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

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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 1-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 1 by plant-mediated RNAi causes moulting impairments and mortality. In the native habitat of *N. attenuata*, β -glucosidase 1 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.

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Plants commonly use lipophilic compounds such as cardenolides, 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^{1–3}. Tissue damage resulting from insect feeding exposes these inactive protoxins to their activating glycosidases resulting in the formation of toxic aglycones^{3–5}. Released aglycones cause cellular damage in herbivores and severely affect their physiology and fitness^{6,7}. Frequently, on ingestion, these glycosides are deglycosylated in the midgut by insect glycosidases^{3,8}. To avoid such deglycosylation, some herbivores downregulate their glycosidases after ingesting glycosides^{9,10}. Several herbivores have even evolved their own glycosylation systems to reglycosylate the formed aglycones^{3,5,11,12}. Glycosides are more water soluble, have higher intracellular mobility and so are excreted faster than their aglycones². Moreover, they are easily transported to vacuolar compartments and the apoplast by membrane-bound transporter systems, which often recognize their glycosyl residues². Hence, deglycosylation or glycoside hydrolysis in insects is commonly thought to result in toxin activation and glycosylation is regarded as a form of detoxification^{11–14}. 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-hydroxygeranylgeranyl di-terpene glycosides (HGL-DTGs) containing multiple sugar moieties¹⁵. HGL-DTGs have been reported to be biocidal and HGL-DTGs of *Nicotiana tabacum* retarded growth of *Heliothis virescens* larvae in artificial diet (AD) experiments¹⁵. 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^{16–18}. *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^{19,20}. In fact, HGL-DTGs were found to be more effective as chemical defences against *M. sexta* than some of *N. attenuata*'s other well-characterized defence compounds, such as nicotine and trypsin proteinase inhibitors¹⁹.

N. attenuata HGL-DTGs are composed of an acyclic C₂₀ 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-6', resulting in additional structures^{20–22}. Heiling *et al.*²⁰ defined lyciumoside I 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, attenoside, nicotianoside III and lyciumoside 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 dimalonylated, based on the number of attached malonyl groups. *Lyc4* with its singly malonylated form,

nicotianoside I (*Nic1*), and its dimalonylated form, nicotianoside II (*Nic2*), constitutes ~80% of the *N. attenuata*'s total HGL-DTG pool²⁰.

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 evolved by *M. sexta* to demalonylate 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)^{23,24}. 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.

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)^{25–27}, midgut content and frass of 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 resulted from 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 alkaline²⁶), 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)²⁸ 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

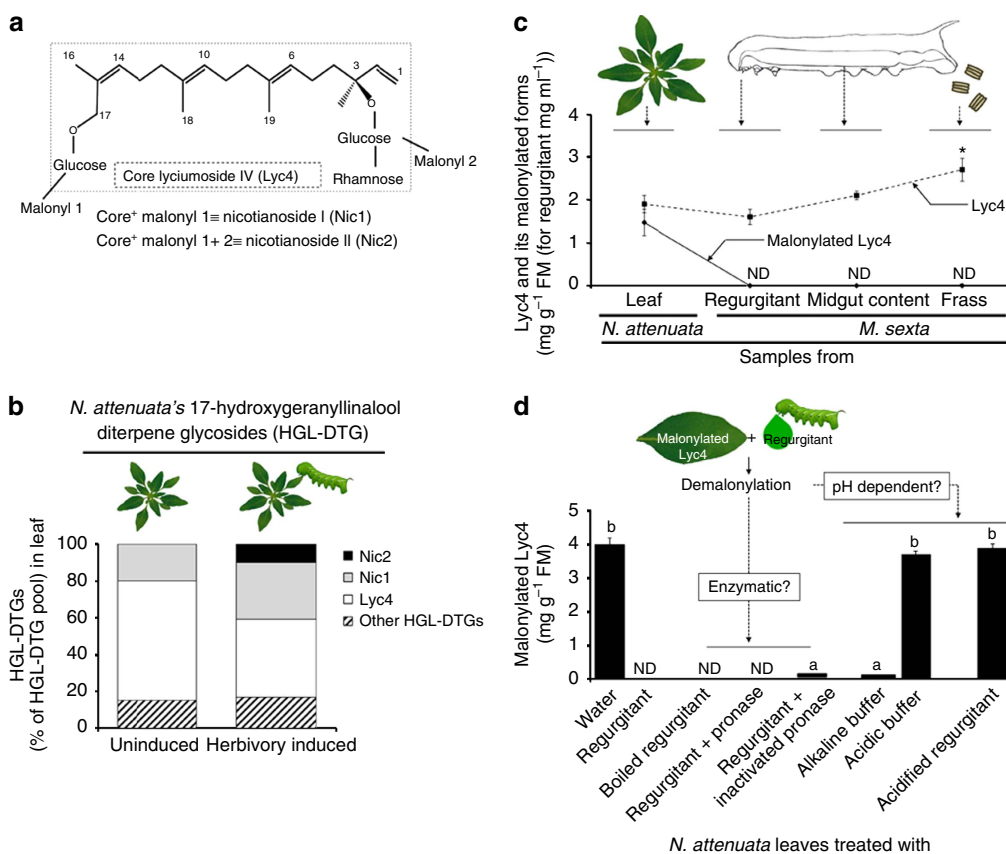


Figure 1 | *N. attenuata*'s malonylated HGL-DTGs are demalonylated non-enzymatically in *M. sexta*'s alkaline regurgitant. (a) Structures of core lyciumoside IV (Lyc4) and its singly and dimalonylated forms, nicotianoside I (Nic1) and nicotianoside II (Nic2), respectively. (b) Proportions of Lyc4 and its malonylated forms Nic1 and Nic2 and other HGL-DTGs in uninduced and *M. sexta* herbivory-induced *N. attenuata* leaves showing that Lyc4 and its derivatives are the major HGL-DTGs (based on data from Heiling *et al.*²⁰). (c) Profile of core and malonylated Lyc4 in *N. attenuata* leaf and in larval regurgitant, midgut content and frass showing that complete demalonylation occurs when the leaf comes into contact with larval regurgitant, at the time of ingestion by larvae ($F_{3,8} = 19.81$, $P \leq 0.0001$; significant differences (threshold: $P \leq 0.05$) between means (\pm s.e.) determined by Games Howell test (Welch's ANOVA); $n = 3$). (d) Analysis to determine whether the demalonylation of Nic1 and Nic2 in larval regurgitant occurs enzymatically or by alkaline hydrolysis in the alkaline pH of the regurgitant; concentration of malonylated Lyc4 (Nic1 and Nic2) in *N. attenuata* leaf when it was crushed in water, regurgitant, boiled regurgitant, pronase-treated regurgitant, heat-inactivated pronase-treated regurgitant, alkaline buffer, acidic buffer and acidified regurgitant ($F_{4,20} = 347.1$, $P \leq 0.001$; significant differences (threshold: $P \leq 0.05$) between means (\pm s.e.) determined by Fisher's LSD test (one-way ANOVA); $n = 5$). ND, not detected. ANOVA, analysis of variance; LSD, least significant difference.

