in eukaryotic cells and the source of dsRNA could be from an inverted repeat (ir) sequence or convergent transcription of transgenes or transposons or viral RNA.
The mechanism of RNAi involves the generation of 21-25 nucleotide short RNA intermediates called small interfering RNAs (siRNAs) with two characteristic unpaired nucleotide overhangs at the 3’ end of each strand. The siRNAs are produced by the endonucleolytic cleavage of the exogenous dsRNA by the multidomain RNAse III enzyme called Dicer in an ATP-dependent process (Hamilton and Baulcombe 1999; Zamore et al. 2000). The siRNAs are then incorporated into a ribo-nucleoprotein complex, 'RNA induced silencing complex' (RISC). The sense strand of siRNA is selectively degraded and then RISC complex with the antisense strand of siRNA scans for the complementary mRNA. An argonaute protein which binds to the RISC complex cleaves the target mRNA molecule leading to down-regulation of the target mRNA accumulation. The PAZ domain of the argonaute protein is involved in the transfer of siRNAs to the RISC complex, and the PIWI domain possess the nuclease activity responsible for siRNA guided cleavage of the target mRNA. The efficiency of gene silencing depends on the amplification of the silencing molecules (siRNAs), i.e. generation of subsequent siRNA molecules (known as secondary siRNA molecules) by RNA-dependent RNA polymerase (RdRP). It has been reported that even a short stretch of homology of 23 nucleotides between the target mRNA and the dsRNA results in gene silencing in plants and few molecules of dsRNA is enough to achieve the silencing due to amplification of the siRNAs by the RdRPs. There is no clear evidence for the presence of RdRP activities in lepidopteran insects. Silencing efficiency is also dependent on the systemic RNA interference deficient-1 (sid-1) gene. Sid1 is responsible for the spreading of the silencing signal systemically (Winston et al. 2007).

RNAi can be induced in insects to silence target genes using different dsRNA introduction routes, such as feeding a droplet containing dsRNA or siRNA, through artificial diet containing dsRNA or siRNA, plant material expressing dsRNA, or injection of dsRNA or soaking of insects in a dsRNA containing solution (Yu et al. 2013). Although injection or feeding of dsRNA through artificial diet is often practiced, this technique has limitations, such as lack of the continuous supply of dsRNA molecules, degradation of dsRNA molecules in artificial diets and difficulty to apply under field conditions, which can be circumvent using plant-mediated RNAi (PMRi).
Plant-mediated RNAi

Figure 3: Plant-mediated RNAi of *M. sexta*’s β-glucosidase gene

Host plants expressing dsRNA targeting an insect gene, the PMRi, continuously supply adequate numbers of dsRNA molecules to insect midguts (Mao et al. 2007) and enhances the silencing efficacy. The application of PMRi to silence insect genes was first demonstrated using transgenic *Arabidopsis thaliana* plants expressing dsRNA of *CYP6AE14*, the gene responsible for gossypol tolerance in *Helicoverpa armigera* (Mao et al. 2007). During insect feeding, dsRNA produced by stable or transient transgenic host plants could penetrate the insect gut cells and reduce the expression of target genes. Several studies have demonstrated successful gene
silencing in various insect orders such as Coleoptera, Lepidoptera and Hemiptera using stable or transient transgenic PMRi (Baum et al. 2007; Mao et al. 2007; Mao et al. 2011; Pitino et al. 2011; Zha et al. 2011; Kumar et al. 2012; Liu et al. 2015; Mamta et al. 2015). A recent advance in PMRi to control insects is achieved by expressing exogenous dsRNAs targeting the β-actin gene of the Colorado potato beetle (CPB) via chloroplast transformation in potato (Zhang et al. 2015). Transplastomic potato plants were shown to be lethal to CPB larvae and were protected from CPB attack (Zhang et al. 2015). PMRi is applied to silence M. sexta's midgut-expressed CYP6B46 using transient or stable N. attenuata plants expressing MsCYP6B46 dsRNA and enabled to understand the role of CYP6B46 in exhaling small amounts of ingested nicotine through spiracles to deter spiders (Kumar et al. 2012; Kumar et al. 2014).

1.5. Scope of the thesis

This thesis consists of two manuscripts. In manuscript I, I described β-glucosidase mediated an unusual detoxification strategy adapted by M. sexta larvae against its host N. attenuata's most abundant multiply glycosylated defense compounds, the HGL-DTGs and the consequences of this counter-adaptation for tritrophic interactions. I used the PMRi approach to unravel this detoxification strategy. And in Manuscript II, I applied PMRi to silence midgut-expressed genes in wild M. quinquemaculata larvae in nature and showed that the PMRi procedure can robustly and specifically silence genes in native congeneric insects that share sufficient sequence similarity and with careful selection of targets, can protect crops from attack by congeneric insects.

N. attenuata plants produce HGL-DTGs in quantities equivalent to starch, in order to defend against its specialist herbivore M. sexta. Lyciumoside IV and its singly- and di-malonylated forms nicotianoside I and nicotianoside II, respectively constitute ~80% of these HGL-DTGs. The overall aim of my thesis was to investigate how M. sexta defends against the most abundant chemical defense metabolites of its natural host N. attenuata, and the ecological consequences of this counter-defense. I used a combination of interdisciplinary methods such as PMRi, various analytical techniques and field experiments to elucidate the counter-defense strategy adapted by M. sexta larvae against host HGL-DTGs and described how a β-glucosidase mediates an unusual counter-adaptation mechanism in M. sexta in Manuscript I.
M. sexta and its closely related species M. quinquemaculata co-occur in N. attenuata's native habitat and M. quinquemaculata is also specialized to feed on N. attenuata plants. M. sexta midgut-expressed counter-defense genes CYP6B46 and β-glucosidase1 shared greater similarity with homologous sequences of M. quinquemaculata. In Manuscript II, I demonstrated homologous silencing of CYP6B46 and β-glucosidase1 genes in native M. quinquemaculata under field conditions using PMRi lines which were originally generated from M. sexta sequences and showed the potential of PMRi in silencing homologous genes with greater sequence similarity in congeneric species.
2. Manuscript Overview

Manuscript I

Detoxification of hostplant’s chemical defence rather than its anti-predator co-option drives β-glucosidase-mediated lepidopteran counteradaptation

Spoorthi Poreddy, Sirsha Mitra, Matthias Schöttner, Jima Chandran, Bernd Schneider, Ian T. Baldwin, Pavan Kumar & Sagar S. Pandit.

Published in Nature Communications, 6:8525/DOI: 10.1038/ncomms9525

In this manuscript, I described Manduca sexta’s unusual counter-adaptation strategy against its host Nicotiana attenuata’s most abundant chemical defenses, 17-hydroxygeranyllinalool diterpene glycosides, namely lyciumoside IV and its malonylated forms, and what are the ecological consequences of this counter-adaptation. The malonyl groups from malonylated lyciumoside IV were lost in the alkaline environment of larval regurgitant and lyciumoside IV was detoxified by deglucosylation via a midgut-expressed β-glucosidase1. Silencing of β-glucosidase1 by plant-mediated RNAi approach suppressed the larval lyciumoside IV deglycosylation and the β-glucosidase1-silenced larvae showed molting impairment and increased mortality. Furthermore, β-glucosidase1 silencing increased larval unpalatability to a native predator, wolf spiders, in N. attenuata’s native habitat. External coating of lyciumoside IV on larvae deterred spiders, suggesting that the defensive co-option of lyciumoside IV could be ecologically advantageous to M. sexta, but M. sexta detoxifies this allelochemical rather than co-opting it perhaps to avoid its deleterious effects.

SP designed and performed the experiments, analyzed the data and wrote the manuscript. SM contributed to the demalonylation experiment, MS contributed to the MicroTOF data analysis, JC and BS carried out the NMR work. PK contributed to the field experiments. ITB coordinated the study and revised the manuscript. SSP coordinated the study and wrote the manuscript.

Note: The following abbreviations are used in the published manuscript; Lyc4- lyciumoside IV, Nic1- Nicotianoside I, Nic2- Nicotianoside II, BG1- β-glucosidase1
Manuscript II

Plant-mediated RNAi silences midgut-expressed genes in congeneric lepidopteran insects in nature

Spoorthi Poreddy, Jiancai Li and Ian T. Baldwin

Manuscript submitted in the Journal of Integrative Plant Biology

In this manuscript, I explained silencing of midgut-expressed genes, namely CYP6B46 and β-glucosidase1 in native M. quinquemaculata larvae under field conditions using Plant-mediated RNAi (PMRi). RNAi induced silencing in lepidopteran insects using dsRNA delivery methods such as injection and feeding via artificial diets remains controversial because of both successful and unsuccessful reports. However, the continuous delivery of dsRNA using PMRi has always resulted in silencing of target genes and has enormous potential for crop protection. The wild tobacco N. attenuata is attacked by two closely related lepidopteran specialist herbivores M. sexta and M. quinquemaculata. The PMRi lines used to silence M. sexta’s midgut-expressed genes, nicotine-ingestion induced CYP6B46 (irCYP6B46 plants) and lyciumoside-IV-ingestion induced β-glucosidase1 (irβ-glucosidase1 plants) also silenced the homologous genes in native M. quinquemaculata larvae feeding on these transgenic plants in nature. The PMRi lines shared 98 and 96% sequence similarity with M. quinquemaculata homologous coding sequences, and CYP6B46 and β-glucosidase1 transcripts were reduced by ca. 90 and 75% in the midguts of irCYP6B46- and irβ-glucosidase1-fed fourth-instar M. quinquemaculata larvae, without reducing the transcripts of their most similar, potential off-target genes, suggesting that PMRi procedure can robustly and specifically silence genes in native congeneric insects that share sufficient sequence similarity.

SP designed and performed the lab and field experiments, analyzed the data and wrote the manuscript. JL contributed to the lab experiments and ITB coordinated the study and wrote the manuscript.
Manuscript I

Detoxification of hostplant’s chemical defence rather than its anti-predator co-option drives β-glucosidase-mediated lepidopteran counteradaptation

Spoorthi Poreddy, Sirsha Mitra, Matthias Schöttner, Jima Chandran, Bernd Schneider, Ian T. Baldwin, Pavan Kumar & Sagar S. Pandit.

Published in Nature Communications, 6:8525/DOI: 10.1038/ncomms9525
Detoxification of hostplant’s chemical defence rather than its anti-predator co-option drives β-glucosidase-mediated lepidopteran counteradaptation

Spoorthi Preddy1, Sirsha Mitra1, Matthias Schöttner1, Jima Chandran2, Bernd Schneider2, Ian T. Baldwin3, Pavan Kumar1,† & Sagar S. Pandit1

The evolutionary plant-herbivore arms race sometimes gives rise to remarkably unique adaptation strategies. Here we report one such strategy in the lepidopteran herbivore Manduca sexta against its hostplant Nicotiana attenuata’s major phytotoxins, 17-hydroxygeranylinalool diterpene glycoside, lyciumoside IV and its malonylated forms. We show that alkalinity of larval regurgitant non-enzymatically demalonylates the malonylated forms to lyciumoside IV. Lyciumoside IV is then detoxified in the midgut by β-glucosidase I-catalysed deglycosylation, which is unusual, as typically the deglycosylation of glycosylated phytochemicals by insects results in the opposite: toxin activation. Suppression of deglycosylation by silencing larval β-glucosidase I by plant-mediated RNAI causes moulting impairments and mortality. In the native habitat of N. attenuata, β-glucosidase I silencing also increases larval unpalatability to native predatory spiders, suggesting that the defensive co-option of lyciumoside IV may be ecologically advantageous. We infer that M. sexta detoxifies this allelochemical to avoid its deleterious effects, rather than co-opting it against predators.
Plants commonly use lipophilic compounds such as cardiac glycosides, iridoids, flavonoids, phenylpropanoids and several cyanogenic compounds as their major defences against their insect herbivores. These compounds are often glycosylated by the plants to increase their stability and to avoid self-intoxication; usually, such compounds are stored in specialized compartments to protect them from endogenous glycosidases. Tissue damage resulting from insect feeding exposes these inactive protoxins to their activating glycosidases resulting in the formation of toxic aglycones. Released aglycones cause cellular damage in herbivores and severely affect their physiology and fitness. Frequently, on ingestion, these glycosides are deglycosylated in the midgut by insect glycosidases. To avoid such deglycosylation, some herbivores downregulate their glycosidases after ingesting glycosides. Several herbivores have even evolved their own glycosylation systems to reglycosylate the formed aglycones. Glycosides are more water soluble, have higher intracellular mobility and so are excreted faster than their aglycones. Moreover, they are easily transported to vacuolar compartments and the apoplast by membrane-bound transporter systems, which often recognize their glycosyl residues. Hence, deglycosylation or glycoside hydrolysis in insects is commonly thought to result in toxic activation and glycosylation is regarded as a form of detoxification. Notably, most of this research has been conducted on compounds harbouring single sugar moieties; deglycosylation of phytochemicals containing more than one sugar moiety remains uninvestigated.

Several Nicotiana (Solanaceae) species produce 17-hydroxy-geranylinalool diterpene glycosides (HGL-DTGs) containing multiple sugar moieties. HGL-DTGs have been reported to be biocidal and HGL-DTGs of Nicotiana tabacum retarded growth of Heliothis virescens larvae in artificial diet (AD) experiments. The biosynthesis of several HGL-DTGs is induced in N. attenuata on attack by its specialist herbivore, Manduca sexta (Ms; Lepidoptera, Sphingidae). The susceptibility of M. sexta larvae to HGL-DTGs was demonstrated using different Nicotiana species such as N. attenuata, N. bigelovii and N. clevelandii, which differ in their HGL-DTG contents and composition, and, more convincingly, with different transgenic isogenic lines of N. attenuata with varying levels of HGL-DTGs. M. sexta larvae grew three times larger than controls when they were fed transgenic N. attenuata plants depleted in their HGL-DTG content by silencing geranylgeranyl pyrophosphate synthase (GGPPS), the gene responsible for the synthesis of the HGL-DTG precursor, geranylgeranyl pyrophosphate. In fact, HGL-DTGs were found to be more effective as chemical defences against M. sexta than some of N. attenuata’s other well-characterised defence compounds, such as nicotine and trypsin proteinase inhibitors.

N. attenuata HGL-DTGs are composed of an acyclic C30 HGL backbone decorated with glucose moieties at OH-3 and OH-17; glucose moieties can further be extended with either glucose or rhamnose moieties at OH-2’, OH-4’ or OH-6’ to produce a large diversity of structures. Frequently, malonyl groups are additionally attached to glucoses at OH-4’, resulting in additional structures. As a precursor HGL-DTG, as it represents the first glycosylation of the HGL backbone at both the OH-3 and OH-17. HGL-DTGs having disaccharides attached to OH-3 and OH-17 are referred to as core HGL-DTGs. N. attenuata produces three core HGL-DTG structures namely, atenidene, nicotianoside III and lycioidene IV (Lyc4). Core HGL-DTGs with malonyl groups on their sugar moieties are defined as malonylated HGL-DTGs and are further classified as singly or dimallyonylated, based on the number of attached malonyl groups. Lyc4 with its singly malonylated form, nicotianoside I (Nic1), and its dimallyonylated form, nicotianoside II (Nic2), constitutes ~80% of the N. attenuata’s total HGL-DTG pool.

Although HGL-DTGs severely affect larval growth, M. sexta larvae survive on the HGL-DTG-rich N. attenuata. We investigated M. sexta’s counteradaptation strategy against HGL-DTGs, using Lyc4 and its malonylated forms as model HGL-DTGs. We discovered unusual mechanisms involved by M. sexta to demalonyleate and then detoxify these allelochemicals. To reveal the significance of this atypical strategy, we applied a modern herbivore reverse genetics approach, plant-mediated RNA interference (PMRI). By silencing M. sexta’s Lyc4 ingestion-induced β-glucosidase (BG), we revealed that it is responsible for Lyc4 deglycosylation in the midgut and such detoxification is vital for larval development. Last, we used the PMRI in the native habitat of N. attenuata, the Great Basin Desert, Utah, USA to elucidate the role of Lyc4 detoxification in M. sexta’s interaction with its predators. We found that Lyc4 is a predator deterrent so avoiding its detoxification could benefit larvae that are vulnerable to predators. We discuss why M. sexta could have chosen the detoxification of Lyc4 in spite of it being ecologically disadvantageous.

**Results**

Nicotianoside I and II are demalonylated by the alkaline pH of Lyc4, Nic1 and Nic2 (Fig. 1a) constitute the major fraction of N. attenuata’s constitutive as well as herbivory-induced HGL-DTGs pool (Fig. 1b; Supplementary Fig. 1); therefore, we focused our study on these three compounds. To determine the fate of Nic1 and Nic2 in M. sexta, regurgitant (or oral secretion, as called by several researchers) was collected from the midgut content and frass by larvae feeding on wild-type (WT) N. attenuata plants were analysed. Neither Nic1 nor Nic2 was detected in any of these samples. Absence of Nic1 and Nic2 in larval regurgitant suggested that demalonylation occurred in the regurgitant (Fig. 1c). To determine whether demalonylation occurred as the action of a plant or larval component, we incubated crushed N. attenuata leaves separately with either water or regurgitant in an in vitro demalonylation assay (Fig. 1d). Demalonylation occurred only in the sample incubated with regurgitant, whereas Nic1 and Nic2 were not found and the levels of Lyc4 increased. To test whether demalonylation was enzymatic, we incubated the leaf with enzyme-inactivated regurgitant; regurgitant was either boiled or treated with pronase to inactivate its enzymes. Complete demalonylation of Nic1 and Nic2 occurred in both treatments (Fig. 1d), suggesting that the reaction was non-enzymatic. Finally, to test whether demalonylation was pH dependent, we incubated the leaf with an alkaline buffer of pH 8.5 (because the regurgitant is alkaline), an acidic buffer (pH 6) or acidified regurgitant (pH 6). Demalonylation occurred in the alkaline buffer but not in the acidic buffer and acidified regurgitant, confirming that ingested Nic1 and Nic2 were non-enzymatically demalonylated to Lyc4 by the alkalinity of larval regurgitant (Fig. 1d).

