The roles of plant activated defenses and specialized herbivore adaptations in multi-trophic interactions

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This dissertation is based on the following research articles:

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1. Introduction

1.1 Plant chemical defenses against herbivores

Plants and herbivorous insects have co-existed for over 350 million years. To cope with the stresses caused by herbivores, plants have developed diverse defense strategies (Gatehouse, 2002). One strategy encompasses external morphological adaptations including thorns, prickles, and trichomes (Hanley *et al.*, 2007), while another involves the production of defensive chemicals: plants synthesize metabolites to either deter or posion insect herbivores or attract herbivore enemies (Chen, 2008; Mithöfer *et al.*, 2012). Plant defensive chemicals are drawn from the huge variety of secondary metabolites produced by plants, estimated to number more than 200,000, with a large degree of structural diversification (Pichersky *et al.*, 2011). This structural diversity allows these metabolites to act on herbivores in many ways as repellants, toxins, anti-digestive compounds, or anti-nutritionals.

Plant defensive metabolites are generally either inducible by herbivore attack or constitutively present in plant tissues (Karban *et al.*, 1999). The utilization of defensive chemicals is considered to be costly for plants due to the resources invested in them, their possible auto-toxicity, and their ecological ramifications, such as when a compound defending against one herbivore attracts another (Purrington, 2000; Strauss *et al.*, 2002). The inducible biosynthesis of plant defensive chemicals solely upon herbivore attack may minimize these costs. However, prior to the induction of defenses, the plant remains vulnerable. In contrast, pre-existing constitutive defense systems arm plants with chemical weapons prior to herbivore attacks, but may be costly due to their deployment in all situations (Wittstock *et al.*, 2002a). In addition, the constant accumulation of high concentrations of toxins in plants can cause auto-toxicity. Glucosylation is one of the major strategies for plants to reduce toxicity by stablilizing defensive compounds in a non-toxic form (pro-toxin), for example as glucosinolates, with the actual toxin (isothiocyanates, ITCs) then being released only upon herbivore attack (Pentzold *et al.*, 2014). Additionally, to enhance defense efficiency, plants are capable of adjusting the distribution of such defensive chemicals in different organs and across developmental stages depending on the frequency of herbivore attacks and the inherent value of the plant tissue (Brown *et al.*, 2003; Shroff *et al.*, 2008; Touw *et al.*, 2020), therefore minimizing the costs of an unnecessary biosynthesis of defensive metabolites.

1.1.1 Defensive glucosides and activating glucosidases

Glucosides exist widely in plants and form several classes. Those serving as defensive chemicals include cyanogenic glucosides (Zagrobelny *et al.*, 2004), benzoxazinoids (Wouters *et al.*, 2016), iridoid glucosides (Pankoke *et al.*, 2012), and glucosinolates (Hopkins *et al.*, 2009). Generally, in intact plant tissue, glucosides are stored spatially separated from β-glucosidases (β-D-glucopyranoside glucohydrolases) to avoid inadvertent activation and auto-toxicity (Morant *et al.*, 2008; Pentzold *et al.*, 2014). Upon plant tissue rupture, for example during herbivore attack, the integrity of cells or cellular compartments is lost. The glucosylated defense chemicals then encounter β-glucosidases resulting in the release of aglucones that are often unstable and are further rearranged to form repellent or toxic metabolites **(Figure 1)**. In addition to pro-toxin stabilization, the increased solubility conferred by glucosylation enables plants to store large amounts of pro-toxic constituents, reaching millimolar concentrations (Jones *et al.*, 2001). Thus, the stored inactive glucosides and β-glucosidases, often referred to as “two-component plant defense systems”, are advantageous both in minimizing auto-toxicity and in increasing the rapidity of the defense response.

β-Glucosidases are enzymes that hydrolyze β-*O*-, or *S*-linked glucosidic bonds to release the terminal glucose residues from glucosides and oligosaccharides (Cairns *et al.*, 2010). These enzymes exist in all domains of living organisms and play a variety of functions, including biomass conversion in microorganisms, breakdown of glycolipids and activating defenses, phytohormones and scent compounds (Cairns *et al.*, 2010). In plant defense against biotic stresses, β-glucosidases have long been known as “detonators” of pro-toxin “bombs”. For example, the cyanogenic glucosides present in more than 2500 plant species are hydrolyzed to release α-hydroxynitrile aglucones, which are further spontaneously or enzymatically degraded to release hydrogen cyanide, an inhibitor of the mitochondrial respiratory pathway **(Figure 1A)** (Morant *et al.*, 2008). The benzoxazinoid DIMBOA glucoside (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one-2-*O*-glucoside) synthesized by several plants in the Poaceae family is activated by specific β-glucosidases. The DIMBOA aglucone thus liberated forms an α-oxo-aldehyde **(Figure 1B),** which is a potent electrophile capable of reacting with nucleophilic residues of proteins, such as thiols and amines, and causing enzymatic inhibition (e.g. of digestive enzymes) (Dixon *et al.*, 2012; Wouters *et al.*, 2016)**.** Iridoid glucosides from plants of the Asteridae family are activated by plant β-glucosidases or by endogenous insect β-glucosidases (Pankoke *et al.*, 2012),

liberating highly reactive aglucones **(Figure 1C)** that irreversibly bind proteins and consequently affect enzyme activity and digestion of dietary proteins (Konno *et al.*, 1999; Dobler *et al.*, 2011)**.** In plants of the order Brassicales, glucosinolates are specifically hydrolyzed by β-thioglucosidases (myrosinases) to form unstable aglucones that spontaneously rearrange into toxic ITCs ("mustard oils") **(Figure 1D)** (Jeschke *et al.*, 2016a). The glucosinolate-myrosinase pair constitutes the well-known two-component defense system referred to as "mustard oil bomb", which protects plants from non-adapted herbivores and pathogens.