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 ^{13}C 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^{20,29,30}. In the ^{13}C 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-hydroxygeranylinalool³⁰ as follows. The ^1H NMR signals at δ 5.23 (1H, H-1a, $^3J_{\text{H1a-H2}} = 17.8$ Hz, $^2J_{\text{H1a-H1b}} = 1.2$ Hz), 5.20 (1H, H-1b, $^3J_{\text{H1b-H2}} = 11.1$ Hz, $^2J_{\text{H1b-H1a}} = 1.2$ Hz) and 5.93 (1H, H-2, $^3J_{\text{H2-H1a}} = 17.8$ Hz, $^3J_{\text{H2-H1b}} = 11.1$ 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 bonds. 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 isochronic methyl signals of H-18 and H-19 at δ 1.59 (6H, s), the signal of H-16 at δ 1.75 (3H, s) and their HMBC correlations with C-6 (δ 125.8), C-7 (δ 136.0), C-10 (δ 125.9), C-11 (δ 135.8), C-14 (δ 128.6) and C-15 (δ 135.7) confirmed the presence of three methyl groups at the olefinic carbons (Supplementary Figs 2–4). The methyl group at δ 1.38 (3H, s , H-20) attached to the quaternary C-3 exhibited HMBC correlations with C-4 (δ 42.8), C-3 (δ 81.6) and C-2 (δ 144.5) (Supplementary Fig. 5).

^1H and ^{13}C NMR signals at δ 4.36 (1H, d , $J = 8.0$ Hz, H-1')/ δ 99.5 (C-1') and 4.85 (1H, d , $J = 1.7$ Hz, H-1'')/ δ 102.9 (C-1'') and

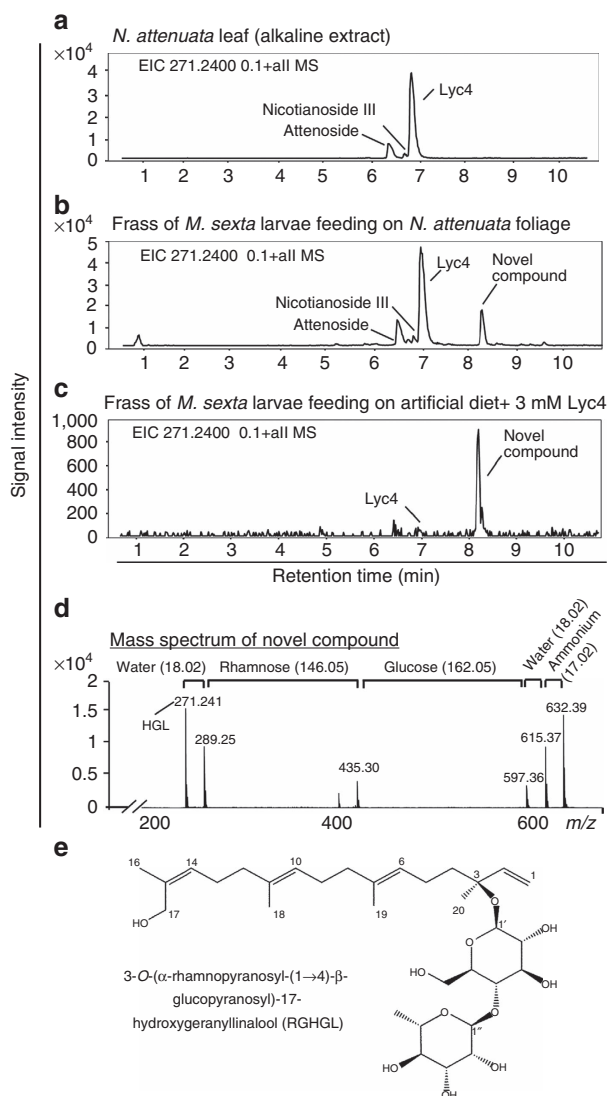


Figure 2 | Lyc4 ingesting *M. sexta* larvae excrete a novel HGL-DTG. Ion (m/z 271.24=HGL) extracted U(H)PLC/ESI-QTOF MS chromatograms showing *N. attenuata*'s major HGL-DTGs in (a) leaf (alkaline extract) and frass of larvae feeding on (b) *N. attenuata* foliage and (c) artificial diet (AD) containing 3 mM Lyc4 (physiological concentration of Lyc4 in an uninduced 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-(α -rhamnopyranosyl-(1 \rightarrow 4)- β -glucopyranosyl)-17-hydroxygeranylinalool (RGHGL).

further ^1H NMR signals between δ 3.20 and 3.96 correlated by HSQC cross peaks to ^{13}C signals characteristic of carbohydrates (δ ~70–80, Supplementary Table 1; Supplementary Fig. 6) confirmed the presence of two sugar moieties as already suggested by 12 corresponding signals in the ^{13}C NMR spectrum. The coupling constants of $^3J_{\text{H}1'-\text{H}2''} = 8.0$ Hz (1H) and $^3J_{\text{H}1''-\text{H}2''} = 1.7$ Hz (H-1'') indicated one sugar unit with β - and one with α -configuration, respectively, at the anomeric positions (Supplementary Table 1). The hydroxymethylene carbon signal at δ 62.2 (C-6'), together with the methyl signal at δ 18.0 (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^{30,31}. 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 \rightarrow 4 linkage between the rhamnose and the glucose units (Supplementary Fig. 7). From these data, the novel compound was identified as 3-O-[α -rhamnopyranosyl-(1 \rightarrow 4)- β -glucopyranosyl]-17-hydroxygeranylinalool (RGHGL; Fig. 2e).

Collected fractions of Lyc4 and RGHGL showed single peaks in U(H)PLC/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 U(H)PLC/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–f)³¹. Extraction efficiency of these compounds from the frass was >90% and it remained linear over a broad range of concentrations (0.02–0.32 $\mu\text{g mg}^{-1}$; 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 BGs⁴, 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)GGPPS 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