**M. sexta does not deglycosylate Lyc4 to form the aglycone.** Usually, glycosides are deglycosylated by herbivores leading to a release of their toxic aglycones. Therefore, we first conducted a gas chromatography–mass spectrometry (GC-MS)-based analysis of foregut (n = 4), midgut (n = 4), hindgut (n = 4), haemolymph (n = 4), fat body along with skin (n = 4) and frass (n = 3) of larvae feeding on Lyc4-replete empty vector-transformed (EV) plants (a transformation control having similar levels of Lyc4, Nic1 and Nic2 to those in WT) to locate 17-HGL, the diterpene backbone of Lyc4, since only Lyc4 enters the larval gut because Nic1 and Nic2 are demalonylated to Lyc4 in the
regurgitant. However, 17-HGL was not found in any of the above-mentioned samples. This led us to the U(H)PLC/ESI-QTOF-MS-based non-targeted Lyc4 metabolite search.

Structure elucidation of a novel HGL-DTG from larval frass. To determine the fate of Lyc4 in the larval digestive system, we analysed the U(H)PLC/ESI-QTOF-MS-generated HGL-DTG profile of the frass of larvae feeding on WT N. attenuata foliage and compared it with that of the alkaline buffer-treated WT leaf (Fig. 2a,b). An unknown compound having an HGL backbone, which was not found in leaf, was detected in the frass. This compound was also found in the frass of larvae fed Lyc4-supplemented AD, clearly indicating that it was a Lyc4 metabolite (Fig. 2c). The monoisotopic mass (m/z = 614.37) of the compound and its fragmentation pattern were revealed by MS/MS analysis (Fig. 2d). The compound was purified from larval frass and its structure was elucidated by one-dimensional (1H and 13C NMR and APT) and two-dimensional (COSY, TOCSY, ROESY, HSQC and HMBC) NMR methods. The 1H NMR data of the novel compound resembled those reported for HGL-DTGs.20-23,39. In the 13C NMR and APT spectra, 20 signals were detected for the aglycone, and 12 signals for two hexose units (Supplementary Table 1). The aglycone was identified as 17-hydroxygeranyllinalool40 as follows. The 1H NMR signals at δ 5.23 (1H, H-1a, JH1a,H1b = 17.8 Hz, JH1a,H1c = 1.2 Hz), 5.20 (1H, H-1b, JH1b,H1c = 11.1 Hz, JH1b,H1a = 1.2 Hz) and 5.93 (1H, H-2, JH2,H1c = 17.8 Hz, JH2,H1a = 11.1 Hz) indicated a terminal double bond. The signals at δ 5.11 (2H, t, J = 7.3 Hz, H-6, H-10) and 5.26 (1H, t, J = 7.1 Hz, H-14) were due to three protons at the tri-substituted double bond. This was also demonstrated by HMBC correlations between the signals of H-6/H-10 with δ 16.2 (C-18/C-19), δ 40.9 (C-12) and δ 41.2 (C-8), respectively, and H-14 with δ 61.5 (C-17) and δ 21.7 (C-16). Vice versa, the isocyclic methyl signals of H-18 and H-19 at δ 1.59 (6H, s) and 5.26 (1H, t, J = 7.1 Hz, H-14) were due to three protons at the tri-substituted double bond. This was also demonstrated by HMBC correlations between the signals of H-6/H-10 with δ 16.2 (C-18/C-19), δ 40.9 (C-12) and δ 41.2 (C-8), respectively, and H-14 with δ 61.5 (C-17) and δ 21.7 (C-16). Vice versa, the isocyclic methyl signals of H-18 and H-19 at δ 1.59 (6H, s) and 5.26 (1H, t, J = 7.1 Hz, H-14) were due to three protons at the tri-substituted double bond. This was also demonstrated by HMBC correlations between the signals of H-6/H-10 with δ 16.2 (C-18/C-19), δ 40.9 (C-12) and δ 41.2 (C-8), respectively, and H-14 with δ 61.5 (C-17) and δ 21.7 (C-16).
Chapter 3 – Manuscript I

Figure 2 | Lyc4 ingesting M. sexta larvae excrete a novel HGL-DTG. Ion (m/z 271.24 ±h) HGL extracted UHPLC/ESI-QTOF MS chromatograms showing N. attenuata’s major HGL-DTGs in (a) leaf (alkaline extract) and frass of larval feeding on (b) N. attenuata foliage and (c) artificial diet (AD) containing 3 mM Lyc4 (physiological concentration of Lyc4 in an untraced leaf). (d) Mass spectrum of the novel compound and prominent losses of functional groups. (e) Structure of the novel compound elucidated by NMR spectroscopy; compound was annotated as 3-O-β-D-glucopyranosyl(1→4)-β-D-glucopyranosyl-17-hydroxygeranyllinalool (RGHGL). Further 1H NMR signals between δ 3.20 and 3.96 correlated by HSQC cross peaks to 13C signals characteristic of carbohydrates (δ 70–80; Supplementary Table 1; Supplementary Fig. 6) confirmed the presence of two sugar moieties as closely suggested by 12 corresponding signals in the 13C NMR spectrum. The coupling constants of JH1-H2 = 8.0 Hz (H1) and JH1-H2 = 1.7 Hz (H1') indicated one sugar unit with β- and one with α-configuration, respectively, at the anomeric positions (Supplementary Table 1). The hydroxymethine carbon signal at δ 62.2 (C-6'), together with the methyl signal at δ 1.80 (C-6'), the chemical shifts and the 1H-1H spin–spin coupling constants (Supplementary Table 1) were compatible with the presence of a β-glucose and an α-rhamnose in the molecule.

The differences between reported HGL-DTGs and the new compound were established as follows. The signal of C-17 at δ 61.5 appeared about 6.5 p.p.m. upfield of the C-17 signal reported for HGL-DTGs (δ 68) bearing a carbohydrate unit at the 17-hydroxyl group. Therefore, it was concluded that in this compound, the 17-hydroxyl group remained unsubstituted and the two hexose units were attached to C-3 as a disaccharide. This was confirmed by the equivalent 1H NMR signal of the protons at C-17, which appeared as a singlet at δ 4.06 while the 1H NMR signals of H-17 of reported HGL-DTGs are inequivalent (δ 8.13). Attachment of the glucose to the 3-hydroxyl group of the diterpene backbone was verified by the HMBC correlation between H-1 and C-3 (δ 81.6) (Supplementary Fig. 7). Interglycosidic HMBC correlations of H-1' with C-4 (δ 79.7) and H-4 with C-1' indicated 1→4 linkage between the rhamnose and the glucose units (Supplementary Fig. 7). From these data, the novel compound was identified as 3-O-β-D-glucopyranosyl(1→4)-β-D-glucopyranosyl-17-hydroxygeranyllinalool (RGHGL; Fig. 2e).

Co-injected fractions of Lyc4 and RGHGL showed single peaks in UHPLC/ESI-QTOF-MS analysis indicating that the isolated compounds were pure. Relative quantification of Lyc4 and RGHGL was achieved using rebaudioside A as an internal standard for UHPLC/ESI-QTOF-MS based measurements (Supplementary Fig. 8a-c) and as an external standard for high-performance liquid chromatography (HPLC; Agilent 1100 series)-based measurements (Supplementary Fig. 8d–e).

Extraction efficiency of these compounds from the frass was > 90% and it remained linear over a broad range of concentrations (0.02–0.32 μg mg⁻¹; Supplementary Fig. 8g–i).

Lyc4 ingestion induces BG1 in the larval midgut. NMR analysis showed that Lyc4 and RGHGL structures were similar, except that the C-17 of RGHGL lacked a glucose moiety (Fig. 2e), and since such deglycosylation reactions are often catalysed by BGs, we searched for the BG involved in RGHGL formation. We identified three BGs (BG1, BG2 and BG3 NCBI accession no: FK816842, FK816724, and FK816837, respectively) from the collection of M. sexta glycoside hydrolases (Supplementary Fig. 9a). Transcript abundance of BG1 (Fig. 3a) but not BG2 and BG3 (Supplementary Fig. 9b,d) was higher in midguts of larvae feeding on EV plants than in the larvae feeding on Lyc4-depleted inverted repeat (ir)GPPS plants. BG1 transcript accumulation was also increased in the midguts of larvae feeding on AD containing 6 mM Lyc4 but not containing 6 mM RGHGL (Fig. 3b), suggesting that BG1 was induced in response to the ingestion of Lyc4 and not involved in the downstream metabolism of RGHGL; this was supported by the fact that when larvae (n = 6) were fed RGHGL in AD, they excreted no other compound having HGL backbone than RGHGL. The abundance of BG2 and BG3 transcripts in the midgut was not elicited in response to Lyc4 and RGHGL ingestions (Supplementary Fig. 9c,e).

Silencing of MsBG1 by PMRI. To understand BG1’s role in larval Lyc4 metabolism and its impact on larval growth and development, we silenced the expression of BG1 in larval midguts, using PMRI (Fig. 3c). Stable transgenic irBG1 lines of N. attenuata expressing 301 bp MsBG1 double-stranded RNA (dsRNA) were generated using Agrobacterium tumefaciens-mediated transformation of an ir of 301 bp MsBG1 complementary DNA (cDNA) fragment. Single transgene insertions in two independently transformed irBG1 lines (375-10 and 379-8) were confirmed by Southern blot hybridization (Supplementary Fig. 10a). Presence of small dsRNA (21–24 nt) in irBG1 leaves and in midguts of irBG1-feeding larvae was confirmed by Northern blot hybridization (Supplementary Fig. 10b); BG1 dsRNA was not detected.
Figure 3 | Silencing of Lyc4 ingestion-induced, midgut-expressed BG1 impairs larval Lyc4 deglucosylation, molting and survival. BG1 transcripts (relative to ubiquitin) in midguts of fourth-instar larval feeding on (a) Lyc4-containing EV and Lyc4-depleted irBGPS plants (F1,10 = 83.35, P ≤ 0.0001; significant difference (P ≤ 0.05) between means (± s.e.) determined by Fisher’s LSD test (one-way ANOVA); n = 6) and (b) AD containing water (control), 6 mM Lyc4 or 6 mM RHGL (F2,10 = 59.12, P ≤ 0.0001; significant differences (P ≤ 0.05) between means (± s.e.) determined by Games Howell test (Welch’s ANOVA); n = 6). (c) Plant-mediated RNAi: pSOL8 binary vector constructed to express 301bp MibGI dsRNA in N. attenuata and the trophic transfer of dsRNA from plant to larvae. (d) BG1 transcripts (relative to ubiquitin) in various tissues of fourth-instar larval feeding on EV, irBG1 and irBGPS plants (midgut: F2,15 = 9.53 P ≤ 0.0001; hindgut: F2,15 = 248, P ≤ 0.0001; significant differences (P ≤ 0.05) between means (± s.e.) determined by Games Howell test (Welch’s ANOVA, separately conducted for each tissue); n = 6 in all bars). (e) RHGL formed in vitro enzyme assays containing midgut extracts of fourth-instar EV- and irBG1-feeding larvae and either (1 mM) Lyc4 (F2,15 = 104.9, P ≤ 0.0001; significant differences (P ≤ 0.05) between means (± s.e.) determined by Fisher’s LSD test (one-way ANOVA); n = 3) or 1 mM Lyc4 + 1 mM Bg inhibitor, β-glucuronidase (n = 3). Excretion (% total lyc4 ingested) of (f) Lyc4 (F2,15 = 13.95, P ≤ 0.001; significant difference (P ≤ 0.05) between means (± s.e.) determined by Fisher’s LSD test (one-way ANOVA); n = 13 (EV) and 14 (irBG1)) and (g) RHGL (F2,15 = 6.26, P ≤ 0.0001; significant difference (P ≤ 0.05) between means (± s.e.) determined by Fisher’s LSD test (one-way ANOVA); n = 13 (EV) and 14 (irBG1)) by fourth-instar EV- or irBG1-feeding larvae. (h) Phenotype (in irBG1-feeding larvae) and (i) percentage of molting impairment (significant difference (P ≤ 0.05) determined by Fisher’s exact test of frequencies; n = 30) and (j) mortality (%) in larval feeding (for 12 d) on EV or irBG1 plants (significant difference (P ≤ 0.05) determined by Fisher’s exact test of frequencies; n = 30). ANOVA, analysis of variance; d, days: LSD, least significant difference.

in EV leaves and in midguts of EV-feeding larvae (controls). Growth, morphology and levels of Lyc4 and other secondary metabolites (nicotiana, rutin, caffeoyl putrescine and chlorogenic acid) of both irBG1 lines were similar to those of EV plants (Supplementary Fig. 10d). Midguts of larvae feeding on irBG1 plants showed >90% lower abundance of BG1 transcripts than that of EV-feeding larvae (Fig. 3d). BG1 transcript abundance was also reduced in foregut and hindgut of irBG1-feeding larvae (Fig. 3d). Efficiencies of silencing by both irBG1 lines 375-10 and 379-8 were similar; therefore, irBG1 (375-10) was randomly
selected for further experiments. To determine whether BGI silencing was specific and had no off-target effects on the expressions of other similar genes, we quantified the transcripts of closely related BGG and BGD (60.2% and 51.5% nucleotide similarity to BGI, respectively). Transcript abundance of BGG and BGD in the midguts of larvae feeding on irBGI plants were similar to those of larvae feeding on EV plants (Supplementary Fig. 10g), suggesting that the BGI silencing by PMIRI was sequence specific. Therefore, we refer the irBGI-feeding larvae as ‘BGI-silenced’ larvae.

MrBGI silencing impairs larval Lyc4 metabolism. The influence of BGI silencing on larval Lyc4 metabolism was tested using an in vitro Lyc4 deglycosylation assay with the midgut tissue extracts of BGI-silenced and control larvae. The amount of RGHGL formed by the midguts of BGI-silenced larvae was significantly lower (55%) than that formed by the midguts of EV-fed larvae (Fig. 3c); similarly reduced RGHGL formation was observed when midguts of control and BGI-silenced larvae were incubated with Lyc4 and a BG inhibitor, α-glucosidase (Fig. 3e). These results revealed that Lyc4 deglycosylation activity was impaired in the irBGI-feeding larvae.

Further, to test the effect of BGI silencing on larval Lyc4 accumulation, we quantified the amounts of Lyc4 and RGHGL in foregut, midgut, hindgut tissues, haemolymph, Malphigian tubules and fat body with skin of fourth-instar BGI-silenced larvae. These larvae showed significantly higher Lyc4 content in their midgut, hindgut, haemolymph and fat body, and lower RGHGL content in midgut compared with those of EV-fed larvae (Supplementary Fig. 11a,b). Excretion efficiency determination assay revealed that although the amounts of Lyc4 ingested by the BGI-silenced and EV-fed larvae were similar, BGI-silenced larvae excreted 40% more Lyc4 and 70% less RGHGL in their frass than did EV-fed larvae (Fig. 3f,g). Before determining these amounts of excreted Lyc4 and RGHGL, we quantified the accuracy of our extraction procedure and also determined that these compounds were stable in larval frass over the 24-h period of the assay. For these examinations, we spiked each compound (separately) into fresh frass of irGGP5s-fed larvae, incubated them for 0 h (control) and 24 h under the assay conditions, extracted the spiked compounds and quantified them. This analysis revealed that the efficiencies of extraction of Lyc4 and RGHGL from frass were 88% and 91%, respectively, and that these compounds were stable in frass over the assay period (Supplementary Fig. 11c).

Suppressed Lyc4 metabolism causes larval moulting impairment. BGI silencing had no effect on larval growth, however, during every moult (until the fourth-instar) we found that some BGI-silenced larvae were unable to shed their exoskeleton; collectively, 21.8% BGI-silenced larvae (n = 32) failed to moult during the first three instar changes (Fig. 3h). Hereafter, we refer to this phenomenon as ‘moultling impairment’. Larvae unable to free themselves from the old exoskeleton stopped feeding, gradually melanized and died within 48 h of (failed) moultling (Fig. 3i,j; Supplementary Fig. 11d); moultling impairment and elevated mortality were not observed in the EV-fed larvae (Fig. 3j). Some BGI-silenced larvae (~10% of the total dead) died soon after moultling but did not show the unshed exoskeleton or melanization phenotypes; these were not counted as moultling-impaired larvae. Although it was demonstrated that BGI silencing suppressed Lyc4 deglycosylation, whether moultling impairment was caused by BGI silencing or by Lyc4 metabolism suppression remained unclear (Fig. 4a). Along with the BGI dsRNA, irBGI plants contained Lyc4 levels equal to those of EV plants so the effects of BGI silencing and Lyc4 deglycosylation suppression could not be uncoupled in the larval feeding on these plants. Therefore, we generated Lyc4-depleted and MrBGI dsRNA-containing plants by crossing irBGI with irGGP5s plants (irBGI × irGGP5s; hereafter called B × G) (Fig. 4b). Levels of HGL-DTGs of B × G plants were similar to the low levels of irGGP5s plants. Although BGI silencing in B × G-fed larvae was similar to that of irBGI-fed larvae (Supplementary Fig. 12a), moultling impairment and resulting mortality were not observed (Fig. 4c,d). Levels of Lyc4 and RGHGL in the bodies (without gut contents) of B × G-fed larvae were similar to those in the bodies of irGGP5s-fed larvae (Supplementary Fig. 12c,d). This indicated that the moultling impairment phenotype did not result from the effect of BGI silencing on larval primary metabolism but was related to Lyc4 deglycosylation by BGI. To test this, we complemented B × G and irGGP5s leaves with Lyc4 and RGHGL by topical coating; water-coated EV, irBGI, irGGP5s and B × G leaves were used as controls. More than 90% of topically coated Lyc4 and RGHGL could be recovered from coated leaves after 24 h, indicating that these compounds were stable on leaves during larval feeding (Supplementary Fig. 12d). Larvae feeding on Lyc4-coated B × G plants (n = 30) showed high moultling impairment (22%) and mortality (28%) did the larvae feeding on water-coated irBGI leaves (Fig. 4c,d). Larvae feeding on RGHGL- or water-coated B × G and irGGP5s leaves and water-coated EV leaves (n = 30) did not show these phenotypes (Fig. 4c,d).

Moultling impairment and mortality associated with the Lyc4 ingestion by BGI-silenced larvae were not observed after the ingestion of Lyc4 or RGHGL alone or after silencing BGI in the absence of Lyc4 ingestion. This clearly showed that the BGI function is vital for larvae ingesting large quantities of Lyc4 produced by N. attenuata.