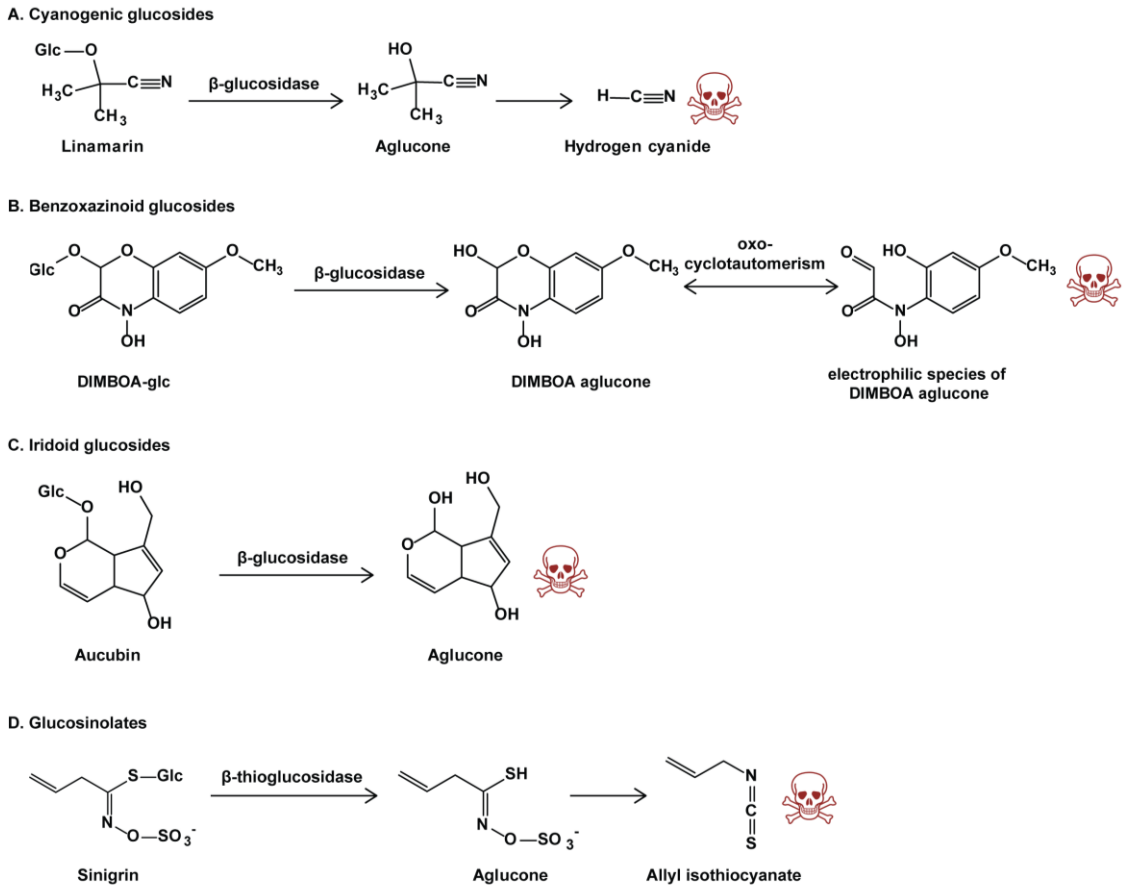

Figure 1. Examples of glucosides involved in plant chemical defense, which are activated by β-glucosidases (or β-thioglucosidases) and converted to the corresponding toxic products. Examples of activation of **(A)** cyanogenic glucosides, **(B)** benzoxazinoid glucosides, **(C)** iridoid glucosides, and **(D)** glucosinolates. Abbreviations: DIMBOA-Glc, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one-2-*O*-glucoside.

1.1.2 Mustard oil bomb: the glucosinolate-myrosinase defense system

Glucosinolates (β-D-thioglucoside-*N*-hydroxysulfates) are glucosylated specialized metabolites in plants of the Brassicales order, encompassing families such as the Capparaceae and Brassicaceae, which includes cabbages, mustards, horseradish, etc (Blažević *et al.*, 2016). Glucosinolates are amino acid-derived phytochemicals comprised of a β-thioglucose moiety and a variable side chain linked to a (*Z*)-*N*-hydroxyiminosulfate ester **(Figure 2)** (Mithen, 2001). Based on the structures of the corresponding amino acid precursors (Ala/Ile/Met/Val/Leu, Phe/Tyr, and Trp) that lead to the variable side chains, the 88 or more naturally occurring glucosinolates are typically classified into three categories, namely aliphatic, benzenic and indolic glucosinolates **(Figure 2)** (Blažević *et al.*, 2020). The variation in the side chain contributes to the variation in the biological activities of these phytochemicals (Blažević *et al.*, 2020). Glucosinolates are genetically variable within plant species and plastically distributed among different plant organs and developmental stages (Brown *et al.*, 2003; Shroff *et al.*, 2008; Touw *et al.*, 2020), which can influence both herbivore feeding choices and physiology (Müller *et al.*, 2010; Bejai *et al.*, 2012). For the model *Arabidopsis thaliana* Columbia-0 (Col-0) accession, the major types of glucosinolates are Met-derived aliphatic glucosinolates and Trp-derived indolic glucosinolates**,** accounting for around 85% and 15% of the total leaf glucosinolate pool, respectively. The most abundant, 4-methylsulfinylbutyl glucosinolate (4MSOB) represents over 70% of the aliphatic glucosinolates (Brown *et al.*, 2003), and has been shown to cause negative effects on the larval development of generalist-feeding insects such as *Spodoptera littoralis* (African cotton leafworm, Lepidoptera: Noctuidae), *Manduca sexta* (tobacco hornworm, Lepidoptera: Sphingidae) and *Trichoplusia ni* (cabbage looper, Lepidoptera: Noctuidae) (Müller *et al.*, 2010; Jeschke *et al.*, 2017).