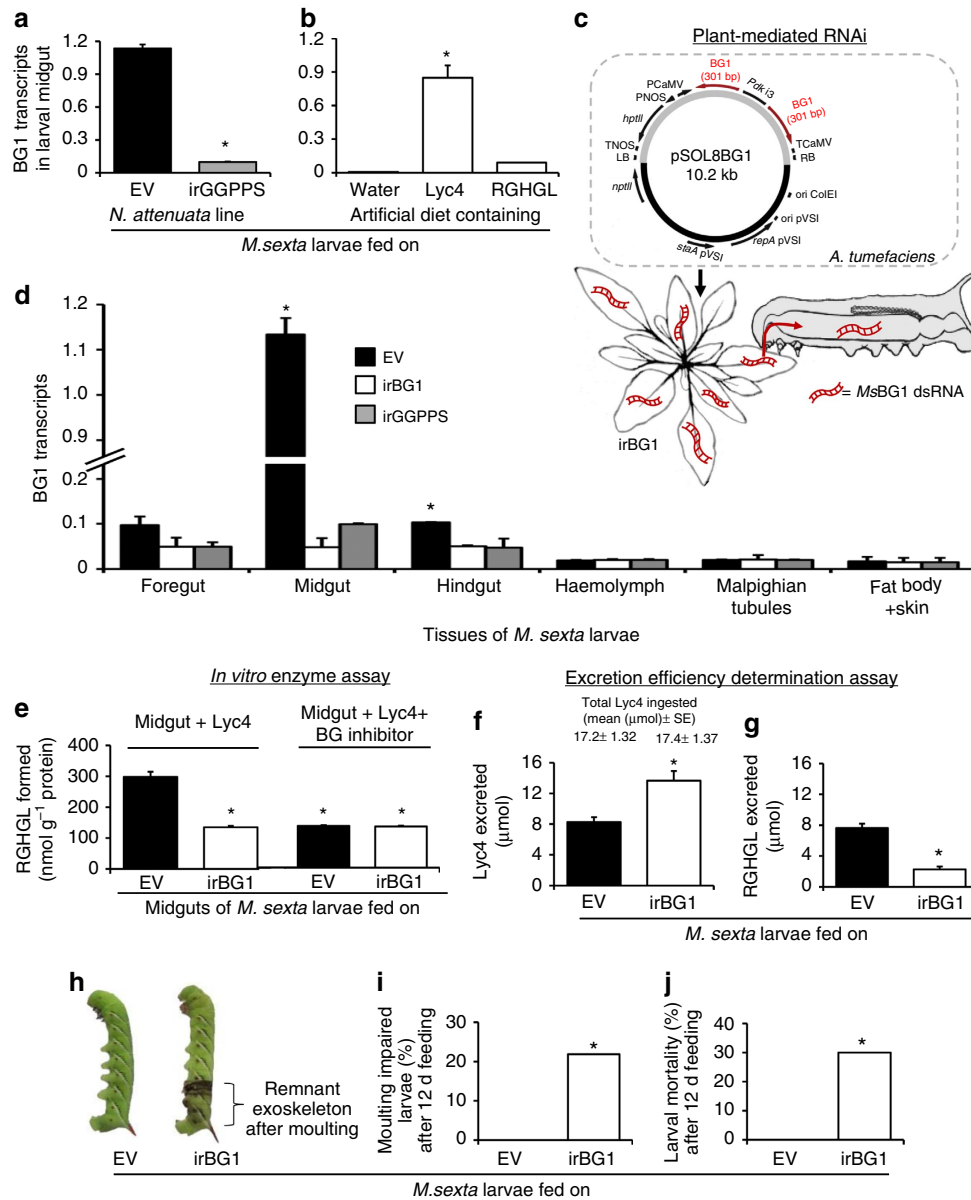


Figure 3 | Silencing of Lyc4 ingestion-induced, midgut-expressed BG1 impairs larval Lyc4 deglycosylation, moulting and survival. BG1 transcripts (relative to ubiquitin) in midguts of fourth-instar larvae feeding on (a) Lyc4-containing EV and Lyc4-depleted irGGPPS plants ($F_{1,10} = 833.5$, $P \leq 0.0001$; significant difference ($P \leq 0.05$) between means (\pm 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 RGHGL ($F_{2,15} = 5812.13$, $P \leq 0.0001$; significant differences ($P \leq 0.05$) between means (\pm s.e.) determined by Games Howell test (Welch's ANOVA); $n = 6$). (c) Plant-mediated RNAi: pSOL8 binary vector constructed to express 301 bp *MsBG1* 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 larvae feeding on EV, irBG1 and irGGPPS plants (midgut: $F_{2,15} = 9.53$, $P \leq 0.002$; hindgut: $F_{2,15} = 248$, $P \leq 0.0001$; significant differences ($P \leq 0.05$) between means (\pm s.e.) determined by Games Howell test (Welch's ANOVA, separately conducted for each tissue); $n = 6$ in all bars). (e) RGHGL formed *in vitro* enzyme assays containing midgut extracts of fourth-instar EV- and irBG1-feeding larvae and either (1 mM) Lyc4 ($F_{3,8} = 104.9$, $P \leq 0.0001$; significant differences ($P \leq 0.05$) between means (\pm s.e.) determined by Fisher's LSD test (one-way ANOVA); $n = 3$) or 1 mM Lyc4 + 1 mM BG inhibitor, δ -gluconolactone ($n = 3$). Excretion (% of total Lyc4 ingested) of (f) Lyc4 ($F_{1, 25} = 13.95$, $P \leq 0.001$; significant difference ($P \leq 0.05$) between means (\pm s.e.) determined by Fisher's LSD test (one-way ANOVA); $n = 13$ (EV) and 14 (irBG1)) and (g) RGHGL ($F_{1, 25} = 62.65$, $P \leq 0.0001$; significant difference ($P \leq 0.05$) between means (\pm 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 larva) and (i) percentage of moulting impairment (significant difference ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 30$) and (j) mortality (%) in larvae feeding (for 12 d) on EV or irBG1 plants (significant difference ($P \leq 0.05$) 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 (nicotine, rutin, caffeoyl putrescine and chlorogenic acid) of both irBG1 lines were similar to those of EV plants (Supplementary Fig. 10d,e). 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 *BG1* silencing was specific and had no off-target effects on the expressions of other similar genes, we quantified the transcripts of closely related *BG2* and *BG3* (60.2% and 51.5% nucleotide similarity to *BG1*, respectively). Transcript abundance of *BG2* and *BG3* in the midguts of larvae feeding on *irBG1* plants were similar to those of larvae feeding on EV plants (Supplementary Fig. 10f,g), suggesting that the *BG1* silencing by PMRi was sequence specific. Hereafter, we refer the *irBG1*-feeding larvae as 'BG1-silenced' larvae.

***MsBG1* silencing impairs larval *Lyc4* metabolism.** The influence of *BG1* silencing on larval *Lyc4* metabolism was tested using an *in vitro* *Lyc4* deglycosylation assay with the midgut tissue extracts of *BG1*-silenced and control larvae. The amount of RGHGL formed by the midguts of *BG1*-silenced larvae was significantly lower (55%) than that formed by the midguts of EV-fed larvae (Fig. 3e); similarly reduced RGHGL formation was observed when midguts of control and *BG1*-silenced larvae were incubated with *Lyc4* and a *BG* inhibitor, δ -gluconolactone (Fig. 3e). These results revealed that *Lyc4* deglycosylation activity was impaired in the *irBG1*-feeding larvae.

Further, to test the effect of *BG1* silencing on larval *Lyc4* accumulation, we quantified the amounts of *Lyc4* and RGHGL in foregut, midgut, hindgut tissues, haemolymph, Malpighian tubules and fat body with skin of fourth-instar *BG1*-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 assays^{32,33} revealed that although the amounts of *Lyc4* ingested by the *BG1*-silenced and EV-fed larvae were similar, *BG1*-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 *irGGPPS*-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.