A native predator fails to ingest the BGI-silenced larvae. Several herbivores defensively co-opt ingested plant metabolites either with or without metabolism11,14,16, so we hypothesized that M. sexta larvae co-opt Lyc4 or RGHGL to use against their natural enemies (Fig. 5a). To test this, we analysed the effect of BGI silencing on larval survival in the host’s predator-temed native habitat24. We introduced EV, irBGI and irGGP5s stable transgenic lines into a field plot in the Great Basin Desert, Utah (Fig. 3b) and allowed M. sexta larvae to feed on these plants. Diurnal and nocturnal survivors of BGI-silenced larvae (68% and 69%, respectively) did not differ significantly from those of larvae feeding on EV (70% and 70%, respectively) and irGGP5s (71% and 70%, respectively) plants (n = 32 larvae per line, in both the experiments; Fig. 5c). However, while recording nocturnal survivorship, we found some carcasses of partially eaten larvae on irBGI plants. Since the moultling-impaired BGI-silenced larvae were not used in these assays, occurrence of such carcasses was likely not related to moultling impairment. Hence, that such carcasses were not found on EV or irGGP5s plants, clearly suggested that the BGI-silenced larvae experienced some distinctive predator behaviour. During the night, M. sexta larvae are mainly preyed on by the wolf spiders (Ctenus ciliatus; Lycosidae), which are abundant in this habitat25,27 (Fig. 5d). Therefore, we hypothesized that these spiders were responsible for the carcasses of partially eaten larvae. To test this, we conducted choice and no-choice predation bioassays using these spiders. During these assays we closely observed spider’s predation behaviour, which generally consists of three steps: (1) prey assessment by tapping it with chemosensory-endowed legs and palps, (2) prey capture followed by killing and (3) ingestion of the prey (Fig. 5e). In choice assays, we offered
two larvae (one each from EV fed and iBG1 fed or EV fed and iGPPS fed or iBG1 fed and iGPPS fed) to each spider (n = 30). Spiders did not show any preference for the given larvae, suggesting that they were unable to differentiate between them (Supplementary Fig. 13a-c). In no-choice assays, each spider was offered only one EV-, iBG1- or iGPPS-fed larva (n = 30). Frequencies of prey capture and killing were similar for EV-, iBG1- and iGPPS-fed larvae. We observed that all the killed EV- and iGPPS-fed larvae were completely eaten; however, spiders did not ingest ~75% of the BG1-silenced larvae that they killed (Fig. 5g,h). These results are consistent with the hypothesis that spiders were responsible for the carcasses of partially eaten larvae we found during the nocturnal survival assays.

High Lyc4 concentration hinders spider’s prey ingestion. To investigate whether Lyc4 or RGHGL content of BG1-silenced larvae prevented spiders from ingesting them, we conducted no-choice assays. We offered each spider a larva fed on water-coated EV, iBG1, iGPPS or B × G leaves, Lyc4-coated iGPPS or B × G leaves or RGHGL-coated iGPPS or B × G leaves. Prey capture and kill frequencies were similar for all these larvae (n = 25 in each treatment) (Fig. 6a). However, prey ingestion frequencies were reduced in the cases of larvae fed on water-coated iBG1 and Lyc4-coated B × G leaves (Fig. 6b); spiders preying on these larvae, but not EV- and iGPPS-fed larvae, showed signs of distress after an unsuccessful attempt to ingest the prey, indicating possible intoxication that influences spider mobility (Supplementary Movies 1–3). To find out whether Lyc4 itself or its unknown metabolite(s) caused this spider behaviour, we conducted choice and no-choice assays using iGPPS- (Fig. 6c–i) and AD-fed (Supplementary Movie 13d–h) larvae (n = 21 in choice assays and n = 30 in no-choice assays) topically coated with water, Lyc4 or RGHGL. More than 90% of these coated compounds could be recovered unmetabolized from the larval body surface after 1 h (n = 3), demonstrating that the compounds were not degraded during the assay period (Supplementary Fig. 13j). Moreover, no Lyc4 or RGHGL was detected in the washes of water-coated iGPPS- or AD-fed larvae and also the EV-, iBG1- and iGPPS-fed larvae that were not coated with any substance, suggesting that these compounds were not externalized by larvae. Prey capture and kill frequency were reduced by at least 80% for Lyc4-coated larvae compared with water- or RGHGL-coated larvae (Fig. 6c–e). Similarly, in choice assays, spiders clearly preferred water- or RGHGL-coated larvae over Lyc4-coated ones, while not showing a clear preference between water- and RGHGL-coated larvae (Fig. 6f–i). Spiders readily captured and killed the water- or RGHGL-coated larvae (Supplementary Movies 4 and 5) but clearly rejected Lyc4-coated larvae after chemosensory assessment (Supplementary Movie 6). In rare incidences during these assays, when spiders tried to ingest the Lyc4-coated larvae, they failed and showed signs of locomotor distress. Together, these results revealed that Lyc4 was the spider deterrent and we infer that the high Lyc4 content of the BG1-silenced larvae hindered spider’s prey ingestion and was associated with spider’s locomotor distress.

**Discussion**

Heiling et al. discovered the occurrence of malonylated HGL-DTGs in N. attenuata. They proposed that malonylation facilitates accumulation and distribution of HGL-DTGs in plant and prevents their deglycosylation by plant glycosidases. However, that the malonylation is instantaneously lost in the
Figure 5 | In the native habitat, spiders capture and kill but do not ingest the BGI-silenced M. sexta larvae. To test (a) whether Lyc4 or RGHGL is defensively co-opted by larvae against the natural enemies in the native habitat, (b) EV, iBG1 and iGGPPS plants were grown in the Great Basin Desert, Utah. (c) Diurnal and nocturnal survival (%) of larvae feeding on these plants (n = 32 larvae per line). (d) Native C. parallelus spider attacking M. sexta larva. (e) Three stages of spider’s predation behaviour. Spider’s (f) prey capture and killing (%; n = 28) and (g) prey ingestion (% of total killed; significant difference (P < 0.05) determined by Fisher’s exact test of frequencies; n = 28) in a no-choice assay (1h) on second-instar M. sexta larvae feeding on EV and iBG1 and iGGPPS plants.

Alkaline larval regurgitant indicates that malonylation’s sole role could be in planta, for example, to ensure the availability of toxic Lyc4’s to the herbivore. Indeed, considering that the lepidopteran herbivore is highly mobile and is likely to devour all parts of the shoot, the concentration and distribution of Lyc4 could be crucial for the optimization of the plant’s defensive strategy. The role of malonylated Lyc4 also appears to be similar to that of phytoantocypins in which, glycosylation ensures the within-plant stability of defence molecules by maintaining them in inactive or rather pretoxin states. However, that the core Lyc4 often occurs in certain tissues of N. attenuata in large quantities implies that its stability and toxicity may not be of concern for the plant. In this way, the malonylated Lyc4 could be considered as a type of phytoanticipin, which is decorated for distribution, rather than stability. From M. sexta’s perspective, demalonylation appears to be unavoidable due to the need to maintain an alkaline midgut.

As mentioned earlier, the large literature on herbivores’ xenobiotic detoxification reveals that glycosylation is an important detoxification mechanism; consequently, the current perception that deglycosylation is often a toxin activation mechanism is widely accepted. On the basis of such literature, we initially hypothesized that Lyc4 is completely deglycosylated by M. sexta to release the non-polar toxic 17-HGL, the diterpene backbone of Lyc4; however, this compound was not found in the body, gut contents or frass of larvae. When we identified RGHGL from the frass of Lyc4-fed larvae, we hypothesized that RGHGL was the activated toxin. High incidences of moulting impairment and mortality in larvae feeding on iBG1 foliage and the reappearance of these phenotypes in larvae feeding on Lyc4-complemented B × G foliage (Fig. 4c,d) falsified this hypothesis, from which we inferred that Lyc4 was the toxin and RGHGL was actually the detoxified product. While detoxification by deglycosylation is uncommon in insects, it has been frequently observed in phytopathogenic fungi. For example, the fungus Gaeumannomyces graminis (Magnaportheae) detoxifies the avenacin saponins (trieterpenoid glycosides containing three sugars) of oat by BG-catalysed deglycosylation. Similarly, various fungi detoxify the tomato steroidal glycoalkaloid α-tomatine (containing four sugars) by removing its C-3 oligosaccharide that renders it incapable of binding to...
3β-hydroxy sterols, which leads to the formation of membrane pores and cell lysis.\textsuperscript{11,42} Notably, in all these cases, fungi completely deglycosylate the toxic glycosides; thus, Lyc4's detoxification is an atypical example of 'detoxification by partial deglycosylation'.

The activity of glycosides can be attributed not only to their aglycones but also to sugar moieties, especially when compounds contain multiple sugars. Such activity mainly depends on the binding specificity of sugars to the target molecules and receptors. For example, aureolic acid group antibiotics bind to DNA and inhibit the DNA-dependent RNA polymerase; aureolic acid variants differing in their sugar moieties show varying degree of inhibitory activities.\textsuperscript{43} But the aglycone is completely unable to bind to DNA.\textsuperscript{44} Similarly, a nonsugar sweetener, glycyrrhizinic acid (ammonium salt), tastes sweeter if its second glucuronic acid is replaced by glucose or xylose, owing to the differential binding capacity of sugars to the receptors in the taste buds.\textsuperscript{45} It is known that binding of glycosides' specific sugars to receptors on the cell surfaces facilitates their active uptake.\textsuperscript{46} Conspicuously, higher amounts of Lyc4 than RGHGL in most of the larval tissues (in EV- as well as irBG1-led larvae) may result from this sugar-specific uptake phenomenon, wherein the uptake of Lyc4 in various tissues is facilitated by the receptor-binding capacity of C-17 glucose, a structural feature absent in RGHGL.

The fact that spider deterrence is caused by Lyc4 and not by RGHGL is consistent with our inference that Lyc4 is the toxin and RGHGL is its detoxified product. That the spiders completely ingest EV- and irGGPPS-fed larvae suggests that M. sexta larvae have not evolved sequestration of Lyc4 for their own defence. Spiders' chemosensory-endowed hair on legs and palps can detect the Lyc4 coated on the larval surface. But the spiders are neither deterred by the control and nor by the BG1-silenced larvae, which have ingested Lyc4, mainly because the larvae do not externalize Lyc4. Moreover, the concentrations of Lyc4 in haemolymph and
fat body, the tissues in which insects usually sequester the xenobiotics, were much lower than those found in the midgut suggesting that Lyc4 is not sequestered. Avoiding self-intoxication could be the reason behind not co-opting Lyc4. However, it would be interesting to study the generalist herbivores of Nicotiana spp. to evaluate if HCL-DTGT co-option has evolved in them; such comparisons between the nicotine metabolism of Manduca spp. and generalist herbivores were highly useful in evaluating the costs and benefits of detoxification and co-option of nicotine37. The observation that spiders that ingest BG1-silenced larvae show signs of locomotor distress suggests that Lyc4 affects spider’s nervous system.

Together, this work reveals that deglycosylation can bring about detoxification and suggests that much remains to be revealed about the detoxification of multiply glycosylated compounds by herbivores and roles of such compounds in tritrophic interactions. It suggests that multiply glycosylated as well as malonylated plant metabolites could be much more useful in pest control than previously thought; consequently, the newly discovered role of insect β-glycosidases in detoxification suggests that these enzymes could be valuable targets for pest control. It demonstrates value of PMRI in molecular ecological research, but given the simplicity and robustness of this trophically mediated gene-silencing technique, it could also become a valuable means of controlling pests in agriculture. The biochemical basis of partial deglycosylations of Lyc4 by BG1 or other β-glycosidases and the mode of action of Lyc4 are interesting future perspectives of this work.

Methods

M. sexta. M. sexta eggs were obtained from an in-house colony in which insects are reared in a growth chamber (Sinnjdem Scientific) at 26°C-18 h light, 24°C-8 h dark and 65% relative humidity, until hatching. M. sexta eggs used in all the field experiments were kindly provided by Carol Miles (Department of Biological Sciences, Binghamton University, Binghamton, NY). Neonates were fed on various N. attenuata lines (Ev, iRGi, N2GSPS or B × G) until they were used in glasshouse experiments. In some experiments, neonates were fed on AD88 containing 6 mM Lyc4 (aqueous; in 100 μg g⁻¹ AD), 6 mM RGHGL (aqueous; in 100 μg g⁻¹ AD) or equivalent amount of water (control), to rear the larvae free from any influence of the host plant.

Plant material. The 30th generation seeds of an inbred line of N. attenuata, which were harvested in 1988 from a native population in Utah (USA), were used for the development of stable transgenic lines59. Seeds were germinated and plants were grown as explained previously60. Field experiments were conducted at Lytle Ranch Preserve in Santa Clara, Utah, 84765 (37°08' 45"N, 114°01' 17" W) in 2013. Seeds of N. attenuata Ev, iRGiGPPS and iRGiGL were imported and released in accordance with Animal and Plant Health Inspection Service notifications (Supplementary Table 3). Planting of transgenic lines in the field plot was performed as described by Kessler et al50.

Selection of glycoside hydrolase gene sequences for PMRI. The complete coding sequences of 12 M. sexta glycoside hydrolases were retrieved from NCBI (accession numbers are given in Supplementary Fig. 9). Their open-reading frames were aligned and the phylogenetic tree was constructed with maximum parsimony method using Clustal W, with 1,000 bootstrap trials to identify the formation of clusters. Three RG sequences (BG1, BG2 and BG3) were selected for the (quantitative PCR with reverse transcription) qRT-PCR-based determination of their transcript levels in larval midgut in response to Lyc4 and RGHGL ingestions. A 301-bp cDNA stretch of BG1, which was upregulated in response to Lyc4 ingestion, was selected for cloning into a PMRI vector as previously described61.

Plant transformation and Southern hybridization. Stable transgenic PMRI plants were generated by A. tumescens-mediated transformation of recombinant pPOLA vector62. The pPOLA vector contained an irf of 301-bp stretch (~44 to ~384) of M6GβG, separated by the pdk 3 intron and a selectable marker, hygromycin phosphotransferase (spdh), for hygromycin resistance63. Transgenic lines were screened as described by Gae et al64. Single transgene insertions in two independently transformed homozygous iRGiGL line (137.5 and 379.6) were confirmed using Southern hybridization. For Southern hybridization, genomic DNA was extracted from the rosette leaves of untransformed W1 and iRGi plants (line no. 375.19 and 379.6) using a modified cetyltrimethylammonium bromide method as described by Rogers and Bendich38. The genomic DNA (10 μg) was completely digested with HindIII and EcoR I separately. The digested DNA was separated on 1% (w/v) agarose gel and blotted onto a nylon membrane (GeneScreen Plus; Perkin-Elmer) by capillary transfer. Hybridization and detection of the transgene copy number were performed as described by Gae et al65. Previously characterized GPPS (NCBI accession no. FE362626) silenced iRGiGPPS line of N. attenuata66 was used as a Lyc4-depleted control to feed M. sexta larvae. An EV-transformed N. attenuata transgenic line (A-04-866-3) was used as a Lyc4-explet control39.

Harvesting larval tissues. Fourth-instar Larvae were immobilized by placing the larvae on ice before dissection. Haemolymph was collected by clipping the larval horn just before dissection, as described by Kumar et al21. Then, larvae were dissected in 0.15 M NaCl to collect malpighian tubules, fat body with skin, foregut, midgut and hindgut. Peritrophic membrane and gut contents were removed before midgut collection. Dissected gut tissues were carefully washed in 0.15 M NaCl to remove any adhering plant material. All the collected tissues were flash-frozen in liquid nitrogen and stored at ~80°C until further use. Lyc4 and RGHGL contents of the entire larval body (without gut contents) were also analysed. For this, all the haemolymph was first collected by opening the larval body with a longitudinal cut on the dorsal side; then, the gut contents were removed by dissecting the gut and all tissues, together with previously collected haemolymph, were stored for further analyses.

Collection of larval regurgitant. The regurgitant from fourth-instar M. sexta larvae was collected with the help of Teflon tubing connected to a vacuum, after gently agitating the larvae52. Collected regurgitant was stored under argon at 4°C until use.

Low-molecular-weight RNA isolation and Northern hybridization. Low-molecular-weight RNA was separated from total RNA isolated from larval midgut as well as from leaves using polyethylene glycol (18%) precipitation in the presence of 1 M NaCl. A 120-bp stretch of the 301-bp-M6GβG fragment cloned from PM4i was PCR amplified using BG1 3′ gene-specific primers (Supplementary Table 2) and used as a template in the labelling reaction. Ten nanogram of PCR product was labelled with a 32P-TPD isotope using the Rediprime II DNA labelling system (Amersham Biosciences). Low-molecular-weight RNA blotting, hybridization and screening of the blot performed53.

RNA isolation and qRT-PCR. Larval foregut, midgut, hindgut, haemolymph, malpighian tubules and fat body along with the skin were collected from the fourth-instar M. sexta larvae and stored in TRI reagent (Invitrogen) at 4°C. Total RNA was isolated from the stored tissues according to the manufacturer’s protocol and was subjected to TURBO DNAse (Ambion) treatment. Ambion’s DNA contamination. Transcript levels of BGs were determined by qRT-PCR conducted in Mx3005P Multiplex qPCR system (Stratagene) using qRT-PCR SYBR Green I kit (Eurogentec). Relative quantification of transcripts was carried out by the comparative D C threshold method. M/Ubiquitin levels were taken as internal controls to normalize the abundance of BG transcripts. All the primer pairs (Supplementary Table 2) used in qRT-PCR were designed using Primer3 software version 4.0 (http://primer3.sourceforge.net/).

Extraction and GC-MS analysis of 17-HGL (aplycnon). Foregut, midgut, hindgut, haemolymph, fat body along with the skin and frass of EV-fed larvae were used to determine whether M. sexta larvae deglycosylated Lyc4 to its aglycone, 17-HGL. EV-fed larvae was also analysed to determine whether the plant glucosidases deglycosylated the HCL-DTGT when herbivores ingest the leaf. Samples of each tissue (100 mg) were homogenized in 1 ml tert-butyl methyl ether and then centrifuged at 13,400 × g for 10 min. The supernatant was dehydrated using anhydrous sodium sulphate at room temperature for 30 min. Subsequently, 100 μl of the supernatant was collected in a glass vial and derivatized by adding 10 μl trimethylsilyl reagent, followed by incubation for 1 hr at 80°C. The resulting extract was analyzed using DB-5 column in a Varian CP-3800 GC-MS 4000 ion trap MS/MS. Peak areas were integrated using an HP Chemstation program.