Glucosinolates are stable and constitutively stored in high mM quantities in sulfur-rich *S*-cells (Koroleva *et al.*, 2011), while their activating β-thioglucosidase enzymes (myrosinases) are stored in protein-abundant idioblasts called myrosin cells (Rask *et al.*, 2000; Andréasson *et al.*, 2003). Upon mixing, glucosinolates are hydrolyzed by myrosinases leading to unstable aglucones, which rearrange into an array of downstream metabolites including ITCs, simple nitriles, thiocyanates and epithionitriles **(Figure 2)**. The outcome of this hydrolysis is dependent on the side chain structure of the parent glucosinolate, the presence of protein modulators (e.g. epithiospecifier, nitrile-specifier, and thiocyanate forming proteins) as well as the reaction conditions and presence of Fe (II)

(Figure 2) (Wittstock *et al.*, 2002b). ITCs contain a reactive –N=C=S functional core group and play major direct roles in plant-herbivore interactions, while simple nitriles are considered to be less directly toxic than ITCs and may function indirectly in defense (Burow *et al.*, 2006). Additionally, certain glucosinolate side chains can further dictate post-hydrolysis reactivities and occasionally lead to additional intramolecular reactions (e.g. cyclization to form oxazolidine-2-thione derivatives) or rapid loss of the –N=C=S group (e.g. to form the carbinol products from indolic glucosinolates) **(Figure 2)** (Blažević *et al.*, 2016), which alter toxicity. These reactivities may explain the stronger detrimental activities of aliphatic and benzenic glucosinolates, which form ITCs upon hydrolysis, than indolic glucosinolates that result in carbinol products (Schlaeppi *et al.*, 2008; Müller *et al.*, 2010; Jeschke *et al.*, 2016b).