BG1 silencing had no effect on larval growth, however, during every moult (until the fourth-instar) we found that some *BG1*-silenced larvae were unable to shed their exoskeleton; collectively, 21.8% *BG1*-silenced larvae ($n = 32$) failed to moult during the first three instar changes (Fig. 3h). Hereafter, we refer to this phenomenon as 'moulting impairment'. Larvae unable to free themselves from the old exoskeleton stopped feeding, gradually melanized and died within 48 h of (failed) moulting (Fig. 3i,j; Supplementary Fig. 11d); moulting impairment and elevated mortality were not observed in the EV-fed larvae (Fig. 3i,j). Some *BG1*-silenced larvae (~10% of the total dead) died soon after moulting but did not show the unshed exoskeleton or melanization phenotypes; these were not counted as moulting-impaired larvae. Although it was demonstrated that *BG1* silencing suppressed *Lyc4* deglycosylation, whether moulting impairment was caused by *BG1* silencing or by *Lyc4* metabolism suppression remained unclear (Fig. 4a). Along with the *BG1* dsRNA, *irBG1* plants contained *Lyc4* levels equal to those of EV

plants so the effects of *BG1* silencing and *Lyc4* deglycosylation suppression could not be uncoupled in the larvae feeding on these plants. Therefore, we generated *Lyc4*-depleted and *MsBG1* dsRNA-containing plants by crossing *irBG1* with *irGGPPS* plants (*irBG1* \times *irGGPPS*; hereafter called $B \times G$) (Fig. 4b). Levels of HGL-DTGs of $B \times G$ plants were similar to the low levels of *irGGPPS* plants. Although *BG1* silencing in $B \times G$ -fed larvae was similar to that of *irBG1*-fed larvae (Supplementary Fig. 12a), moulting impairment and resulting mortality were not observed (Fig. 4c,d). Levels of *Lyc4* and RGHGL in the bodies (without gut contents) of $B \times G$ -fed larvae were similar to those in the bodies of *irGGPPS*-fed larvae (Supplementary Fig. 12b,c). This indicated that the moulting impairment phenotype did not result from the effect of *BG1* silencing on larval primary metabolism but was related to *Lyc4* deglycosylation by *BG1*. To test this, we complemented $B \times G$ and *irGGPPS* leaves with *Lyc4* and RGHGL by topical coating; water-coated EV, *irBG1*, *irGGPPS* and $B \times G$ 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 $B \times G$ plants ($n = 30$) showed high moulting impairment (22%) and mortality (28%) as did the larvae feeding on water-coated *irBG1* leaves (Fig. 4c,d). Larvae feeding on RGHGL- or water-coated $B \times G$ and *irGGPPS* leaves and water-coated EV leaves ($n = 30$) did not show these phenotypes (Fig. 4c,d).

Moulting impairment and mortality associated with the *Lyc4* ingestion by *BG1*-silenced larvae were not observed after the ingestion of *Lyc4* or RGHGL alone or after silencing *BG1* in the absence of *Lyc4* ingestion. This clearly showed that the *BG1* function is vital for larvae ingesting large quantities of *Lyc4* produced by *N. attenuata*.

A native predator fails to ingest the *BG1*-silenced larvae.

Several herbivores defensively co-opt ingested plant metabolites either with or without metabolism^{3,11,34–36}; 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 *BG1* silencing on larval survival in the host's predator-teemed native habitat³². We introduced EV, *irBG1* and *irGGPPS* stable transgenic lines into a field plot in the Great Basin Desert, Utah (Fig. 5b) and allowed *M. sexta* larvae to feed on these plants. Diurnal and nocturnal survivorships of *BG1*-silenced larvae (68% and 69%, respectively) did not differ significantly from those of larvae feeding on EV (70% and 70%, respectively) and *irGGPPS* (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 *irBG1* plants. Since the moulting-impaired *BG1*-silenced larvae were not used in these assays, occurrence of such carcasses was likely not related to moulting impairment. Hence, that such carcasses were not found on EV or *irGGPPS* plants, clearly suggested that the *BG1*-silenced larvae experienced some distinctive predator behaviour. During the night, *M. sexta* larvae are mainly preyed on by the wolf spiders (*Camptocosa parallela*; Lycosidae), which are abundant in this habitat^{32,37} (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

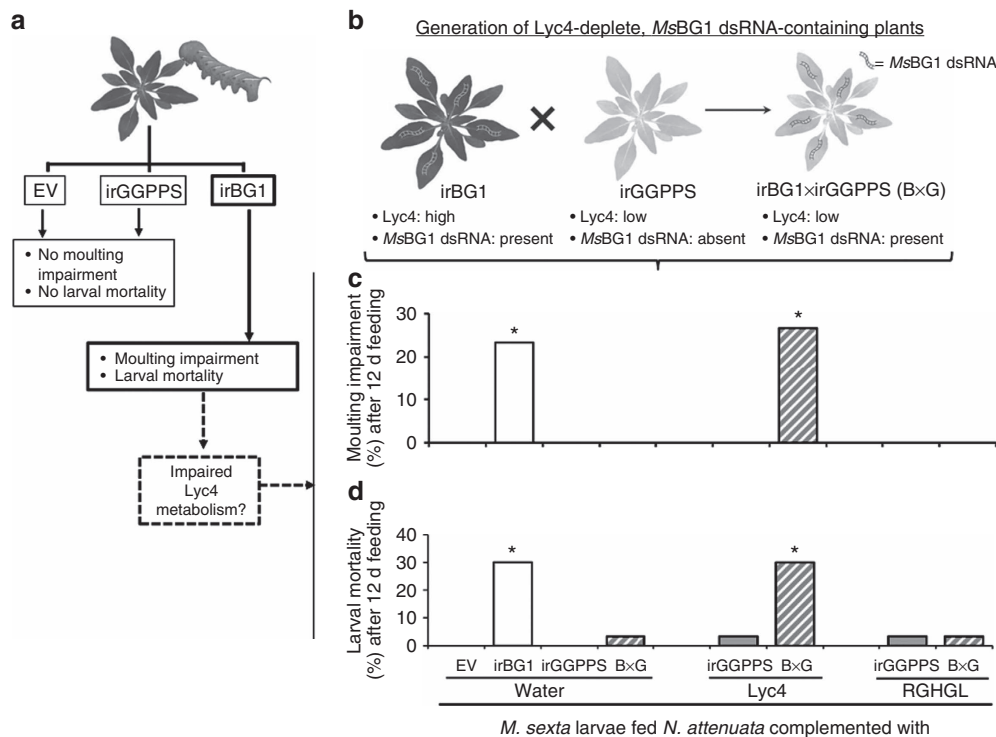


Figure 4 | Mothing impairment and mortality in BG1-silenced larvae is associated with suppressed *Lyc4* metabolism. (a) Schematic showing the motivation for the hypothesis that suppressed *Lyc4* metabolism in *irBG1*-feeding larvae causes mothing impairment and mortality. (b) Generation of *MsBG1*-containing and *Lyc4*-depleted transgenic *N. attenuata* plants (B × G) by crossing *irBG1* with *irGGPPS* plants. (c) Mothing impairment (%) (significant difference ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 30$) and (d) mortality (%) (significant difference ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 30$) in larvae after 12 d 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, days.