Extraction and purification of metabolites from larval frass. Frass of EV-fed M. sexta larvae was collected and stored at ~80°C until further use. It was ground in liquid nitrogen and then extracted using 100% methanol (1 ml methanol for 100 mg of frass). Samples were centrifuged at 13,400 × g for 30 min at 4°C. The supernatants were incubated at 65°C for 20 min for precipitating proteins and were collected by centrifugation at 13,400 × g for 30 min at 4°C and the clear supernatants were collected. Further, the extract was evaporated to a rotary evaporator to one-tenth of its original volume. The concentrated extract was centrifuged at 13,400 × g for 20 min at 4°C and the supernatant was subjected to fractionation using a reverse-phase
**Chapter 3 — Manuscript I**

**HPLC** (Luna C-18 (2), 250 × 10 mm; Phenomenex) at a 3 μl/min 1 flow rate. The solvents (A and B) were 100% acetonitrile, and 100% water, respectively. The mobile phase was obtained using a Millipore system (Milli-Q Advantage A10). Chromatographic solvent gradients were, from 0 to 20 min (50% of B and from 20 to 40 min (100% of B). Lyce and RHGL were collected from fractions 46 (23 min) and 48 (24 min), respectively. Collected fractions were dried completely in a vacuum concentrator (3.7 mmivaporizer; Eppenheur), and thus obtained pure metabolites were used for further experiments.

**Extraction and UHPLC/EI-TOF-QMS analysis of HGL-DTGs.** For analysing Lyce and its malodorous forms of Lyce in the leaf, larval tissues and larval frass, samples were homogenized and extracted in an acidic extraction buffer A (60% solution (1.213 ml) of acetic acid, 3.41 g/l ammonium phosphate, pH 4.8 using 1 M NH4OH and 40% methanol). An internal standard rebaudioside A was always spiked into extraction buffer A (10 ng/ml) before extraction. A amount of 100 mg of homogenized sample was extracted in 1 ml of buffer A. Extracted samples were eluted 1.50 with buffer B (one part of buffer A and nine parts of 40% methanol) and analysed using a UHPLC/EI-TOF-QMS(MS). Retention times and molecular ions of Lyce, RHGL, and rebaudioside A were determined by loading 1 μg of each of these compounds onto a Fisher Scientific Acclaim C18 (2.1 × 150 mm) UHPLC column (particle size 2.2 μm) with solvent A (0.1% acetic acid, 0.05% phosphoric acid (v/v) in methanol (MeOH)) and solvent B (0.05% formic acid in acetonitrile (ACN)). The gradient of 0 min 10% B, 1 min 10% B, 9 min 80% B, 10 min 80% B, with a flow rate of 0.3 ml/min 1 was used. Compounds were detected using Electrospray Mass Spectrometer (MicrOMASS TOF QII Bruker Daltonik, Germany) equipped with an ESI source in positive ion mode (instrument settings: capillary voltage, 4500 V; capillary exit, 130 V; dry gas temperature, 200 °C; dry gas flow 8100, 100 °C). Calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% (v/v) isopropanol/water containing 0.2% formic acid (v/v)).

**NMR analysis.** 1H NMR, 13C NMR, 1H-13C COSY, ROESY, TOCSY, HSQC, and HMBC experiments were recorded on a Bruker Avance 500 NMR spectrometer (Bruker Biospin, Germany), operating at 500.13 MHz for 1H and 125.75 MHz for 13C. Protons and carbon peaks were allocated in a TCI cryoprobe (5 mm) at a probe temperature of 300 K. Samples were prepared in methanol-d4, solvent (85%). 1H and 13C chemical shifts were referenced to the residual solvent signals of methanol-d4 at 3.31 and 45.15, respectively.

**Extraction and HPLC analysis of Lyce and other metabolites.** Stored leaf and frass samples were homogenized in liquid nitrogen using pre-chilled mortar and pestle. Extraction buffer A (11 ml, 60% methanol containing 0.05% glacial acetic acid) and ceramic beads (0.9 g; Sili GmbH, Germany) were added to each aliquot (100 mg) sample, which was again homogenized using Geno/Grind 2000 (Spex, USA) for 5 min with 600 strokes per min. Homogenized samples were centrifuged at 13,400 g for 20 min, at 4 °C. The supernatants were re-centrifuged at 13,400 g for 20 min at 4 °C for separating undissolved particles. Clear supernatants were analyzed using UHPLC (Agilent 1100 series) Lyce from leaf and Lyce and RHGL from frass were quantified using rebaudioside A (Sigma, Germany) as an external standard. The linearity of the extraction of Lyce and RHGL from frass was determined and charged dried frass (100 mg) of Frass was injected into the 4, 6, 8, 10, and 12 μg of each compound. Spiked frass samples were extracted and analysed as mentioned above. The detected quantity of the recovered compound was calculated relative to rebaudioside A. Linearity was determined using the standard curve method, as previously described.

Nicotine, chlorogenic acid, caffeine butanone and rutin were also extracted from the leaf and frass extracts. These were subsequently clean-up and derivatized with ethereal diazomethane. The reaction products were saponified in base, and the resulting products were extracted with dichloromethane. The extracts were successively washed with 1 N HCl, 0.1 M NaOH, and water, and the organic extracts were dried over sodium sulfate. The solvent was evaporated under vacuum.

**Extraction efficiency determination assays.** Injection of Lyce and extraction of its metabolites were performed using the extraction efficiency determination assays. Freshly harvested M. sexta neonates were allowed to feed on EV and irBG1 rosette-stage plants until they reached the fourth-instar. The mass of each larva was recorded until the larva reached 16 h light, 24°C/18°C dark and 60% relative humidity. To absorb the excised liquids, blotting paper disks of known masses were laid in each assay container. The mass of each larva and that of remaining leaf material were recorded at 16 h feeding. All the larvae were again starved for 4 h to empty their guts. Frass excreted by each larva, during the 24 h of feeding, and the 4 h of starvation were collected, weighed along with the paper disk and stored at −80°C until further use. Lyce levels of leaves of each line were measured by HPLC (Agilent 1100 series). Actual amount of Lyce ingested by each larva was calculated based on the amount of foliage ingested. Lyce and RHGL levels in the collected frass samples (along with the blotting paper disk) were also measured and corrected for the mass of the blotting paper disk. The percentage of Lyce or RHGL extracted was determined by calculating the ratio of the amount of compound excreted/amount of Lyce in the ingested food × 100.

**Generation of BG1 × iGPP3 (B × G) plants.** Lyce-depleted and BG1 dA-dDNA-producing plants were generated by crossing irBG1 (375-10) with iGPP3 (230-5) plants. Crossing was performed by pollinating the stigmas of atherocomenized (before pollen maturation) irBG1 flowers with iGPP3 pollen. B × G seeds were collected and germinated. Lyce was extracted from the rosette B × G leaves and was analysed using HPLC (Agilent 1100 series). Genomic DNA was isolated from B × G leaves and was sequenced to PCR using BG1 primers (Supplementary Table 2) to confirm the presence of irBG1 cDNA fragment inherited from the irBG1 parent.

**Moulting impairment and mortality analyses.** To determine the effect of Lyce detoxication suppression on larvae, we carefully monitored the larvae feeding on EV and irBG1 plants for 12 days (until they reached fourth-instar). After moulting impairment and mortality observations were performed, 10 larvae were observed during each instar change, to determine whether BG1 silencing or suppressed Lyce deacetylation caused these phenotypes, we used the complementation method. Detached fully expanded rosette leaves of irBG1 and B × G were coated with Lyce and RHGL (separately; final concentration 6 mM; 4.66 μg Lyce and 3.68 μg RHGL in 10 μl water per gram leaf, respectively) and EV, irBG1, iGPP3 and B × G leaves were coated with 100 μl water (control) using a paint brush. Freshly harvested leaves were reared on these leaves for 12 days (until they reached fourth-instar). Fresh leaf material (1 g, RHGL or water coated) was daily provided to the larvae. Larvae were daily monitored for moulting impairment and mortality. After 12 days, surviving larvae from both control and treatments were used to quantify BG1 transcripts in their midguts by qRT-PCR and Lyce and RHGL levels in their bodies using UHPLC/ESI-QTOF-MS.

**Survivability assays in the field.** Diurnal and nocturnal survivability assays were conducted as described by Kumar et al. M. sexta neonates were fed on EV, irBG1 and iGPP3 plants until they reached second instar. Thirty larvae from each N. attenuata line were placed on respective rosette-stage plants that were planted across a predator-teemed field in a random spatial array. To analyse diurnal survivability, larvae placed on plants in the field at 9000 hours and the number of larvae surviving on each plant was counted at 24 hours; to analyse nocturnal survivability, larvae were placed on plants in the field at 2000 hours and surviving larvae were counted at 0600 hours. Moulting-impaired BG1-silenced larvae were not used in these assays.

**Spider predation bioassays.** All choice and no-choice assays were conducted for 1 h in a 100 cm Cylindrical plastic chamber, as described by Kumar et al. Twelve spiders were collected from in and around the N. attenuata field were used as predators in all assays. Spiders were starved for 12 h before conducting assays. Spiders were not reared in these assays. M. sexta neonates were fed with their respective experimental diets until they reached late second-instar. Late second-instar M. sexta larvae were used as prey in all assays. During the assay, only if the spider captured and killed a larva it was recorded as a spider’s choice in a choice assay or as a spider’s predation in a no-choice assay. Ingestion of larva was recorded only if the spider consumed an entire larva during the assay.

**No-choice assay.** In these assays, only one larva was given to a spider in each assay container. Assays with test and respective control larvae were always performed simultaneously. Whether spiders were the predators that left the killed larva on irBG1 plants was tested with either 1 mM of Lyce or 1 mM of LYXe + 1 mM 6-glucosidase (BG1 inhibitor; Sigma, Germany)34; for 1 h. Then, the assay contents were extracted and their Lyce and RHGL concentrations were determined using UHPLC/ESI-QTOF-MS.

**In vitro BG enzyme assay.** Midguts of larvae feeding on EV and irBG1 plants were homogenized using Dounce homogenizer and incubated with either 1 mM of Lyce or 1 mM of LYXe + 1 mM 6-glucosidase (BG1 inhibitor; Sigma, Germany)34; for 1 h. Then, the assay contents were extracted and their Lyce and RHGL concentrations were determined using UHPLC/ESI-QTOF-MS.

**In vitro BG enzyme assay.** Midguts of larvae feeding on EV and irBG1 plants were homogenized using Dounce homogenizer and incubated with either 1 mM of Lyce or 1 mM of LYXe + 1 mM 6-glucosidase (BG1 inhibitor; Sigma, Germany)34; for 1 h. Then, the assay contents were extracted and their Lyce and RHGL concentrations were determined using UHPLC/ESI-QTOF-MS.
behaviour, larval fed on water-coated EV, irBG1, irGPPS or B or G leaves. Lyc4-coated irGPPS or B or G leaves or RGH4-coated irGPPS or B or G leaves were used. In the assays conducted to evaluate whether externalized Lyc4 or RGH4 can influence spirea’s predator behaviour, water-Lyc4- and RGH4-coated AD-fed larvae were used instead of irGPPS-fed larvae to remove the effect of nicotine present in irGPPS plants because M. sexta larvae exhalate the ingested nicotine to deter spiders.25

Choice assay. In these assays, each spider was allowed to choose between one test and one control larva. Following combinations of test and control larvae were used in the assays conducted to determine whether spiders can choose between control, Lyc4-coated and Lyc4 metabolism-suppressed larva: irBG1 fed + EV fed, irBG1 fed + irGPPS fed and irGPPS fed + EV fed. Similarly, the following combinations of test and control larvae were used in the assays conducted to find larvae: water-coated + water coated and Lyc4 coated + RGH4 coated, AD-fed larvae were used in these assays. Each spider was allowed only one choice during the 1-h assay. Spiders’ choices of larvae were expressed in terms of the percentage of spiders that chose larva from each test treatment.

Feeding M. sexta larvae with water, Lyc4, or RGH4. AD-fed M. sexta larvae (50 ± 5 mg FM) were coated with 50 μl water (control) or 50 μl of 4 mM sodium Lyc4 or RGH4 using a 10-μl microsyringe tip. Coated compounds were allowed to air dry for 5 min, before using the larvae for assays.

Stability of Lyc4 and RGH4 in frass. To test whether Lyc4 and RGH4 degrade in the excrated frass during the 24 h of excetration efficiency determination assay, these metabolites were separately spiked onto fresh frass (0.5% in 50 mg FM frass) and incubated for 24 h at the excetration efficiency determination assay. Samples immediately after spiking (0 h incubated) were used as controls. Metabolites were extracted and quantified from 0 and 24 h samples using HPLC (Agilent 1100 series).

Stability of Lyc4 and RGH4 in coated leaf and larvae. To test whether Lyc4 and RGH4 coated on leaves degraded during assays, these metabolites were extracted from the coated leaves after 0 (control) and 24 h (period for which these leaves were fed to larvae) incubations. Extracted metabolites were analysed using UHPLC/QTOF-MS.

Similarly, to test whether Lyc4 and RGH4 coated on larval body surface degraded during assays, these metabolites were extracted from the coated larvae after 0 h (control) and 1 h (predation assay period) incubations. After 0 and 1 h larvae were washed in 1 ml buffer A (containing 10 mM NaH2PO4, 1 mM Na2EDTA A1) and these washes were analysed for metabolites using UHPLC/QTOF-MS.

Analysis of Lyc4 or RGH4 externalization by larvae. To test whether the larvae externalized Lyc4 or RGH4, second instar EV, irBG1- and irGPPS-fed and water-coated irGPPS- and AD-fed larvae (n = 3) were washed with 1 ml buffer A (containing 10 mM NaH2PO4 1 mM Na2EDTA A1) and the washes were analysed using UHPLC/QTOF-MS, as described above.

Statistical analyses. All the quantitative data (metabolites in leaf material, larval tissues, and larval frass and B1 transcript levels) were analysed by one-way analysis of variance and the statistical significance (P < 0.05) was determined by Fisher’s least significant difference or Games Howell post hoc test. Mean’s resource equation method26 was used to determine the adequate sample sizes. Homogeneity of variance of sample groups was tested using Levene’s test24; sample groups with unequal variances were analysed using Welch’s test26. Significance (P < 0.05) of the binary results of all molting impairment, mortality, survivorship and predation assays was evaluated using Fisher’s exact test. These data were normalized by calculating the percentages for each column to facilitate visual comparisons. Percentages were also analysed by the Fisher’s exact test and the significance applicable to both frequencies and percentages are shown in the figures.

References


60. Welch, D. L. The generalization of Student’s problem when several different population variances are involved. Biometrika 24, 28-35 (1947).

Acknowledgements

We thank the Max Planck Society for the financial support, the Brigham Young University for use of their awesome Little Ranch Preserve field station, Danny Kessler, and Celia Dietzel for support with the field experiments; Dr Klaus Gane, Thomas Hahn and Antje Wissgott for construct generation, plant transformation and technical support; Dr Axel Schmidt, Meredith Scherman, Marlin Kallenbach, Marion Stanton and Mr Sven Heiling for their valuable comments on manuscript; Andreas Schuermann and the MPI-E COB greenhouse team for nurturing the plants in the greenhouse.

Author contributions


Additional information

Supplementary information accompanies this paper at http://www.nature.com/

Reprints and permission information is available online at http://npg.nature.com/