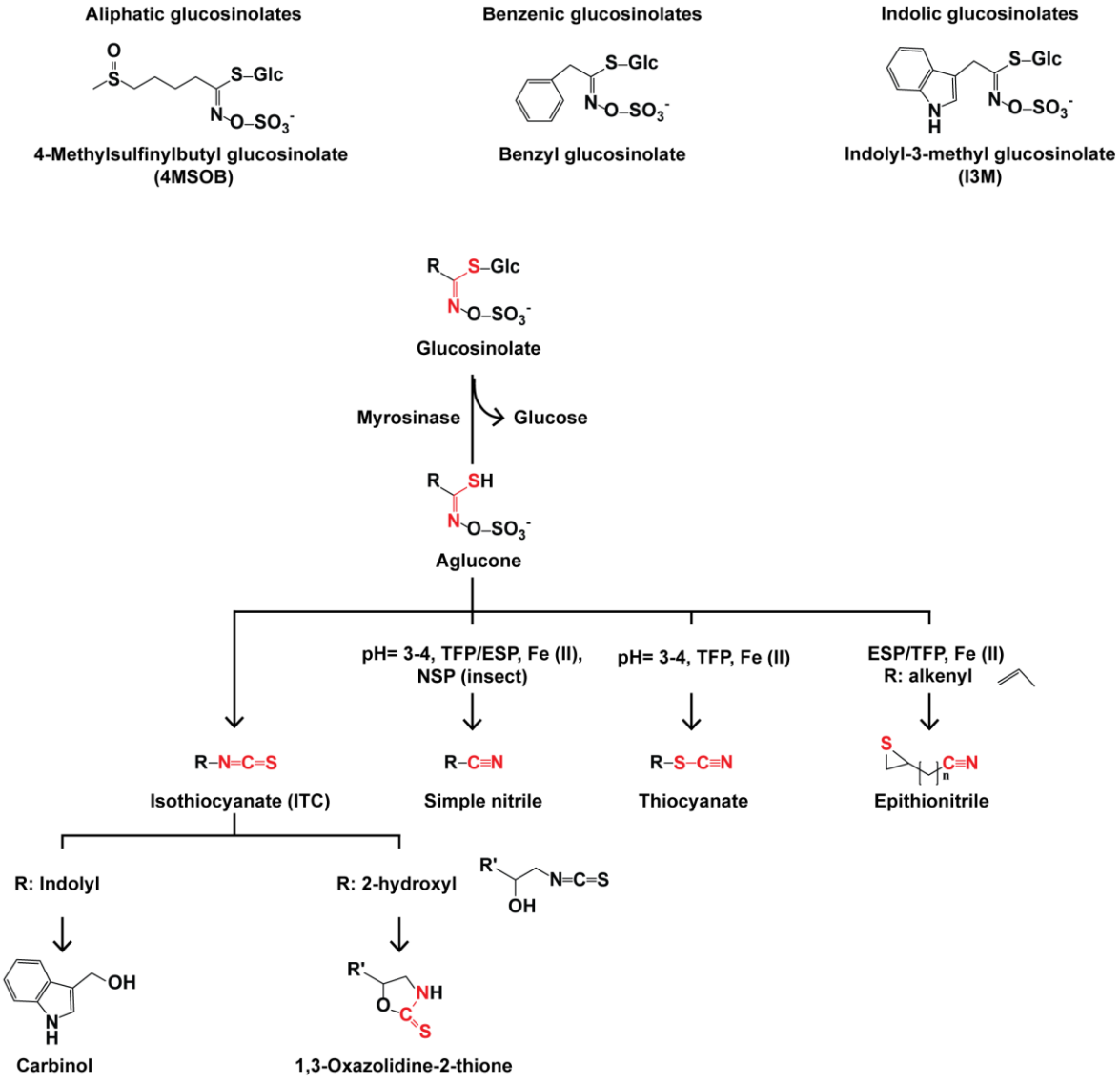

Figure 2. Glucosinolates and their degradation by myrosinases. Abbreviations: ESP, epithiospecifier protein; NSP, nitrile-specifier protein; TFP, thiocyanate forming protein.

1.1.3 Toxicity of isothiocyanates

The mustard oil bomb defense system of Brassicales plants generates as hydrolysis products the biologically active ITCs, an important class of glucosinolate metabolites that has been intensively studied for its toxicology (Rask *et al.*, 2000). The electrophilicity of the –N=C=S functional core group enables ITCs to react quickly and at times spontaneously with biological nucleophiles (Kawakishi *et al.*, 1982). Physiologically speaking, potential nucleophilic targets of ITCs include glutathione (GSH) (Schramm *et al.*, 2012), proteins containing reactive cysteine, selenocysteine or amine groups (Nakamura *et al.*, 2009; Hanschen *et al.*, 2012), and nucleic acids (Baasanjav-Gerber *et al.*, 2011). Protein modification and depletion of GSH are central to the effects of ITCs in biological systems. Moreover, the lipophilicity of the side chains can facilitate the diffusion of ITCs through the lipid bilayer membranes to reach the intracellular environment. While electron-withdrawing groups on the side chain will increase overall electrophilicity, and thus reactivity, steric attraction or hindrance between the side chain and a target protein will also promote selective reactivity (Brown *et al.*, 2011). Hence, in addition to the general properties of the electrophilic –N=C=S functional group, the structural diversity linked to the various type of ITC side chains can expand their potency to targets in a broad range of organisms, e.g. molluscan herbivores (Giamoustaris *et al.*, 1995; Falk *et al.*, 2014), insect herbivores (Brown *et al.*, 2011; Jeschke *et al.*, 2017), plant pathogens (Tierens *et al.*, 2001; Vela-Corcía *et al.*, 2019) and even plants (Bones *et al.*, 1996; Øverby *et al.*, 2015).

ITCs have toxic effects and impact the growth of generalist herbivorous insects feeding on plants of the Brassicales (Bruce, 2014). The larval growth and development of generalists such as *S. littoralis* and *Mamestra brassicae* (cabbage moth, Lepidoptera: Noctuidae) are negatively affected by the ingestion of the aliphatic 4-methylsulfinylbutyl ITC (4MSOB-ITC) (Jeschke *et al.*, 2017), and the larval growth of the generalist *Spodoptera frugiperda* (fall armyworm, Lepidoptera: Noctuidae) was slowed by the intake of the benzenic 3-methoxybenzyl ITC (Robert *et al.*, 1989). Specialized herbivores are, on the other hand, often armed with specific counter-mechanisms to avoid activation of plant glucosinolates (see section 1.2.2). Nevertheless, the development of specialists can also be negatively affected by the presence of glucosinolate-derived ITCs. For example, allyl ITC was lethal to neonate *Plutella xylostella* (diamondback moth, Lepidoptera: Plutellidae) larvae (Li *et al.*, 2000), and impacted the survival and growth of *Pieris rapae* (small cabbage

white, Lepidoptera: Pieridae) larvae (Agrawal *et al.*, 2003). Hence, the formation of ITCs by the glucosinolate-myrosinase system helps to protect Brassicales plants against attacks from a large diversity of herbivores. Correspondingly, in order to feed on those plants, some herbivores have developed counter-adaptations to this plant defense system, either by circumventing the formation of ITCs or modifying the ITCs formed to yield less toxic products.

1.2 Herbivore mechanisms for avoiding the toxicity of plant defenses

Faced with plant chemical defense systems, some herbivorous insects have developed successful strategies for avoiding the toxic effects of these compounds. Insects with restricted dietary preferences or specificities often have more specialized toxin-avoidance mechanisms compared to generalists. In contrast, generalist herbivores, which feed on a variety of plants, likely encounter several complex chemical defensive cocktails (Barrett *et al.*, 2012). Toxin avoidance mechanisms include modified feeding behavior, enzymatic detoxification, rapid sequestration, efficient excretion, target site insensitivity, and symbiont metabolism (Pentzold *et al.*, 2014; War *et al.*, 2018).