two larvae (one each from EV fed and *irBG1* fed or EV fed and *irGGPPS* fed or *irBG1* fed and *irGGPPS* 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-, *irBG1*- or *irGGPPS*-fed larva ($n = 30$). Frequencies of prey capture and killing were similar for EV-, *irBG1*- and *irGGPPS*-fed larvae. We observed that all the killed EV- and *irGGPPS*-fed larvae were completely eaten; however, spiders did not ingest ~75% of the BG1-silenced larvae that they killed (Fig. 5f,g). These results are consistent with the hypothesis that spiders were responsible for the carcasses of partially eaten larvae we found during the nocturnal survivorship 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, *irBG1*, *irGGPPS* or B × G leaves, *Lyc4*-coated *irGGPPS* or B × G leaves or RGHGL-coated *irGGPPS* 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 *irBG1* and *Lyc4*-coated B × G leaves (Fig. 6b); spiders preying on these larvae, but not EV- and *irGGPPS*-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 *irGGPPS*- (Fig. 6c–i) and AD-fed (Supplementary Fig. 13d–h) larvae ($n = 21$

in choice assays and $n = 30$ in no-choice assays) typically 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. 13i). Moreover, no *Lyc4* or RGHGL was detected in the washes of water-coated *irGGPPS*- or AD-fed larvae and also the EV-, *irBG1*- and *irGGPPS*-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.*²⁰ 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

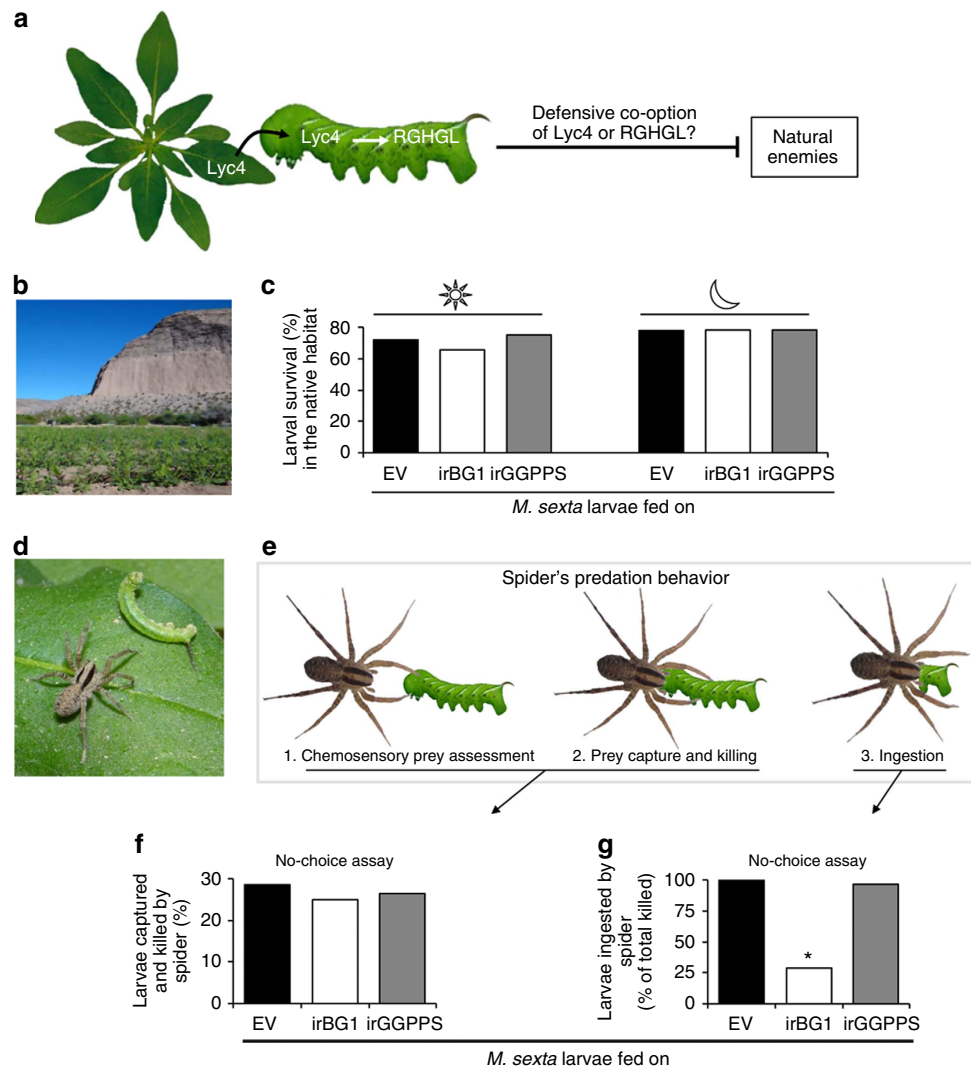


Figure 5 | In the native habitat, spiders capture and kill but do not ingest the BG1-silenced *M. sexta* larvae. To test (a) whether Lyc4 or RGHGL is defensively co-opted by larvae against the natural enemies in the native habitat, (b) EV, irBG1 and irGGPPS plants were grown in the Great Basin Desert, Utah. (c) Diurnal and nocturnal survival (%) of larvae feeding on these plants ($n = 32$ larvae per line). (d) Native *C. parallela* 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 ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 28$) in a no-choice assay (1 h) on second-instar *M. sexta* larvae feeding on EV and irBG1 and irGGPPS 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 the distribution of Lyc4 could be crucial for the optimization of the plant's defensive strategy³⁸. The role of malonylated Lyc4 also appears to be similar to that of phytoanticipins in which, glycosylation ensures the within plant stability of defence molecules by maintaining them in inactive or rather pretoxin states^{1,2}. 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^{3,11,12} 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 irBG1 foliage and the reappearance of these phenotypes in larvae feeding on Lyc4-complemented B \times 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* (Magnaporthaceae) detoxifies the avenicin saponins (triterpenoid glycosides containing three sugars) of oat by BG-catalysed deglycosylation^{39,40}. 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