This work is licensed under a Creative Commons Attribution 4.0

International License. The images or other third party material in this

article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/
Supplementary Figure 1. HGL-DTG levels in uninduced and herbivory induced leaves. Total HGL-DTG concentrations \(F_{1,4}=36.88, P \leq 0.0037\); significant differences (threshold: \(P \leq 0.05\)) between means (± SE) determined by Fisher’s LSD test (one-way ANOVA); \(n=3\) and proportions of malonylated and non-malonylated forms in uninduced and \(M.\ sexta\) herbivory-induced \(N.\ attenuata\) leaves. Together with the data in Fig. 1b, which shows proportions of various HGL-DTGs in uninduced and induced leaves, this data shows the quantitative increase contributed by increases in Lyc4, Nic1 and Nic2 contents in herbivory-induced leaves.
Supplementary Figure 2: Structure and key HMBC correlations (\(^1\text{H} \rightarrow \text{C}^{13}\)) of 3-\(\text{O}-[\alpha\text{-rhamnopyranosyl-(1}\rightarrow\text{4}-\beta\text{-glucopyranosyl]-17-hydroxygeranyllinalool (RGHGL)}\). The colors of the arrows correspond to the colors of the cross signals in the spectra shown in Supplementary Fig. 3-7.
Supplementary Figure 3: Partial HMBC spectrum (500 MHz, MeOH-\textit{d}_4) of RGHGL
Supplementary Figure 4: Partial HMBC spectrum (500 MHz, MeOH-$_d_4$) of RGHGL
Supplementary Figure 5: Partial HMBC spectrum (500 MHz, MeOH-$d_4$) of RGHGL
Supplementary Figure 6: Partial HSQC spectrum (500 MHz, MeOH-\textit{d}_4) of RGHGL
Supplementary Figure 7: Partial HMBC spectrum (500 MHz, MeOH-δ4) of RGHGL
Supplementary Figure 8. U(H)PLC/ ESI-QTOF-MS based analysis of Lyc4, RGHGL and rebaudioside A. Standard curves of (a) Lyc4 (b) RGHGL and (c) rebaudioside A (internal standard) revealed linear responses of U(H)PLC/ESI-QTOF MS to them (n= 3 for each concentration of a compound); limit of detection of rebaudioside A was 0.3 ng. Standard curves of (d) Lyc4 and (e) RGHGL (f) rebaudioside A revealed linear responses of HPLC-ELS1A to them (n= 3 for each concentration of a compound); rebaudioside A was used as an external standard for the relative quantification of these compounds by HPLC-ELS1A and its limit of detection was 15 ng. Plots showing linearity in extraction efficiency of (g) Lyc4 and (h) RGHGL (i) rebaudioside A, from standard addition experiments with frass; for both the compounds, extraction efficiency was >90% when 2, 4, 6, 8, 16 and 32 µg of each compound was spiked to 100 mg frass (n= 3 for each spiking concentration) before extraction and analyzed by HPLC-ELS1A.
Supplementary Figure 9. *M. sexta* possesses three *BGs; MsBG2 and MsBG3 transcripts are not upregulated upon Lyc4 ingestion. (a) Phylogeny of *M. sexta* glycoside hydrolases (only ORFs) deciphered using Clustal-W (thousand bootstrapping trials; only the bootstrap values >50 displayed) showing that *M. sexta* transcriptome contains three *BGs; MsBG2 and MsBG3 are 60.2% and 51.5% similar to MsBG1, respectively. BG2 transcripts (relative to ubiquitin) in midguts of fourth-instar larvae feeding on (b) Lyc4-containing EV and Lyc4-deplete irGGPPS plants (n= 6) and (c) artificial diet containing water (control), 6 mM Lyc4 or RGHGL (n= 6). BG3 transcripts (relative to ubiquitin) in midguts of fourth-instar larvae feeding on (d) Lyc4-containing EV and Lyc4-deplete irGGPPS plants (n= 6) and (e) artificial diet containing water (control), 6 mM Lyc4 or RGHGL (n= 6).
Supplementary Figure 10. Characterization of transgenic irBG1 *N. attenuata* lines and BG1-silenced *M. sexta* larvae. (a) Southern hybridization over the HindIII digested genomic DNA, showing the presence of a single copy of transgene fragment inserted in two independently generated transgenic irBG1 *N. attenuata* lines (375-10 and 379-8); wild type control shows the absence of transgene insertion. 1kb DNA ladder was used as a size marker. (b) Northern hybridization showing the presence of BG1 small RNA in irBG1 (375-10) and irBG1 (379-8) *N. attenuata* leaves and in the midguts of fourth-instar larvae feeding on the respective stable transgenic lines; RNA from EV leaves and midgut of larvae those were feeding on EV leaves were used as negative controls, respectively. (c) BG1 transcripts (relative to ubiquitin) in midguts of fourth-instar larvae feeding on the second independently transformed irBG1 line (379-8) \( F_{1,10} = 940.5, P \leq 0.0001 \); significant differences (threshold: \( P \leq 0.05 \)) between means (± SE) determined by Fisher’s LSD test (one-way ANOVA); n = 6], showing equal silencing as that achieved by feeding on irBG1 (375-10) (shown in Fig. 3E). (d) Flowering EV and independently transformed irBG1 plants (375-10 and 379-8) plants showing that the transformation of PMRi construct did not affect plant morphology. (e) Concentrations of various secondary metabolites in leaves of EV and independently transformed irBG1 lines (375-10 and 379-8) (n = 5); since morphologies and the secondary metabolite concentrations and *MsBG1* silencing efficiencies of 375-10 and 379-8 did not differ, 375-10 was randomly selected for the further experimentation. Transcripts (relative to ubiquitin) of (f) *MsBG2* (60.2% similar to *MsBG1*) and (g) *MsBG3* (51.5% similar to *MsBG1*) in the midguts of fourth-instar EV- and irBG1-feeding larvae showing that the off-target silencing of *MsBG2* and *MsBG3* had not occurred while silencing *MsBG1* (n = 6).
Supplementary Figure 11. Characterization of BG1-silenced *M. sexta* larvae. Concentration of (a) Lyc4 [midgut: $F_{1,11} = 2.96$, $P \leq 0.05$; hindgut: $F_{1,11} = 6.41$, $P \leq 0.02$; hemolymph: $F_{1,8} = 14.9$, $P \leq 0.005$; fat body+ skin: $F_{1,11} = 5.926$, $P \leq 0.03$; significant differences (threshold: $P \leq 0.05$) between means ($\pm$ SE) determined by Fisher’s LSD test (one-way ANOVA, separately conducted for each tissue);] and (b) RGHGL [ midgut: $F_{1,11} = 13.7$, $P \leq 0.005$; significant differences (threshold: $P \leq 0.05$) between means ($\pm$ SE) determined by Fisher’s LSD test (one-way ANOVA, separately conducted for each tissue);] in foregut, midgut, hindgut, hemolymph, Malpighian tubules and skin with fat body of fourth-instar larvae feeding on EV and irBG1 (375-10) *N. attenuata* plants. For Lyc4 as well as RGHGL concentrations in foregut, midgut, hindgut, hemolymph, Malpighian tubules and skin with fat body of EV-fed larvae, n= 7, 7, 7, 6, 5 and 7, respectively and for Lyc4 concentration in foregut, midgut, hindgut, hemolymph, Malpighian tubules and skin with fat body of irBG1-fed larvae, n= 7, 6, 6, 4, 5 and 6, respectively. (c) Lyc4 and RGHGL are not degraded in frass over the 24 h period of the excretion efficiency determination assays. Fresh frass was spiked with each metabolite to attain the final concentration of 0.5%; the spiked frass was extracted and analyzed after zero and 24 h of incubation to quantify the recovered metabolite (n= 3). (d) Stages of molting impairment leading to mortality in *M. sexta* larvae feeding on irBG1 plants.
Supplementary Figure 12. BG1 transcripts and Lyc4 and RGHGL concentrations in larvae feeding on Lyc- and RGHGL-supplemented leaves. (a) BG1 transcripts (relative to ubiquitin) in midguts \([F_{7,40} = 9253, P \leq 0.0001; \text{significant differences (threshold: } P \leq 0.05)\) between means (± SE) determined by Games Howell test (Welch’s ANOVA); n= 6] and (b) Lyc4 \([F_{7,24} = 75.39, P \leq 0.0001; \text{significant differences (threshold: } P \leq 0.05)\) between means (± SE) determined by Games Howell test (Welch’s ANOVA); n= 4] and (c) RGHGL \([F_{7,24} = 50.77, P \leq 0.0001; \text{significant differences (threshold: } P \leq 0.05)\) between means (± SE) determined by Games Howell test (Welch’s ANOVA); n= 4] concentrations in the bodies of *M. sexta* larvae after 8d feeding on water coated EV, irBG1, irGGPPS and B×G leaves, Lyc4 coated (final concentration 6 mM) irGGPPS and B×G leaves and RGHGL coated (final concentration 6 mM) irGGPPS and B×G leaves. (d) Lyc4 and RGHGL coated on irGGPPS leaf are not degraded over the 24 h period. Each detached leaf was coated with Lyc4 or RGHGL to attain the final concentration of 6 mM and was extracted and analyzed after zero and 24 h of incubation to quantify the recovered metabolite (n= 5).
Supplementary Figure 13. Spiders do not choose between EV- irBG1- and irGGPPS-fed larvae.

Spider’s prey capture and killing (%) in choice assays (1 h) on second-instar *M. sexta* larvae feeding on (a) EV or irBG1, (b) EV or irGGPPS and (c) irBG1 and irGGPPS plants (n = 30 in all assays). (d) Spider’s prey capture and killing (%) [significant differences (P ≤ 0.05) determined by Fisher’s exact test of frequencies; n = 30] and (e) prey ingestion (% of total killed) [significant differences (P ≤ 0.05) determined by Fisher’s exact test of frequencies; n = 30] in no-choice assays (1 h) on second-instar water-, Lyc4- or RGHGL-coated (final concentration 6 mM for both Lyc4 and RGHGL) *M. sexta* larvae feeding on AD. Spider’s prey capture and killing (%) in choice assays (1 h) on AD-feeding second-instar *M. sexta* larvae coated with (f) water or Lyc4 (final concentration 6 mM) [significant difference (P ≤ 0.05) determined by Fisher’s exact test of frequencies; n = 21], (g) water or RGHGL (final concentration 6 mM) (n = 21) and (h) Lyc4 (final concentration 6 mM) or RGHGL (final concentration 6 mM) [significant difference (P ≤ 0.05) determined by Fisher’s exact test of frequencies; n = 21]. Please note that prey capture and killing percentages using AD-fed larvae are higher than those obtained using irGGPPS-fed larvae (Fig. 6 d-f and h-i) because AD does not contain nicotine, which larvae ingest from irGGPPS plants and exhale to deter spiders. (i) Lyc4 and RGHGL topically coated to the larval body are not degraded over the period of choice or no-choice assays (1 h). Each larva was coated with either Lyc4 or RGHGL to attain the final concentration of 6 mM; it was washed after zero and 1 h of incubation and the wash was analyzed to quantify the recovered metabolite (n = 3).
Supplementary Table 1. $^1$H NMR (500 MHz) and $^{13}$C NMR (125 MHz) chemical shifts for RGHGL in MeOH-$d_4$

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_H, \text{multiplet, } J , (\text{Hz})$</th>
<th>$\delta_C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>5.23, $dd$, 17.8, 1.2</td>
<td>116.0</td>
</tr>
<tr>
<td>1b</td>
<td>5.20, $dd$, 11.1, 1.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.93, $dd$, 17.8, 11.1</td>
<td>144.5</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>81.6</td>
</tr>
<tr>
<td>4</td>
<td>1.59, $m$</td>
<td>42.8</td>
</tr>
<tr>
<td>5</td>
<td>2.05, $m$</td>
<td>23.7</td>
</tr>
<tr>
<td>6</td>
<td>5.11, $t$, 7.3</td>
<td>125.8*</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>136.0</td>
</tr>
<tr>
<td>8</td>
<td>1.99, $m$</td>
<td>41.2**</td>
</tr>
<tr>
<td>9</td>
<td>2.08, $m$</td>
<td>27.6</td>
</tr>
<tr>
<td>10</td>
<td>5.11, $t$, 7.3</td>
<td>125.9*</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>135.8</td>
</tr>
<tr>
<td>12</td>
<td>1.99, $m$</td>
<td>40.9**</td>
</tr>
<tr>
<td>13</td>
<td>2.15, $m$</td>
<td>27.4</td>
</tr>
<tr>
<td>14</td>
<td>5.26, $t$, 7.1</td>
<td>128.6</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>135.7</td>
</tr>
<tr>
<td>16</td>
<td>1.75, $s$</td>
<td>21.7</td>
</tr>
<tr>
<td>17</td>
<td>4.06, $s$</td>
<td>61.5</td>
</tr>
<tr>
<td>18</td>
<td>1.59, $s$</td>
<td>16.2</td>
</tr>
<tr>
<td>19</td>
<td>1.59, $s$</td>
<td>16.2</td>
</tr>
<tr>
<td>20</td>
<td>1.38, $s$</td>
<td>23.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_H, \text{multiplet, } J , (\text{Hz})$</th>
<th>$\delta_C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1'</td>
<td>4.36, $d$, 8.0</td>
<td>99.5</td>
</tr>
<tr>
<td>2'</td>
<td>3.20, $dd$, 8.0, 9.2</td>
<td>75.4</td>
</tr>
<tr>
<td>3'</td>
<td>3.42, $dd$, 9.2, 9.4</td>
<td>77.0</td>
</tr>
<tr>
<td>4'</td>
<td>3.52, $dd$, 9.4, 9.4</td>
<td>79.7</td>
</tr>
<tr>
<td>5'</td>
<td>3.24, $m$</td>
<td>76.6</td>
</tr>
<tr>
<td>6'a</td>
<td>3.76, $dd$, 12.0, 2.2</td>
<td>62.2</td>
</tr>
<tr>
<td>6'b</td>
<td>3.64, $dd$, 12.0, 4.3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_H, \text{multiplet, } J , (\text{Hz})$</th>
<th>$\delta_C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&quot;</td>
<td>4.85, $d$, 1.7</td>
<td>102.9</td>
</tr>
<tr>
<td>2&quot;</td>
<td>3.83, $dd$, 3.3, 1.7</td>
<td>72.5</td>
</tr>
<tr>
<td>3&quot;</td>
<td>3.63, $dd$, 9.6, 3.3</td>
<td>72.3</td>
</tr>
<tr>
<td>4&quot;</td>
<td>3.40, $dd$, 9.6, 9.4</td>
<td>73.9</td>
</tr>
<tr>
<td>5&quot;</td>
<td>3.96, $dq$, 9.4, 6.2</td>
<td>70.7</td>
</tr>
<tr>
<td>6&quot;</td>
<td>1.26, $d$, 6.2</td>
<td>18.0</td>
</tr>
</tbody>
</table>

*, **: May be interchanged
Supplementary Table 2. *M. sexta* gene primers used in various experiments

<table>
<thead>
<tr>
<th>Primer pair No</th>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MsBG1.1</td>
<td>For- CTCGCTTGTATGGCGGGT</td>
<td>Amplification of 301bp fragment to clone in PMRi vector</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev- GCGGCAGTGGCTGCAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>For- TCGTCCTTCTCGTTGTGAT</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>MsBG1.2</td>
<td>Rev- GCTGCACCAACAGAAATCC</td>
<td>Transcript quantification and testing the silencing efficiency of <em>M. sexta</em> BG1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For- TCGAGTCTCTAGCTCTCATCA</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MsBG1.3</td>
<td>Rev- GGACAAAAACGTGTCACATGGTA</td>
<td>Generation of probe for northern hybridization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For- CCACGGTCACAAGTTAAGGC</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MsBG2</td>
<td>Rev- CTTTTGCGTCCTCATCATCC</td>
<td>Transcript quantification and testing the co-silencing efficiency of <em>M. sexta</em> BG2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For- GGATCTGCCGCAAAGACTG</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MsBG3</td>
<td>Rev- TCACTCTATCGCCGAAAGTTTCTC</td>
<td>Transcript quantification and testing the co-silencing efficiency of <em>M. sexta</em> BG3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>For- GGAATCCTTGATTGGCGGTT</td>
<td></td>
</tr>
</tbody>
</table>
Supplementary Table 3. APHIS notification numbers for importing transgenic *N. attenuata* seeds and releasing plants at the field station, Utah, USA.

<table>
<thead>
<tr>
<th>Line</th>
<th>Import #</th>
<th>Year</th>
<th>Release#</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV</td>
<td>07-341-101n</td>
<td>2013</td>
<td>13-051-101r</td>
</tr>
<tr>
<td>irGGPPS</td>
<td>07-341-101n</td>
<td>2013</td>
<td>13-051-101r</td>
</tr>
<tr>
<td><em>(NaGGPPS</em> NCBI accession no. EF382626)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>irBG1</td>
<td>10-004-105m</td>
<td>2013</td>
<td>13-051-101r</td>
</tr>
<tr>
<td><em>(MsBG1</em> NCBI accession no. FK816842)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Manuscript II

Plant-mediated RNAi silences midgut-expressed genes in congeneric lepidopteran insects in nature

Spoorthi Poreddy, Jiancai Li and Ian T. Baldwin
Manuscript submitted in the Journal of Integrative Plant Biology
Plant-mediated RNAi silences midgut-expressed genes in congeneric lepidopteran insects in nature

Running title: Homologous gene silencing in Manduca spp by PMRi

Spoorthi Poreddy, Jiancai Li and Ian T. Baldwin*

Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Str. 8, DE-07745 Jena, Germany

*Correspondence:

Prof. Ian T. Baldwin
Department of Molecular Ecology
Max Planck Institute for Chemical Ecology
Hans-Knöll-Straße 8, D-07745 Jena
email: baldwin@ice.mpg.de
Phone: +49 (0)3641 57 1100
Fax: +49 (0)3641 57 1102
Abstract

Plant-mediated RNAi (PMRi) silencing of insect genes has enormous potential for crop protection, but whether it works robustly under field conditions, particularly with lepidopteran pests, remains controversial. Wild tobacco *Nicotiana attenuata* and cultivated tobacco (*N. tabacum*) (Solanaceae) is attacked by two closely related specialist herbivores *Manduca sexta* and *M. quinquemaculata* (Lepidoptera, Sphingidae). When *M. sexta* larvae attack transgenic *N. attenuata* plants expressing double-stranded RNA (dsRNA) targeting *M. sexta’s* midgut-expressed genes, the nicotine-ingestion induced *cytochrome P450 monooxygenase* (irCYP6B46-plants) and the lyciumoside-IV-ingestion induced *β-glucosidase*1 (irBG1-plants), these larval genes which are important for the larvae’s response to ingested host toxins, are strongly silenced. Here we show that the PMRi procedure also silences the homologous genes in native *M. quinquemaculata* larvae feeding on these transgenic plants in nature. The PMRi lines shared 98 and 96% sequence similarity with *M. quinquemaculata* homologous coding sequences, and CYP6B46 and BG1 transcripts were reduced by ca. 90 and 75%, without reducing the transcripts of the larvae’s most similar, potential off-target genes. We conclude that the PMRi procedure can robustly and specifically silence genes in native congeneric insects that share sufficient sequence similarity and with the careful selection of targets, can protect crops from attack by congeneric-groups of insect pests.

Keywords: RNA interference, Plant-mediated RNAi, *Manduca quinquemaculata*, *CYP6B46*, *β-glucosidase*, transgenic tobacco plants.
INTRODUCTION

RNA interference (RNAi) is a sequence-specific, double stranded RNA (dsRNA) induced gene-silencing mechanism that operates at transcriptional or post-transcriptional levels and is conserved across all the eukaryotes (Fire et al. 1998), including lepidopteran insects (Terenius et al. 2011). Since the RNAi mechanism was first demonstrated in Caenorhabditis elegans in 1998, RNAi has emerged as a potent gene-silencing tool for loss-of-function analyses in a wide range of organisms (Fire et al. 1998; Kalleda et al. 2013; Younis et al. 2014). RNAi has emerged as the most powerful technique to analyze gene function in insects, for which stable transgenesis is not available (Gordon and Waterhouse 2007; Terenius et al. 2011). The success of RNAi in insects is highly dependent on the insect-species, target gene and its function, organ of gene expression and mode of delivery of silencing molecules (Huvenne and Smagghe 2010; Terenius et al. 2011). RNAi-mediated gene silencing in some insect species is quite robust and can even be transmitted to subsequent generations via germ line transmission (Bucher et al. 2002; Lynch and Desplan 2006; Mito et al. 2008). In contrast, lepidopteran species, particularly in laboratory experiments with injected dsRNA are recalcitrant to the procedure (Terenius et al. 2011; Garbutt and Reynolds 2012).

Although the silencing of target genes in insects is achieved by various dsRNA introduction strategies, such as microinjection or oral delivery via artificial diets (Yu et al. 2013), gene silencing can be enhanced and possibly achieved under field conditions by engineering host plants to produce dsRNAs (Gordon and Waterhouse, 2007). An efficient method to down-regulate cotton bollworm defense genes using plant-mediated RNAi (PMRi) has been described by engineering host plants to produce dsRNAs directed against bollworm larvae's P450 monooxygenase (CYP6AE14) gene in laboratory experiments (Mao et al. 2007). Several studies have demonstrated successful gene silencing in various insect orders such as Coleoptera, Lepidoptera and Hemiptera using stable or transient transgenic PMRi plants (Baum et al. 2007; Mao et al. 2007; Mao et al. 2011; Pitino et al. 2011; Zha et al. 2011; Kumar et al. 2012; Liu et al. 2015; Mamta et al. 2015). A recent advance in PMRi to control insects was achieved by expressing exogenous dsRNAs targeted against the β-actin gene of the Colorado potato beetle (CPB) via chloroplast transformation in potato. Transplastomic potato plants were shown to be
lethal to CPB larvae and were protected from CPB attack in glasshouse experiments (Jin et al. 2015).