1.2.1 General mechanisms

Behavioral modifications

Herbivorous insects are not blind when foraging: some are found to selectively ingest certain parts of plants that contain less toxins. For instance, *Helicoverpa armigera* (cotton bollworm, Lepidoptera: Noctuidae) larvae avoid the intake of the midvein and periphery of *A. thaliana* leaves while feeding almost exclusively on the inner lamina, where lower glucosinolate concentrations are present (Shroff *et al.*, 2008), and also move among plant parts to avoid the ingestion of increased levels of plant defenses in induced tissues (Perkins *et al.*, 2013). In fact, insects forage on plants through multiple strategies, such as piercing-sucking, leaf-snipping, leaf-chewing, and leaf-mining. Piercing-sucking herbivores, with their non-disruptive type of feeding, might largely prevent tissue damage and, therefore, circumvent two-compound defense systems. Such a feeding type is characteristic of aphids, such as *Brevicoryne brassicae* (cabbage aphid, Hemiptera: Aphididae) and *Myzus persicae* (green peach aphid, Hemiptera: Aphididae), which ingest intact glucosinolates from phloem sap while causing little damage to plant tissues (Bridges *et al.*, 2002; Barth *et al.*, 2006).

Properties of the alimentary canal

After intake, the lining of the alimentary canal protects herbivorous insects from plant

defensive metabolites. Accordingly, adaptations are though to be present to digest plant materials and to absorb nutrients, while presumably minimizing the effects of toxic plant chemicals via compartmentalization, pH regulation, and detoxification (Linser *et al.*, 2014). The peritrophic membrane **(Figure 3)** provides a physical barrier between the ingested plant materials and the epithelial cells of the midgut, obstructing the access of certain toxic materials to the gut cells and hemolymph (Linser *et al.*, 2014). Moreover, the lumen of the insect herbivore gut can exhibit a range of pHs and can reach extreme levels of basicity, e.g. in *M. sexta* where the luminal pH may exceed 12 (Wieczorek, 1992). Such an alkaline environment affects catalysis and may inhibit plant defense metabolism (Berenbaum, 1980; Harrison, 2001). For instance, *Hyphantria cunea* (the fall webworm, Lepidoptera: Erebidae) larvae suppress the formation of toxic cyanide in their alkaline foregut, in which the pH is raised to 11 (Fitzgerald, 2008). Different gut cell types also create distinct components in insect digestive systems. For example, compared with the anterior midgut, the posterior midgut is enriched in the number of microvilli cells **(Figure 3)** that produce a plethora of enzymes and transporters engaging in plant defense detoxification (Linser *et al.*, 2014).

Metabolism by dedicated enzymes

Many ingested plant defense metabolites are metabolized by a variety of herbivore enzymes **(Figure 3)**. In general, such metabolism is divided in three phases. In phase I, enzymes such as P450 mono-oxygenases (P450s) and carboxylesterases (COEs) alter the reactivity and toxicity of defenses mainly through oxidation, reduction or hydrolysis (Brattsten, 1988; Feyereisen, 2012). These reactions increase the polarity and thus the excretion potential, or create new chemical functional groups that render molecules more suitable for phase II enzymatic reactions (Kennedy *et al.*, 2013). In phase II, the metabolism of toxins is mediated by conjugation to residues such as GSH, sulfate, and sugars by a broad range of enzyme families such as glutathione *S*-transferases (GSTs), sulfotransferases, acyltransferases, methyltransferases, and UDP-glucosyltransferases (UGTs). As a consequence, excretion is facilitated due to decreased reactivity and enhanced compound polarity. Several GSTs, for instance, catalyze the conjugation of GSH with ITCs, altering their electrophillicity and prompting a rapid efflux from cells (Brown *et al.*, 2011). This detoxification cascade is present in many generalist herbivores (Schramm *et al.*, 2012; Novoselov *et al.*, 2015; Krempl *et al.*, 2016), and also in certain mammals (Zhang *et al.*, 1995). In phase III, toxins and/or conjugated toxins are transported out of herbivore cells. This process recruits important membrane-bound proteins such as the ATP-binding

cassette (ABC) transporters. Beyond mediating the excretion of defenses out of cells, transporters are also indispensible for transferring compounds to defensive glands, spiracles or cuticular cavities of the integument. A pertinent example is the ABC transporter CpMRP identified in *Chrysomela populi* (poplar leaf beetle, Coleoptera: Chrysomelidae) that delivers the plant-derived phenolglucoside salicin from the hemolymph to the defensive gland (Strauss *et al.*, 2013).