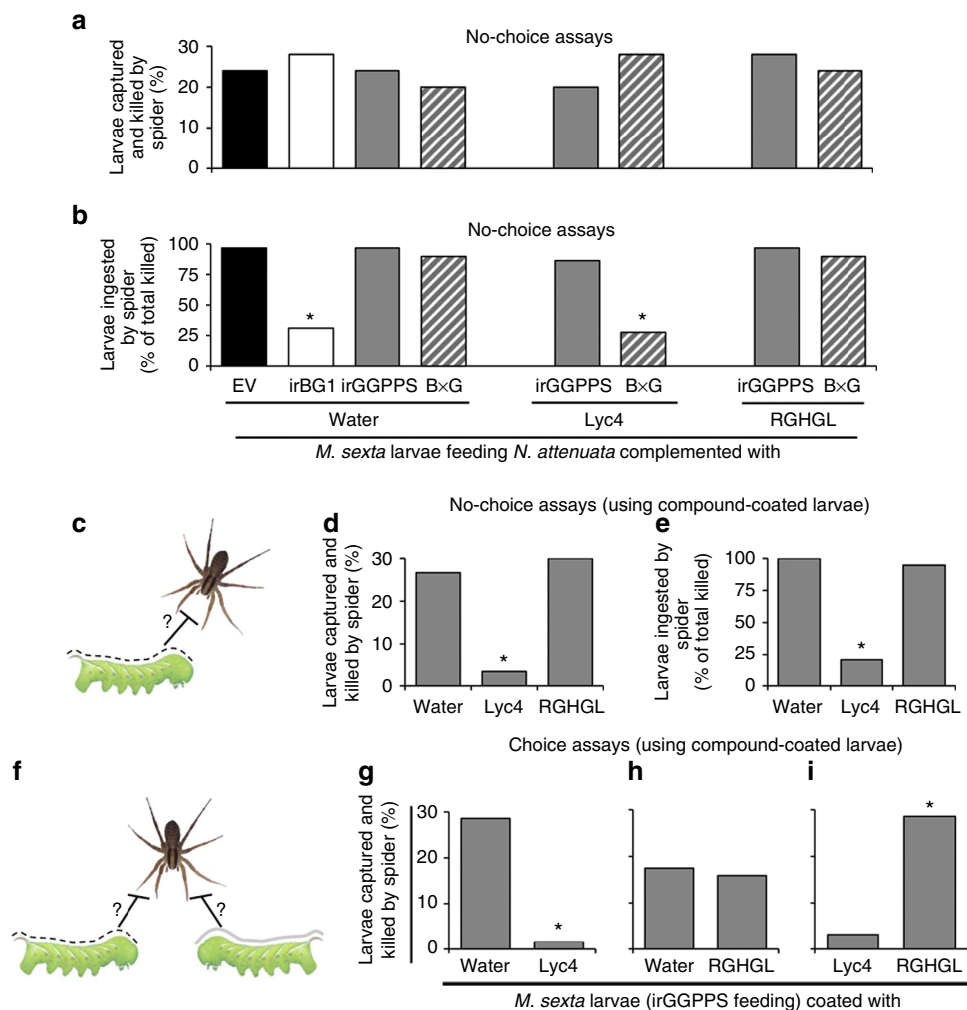


Figure 6 | High larval body Lyc4 concentration hinders spiders' prey ingestion and topically coated Lyc4 deters spiders. Spider's (a) prey capture and killing (%; $n = 25$) and (b) prey ingestion (% of total killed; significant difference ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 25$) in a no-choice assay (1 h) using second-instar *M. sexta* larvae feeding on EV and irBG1 leaves coated with water (control) and irGGPPS and B × G leaves coated with water (control), Lyc4 (final concentration 6 mM) and RGHGL (final concentration 6 mM). (c) Schematic of a no-choice assay. (d) Spider's prey capture and killing (%; significant difference ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 30$) and (e) prey ingestion (% of total killed; significant difference ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 30$) in no-choice assays (1 h) using second-instar water-, Lyc4- or RGHGL-coated (final concentration 6 mM for both Lyc4 and RGHGL) *M. sexta* larvae feeding on irGGPPS plants and (f) schematic of a choice assay. Spider's prey capture and killing (%) in choice assays (1 h) using irGGPPS-feeding second-instar *M. sexta* larvae coated with (g) water or Lyc4 (final concentration 6 mM; significant difference ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 21$), (h) water or RGHGL (final concentration 6 mM; $n = 21$) and (i) Lyc4 (final concentration 6 mM) or RGHGL (final concentration 6 mM; significant difference ($P \leq 0.05$) determined by Fisher's exact test of frequencies; $n = 21$).

3 β -hydroxy sterols, which leads to the formation of membrane pores and cell lysis^{41,42}. 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 activities⁴³, but the aglycone is completely unable to bind to DNA⁴⁴. Similarly, a nonsugar sweetener, glycyrrhizic 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 buds⁴⁵. It is known

that binding of glycosides' specific sugars to receptors on the cell surfaces facilitates their active uptake⁴⁶. Conspicuously, higher amounts of Lyc4 than RGHGL in most of the larval tissues (in EV- as well as irBG1-fed 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^{36,47}, 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 HGL-DTG 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 (Snijders Scientific) at 26 °C:16-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, irBG1, irGGPPS or B × G) until they were used in glass-house experiments. In some experiments, neonates were fed on AD⁴⁸ containing 6 mM *Lyc4* (aqueous; in 100 $\mu\text{g g}^{-1}$ AD), 6 mM RGHGL (aqueous; in 100 $\mu\text{g g}^{-1}$ AD) or equivalent amount of water (control), to rear the larvae free from any influence of the hostplant.

Plant material. The 30th generation seeds of an inbred line of *N. attenuata*, which were originally harvested in 1988 from a native population in Utah (USA), were used for the development of stable transgenic lines⁴⁹. Seeds were germinated and plants were grown as explained previously²⁴.

Field experiments were conducted at Lytle Ranch Preserve in Santa Clara, Utah, 84765 (37° 08' 45" N, 114° 01' 11" W) in 2013. Seeds of *N. attenuata* EV, irGGPPS and irBG1 lines 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 al.*⁵⁰

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 bootstrapping trials to identify the formation of clusters. Three BG 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 described²⁴.

Plant transformation and Southern hybridization. Stable transgenic PMRi plants were generated by *A. tumefaciens*-mediated transformation of recombinant pSOL8 vector⁴⁹. The pSOL8 vector contained an ir of 301-bp stretch (+84 to +384) of *MsBG1* separated by the *pdk i3* intron and a selectable marker, hygromycin phosphotransferase (*hptII*), for hygromycin resistance⁵¹. Transgenic lines were screened as described by Gase *et al.*⁵² Single transgene insertions in two independently transformed homozygous irBG1 lines (375-10 and 379-8) were confirmed using Southern hybridization. For Southern hybridization, genomic DNA was extracted from the rosette leaves of untransformed WT and irBG1 plants (line no. 375-10 and 379-8) using a modified cetyltrimethylammonium bromide

method as described by Rogers and Bendich⁵³. The genomic DNA (10 μg) was completely digested with HindIII and EcoRV, 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 Gase *et al.*⁵²

Previously characterized GGPPS (NCBI accession no. EF382626)-silenced irGGPPS line of *N. attenuata*²⁰ was used as a *Lyc4*-depleted control to feed *M. sexta* larvae. An EV-transformed *N. attenuata* transgenic line (A-04-266-3) was used as a *Lyc4*-replete control²⁸.

Harvesting larval tissues. Fourth-instar Larvae were immobilized by placing them on ice before dissection. Haemolymph was collected by clipping the larval horn just before dissection, as described by Kumar *et al.*²⁴. 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 $-80\text{ }^{\circ}\text{C}$ until further use²⁴. *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 skin; 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 larvae²⁵. Collected regurgitant was stored under argon at $-80\text{ }^{\circ}\text{C}$ 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 (10%) precipitation in the presence of 1 M NaCl²⁴.