The capacity of insect pests to adapt to conventional insecticides (Kranthi et al. 2002) or to *Bacillus thuringiensis* (Bt) expressing plants (Storer et al. 2010) is an ongoing concern for the long-term pest management of crop plants. The PMRi approach offers the potential to develop insect-resistant crops that produce insecticidal dsRNAs, but only a few reports have demonstrated its potential under field conditions (Pitino et al. 2011; Kumar et al. 2014; Liu et al. 2015; Poreddy et al. 2015). Crop plants are commonly attacked by congeneric insect pests, for instance *Spodoptera litura* and *S. exigua* are both major pests of various crops (Xiu et al. 2008); *Helicoverpa armigera* and *H. punctigera* are major pests of cotton (Liao et al. 2002); *H. armigera* and *H. zea* attack maize plants (Fitt 1989). Hence whether PMRi can silence conserved essential genes in congeneric insect pests under field condition would be an important test of this procedures utility as a crop protection tool.

The congeneric species, tobacco hornworm (*Manduca sexta*, Linnaeus, 1763) and tomato hornworm (*M. quinquemaculata*, Haworth, 1803) (Lepidoptera: Sphingidae) are closely related sympatric sibling species (Halitschke et al. 2001; Kessler and Baldwin 2004; Kawahara et al. 2013) that both attack solanaceous crops in North America (Kester and Barbosa 1994). These two insects have similar morphologies (Sannino et al. 1995), behavior (Peterson et al. 1993), and ecology (Bossart and Gage 1990) and their host-plant interactions have been intensely studied (Raguso and Willis 1997; Schittko et al. 2000; Kessler and Baldwin 2002, 2004). Both of these hornworm species are significant pests on solanaceous crops in North America. In nature, the annual tobacco plant *Nicotiana attenuata* Torr. ex Wats, native to Great Basin Desert of southwestern Utah is a common host plant for both of these pest insects and both also attack cultivated tobacco.

Recently, we elucidated *M. sexta*’s counter-adaptation strategies against two of *N. attenuata*’s major chemical defenses, namely nicotine and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs), by silencing *M. sexta*’s midgut-expressed *cytochrome P450 monooxygenase* (*CYP6B46*) and *β-glucosidase1* (*BG1*) genes. This was accomplished by developing transgenic *N. attenuata* PMRi lines that expressed inverted repeat (ir) constructs of CYP6B46 and BG1 and planting these plants into field plots in the plants and insects native
habitat (Kumar et al. 2014; Poreddy et al. 2015). Since *M. sexta* and *M. quinquemaculata* are closely related, co-occurring species in *N. attenuata*'s native habitat (Kessler and Baldwin 2001), we hypothesized that PMRi plants producing *M. sexta*'s CYP6B46 and BG1 dsRNA could silence the homologous genes in native *M. quinquemaculata*.

**RESULTS**

**Experimental system used to evaluate homologous gene silencing**

*M. sexta* and *M. quinquemaculata* are two specialist herbivores of *N. attenuata* (Figure 1A). Previously, we silenced the expression of CYP6B46 and BG1 in *M. sexta* larval midguts using PMRi lines irCYP6B46 (Kumar et al. 2014) and irBG1 (Poreddy et al. 2015), respectively. These stable transgenic irCYP6B46 and irBG1 *N. attenuata* lines were generated using *Agrobacterium tumefaciens*-mediated transformation with an inverted repeat of 312 and 301 bp *MsCYP6B46* and *MsBG1* complementary DNA (cDNA), respectively. Here, we utilized these PMRi plants generated from *M. sexta* genes to examine the silencing of the homologous genes in a feral population of *M. quinquemaculata* (Figure 1B).

*M. sexta CYP6B46 and BG1 genes share high sequence similarity with *M. quinquemaculata* homologous genes*

Since PMRi operates in a sequence specific manner to silence target gene transcripts, homologous gene silencing depends on sequence similarities between homologous genes particularly in the regions that are used in the generation of the transgenic plants. To test the sequence similarities of *MsCYP6B46* and *MsBG1* with their corresponding homologous genes in *M. quinquemaculata*, we aligned the partial coding sequences of *MsCYP6B46* (312 bp) and *MsBG1* (301 bp) used in the generation of the irCYP6B46 and irBG1 PMRi plants with the coding regions of *M. quinquemaculata*’s homologous genes. We found that the *M. sexta* CYP6B46 fragment cloned to generate the irCYP6B46 plants shared 98% sequence similarity with the *M. quinquemaculata* CYP6B46 sequence (Figure 2A) and that the *M. sexta* BG1 fragment cloned to generate the irBG1 plants shared 96% sequence similarity with the *M. quinquemaculata* BG1 sequence (Figure 2B). Notably, several identical homologous regions of >21nt were identified in the alignments (Figure 2A, B).
Silencing of *CYP6B46* and *BG1* in wild *M. quinquemaculata*

Naturally oviposited wild *Manduca* spp eggs (Figure 3A) were collected from the study area and larvae were reared on EV, irPMT, irGGPPS, irCYP6B46 and irBG1 plants (Table 1) that were planted in a field plot located in *N. attenuata*'s native habitat (Figure 3B), until they had reached the fourth-instar. The two species are easily distinguished when they attain the third-instar. In *M. sexta* larvae CYP6B46 and BG1 are the midgut-expressed genes that are induced in response to the ingestion of nicotine and lyciumoside IV (a major HGL-DTG), respectively. To test whether these candidate genes were midgut expressed in *M. quinquemaculata*, and are induced by the ingestion of nicotine and lyciumoside IV, we profiled their transcript levels in various tissues, including foregut, midgut, hindgut, hemolymph, Malpighian tubules and fat body along with skin, in nicotine and lyciumoside IV containing EV-fed control, nicotine depleted irPMT-fed, and lyciumoside IV depleted irGGPPS-fed larvae. Both the genes, namely CYP6B46 (P≤0.001; Figure 3C) and BG1 (P≤0.001; 3D) were found to have relatively higher expression levels in the midguts of larvae feeding on EV plants than in the larvae feeding on irPMT and irGGPPS plants, respectively suggesting that feeding on nicotine- and lyciumoside IV-containing EV plants strongly induced the midgut expression of CYP6B46 and BG1 transcripts. Although the transcript abundance of CYP6B46 was higher (P≤0.005; Figure 3C) in hindguts and Malpighian tubules of larvae feeding on EV plants than in the larvae feeding on irPMT plants, the CYP6B46 expression in these tissues is significantly lower (six fold) as compared to that of midguts.

Midguts of irCYP6B46-fed larvae showed >90% lower abundance of CYP6B46 transcripts than that of EV-fed larvae. Feeding on the irCYP6B46 PMRi plants which contain nicotine levels similar to those of EV plants reduced the CYP6B46 transcript levels to that found in larvae feeding on nicotine-depleted irPMT plants (Figure 3C; $F_{2,15}=7.219$ P≤ 0.006). Transcript abundance of BG1 in the midguts of irBG1-fed larvae was >80 % lower than that of EV-fed larvae and the reduction in BG1 transcripts was not observed in other tissues of irBG1-fed larvae. Feeding on the irBG1 PMRi plants which contain HGT-DTGs similar to those of EV plants reduced the BG1 transcript abundance to levels found in larvae feeding on HGT-DTG-depleted irGGPPS plants (Figure 3D; $F_{2,14} = 9.458$ P≤ 0.002).
Sequence analysis of *MqCYP6B46* and *MqBG1* with their potential off-target genes

Gene silencing may also result in silencing of non-target genes that belong to closely related gene families if they share high sequence similarities in the targeted regions. Off-target effects could be minimized by using a region of coding sequence for inverted repeat construct preparation which is specific to the targeted gene and do not share high sequence similarity with other closely related genes. Sequence similarities of *MqCYP6B46* and *MqBG1* coding sequences with their corresponding potential off target genes *MqCYP6B45* and *MqBG2*, respectively were determined by aligning their coding regions. *MqCYP6B46* partial coding sequence had 82% sequence similarity to *MqCYP6B45* and one identical homologous region with a continuous stretch >21 nt (Figure 4A). The *MqBG1* partial coding sequence shared 71% sequence similarity with *MqBG2* and no identical homologous regions with a continuous stretch >21 nt (Figure 4B).

**Silencing of *MqCYP6B46* and *MqBG1* is target gene specific**

To determine whether silencing of *MqCYP6B46* and *MqBG1* was target-gene specific, we quantified the transcript levels of *MqCYP6B45* and *MqBG2*, respectively. CYP6B45 transcript levels in the midguts of larvae feeding on irCYP6B46 were as not reduced in comparison to those of EV-fed larvae (Figure 4C). From these results, we infer that the one identical continuous stretch >21 nt was not used in the production of potential small interfering RNA or that the silencing signal was not sufficient to reduce CYP6B45 transcript levels. Transcript abundance of BG2 in the midguts of larvae feeding on irBG1 plants was also not reduced and even slightly higher than those of EV-fed larvae (Figure 4D), suggesting that the silencing of BG1 may have up-regulated its closely related off-target gene to compensate for the loss of function of the target gene (Kalleda et al. 2013). These results suggest that *MqCY6B46* and *MqBG1* silencing by PMRi was highly sequence-specific and free of off-target effects.

**DISCUSSION**

RNAi is conserved in all eukaryotic organisms. The RNAi-mediated gene silencing in insects can be achieved by various dsRNA delivery methods. In this study, we showed that the homologous target genes in the midgut of the lepidopteran *M. quinquemaculata* can be suppressed by feeding native *M. quinquemaculata* larvae on transgenic *N. attenuata* plants producing dsRNA against *M. sexta* midgut-expressed genes, under field conditions.
The RNAi-induced silencing in insects is initially achieved majorly via three dsRNA-delivery methods, such as injection (Mutti et al. 2006), ingestion (Baum et al. 2007) and feeding dsRNA producing bacteria (Tian et al. 2009). These delivery methods were not efficient in silencing of target genes in lepidopteran insects perhaps due to the instability of dsRNAs upon exposure to the nuclease rich environment of the larval gut. In *M. sexta*, RNAi-mediated gene silencing was first achieved by injection of dsRNAs targeting the integrin-beta1 gene, a haemocyte-specific integrin required for haemocytic encapsulation (Levin et al. 2005). Silencing of integrin-beta1 gene using RNAi decreased the encapsulation of haemocytes. Injection of the pathogenic bacteria, *Photorhabdus luminescens* into *M. sexta* haemolymph induces the expression of an antimicrobial protein nitric oxide synthase in the fat body and haemocytes (Eleftherianos et al. 2009). Knock-down of nitric oxide synthase expression throughout the larvae using RNAi increased the mortality of infected larvae, suggesting that nitric oxide synthesis is important for larval immune defense (Eleftherianos et al. 2009). RNAi-induced silencing in larval chemosensory tissues was achieved by Howlett and colleagues by feeding dsRNA corresponding to a coding region of the *M. sexta* olfactory coreceptor, a chemosensory receptor gene (Howlett et al. 2012).

In addition to the abovementioned successful RNAi-induced silencing studies in *M. sexta*, Terenius and colleagues have also reported the variable sensitivity of *M. sexta* larvae to RNAi techniques, particularly when dsRNA is delivered by injection (Terenius et al. 2011). The efficiency of target gene silencing may be enhanced by the continuous supply of dsRNA, because the core RNAi genes in *M. sexta* larvae are known to be induced in response to the dsRNA injection and elevated when contact with exogenous dsRNA is prolonged (Garbutt and Reynolds 2012). Injected dsRNA into *M. sexta* hemolymph is unstable and rapidly degraded by RNAses (Garbutt et al. 2013).

The PMRi approach, in which the insect’s host plant is utilized as an oral delivery vehicle of dsRNAs could circumvent the problem of dsRNA degradation by continuously supplying dsRNA to feeding larvae (Baum et al. 2007; Mao et al. 2011). *M. sexta* midgut-expressed, nicotine-ingestion induced *CYP4B46, CYP4M1* and *CYP4M3* genes are efficiently silenced without any co-silencing of closely related non target genes, using plant virus based dsRNA-producing system (VDPS) (Kumar et al. 2012). VDPS based target gene silencing is rapid and
does not require the laborious generation of stable transgenic plants; however, this method is transient and cannot yet be used under field conditions.

MATERIALS AND METHODS

Plant material

All the stable transgenic *N. attenuata* lines EV (Schwachtje et al. 2008), irPMT (Steppuhn et al. 2004), irGGPPS (Heiling et al. 2010), irCYP6B46 (Kumar et al. 2012) and irBG1 (Poreddy et al. 2015) used in the study were previously characterized. irPMT *N. attenuata* plants (A-03-108-3) were used as nicotine deficient host plants, irGGPPS *N. attenuata* plants (A-07-230-5) were used as HGL-DTG depleted host plants, irCYP6B46 transgenic *N. attenuata* PMRi plants (A-09-30-2) were used to silence *M. quinquemaculata* CYP6B46, irBG1 transgenic *N. attenuata* PMRi plants (A-08-375-10) were used to silence *M. quinquemaculata* BG1 and EV-transformed plants (A-04-266-3) were used as transgenic controls. Silencing of *M. sexta* CYP6B46 and BG1 using irCYP6B46 and irBG1 PMRi plants, respectively was reported previously (Kumar et al. 2014; Poreddy et al. 2015).

These transgenic lines were developed from *N. attenuata* 30× inbred seeds, which was originally collected in 1988 from a native population in Utah (USA) (Krügel et al. 2002). Field experiments were conducted at the Lytle Ranch Preserve in Santa Clara, Utah, 84765 (37° 08' 45" N, 114° 01' 11"W) in 2014. Seeds of *N. attenuata* EV, irPMT, irGGPPS, irCYP6B46 and irBG1 lines were imported and released in accordance with Animal and Plant Health Inspection Service notifications (Table 1). Seedlings of *N. attenuata* were transferred to 50 mm peat pellets (Jiffy) 15 days after germination, then seedling were kept in the shade to get acclimatized to the environmental conditions of high sunlight and low relative humidity over 14 days. Adapted early rosette-stage plants were transplanted into the field plot and plants were watered regularly until roots were established.

*M. quinquemaculata*

Wild *Manduca spp.* eggs were collected from natural ovipositions on native *N. attenuata* and *Datura* populations in the study area during May, 2014. The hatched neonates were transferred to rosette-stage *N. attenuata* plants and reared until they had reached the third instar
when the two species, the tobacco hornworm (*M. sexta*) and the tomato hornworm (*M. quinquemaculata*) can be morphologically distinguished. When the *M. quinquemaculata* larvae had reached the fourth-instar they were used in all the experiments.

**Harvesting larval tissues**

Fourth-instar *M. quinquemaculata* larval tissues such as foregut, midgut, hindgut, hemolymph, Malpighian tubules and fat body along with skin were collected in RNA-later for transcript profiling of target and off-target genes. Larvae were immobilized by placing them on ice before dissection. First, haemolymph was collected by clipping the larval horn, as previously described (Kumar et al. 2012). Then, larvae were dissected to collect various tissues. Gut content and peritrophic membrane was removed before midgut collection. Dissected tissues were immediately transferred to RNA-later solution (Ambion) and stored at -80°C as recommended by the manufacturer, until further use.

**Total RNA isolation and cDNA synthesis**

Tissues stored in RNA later solution were recovered and total RNA was extracted using TRI reagent (Invitrogen) according to manufacturer’s protocol. Isolated total RNA was subjected to TURBO DNase (Ambion) treatment to remove genomic DNA contamination. For each sample, 500 ng of total RNA was used for cDNA synthesis using oligo(dT)18 primer and SuperScript II enzyme (Invitrogen) according to manufacturer’s instructions.

**Sequencing of partial coding regions**

*M. quinquemaculata* *Ubiquitin*, *CYP6B46*, *CYP6B45*, *BG1* and *BG2* gene sequences were initially retrieved from *M. quinquemaculata* midgut transcriptome and partial coding sequences of all these genes were sequenced by Sanger dideoxy sequencing method. The obtained partial coding sequences of *Ubiquitin*, *CYP6B46*, *CYP6B45*, *BG1* and *BG2* were submitted to NCBI under the accession numbers KX074011, KX074015, KX074014, KX074013 and KX074012, respectively. The primer sequences used for amplification of partial coding sequences were listed in Table 2.
Real time quantitative PCR

Transcript levels of *Ubiquitin, CYP6B46, CYP6B45, BG1* and *BG2* were determined by qRT–PCR conducted in Mx3005P Multiplex qPCR system (Stratagene) using qRT–PCR SYBR Green I kit (Eurogentec) (Kumar et al. 2012). Relative quantification of transcripts was carried out by the comparative D cycle threshold method. *Mq*Ubiquitin levels were used as internal controls to normalize the abundance of other transcripts. All the primer pairs (Table 2) used in qRT–PCR were designed using Primer3 software version 4.0 (http://primer3.ut.ee/).

Sequence similarity

*M. sexta's CYP6B46* and *BG1* coding sequences used to generate PMRi lines with corresponding homologous coding sequences of *M. quinquemaculata* were aligned and sequence similarities were determined using nucleotide BLAST (www.ncbi.nlm.nih.gov). Aligned regions were scrutinized for continuous stretches >21nt. In a similar way, *Mq*CYP6B46 and *Mq*BG1 partial coding sequences were aligned with potential off-target genes to determine their sequence similarity.

Statistical analysis

All statistical analyses were performed with StatView version 5 (SAS Institute Inc.). All transcript levels were analyzed by one-way analysis of variance and the statistical significance (P ≤ 0.05) was determined by Fisher’s least significant difference post hoc tests.

ACKNOWLEDGEMENTS

This research was supported by Max Plank Society. We thank Brigham Young University for use of their Lytle Ranch Preserve field station, and Danny Kessler, Celia Diezel and Pia Backmann for support with the field experiments, and all Utah folks of field season 2014 for their assistance in collecting wild *Manduca spp* eggs, and Sagar Pandit and Shuquing Xu for sharing *M. quinquemaculata* midgut transcriptome, and Mariana Stanton and Sagar Pandit for the scientific discussions, and Klaus Gase and Thomas Hahn for assistance with molecular work.
AUTHOR CONTRIBUTIONS

SP designed and performed the lab and field experiments, analyzed the data and drafted the manuscript. JL performed the lab experiments and ITB supervised the study and revised the manuscript.

REFERENCES


### TABLES

Table 1. APHIS notification numbers for importing seeds and releasing transgenic *N. attenuata* plants.