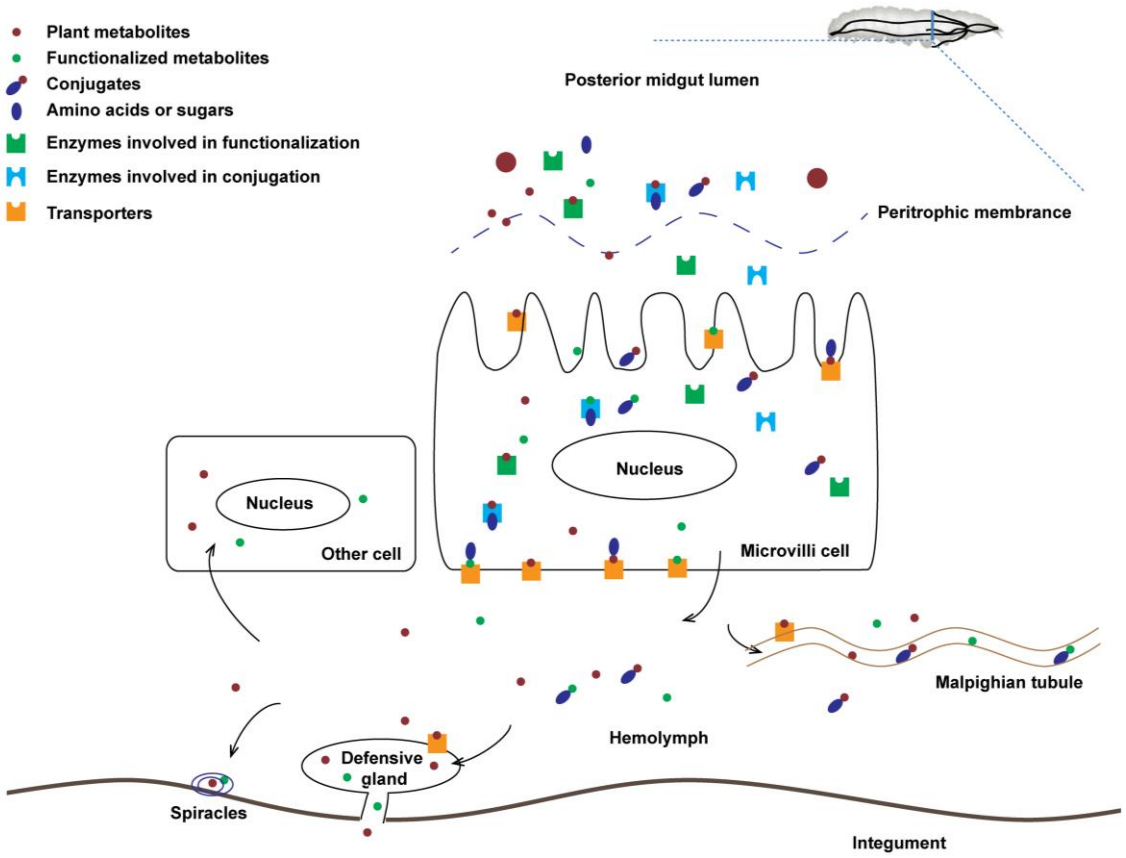

Figure 3. A general schematic representation of herbivore mechanisms to avoid the effects of plant toxins in the midgut.

Sequestration

The sequestration of plant toxins is a strategy exploited by specialized insect herbivores to redirect store specific plant defensive chemicals in tissues like hemolymph or defensive glands **(Figure 3)** as protection against antagonists (Rowell-Rahier *et al.*, 1992; Erb *et al.*, 2016). The monarch butterfly (*Danaus plexippus*, Lepidoptera: Nymphalidae), for instance, sequesters cardiac glycosides from host plants and stores them in sophisticated storage compartments, and excretes the glycosides upon predator attack (Bramer *et al.*, 2017).

Simultaneously, monarch larvae and butterflies are tolerant to cardiac glycosides via target site insensitivity, a series of amino acid substitutions in the binding site of the cardiac glycoside target enzyme, Na^+, K^+-ATPase, that disrupts binding of those molecules and thus lowers their toxic effects (Holzinger *et al.*, 1996). On the other hand, the turnip sawfly *Athalia rosae* (Hymenoptera: Tenthredinidae) rapidly sequesters glucosinolates into its hemolymph, thus avoiding hydrolysis and keeping the intact glucosinolate in the hemolymph as a defense against predators (Müller *et al.*, 2001; Abdalsamee *et al.*, 2014). To function as effective defenses, certain stabilized toxins need to be activated. *B. brassicae* and *Phyllotreta striolata* (striped flea beetle, Coleoptera: Chrysomelidae) produce β-thioglucosidases that hydrolyze sequestered glucosinolates and release ITC upon tissue disruption (Kazana *et al.*, 2007; Beran *et al.*, 2014).

1.2.2 Mechanisms to circumvent the glucosinolate-myrosinase defense system

Insect herbivores that feed on Brassicales plants have developed a multiplicity of mechanisms to overcome the glucosinolate-myrosinase defense system (Winde *et al.*, 2011; Jeschke *et al.*, 2016a) that act on different steps during the processes of ingestion and activation of these two-component defenses **(Figure 4)**.

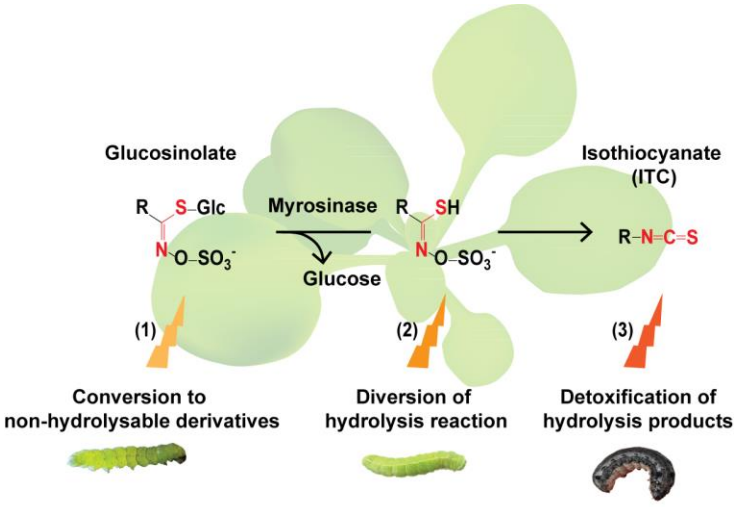

Figure 4. Herbivores interfere with different steps of glucosinolate activation.