A 120-bp stretch of the 301-bp-*MsBG1* fragment cloned for PMRi 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 α -³²P isotope using the Rediprime II DNA labelling system (Amersham Biosciences). Low-molecular-weight RNA blotting, hybridization and screening of the blot performed²⁴.

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 to eliminate genomic 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)²⁴. Relative quantification of transcripts was carried out by the comparative D cycle threshold method. *MsUbiquitin* 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.ut.ee/>).

Extraction and GC-MS analysis of 17-HGL (aglycone). 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 leaf was also analysed to determine whether the plant glucosidases deglycosylate the HGL-DTGs when herbivores ingest the leaf. Samples of each tissue (100 mg) were homogenized in 1 ml *tert*-butyl methyl ether and then centrifuged at 13.4g 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 trimethyl sulfonium hydroxide. Derivatized samples were analysed for aglycones using DB5 column in Varian CP-3800 GC-Saturn 4000 ion trap MS^{54,55}. Leaf tissue was crushed in the alkaline buffer (sodium phosphate buffer, pH-8.5), acidic buffer (sodium phosphate buffer, pH-6) and acidified regurgitant (pH-6), separately, and was incubated at 37 °C for 1 h before extraction.

Extraction and purification of metabolites from larval frass. Frass of EV-fed *M. sexta* larvae was collected and stored at $-80\text{ }^{\circ}\text{C}$ 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 13.4g for 30 min at 4 °C. The supernatants were incubated at $-20\text{ }^{\circ}\text{C}$ for 2 h for precipitating proteins and were again centrifuged at 13.4g for 30 min at 4 °C and the clear supernatants were collected. Further, the extract was evaporated in a rotary evaporator to one-tenth of its original volume. The concentrated extract was centrifuged at 13.4g for 20 min at 4 °C and the supernatant was subjected to fractionation using a reverse-phase

HPLC (Luna C-18 (2), 250 × 10 mm; Phenomenex) at a 3-ml min⁻¹ flow rate. Two types of solvents (A and B) were used to elute analytes from the column. Solvent A: HPLC grade methanol (Fluka, Germany) and solvent B: millipore water (obtained using a Millipore model Milli-Q Advantage A10). Chromatographic solvent gradients were, from 0 to 20 min (15% of B) and from 20 to 40 min (100% of B). Lyc4 and RGHL were collected from fractions 46 (23 min) and 48 (24 min), respectively. Collected fractions were dried completely in a vacuum concentrator (3.7 mbar; Concentrator 5301; Eppendorf), and thus obtained pure metabolites were used for further experiments.

Extraction and U(H)PLC/ESI-QTOF-MS analysis of HGL-DTGs. For analysing Lyc4 and its malonylated forms of Lyc4 in the leaf, larval tissues and larval frass, samples were homogenized and extracted in an acidic extraction buffer A (60% solution 1 (2.3 ml l⁻¹ of acetic acid, 3.41 g l⁻¹ ammonium acetate, pH-4.8 using 1 M NH₄OH) and 40% methanol). An internal standard rebaudioside A was always spiked into extraction buffer A (10 ng μl⁻¹) before extraction. A amount of 100 mg of homogenized sample was extracted in 1 ml of buffer A. Extracted samples were diluted 1:50 with buffer B (one part of buffer A and nine parts of 40% methanol) and analysed using a U(H)PLC/ESI-QTOF-MS²⁰. Retention times and molecular ions of Lyc4, RGHL and rebaudioside A were determined by loading 1 ng of each of these compounds onto a Fisher Scientific Acclaim C18 (2.1 × 150 mm) U(H)PLC column (particle size 2.2 μm) with solvent A (0.1% acetonitrile, 0.05% formic acid (vol/vol) in ultrapure Millipore water) and solvent B (0.05% formic acid in acetonitrile). 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 a qTOF-mass spectrometer (micro TOF QII Bruker Daltonik, Germany) equipped with an ESI source in positive ion mode (instrument settings: capillary voltage, 4,500 V; capillary exit, 130 V; dry gas temperature, 200 °C; dry gas flow, 8 l min⁻¹). Calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% (vol/vol) isopropanol/water containing 0.2% formic acid (vol/vol)).

NMR analysis. ¹H NMR, ¹³C NMR, ¹H-¹H COSY, ROESY, TOCSY, HSQC and HMBC experiments were measured on a Bruker Avance 500 NMR spectrometer (Bruker Biospin, Germany), operating at 500.13 MHz for ¹H and 125.75 MHz for ¹³C. Capillary tubes (2-mm-internal diameter) were used to measure spectra in a TCI cryoprobe (5 mm) at a probe temperature of 300 K. Samples were prepared in methanol-*d*₄ solvent (85 μl). ¹H and ¹³C chemical shifts were referenced to the residual solvent signals of methanol-*d*₄ at δ 3.31 and δ 49.15, respectively.

Extraction and HPLC analysis of Lyc4 and other metabolites. Stored leaf and frass samples were homogenized in liquid nitrogen using pre-chilled mortar and pestle. Extraction buffer A (1 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/Grinder 2000 (Spex, UK) for 2 min with 600 strokes per min. Homogenized samples were centrifuged at 13.4g for 20 min, at 4 °C. The supernatants were re-centrifuged at 13.4g for 20 min at 4 °C for separating undissolved particles. Clear supernatants were analysed using HPLC (Agilent 1100 series)²⁹. Lyc4 from leaf and Lyc4 and RGHL from frass were quantified using rebaudioside A (Sigma, Germany) as an external standard.

The linearity of the extraction of Lyc4 and RGHL from frass was determined as follows. Crushed dried frass (100 mg) of irGGPPS-fed larvae was spiked with 2, 4, 6, 8, 16 and 32 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 described³².

Nicotine, chlorogenic acid, caffeoyl putrescine and rutin were also extracted from the EV, irBG1 (375-10) and irBG1 (379-8) rosette leaves using this method and were analysed by HPLC (Agilent 1100 series) as described by Keinaenen *et al.*²⁹

In vitro demalonylation assay. Leaf discs (10 mm) from fully expanded node-2 rosette leaf³⁶ of EV plants were collected in liquid nitrogen and homogenized using Dounce homogenizers. A amount of 50 mg of leaf material was used in each assay and incubated with 100 μl of water (control), regurgitant, boiled regurgitant, regurgitant + pronase E (4 units per microlitre of regurgitant; Sigma), regurgitant + inactivated pronase E (heated at 85 °C for 20 min), alkaline buffer (sodium phosphate buffer, pH-8.5), acidic buffer (sodium phosphate buffer, pH-6) and acidified regurgitant (pH-6) in separate treatments at 37 °C for 1 h. Regurgitant was boiled or treated with pronase E to inactivate enzymes in it. During the assay period, assay contents were mixed intermittently. After 1 h, metabolites were extracted and analysed for Lyc4 and its malonylated forms using HPLC.

In vitro BG enzyme assay. Midguts of larvae feeding on EV and irBG1 plants were homogenized using Dounce homogenizers and incubated with either 1 mM of Lyc4 or 1 mM of Lyc4 + 1 mM δ-gluconolactone (BG inhibitor; Sigma, Germany)⁵⁷, for 1 h. Then, the assay contents were extracted and their Lyc4 and RGHL concentrations were determined using U(H)PLC/ESI-QTOF-MS.