<table>
<thead>
<tr>
<th>Line</th>
<th>Import #</th>
<th>Year</th>
<th>Release #</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV</td>
<td>07-341-101n</td>
<td>2014</td>
<td>13-350-101r</td>
</tr>
<tr>
<td>irPMT (NaPMT NCBI accession no. AF280402)</td>
<td>07-341-101n</td>
<td>2014</td>
<td>13-350-101r</td>
</tr>
<tr>
<td>irGGPS (NaGGPS NCBI accession no. EF382626)</td>
<td>07-341-101n</td>
<td>2014</td>
<td>13-350-101r</td>
</tr>
<tr>
<td>irCYP6B46 (MsCYP6B46 NCBI accession no. GU731529)</td>
<td>10-004-105m</td>
<td>2014</td>
<td>13-350-101r</td>
</tr>
<tr>
<td>irBG1 (MsBG1 NCBI accession no. FK816842)</td>
<td>10-004-105m</td>
<td>2014</td>
<td>13-350-101r</td>
</tr>
<tr>
<td>Primer pair No</td>
<td>Gene</td>
<td>Primer sequences (5’-3’)</td>
<td>Use</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------</td>
</tr>
</tbody>
</table>
| 1             | MqUbiquitin   | For- CAAGAAGCGCAAGAAGAAGAAGAAGAAC  
Rev- CGTCCACCTTGTAGAAGCCCTAAG | Internal control for *M. quinquemaculata* transcript quantification     |
| 2             | MqBG1         | For- CCAACCCGCTATGCTGATAAAA  
Rev- GTGACCATGGGGTTGGATGTT | Transcript quantification and testing the silencing efficiency of *M. quinquemaculata* BG1 |
| 3             | MqBG2         | For- GCTGTATGTTACGCGAAGAAGAAGAAGGAG  
Rev- CACGCGCTTCTCAGTGATGATA | Transcript quantification and testing the co-silencing efficiency of *M. quinquemaculata* BG2 |
| 4             | MqCYP6B46     | For- GTGCCTATTACTCCGCGATCTA  
Rev- CAAGCCTTCTTTGCTAAACTCTC | Transcript quantification and testing the silencing efficiency of *M. quinquemaculata* CYP6B46 |
| 5             | MqCYP6B45     | For- GAAATGGGATAAAATGTTTGGTACC  
Rev- TTATTTGACAGAAGAGATGGAGG | Transcript quantification and testing the co-silencing efficiency of *M. quinquemaculata* CYP6B45 |
| 6             | MqUbiquitin   | For- CGACTACAACATCCGAGAAGAAGAAGAAGGAG  
Rev- GGCTTCAGCTACATCTTAGTC | Amplification of partial coding sequence of *M. quinquemaculata* Ubiquitin |
| 7             | MqBG1         | For- GAAGTTTGTAGTGCCTCGCC  
Rev- GTGACCATGGGGTTGGATGTT | Amplification of partial coding sequence of *M. quinquemaculata* BG1 |
| 8             | MqBG2         | For- GCTGTATGTTACGCGAAGAAGAAGAAGAAGGAG  
Rev- GGCTGAATATTGTATTTAGC | Amplification of partial coding sequence of *M. quinquemaculata* BG2 |
| 9             | MqCYP6B46     | For- TGCCTATTACTCCGCGATCTA  
Rev- TCAATTCGCTCTGCTAGGT | Amplification of partial coding sequence of *M. quinquemaculata* CYP6B46 |
| 10            | MqCYP6B45     | For- GATCAAAGATTTCGACGTTTACCATTTAGTTTACATTTAGTTAAGGAG  
Rev- ACTTTGAGAGGGAAGAGATTGAAA | Amplification of partial coding sequence of *M. quinquemaculata* CYP6B45 |
Figure 1. Experimental system used to evaluate if the PMRi lines generated from *Manduca sexta* genes can silence *M. quinquemaculata* homologous genes.

(A) Fifth-instar *M. sexta* and its closely related species *M. quinquemaculata* larvae on their native host plant, the wild tobacco *Nicotiana attenuata* in Great Basin Desert of southwestern Utah. (B) Schematic overview of plant-mediated RNAi: the binary vector constructed to independently express ~300 bp dsRNA of *M. sexta*'s target genes such as cytochrome P450 monooxygenase (*CYP6B46*) and β-glucosidase 1 (*BG1*) in *N. attenuata*. The trophic transfer of these dsRNA from plant to *M. sexta* larvae silences their respective target gene expression. PMRi lines generated with *M. sexta* dsRNA were used to test if the trophic transfer can also silence homologous gene expression in *M. quinquemaculata* larvae in nature.
Figure 2. Alignments of *M. sexta* cDNA regions selected to generate PMRi lines with *M. quinquemaculata* homologs.

(A) *M. sexta* CYP6B46 fragment cloned to generate inverted-repeat (ir) CYP6B46 showed 98.1% sequence similarity with *M. quinquemaculata* CYP6B46 and (B) *M. sexta* BG1 fragment cloned to generate irBG1 showed 96% similarity with *M. quinquemaculata* BG1. Identical homologous regions of >21nt were identified in the alignment of *M. sexta* CYP6B46 and BG1 cDNA regions selected to generate PMRi lines with that of *M. quinquemaculata* CYP6B46 and BG1, respectively.
**Figure 3**

(A) Wild *Manduca* spp eggs oviposited on *Datura wrightii* provided the source of *M. quinquemaculata* larvae which can be distinguished from *M. sexta* larvae when the larvae reach the third-instar. (B) PMRi *N. attenuata* lines and *N. attenuata* plants transformed by RNAi to

---

**Figure 3.** Silencing of midgut-expressed *CYP6B46* and *BG1* genes in wild *M. quinquemaculata* larvae feeding on WT, PMRi, and nicotine and DTG-depleted plants

(A) Wild *Manduca* spp eggs oviposited on *Datura wrightii* provided the source of *M. quinquemaculata* larvae which can be distinguished from *M. sexta* larvae when the larvae reach the third-instar. (B) PMRi *N. attenuata* lines and *N. attenuata* plants transformed by RNAi to
silence: nicotine biosynthesis, by expressing an inverted repeat (ir) construct of the host plant’s putrescine N-methyl transferase (irPMT) and 17-hydroxygeranyllinalool diterpene glycoside (HGL-DTGs) biosynthesis, by expressing an ir construct of geranylgeranyl pyrophosphate synthase (irGGPPS); planted in a field plot in Great Basin Desert of southwestern Utah. (C) M. quinquemaculata CYP6B46 transcripts (relative to ubiquitin) in various tissues of fourth-instar larvae fed on EV, irCYP6B46 and irPMT plants (midgut: $F_{2,15} = 7.219$ $P \leq 0.006$; hindgut: $F_{2,15} = 6.651$ $P \leq 0.008$; Malpighian tubules: $F_{2,15} = 10.604$ $P \leq 0.001$; n = 6 in all bars). Note that feeding on nicotine-containing WT plants strongly induces the midgut expression of CYP6B46 transcripts and that feeding on the PMRi plants which contain WT levels of nicotine deplete CYP6B46 transcript abundance to levels found in larvae feeding on nicotine-depleted irPMT plants. (D) M. quinquemaculata BG1 transcripts (relative to ubiquitin) in various tissues of fourth-instar larvae feeding on EV, irBG1 and irGGPPS plants (midgut: $F_{2,14} = 9.458$ $P \leq 0.002$; n = 6 in EV, GGPPS and 5 in BG1 group). Note that feeding on HGL-DTG-containing WT plants strongly induces the midgut expression of BG1 transcripts and that feeding on the PMRi plants which contain WT levels of HGT-DTGs deplete BG1 transcript abundance to levels found in larvae feeding on HGT-DTG-depleted irGGPPS plants. Asterisks indicate significant differences between means (± SE) in comparison to EV, determined by one way ANOVA and Fisher LSD post hoc, which was conducted separately for each tissue.
Figure 4: Silencing of *MqCYP6B46* and *MqBG1* does not silence the most closely related, likely non-target genes.

(A) Alignment of *MqCYP6B46* partial coding sequence with *MqCYP6B45* showed 83% sequence similarity and one identical homologous region >21nt was identified. (B) Alignment of *MqBG1* coding sequence with *MqBG2* showed 71% sequence similarity and no identical homologous regions >21nt were identified. Transcripts (relative to ubiquitin) of (C) *MqCYP6B45* and (D) *MqBG2* in the midguts of fourth-instar EV-, irCYP6B46 and EV-, irBG1-feeding larvae did not show off-target silencing of CYP6B45 and BG2, respectively, (n= 6).
Discussion

In the course of co-evolution, plants and insects have developed an impressive array of defense and counter-defense strategies to deal with each other. Plants produce several secondary defense metabolites to thwart herbivory. In response, herbivores use adaptation strategies such as detoxification, rapid excretion and sequestration of plant defense compounds (Self et al. 1964; Nishida 2002; Despres et al. 2007). I discovered \( \beta \)-glucosidase mediated an unusual detoxification mechanism adapted in \( M. \) sexta larvae against their host \( N. \) attenuata's diterpene glycosides, lyciumoside IV and its malonylated forms, using PMRi. Silencing of \( M. \) sexta's lyciumoside IV-ingestion induced \( \beta \)-glucosidase1 impaired the detoxification of lyciumoside IV and \( \beta \)-glucosidase1-silenced larvae experienced molting impairments and higher mortality. Moreover, wolf spiders, the nocturnal native predator of \( M. \) sexta larvae has showed increased unpalatability to \( \beta \)-glucosidase1-silenced larvae because of higher lyciumoside IV content in their bodies. This suggests that co-option of lyciumoside IV would be advantageous to \( M. \) sexta larvae but larvae have adapted to detoxify this allelochemical perhaps to avoid its harmful effects (Manuscript I). Further, I applied PMRi to silence midgut genes in \( M. \) sexta's closely related species \( M. \) quinquemaculata under field conditions and showed that the PMRi procedure can robustly and specifically silence genes in native congeneric insects that share sufficient sequence similarity (Manuscript II).

Functional genomics using RNAi technology has developed as a powerful tool in plant biology (Younis et al. 2014). Since the first discovery of RNAi mechanism in \( Caenorhabditis \) elegans in 1998, RNAi has emerged as a potential gene-silencing tool for loss-of-function analyses in a wide range of organisms, including lepidopteran insects (Terenius et al. 2011). The success of RNAi in insects is highly dependent on insect-species, target gene and function, organ of gene expression and mode of delivery of silencing molecules (Huvenne and Smagghe 2010; Terenius et al. 2011). In most of the early reports of RNAi-induced silencing in insects, the dsRNA is delivered using three methods such as injection (Mutti et al. 2006), ingestion (Baum et al. 2007) and feeding via bacteria that produce dsRNA (Tian et al. 2009). However, efficiency of these delivery methods is not satisfactory because of the instability of dsRNAs in nuclease-rich environments.
M. sexta has been a model organism of entomologists for several years (Diamond et al. 2010). In M. sexta, RNAi-induced silencing was first achieved by injection of dsRNAs targeted against integrin-beta1 gene, a haemocyte-specific integrin which is required for haemocytic encapsulation (Levin et al. 2005). Silencing of integrin-beta1 gene using RNAi has decreased the encapsulation of haemocytes. Injection of pathogenic bacteria, Photobacterium luminescens into M. sexta haemolymph induces the expression of an antimicrobial protein nitric oxide synthase in fat body and haemocytes (Eleftherianos et al. 2009). Knock-down of nitric oxide synthase expression throughout the larvae using RNAi has increased the mortality of infected larvae, suggesting that nitric oxide synthesis is important for larval immune defense (Eleftherianos et al. 2009). RNAi induced silencing in larval chemosensory tissues was achieved by Howlett et al. by feeding dsRNA corresponding to a coding region of the M. sexta olfactory coreceptor, a chemosensory receptor gene (Howlett et al. 2012). In addition to the above mentioned successful RNAi-induced silencing studies in M. sexta, Terenius and colleagues have reported the variable sensitivity of M. sexta larvae to RNAi technique particularly when dsRNA is delivered by injection (Terenius et al. 2011). Efficiency of silencing could be enhanced by continuous supply of dsRNA, because the core RNAi genes in M. sexta larvae are induced in response to the dsRNA injection and the expression of core RNAi genes is elevated when the duration of contact with exogenous dsRNA is prolonged (Garbutt and Reynolds 2012). The dsRNA injected into the M. sexta hemolymph is found to be unstable and rapidly degraded by RNase (Garbutt et al. 2013).

In addition to the abovementioned dsRNA delivery methods, RNAi is induced in M. sexta larvae using PMRi, where transient or stable transgenic plants expressing insect-specific dsRNAs are used to feed the insects (Kumar et al. 2012). M. sexta's midgut-expressed, nicotine-ingestion induced CYP4B46, CYP4M1 and CYP4M3 genes can be silenced without any co-silencing of their closely related non target genes, using plant virus based dsRNA-producing system (VDPS) (Kumar et al. 2012). VDPS based target gene silencing is rapid and did not require generation of laborious stable transgenic plants; however, this method is transient and can hardly be applied under field conditions. M. sexta’s unique mechanism of exhaling the ingested nicotine to deter predatory spiders was discovered recently using transgenic stable N. attenuata PMRi lines generated by Agrobacterium tumifaciens-mediated transformation of an inverted repeat sequence of M. sexta's CYP6B46 (Kumar et al. 2014). PMRi enabled the
generation of loss-of-function phenotypes of \textit{M. sexta} and more importantly, examination of their effects in the plants’ and herbivores’ native habitat (Kumar et al. 2014). Certainly, PMRi has the potential to revolutionize insect functional genomics as it confers the highly desired \textit{in vivo} dimension to this field. This thesis provides a valuable example of PMRi’s usefulness in unraveling a unique detoxification mechanism evolved by \textit{M. sexta} larvae and also emphasize the application of PMRi to silence homologous genes that share high sequence similarity in congeneric insects.

Among \textit{N. attenuata}'s induced defense compounds, HGL-DTGs are known to thwart the growth of \textit{M. sexta} larvae (Jassbi et al. 2008). Lyciumoside IV and its malonylated compounds are the major HGL-DTGs produced by \textit{N. attenuata} both in herbivory-induced and un-induced states (Heiling et al. 2010). I selected lyciumoside IV and its malonylated forms as model HGL-DTGs to study \textit{M. sexta}'s counter-adaptation against HGL-DTGs. OS is the first digestive substance that comes in contact with plant material during herbivore feeding. The instantaneous loss of malonyl groups from malonylated lyciumoside IV in the alkaline environment of \textit{M. sexta} larval OS (Figure 1, Manuscript I) suggests that malonylation of HGL-DTGs could be a strategy of \textit{N. attenuata} to ensure the availability of toxic lyciumoside IV to the larvae. The role of malonylated lyciumoside IV could be similar to that of phytoanticipins in which, glycosylation provides the within-plant stability of defense molecules by maintaining them in inactive or, rather, pre-toxin states (Jones and Vogt 2001; Bowles et al. 2006). However, the greater amount of free lyciumoside IV than its malonylated forms implies that the role of malonylation might be for distribution within the plant, rather than stability.

Based on various studies in insects which show that deglycosylation of glycosylated compounds is often atoxin activation process, and reglycosylation of the formed aglycones serves detoxification (Despres et al. 2007; Ahn et al. 2012; Pentzold et al. 2014), I hypothesized that lyciumoside IV is completely deglycosylated by \textit{M. sexta} larvae to form the aglycone 17-HGL. But the 17-HGL aglycone was never found in larval bodies or frass. Instead, a novel compound which we identified as a partially deglycosylated form of lyciumoside IV was found in larval tissues and frass (Figure 2, Manuscript I). Mass spectrometry analysis of this compound has revealed that it contains one glucose and one rhamnose along with 17-HGL backbone. I isolated and purified the novel compound from larval frass and the structure of the novel
compound was elucidated using NMR spectroscopy as 3-O-[α-rhamnopyranosyl-(1-4)-β-glucopyranosyl]-17-hydroxygeranyllinalool (RGHGL).

In response to the ingestion of plant allelochemicals, insect herbivores induce several detoxification enzymes including cytochrome P450 monooxygenases, esterases, glutathione-S-transferases and glucosyl-transferases. (Krieger et al. 1971; Ahmad 1983; Snyder et al. 1995b; Scott 1999; Ahn et al. 2011). In order to test *M. sexta*’s response to lyciumoside IV, *M. sexta* larvae were fed on HGL-DTG-depleted irGGPPS plants, or on empty vector transformed controls (EV) and also on lyciumoside IV containing artificial diet. Govind et al., reported that three β-glucosidases were up-regulated in *M. sexta* larvae when they fed on *N. attenuata* plants (Govind et al. 2010). I measured the relative abundance of transcripts for these three β-glucosidases and found that β-glucosidase1 was induced in larval midguts in response to lyciumoside IV ingestion. We used PMRi to silence *M. sexta*’s midgut-expressed β-glucosidase1: we generated stable transgenic *N. attenuata* plants harboring a 301 bp fragment of *Msβ-glucosidase1* in an inverted repeat (ir) orientation (irβ-glucosidase1) to produce β-glucosidase1 dsRNA. An *in vitro* enzyme assay with the midgut tissue extracts of β-glucosidase1-silenced and control larvae showed that the amount of RGHGL formed by midgut tissue extracts of β-glucosidase1-silenced larvae was 55% lower than that formed in control samples. The amount of lyciumoside IV ingested and the amount of lyciumoside IV and RGHGL excreted by β-glucosidase1-silenced and control larvae were determined by an excretion efficiency determination assay. β-glucosidase1-silenced larvae excreted 40% more lyciumoside IV and 70% less RGHGL than did EV-fed control larvae (Figure 3, Manuscript I). Both of these assays suggested that β-glucosidase1 silencing suppressed the lyciumoside IV deglycosylation in *M. sexta* larvae.

β-glucosidase1-silenced larvae showed molting impairment, a phenomenon in which larvae are unable to free themselves from old exoskeleton during molting and higher mortality than control larvae feeding on EV plants. I used irβ-glucosidase1 plants crossed with irGGPPS (B×G) to test the hypothesis that reduced lyciumoside IV deglucosylation to RGHGL causes molting impairments and mortality. Occurrence of molting impairments and mortality in larvae feeding on irβ-glucosidase1 plants and on lyciumoside IV-coated B×G plants suggested that lyciumoside IV deglucosylation is a detoxification process and is important for *M. sexta* larvae.
In insects, detoxification by deglycosylation is unusual, but this type of detoxification has been observed in phytopathogenic fungi. For example, the fungus *Gaeumannomyces graminis* (Magnaporthaceae) detoxifies the avenicin saponins of oat (triterpenoid glycosides containing three sugars) by $\beta$-glucosidase-catalyzed deglucosylation (Turner 1961; Crombie et al. 1986). Similarly, various fungi detoxify the tomato steroidal glycoalkaloid $\alpha$-tomatine (containing four sugars) by removing its C-3 oligosaccharide, which renders it incapable of binding to 3$\beta$-hydroxy sterols, preventing the formation of membrane pores and cell lysis which would result from this binding (Sandrock and Vanetten 1998; Seipke and Loria 2008). In the above cases, fungi detoxify the glycosides by complete deglycosylation. Whereas *M. sexta* larvae detoxify lyciumoside IV by partial deglycosylation.