Strategies include the reduced ingestion of glucosinolates through the choice of plant parts with low glucosinolate content (Perkins *et al.*, 2013), minimizing the decompartmentalization of glucosinolates and myrosinase by feeding without causing much tissue damage, e.g. sucking phloem-sap (Bridges *et al.*, 2002; Barth *et al.*, 2006) or rapid

sequestration. *Plutella xylostella* exploits another strategy converting glucosinolates prior to their hydrolysis into non-hydrolysable derivatives. For this purpose, glucosinolates are desulfated by endogenous sulfatases, which could outcompete hydrolysis by plant myrosinases **(Figure 5)**. This strategy is also seen in other herbivores, such as *Schistocerca gregaria* (desert locust, Orthoptera: Acrididae), *Bemisia tabaci* (silverleaf whitefly, Hemiptera: Aleyrodidae), and *Psylliodes chrysocephala* (cabbage stem flea beetle, Coleoptera: Chrysomelidae) (Falk *et al.*, 2007; Malka *et al.*, 2016; Ahn *et al.*, 2019).

Alternatively, certain herbivores may divert the hydrolysis reaction to give products less toxic than ITCs. The presence of nitrile-specifier proteins (NSPs) in species of *Pieris* enables the rearrangement of unstable aglucones into simple nitriles instead of toxic ITCs, as exemplified by the specialist *P. rapae* **(Figure 5)** (Wittstock *et al.*, 2004). The simple nitriles thus formed have lower direct toxicity to herbivores than ITCs (Wittstock *et al.*, 2003; Burow *et al.*, 2006), but can still benefit plants by attracting parasitoids or deterring oviposition by *P. rapae* (Mumm *et al.*, 2008).

If ITCs are formed, they may be detoxified via conjugation to the tripeptide glutathione (GSH, ɣ-Glu-Cys-Gly) following the canonical mercapturic acid pathway, as demonstrated in many generalist herbivores and mammals **(Figure 5)** (Kassahun *et al.*, 1997; Schramm *et al.*, 2012; Gonzalez *et al.*, 2018). ITC-GSH conjugates can be rapidly effluxed from cells, and the removal of glutamate and glycine followed by *N*-acetylation leads to the sequential formation of the ITC-cysteinylglycine (ITC-CG), ITC-cysteine (ITC-Cys), and ITC-*N*-acetylcysteine conjugates (ITC-NAC) (Brown *et al.*, 2011). Although the cost of biosynthesizing the GSH necessary for ITC detoxification leads to delayed larval development (Jeschke *et al.*, 2016b), the conjugation of glucosinolate-derived ITCs to GSH reduces the reactivity of these plant toxins and thus suppresses their toxicity.

1.2.3 *Plutella xylostella* and its glucosinolate sulfatases

The diamondback moth *P. xylostella* is the most devastating specialized pest of cultivated brassicaceous crops in the world, causing losses of US$ 4 – 5 billion per year (Zalucki *et al.*, 2012). Its high potential for crop devastation can be attributed to its extremely short (14 days) life cycle, which allows it to complete more than 20 generations per year. Adding to the trouble, *P. xylostella* is capable of rapidly developing resistance to insecticides (Sarfraz *et al.*, 2005a). Notably, it has developed resistance to almost all classes of synthetic chemical insecticides, as well as to modern biological pesticides like *Bacillus thuringiensis* (Bt) toxins (Sarfraz *et al.*, 2005b). Thus, it has become one of the most difficult pests to

control. The enzymatic desulfation of glucosinolates by *P. xylostella* (Ratzka *et al.*, 2002) has been long thought to allow these insects to suppress the activation of glucosinolates and thus circumvent the glucosinolate-myrosinase defense system of their host plants.

Glucosinolate sulfatases predominantly expressed in the midgut of *P. xylostella* larvae desulfate glucosinolates to form non-toxic desulfo-glucosinolates **(Figure 5)** (Ratzka *et al.*, 2002), which are no longer substrates for plant myrosinases. This process is controlled by a small gene family encoding glucosinolate sulfatases (GSSs) with varying specificities for different types of glucosinolates. GSS1 desulfates all glucosinolates tested in vitro except for 1-methoxyindolyl-3-methyl glucosinolate (1MOI3M), GSS3 activity is restricted to indolic and benzenic glucosinolate detoxification, and GSS2 uses only a subset of long-chain aliphatic glucosinolates (Heidel-Fischer *et al.*, 2019). GSSs thus help to avoid the toxicity of the glucosinolate-myrosinase system, and indeed it was reported that *P. xylostella* contains sufficient GSS to readily desulfate all ingested glucosinolates (Ratzka *et al.*, 2002). Accordingly, when isotopically labeled glucosinolates were administered to *P. xylostella*, over 80% were converted to desulfo-glucosinolates (Jeschke *et al.*, 2017). However, it cannot be automatically assumed that desulfation is an effective detoxification strategy without knowing whether it is of net benefit to insect performance and fitness, since desulfation may incur unanticipated physiological costs. Besides, there may be other mechanisms to circumvent glucosinolate toxicity that are more important than desulfation in allowing *P. xylostella* to feed on glucosinolate-containing plants. In this thesis we therefore attempted to down-regulate *P. xylostella* GSS activity to determine if glucosinolate desulfation fundamentally benefits this herbivore.

1.2.4 *Brevicoryne brassicae* and glucosinolate sequestration

The cabbage aphid *B. brassicae* is a destructive herbivore pest that specifically feeds on plants of Brassicaceae family. This aphid is of agricultural concern not only because of the wilting, yellowing and general stunting of the plants it causes, but it also vectors at least 20 viral pathogens that can induce diseases in Brassicaceae (Gill *et al.*, 2013). In warm climates, the rapid reproduction caused by aphid apomixia makes this herbivore more devastating. The piercing-sucking feeding behavior of aphids largely prevents plant tissue damage, and therefore mostly avoids the decompartmentalization of the host glucosinolate-myrosinase defense system, suppressing hydrolysis and thus minimizing ITC production.