Excretion efficiency determination assays. Ingestion of Lyc4 and excretion of Lyc4 and RGHL by *M. sexta* larvae were budgeted using the excretion efficiency determination assays^{32,33}. Freshly hatched *M. sexta* neonates were allowed to feed on EV and irBG1 rosette-stage plant leaves until they had reached the fourth-instar. The mass of each larva was recorded and then the larvae were starved for 4 h to empty their guts. Larval mass was measured again after starvation. Each larva was then provided with a known mass of leaf material from the same genotype of plant that they had been fed previously. Larvae were then fed for 24 h at 26 °C:16-h light, 24 °C:8-h dark and 60% relative humidity. To absorb the excreted 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 after 24 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. Lyc4 levels of leaves of each line were measured by HPLC (Agilent 1100 series). Actual amount of Lyc4 ingested by each larva was calculated based on the amount of foliage ingested. Lyc4 and RGHL levels in the collected frass samples (along with the blotting paper discs) were also measured and corrected for the mass of the blotting paper disk. The percentage of Lyc4 or RGHL excreted was determined by calculating the ratio of the amount of compound excreted/amount of Lyc4 in the ingested food × 100.

Generation of irBG1 × irGGPPS (B × G) plants. Lyc4-depleted and BG1 dsRNA-producing plants were generated by crossing irBG1 (375-10) with irGGPPS (230-5) plants. Crossing was performed by pollinating the stigmas of atherectomized (before pollen maturation) irBG1 flowers with irGGPPS pollen. B × G seeds were collected and germinated. Lyc4 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 subjected to PCR using BG1 primers (Supplementary Table 2) to confirm the presence of *MsBG1* cDNA fragment inherited from the irBG1 parent.

Moulting impairment and mortality analyses. To determine the effect of Lyc4 detoxification 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 were observed in irBG1-feeding larvae during their each instar change, to determine whether *BG1* silencing or suppressed Lyc4 deglycosylation caused these phenotypes, we used the complementation method. Detached fully expanded rosette leaves of irGGPPS and B × G were coated with Lyc4 and RGHL (separately; final concentration 6 mM; 4,656 μg Lyc4 and 3,684 μg RGHL in 100 μl water per gram leaf, respectively) and EV, irBG1, irGGPPS and B × G leaves were coated with 100 μl water (control) using a paint brush. Freshly hatched neonates were reared on these leaves for 12 days (until they reached fourth-instar). Fresh leaf material (Lyc4, RGHL 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 Lyc4 and RGHL levels in their bodies using U(H)PLC/ESI-QTOF-MS.

Survivorship assays in the field. Diurnal and nocturnal survivorship assays were conducted as described by Kumar *et al.*³². *M. sexta* neonates were fed on EV, irBG1 and irGGPPS plants until they reached second-instar. Thirty-two 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 array³². To analyse diurnal survivorship, larvae were placed on plants in the field at 0600 hours and the number of larvae surviving on each plant was counted at 2000 hours; to analyse nocturnal survivorship, 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 polypropylene container (60 cc), as described by Kumar *et al.*³². *C. parallela* spiders 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 reused 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 larvae on irBG1 plants was tested using no-choice assays. Percentages of larvae captured and killed by spiders and larvae ingested (of total captured) by spiders in 1 h were calculated for EV-, irBG1- and irGGPPS-fed larvae. To determine whether Lyc4 or RGHL content of BG1-silenced larvae influenced spider's prey ingestion

behaviour, larvae fed on water-coated EV, irBG1, irGGPPS or B × G leaves, Lyc4-coated irGGPPS or B × G leaves or RGHGL-coated irGGPPS or B × G leaves were used. In the assays conducted to evaluate whether externalized Lyc4 or RGHGL can influence spider's predation behaviour, water-Lyc4- and RGHGL-coated AD-fed larvae were used; AD-fed larvae were used instead of irGGPPS-fed larvae to remove the effect of nicotine present in irGGPPS plants because *M. sexta* larvae exhale the ingested nicotine to deter spiders³².

Choice assay. In these assays, each spider was allowed to choose between one test and one control larvae. Following combinations of test and control larvae were used in the assays conducted to determine whether spiders can choose between control, Lyc4-depleted and Lyc4 metabolism-suppressed larvae: irBG1 fed + EV fed, irBG1 fed + irGGPPS fed and irGGPPS fed + EV fed. Similarly, the following combinations of test and control larvae were used in the assays conducted to find whether Lyc4 or RGHGL deterred spiders: Lyc4 coated + water coated, RGHGL coated + water coated and Lyc4 coated + RGHGL 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 larvae from each test treatment.

Coating *M. sexta* larvae with water, Lyc4 or RGHGL. AD-fed *M. sexta* larvae (50 ± 5 mg FM) were coated with 50 µl water (control) or 50 µl of 6 mM aqueous Lyc4 or RGHGL, using a 10-µl-micropipette tip. Coated compounds were allowed to air dry for 5 min, before using the larvae for assays.

Stability of Lyc4 and RGHGL in frass. To test whether Lyc4 and RGHGL degrade in the excreted frass during the 24 h of excretion 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 excretion efficiency determination assay. Samples immediately extracted 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 RGHGL in coated leaf and larvae. To test whether Lyc4 and RGHGL coated on leaves degraded during assays, these metabolites were extracted from the coated leaves after 0 h (control) and 24 h (a period for which these leaves were fed to larvae) incubations. Extracted metabolites were analysed using U(H)PLC/ESI-QTOF-MS.

Similarly, to test whether Lyc4 and RGHGL 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 ng µl⁻¹ rebaudioside A) and these washes were analysed for metabolites using U(H)PLC/ESI-QTOF-MS.

Analysis of Lyc4 or RGHGL externalization by larvae. To test whether the larvae externalize Lyc4 or RGHGL, second-instar EV-, irBG1- and irGGPPS-fed and water-coated irGGPPS- and AD-fed larvae (*n* = 3) were washed with 1 ml buffer A (containing 10 ng µl⁻¹ rebaudioside A) and the washes were analysed using U(H)PLC/ESI-QTOF-MS, as described above.

Statistical analyses. All the quantitative data (metabolites in leaf material, larval tissues, and larval frass and BG1 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. Mead's resource equation method⁵⁸ was used to determine the adequate sample sizes. Homogeneity of variance of sample groups was tested using Levene's test⁵⁹; sample groups with unequal variances were analysed using Welch's test⁶⁰. Significance (*P* ≤ 0.05) of the binary results of all moulting 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.

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Author contributions

S.P., S.S.P., I.T.B., S.M., B.S. and J.C. designed the study. S.P., S.M., S.S.P., M.S., J.C. and P.K. carried out experiments. S.P., S.M., S.S.P., M.S., J.C., B.S. and P.K. analysed data; S.S.P., S.P., I.T.B., S.M., B.S., J.C. and P.K. prepared the manuscript.

Additional information

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