The biochemical action of lyciumoside IV responsible for its association with molting impairment and consequent mortality is not yet understood. In fact, the mode of action is not completely known for any diterpene glycosides (DTGs). However, negative effects of various DTGs on various organisms have been reported. For instance, pseudopterosin A (a monoglycosylated DTG) inhibits cell division, DNA, and protein synthesis to arrest embryogenesis in sea urchin (Ettouati and Jacobs 1987). Various capsianosides (monomeric and dimeric DTGs containing multiple sugars) negatively affect the cytoskeletal function in human intestinal cells by interfering in the reorganization of actin filaments (Hashimoto et al. 1997). In rat, steviosides inhibit cross-membrane sugar transport in various tissues (Ishii et al. 1987; Lailerd et al. 2004; Rizzo et al. 2013). One possibility is that lyciumoside IV could play a similar role in sugar transport. In $\beta$-glucosidase1-silenced larvae, lyciumoside IV accumulates in the fat body in considerable concentrations in comparison to EV-fed larvae (Figure S11, Manuscript I). During the molting process, the fat body is the site of gluconeogenesis (Thompson 1997). Hydrolytic products of this reaction are mobilized to serve as precursors required for chitin biosynthesis, which is fundamental to the molting process of insects because it ensures the formation and expansion of new exoskeleton by the addition of sugars to the existing chitin polymer. If lyciumoside IV accumulated in the fat body inhibits the sugar transport during chitin synthesis, it could easily hamper the formation of new exoskeleton. However, the stuck exoskeleton is a common problem in molting caterpillars that are weak or stressed for a variety of reasons.
Usually insect herbivores use the ingested plant allelochemicals or their metabolized products against their natural enemies. *M. sexta* larvae exhale nicotine through spiracles in order to deter spiders and nearly 60% of the spiders tested were deterred by the exhaled nicotine. We hypothesized that *M. sexta* larvae co-opt either lyciumoside IV or RGHGL to use against their natural enemies. We introduced irβ-glucosidase1, irGGPPS and EV plants in a field plot in *N. attenuata*'s native habitat and determined the larval survival on these lines (Figure 5, Manuscript I). Diurnal and nocturnal survivorship of larvae feeding on all three lines did not differ significantly, but we found carcasses of partially eaten larvae on irβ-glucosidase1 plants while recording the diurnal survivorship, suggesting that β-glucosidase1-silenced larvae experienced a distinctive predator behavior during night. The main nocturnal predator, *C. parallela* spiders completely ingested EV- and irGGPPS- fed larvae but seemed to find β-glucosidase1-silenced larvae unpalatable. *M. sexta* larvae do not externalize the ingested lyciumoside IV or its detoxified product RGHGL. Complete ingestion of EV-fed larvae by spiders suggests that *M. sexta* larvae have not evolved the co-option of lyciumoside IV for their own defense. Spiders’ chemosensory-endowed hair on legs and palps could detect lyciumoside IV coated on the larval surface. But the spiders are not deterred by the β-glucosidase1-silenced and control larvae, which have ingested lyciumoside IV, suggesting that *M. sexta* larvae did not co-opt lyciumoside IV perhaps to avoid self-intoxication. However, it would be interesting to study the other *Manduca* spp. or even the generalist herbivores of *Nicotiana* spp. to evaluate if HGL-DTG co-option has evolved in either generalist or specialist herbivores of taxa that produce HGL-DTGs. Moreover, it would also be interesting to study the effect of lyciumoside IV and RGHGL on other predators and hemolymph-dwelling parasitoids.

Counter-defense strategies of *M. quinquemaculata* larvae against *N. attenuata*'s chemical defenses are largely elusive. Suppression of midgut-expressed counter-defense genes using PMRi is an ideal approach to unravel their role in *M. quinquemaculata*'s counter adaptations to its host's chemical defenses. PMRi experiments are often conducted in laboratory conditions, but very few reports demonstrated successful application of PMRi in nature (Pitino et al. 2011; Kumar et al. 2014; Liu et al. 2015; Poreddy et al. 2015). Moreover, PMRi works in a sequence-specific manner, so it is feasible to target congeneric insect pests that attack the same plant by selecting a conserved target gene region to generate transgenic PMRi plants.
Since *M. sexta* and *M. quinquemaculata* are closely related species and they both co-occur in *N. attenuata*'s native habitat (Kessler and Baldwin 2001; Schuman et al. 2012), I hypothesized that irCYP6B46 and irβ-glucosidase1 PMRi plants which were originally generated to silence *M. sexta* genes *CYP6B46* and *β-glucosidase1*, respectively could possibly also silence homologous genes in *M. quinquemaculata*. To test this hypothesis, the PMRi plants irCYP6B46 and irβ-glucosidase1 were planted in their native habitat and native *M. quinquemaculata* larvae were allowed to feed on these plants until larvae had reached the fourth-instar. The RNA was extracted from various tissues of these larvae and relative transcript abundance was measured for the homologous genes. I found that *M. sexta*'s *CYP6B46* and *β-glucosidase1* coding sequences shared 98 and 96% sequence similarity with *M. quinquemaculata* homologous coding sequences in the regions used for cloning to generate PMRi lines (Figure 2; Manuscript II). Both the genes, *CYP6B46* and *β-glucosidase1* found to have relatively higher expression levels in the midguts of larvae feeding on EV plants than in the larvae feeding on nicotine-depleted, and HGL-DTG-depleted, transgenic *N. attenuata* plants, respectively, suggesting that CYP6B46 and BG1 expression is strongly induced in the midguts of *M. quinquemaculata* larvae in response to the ingestion of corresponding toxic compounds. CYP6B46 and BG1 transcripts were reduced by ca. 90 and 75% in the midguts of irCYP6B46- and irBG1-fed fourth-instar *M. quinquemaculata* larvae (Figure 3, Manuscript II), without reducing the transcripts of their most similar, potential off-target genes. The *CYP6B46* and *β-glucosidase1* genes in wild *M. quinquemaculata* larvae were efficiently silenced using PMRi lines irCYP6B46 and irβ-glucosidase1 in *N. attenuata*'s native habitat without any off-target effects detected.

In summary, I first described the counter-adaptation of *M. sexta* larvae to *N. attenuata*’s major HGL-DTG defenses, lyciumoside IV and its malonylated forms. Malonylated lyciumoside IV readily lose their malonyl groups in the alkaline larval OS, reverting to their core compound lyciumoside IV. Midgut expressed *β-glucosidase1* deglucosylates lyciumoside IV to RGHGL. Suppression of this deglucosylation by *β-glucosidase1* silencing results in high lyciumoside IV concentration in the larval body, which further causes molting impairment and mortality. Lastly, I showed that in their native habitat, *M. sexta*’s natural enemy, the wolf spider fails to completely ingest *β-glucosidase1*-silenced larvae, due to their high lyciumoside IV content. Although lyciumoside IV is spider deterrent, *M. sexta* larvae do not co-opt it, most likely to avoid self-
intoxication, which leads to deleterious effects such as molting impairment and mortality. Thus, our results show that deglycosylation can bring about detoxification; they imply that much remains to be revealed about detoxification of multiply glycosylated compounds by herbivores and roles of such compounds in tritrophic interactions. Further, I applied PMRi for silencing midgut-expressed genes in native *M. quinquemaculata* larvae in nature using *N. attenuata* PMRi plants producing *M. sexta's* CYP6B46 and β-glucosidase1 dsRNAs. Silencing of CYP6B46 and β-glucosidase1 genes in *M. quinquemaculata* was achieved without co-silencing of potential off-target genes. Silencing of counter-defense genes of *M. quinquemaculata* may shed light on its counter-adaptation strategies and our study also demonstrates the potential of PMRi to control congeneric insect pests in agriculture.
References

(Introduction & Discussion)


**Zavala JA, Baldwin IT** (2004) Fitness benefits of trypsin proteinase inhibitor expression in *Nicotiana attenuata* are greater than their costs when plants are attacked. BMC Ecol 4: 11.


Summary

Glycosylated phytochemicals are typically thought to release antiherbivore toxins upon deglycosylation. I discovered an exception: an herbivore that detoxifies a glycoside by deglycosylation. *Nicotiana attenuata* (Solanaceae) produces 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) in starch-equivalent concentrations that thwart the growth of its specialist lepidopteran herbivore *Manduca sexta* (Lepidoptera, Sphingidae). Lyciumoside IV and its malonylated forms, nicotianoside I and II, constitute ~80% of *N. attenuata*'s HGL-DTG pool. Upon ingestion, the malonylated forms are rapidly and non-enzymatically demalonylated to lyciumoside IV by the alkalinity of larval oral secretion. Ingested lyciumoside IV (44%) is excreted as a novel compound, 3-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-17-hydroxygeranyllinalool (RGHGL). It differs from lyciumoside IV only in its lack of the C-17-glucose. Of *M. sexta*’s midgut-expressed β-glucosidases, only β-glucosidase 1 is upregulated upon lyciumoside IV ingestion, and when silenced by plant-mediated RNAi (PMRi), larvae are impaired in lyciumoside IV deglucosylation. The body and frass of lyciumoside IV-ingesting β-glucosidase 1-silenced larvae contained 42% and 40% more lyciumoside IV and 63% and 70% less RGHGL, respectively, than controls. Lyciumoside IV, but not RGHGL ingestion, is associated with molting impairment and mortality in β-glucosidase 1-silenced larvae, indicating that RGHGL is detoxification product of lyciumoside IV. To examine the consequences of this detoxification process on natural tritrophic interactions, β-glucosidase 1-silencing PMRi plants were planted into native habitats; control and β-glucosidase 1-silenced larvae’s survival was similar; however, while *Camptocosa parallela* spiders captured and killed the control and lyciumoside IV-replete β-glucosidase 1-silenced larvae similarly, the spiders ingested only 25% of β-glucosidase 1-silenced larvae and ingestion resulted in locomotor distress in the spiders. While spiders attacked and ingested RGHGL-coated or -ingested larvae equally, they were deterred by the lyciumoside IV-coated larvae. Although lyciumoside IV deters spiders, it is not defensively co-opted by *M. sexta* larvae, perhaps to avoid its deleterious effects such as molting impairment and mortality.

The congeneric species *M. sexta* and *M. quinquemaculata* are closely related and co-occur in *N. attenuata*'s native habitat. When *M. sexta* larvae attack transgenic *N. attenuata* plants...
irCYP6B46 and irβ-glucosidase1 plants expressing dsRNA targeting *M. sexta*'s midgut-expressed genes, nicotine-ingestion induced *CYP6B46* and lyciumoside-IV-ingestion induced β-glucosidase1 genes, these larval genes which are important in adaptation to ingested host toxins, are strongly silenced. I show that these PMRi plants also silence the homologous genes in native *M. quinquemaculata* larvae feeding on these transgenic plants in nature. The target gene sequences in the PMRi lines shared 98 and 96% sequence similarity with *M. quinquemaculata* homologous coding sequences, and *CYP6B46* and β-glucosidase1 transcripts were reduced by ca. 90 and 75% in the midguts of irCYP6B46- and irβ-glucosidase1-fed fourth-instar *M. quinquemaculata* larvae, without reducing the transcripts of their most similar, potential off-target genes. We conclude that the PMRi procedure can robustly and specifically silence genes in native congeneric insects that share sufficient sequence similarity and with the careful selection of targets, can protect crops from attack by congeneric groups of insect pests.
Zusammenfassung

Glukosidase1–Gen fraßen. Fraßen die Spinnen Raupen mit reichlich Lyciumosid IV, hatte dies motorische Störungen zur Folge. Während die Spinnen Raupen, die mit RGHGL bestrichen worden waren oder dieses aufgenommen hatten, gleichermaßen angriffen und fraßen, wurden sie von Raupen abgeschreckt, die mit Lyciumosid IV bestrichen worden waren. Obwohl Lyciumosid IV Spinnen abschreckt, wird es von M. sexta Raupen nicht für die eigene Abwehr genutzt, womöglich um eigene Schädigungen durch diese Substanz, wie Häutungsschäden oder erhöhte Sterblichkeit, zu vermeiden.

Eigenständigkeitserklärung


Spoorthi Poreddy
Jena, February 04, 2016

Erklärung über laufende und frühere Promotionsverfahren


Spoorthi Poreddy
Jena, February 04, 2016
Spoorthi Poreddy

Born 11th November 1986, India

Nationality: Indian

Email: poreddyspoorthi@gmail.com

---

**Education**

- 2013-2016, Ph. D. Molecular Biology
  International Max Planck Research School, MPI for Chemical Ecology, Jena, Germany
  Dissertation: Probing the herbivore’s responses to plant defenses using plant-mediated RNAi

- 2006-2008, M. Sc Biotechnology, University 6th Rank
  Osmania University, Hyderabad, India
  Dissertation: *In vitro* plant regeneration and genetic transformation of *Vigna radiata*

- 2003-2006, B. Sc Life sciences, Distinction
  Osmania University, Hyderabad, India

- 2001-2003, Senior School Education, Biology
  Central Board of Secondary Education, India

**Research Experience**

- Ph. D. student, 2013-2016, Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Jena, Germany

- Senior research fellow, 2011-2012, Germplasm Evaluation Division, National Bureau of Plant Genetic Resources, New Delhi, India
  Characterization of the etiological agent of leaf deformity disease of sunn-hemp and development of viral gene constructs for detection and management

- Research assistant, 2009-2010, Osmania university, Hyderabad

**Publications**

Curriculum Vitae

- **Poreddy, S.,** Li, J., and Baldwin, I.T. (submitted to *JIPB*) Plant-mediated RNAi silences midgut-expressed genes in congeneric lepidopteran insects in nature.
- **Poreddy, S.,** Kallenbach, M., and Baldwin, I.T. (in preparation) The wild tobacco *Nicotiana attenuata* distinguishes attack by two closely related specialist herbivores, *Manduca sexta* and *M. quinquemaculata*

**Book chapters**


**Oral Presentations**

- **Poreddy, S.** (2015) Reverse genetics reveals a lepidopteran herbivore's β-glucosidase mediated unusual counter defense against its host's most abundant defenses. PRIR 2015, Aachen, Germany.
- **Poreddy, S.** (2015) *Manduca sexta*’s β-glucosidase mediated unusual counter-defense against its host’s most abundant chemical defenses, the diterpene glycosides. 14th IMPRS symposium, MPI for Chemical Ecology, Dornburg, Germany.
Poster Presentations


Language Proficiency

Telugu: mother language
Hindi: fluent
English: fluent
German: Basic

Spoorthi Poreddy
Jena
List of Publications

Scientific articles

- Poreddy, S., Kallenbach, M., and Baldwin, I.T. (in preparation) The wild tobacco *Nicotiana attenuata* distinguishes attack by two closely related specialist herbivores, *Manduca sexta* and *M. quinquemaculata*
- Poreddy, S., Li, J., and Baldwin, I.T. (submitted to JIPB) Plant-mediated RNAi silences midgut-expressed genes in congeneric lepidopteran insects in nature.

Book chapters

Oral Presentations

- **Poreddy, S.** (2015) Reverse genetics reveals a lepidopteran herbivore's β-glucosidase mediated unusual counter defense against its host's most abundant defenses. PRIR 2015, Aachen, Germany.

- **Poreddy, S.** (2015) *Manduca sexta*’s β-glucosidase mediated unusual counter-defense against its host’s most abundant chemical defenses, the diterpene glycosides. 14th IMPRS symposium, MPI for Chemical Ecology, Dornburg, Germany.

Poster Presentations


- **Poreddy, S.** (2013) Revealing *Manduca sexta*’s diterpene glycoside tolerance using plant mediated RNAi. 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany.

- **Poreddy, S., and Roy, A.** (2012) Molecular evidence for association of a begomovirus and betasatellite with a recently emerged leaf deformity disease of Sunn-hemp in eastern India. 3rd world congress on Biotechnology, Omics group conferences, Hyderabad, India.
Acknowledgments

First of all, I would like to thank Prof. Ian T Baldwin for giving me the opportunity to perform my PhD work under his supervision and introducing me into the exciting world of Molecular Ecology. I am very grateful for his constant support and encouragement, and for giving me the opportunity to broaden my horizons by allowing me to carry out field work in Utah, USA.

Thanks to Sagar and Pavan for introducing me to Manduca and for helping me in my first year, and for always motivating me. Thanks to Axel Schmidt, Shuqing Xu and Mariana Stanton for the scientific discussions.

Thanks to Matthias Schöttner and Mario Kallenbach for their ever ready help with analytical instruments. Thanks to Klaus, Wibke, Antje, Eva, Celia, Danny and Thomas for the technical help. Thanks to Jima and Dr. Bernd Schneider for their support with NMR work.

I would also like to thank the International Max Planck Research School (IMPRS) and the Max-Planck-Gesellschaft for funding. Thanks to Evelyn Claußen and Karin Groten and for their support and guidance that I received since the day I arrived in Jena. Thanks to the IMPRS coordinators, Karin and Claudia, for their personal support and thanks to Angela Overmeyer for helping me to fulfill PR requirements. Thanks to Merry for her constant support in many things.

Thanks to the greenhouse team, especially Andreas, for taking care of hundreds of plants.

Thanks to the Utah crew of 2014 field season for helping me in collecting Manduca eggs and, in particular Merry, Karin and Sang-yu, for all your support in the field.

I would also like to thank Nora and Jyotasana; and my previous and present office mates, Preeti, Sirsha, Ting, Ming, Henrique and Hahn; and lab 17 members, mainly Jiancai; and the lunch
batch, Van, Wenwu, Dechang and Rakesh for providing a helpful and friendly environment.

I am deeply thankful to all the members of the department of Molecular Ecology for providing a very nice working atmosphere, for helpful and constructive discussions and for excellent technical assistance.

My parents, my sister and her family and my friends and relatives deserve special mention for their endless support and for believing in me. Finally, I want to express my gratitude to my husband, Natraj for his love, endless patience, support, and for always believing in me.