Brevicoryne brassicae not only feeds on its Brassicaceae host plants without appearent negative effects, but also sequesters glucosinolates from them **(Figure 5)** for use

as defenses against its own antagonists. This aphid accumulates ingested glucosinolates in its hemolymph and concomitantly produces an endogenous myrosinase (BMY, *B. brassicae* β-thioglucoside glucohydrolase) in its head and thoracic muscles (Kazana *et al.*, 2007). Once the tissues are disrupted by predators, glucosinolates and BMY generate toxic ITCs, giving *B. brassicae* the description of "walking mustard oil bombs" **(Figure 5)** (Kazana *et al.*, 2007). *B. brassicae* preferentially accumulates aliphatic glucosinolates instead of indolic glucosinolates, even though many of its host plants are rich in the latter (Kos *et al.*, 2011). As Brassicales plants vary substantially in glucosinolate content, it is questionable if the glucosinolate selectivity of *B. brassicae* fed on one plant applies to other plants as well. The selectivity of sequestration gives rise to a further question: to what extent does the catalytic activity of endogenous BMY correspond to the glucosinolates actually sequestered? Thus, in this study, we wanted to clarify some details concerning glucosinolate accumulation and metabolism in *B. brassicae* aphids.

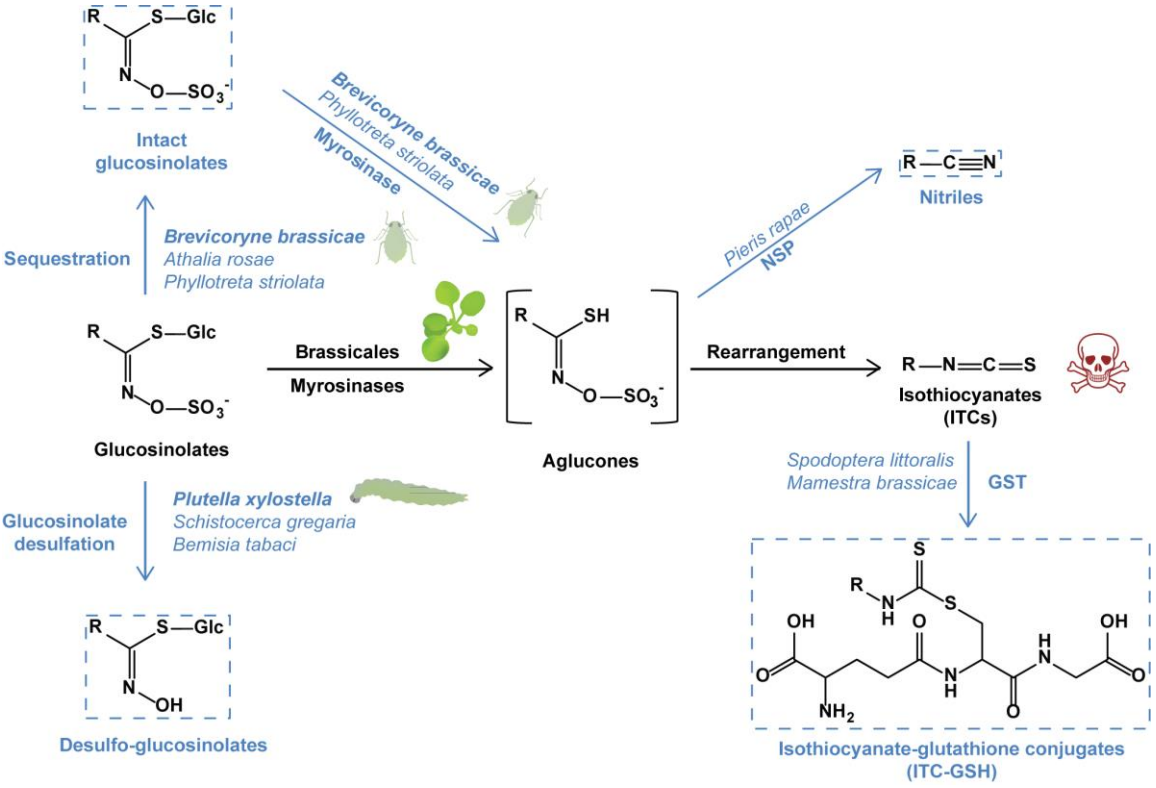

Figure 5. Glucosinolate activation in plants and metabolism in herbivores. Glucosinolates are hydrolyzed by plant myrosinases, forming glucosinolate-derived metabolites (black arrows). Herbivores can counter-act the plant glucosinolate-myrosinase defense system by sequestration, desulfation, and detoxification (blue arrows). Abbreviation: NSP, nitrile specifier proteins; GST,

glutathione *S*-transferases.

1.3 Tri-trophic interactions

Multi-trophic interactions involving plants, herbivores, and their antagonists are ubiquitous in terrestrial ecosystems and underpin our understanding of the structure and function of ecological communities. Most plants in nature are attacked by insect herbivores, and high infestations can severely damage plant tissues and thus reduce fitness (Johnson *et al.*, 2006). In response, plants employ an array of strategies to reduce herbivory, including the production of chemical defenses that not only act directly against herbivores, but that can indirectly attract herbivore antagonists. Glucosinolate-derived ITCs for instance are toxic to a variety of herbivores (Brown *et al.*, 2011; Jeschke *et al.*, 2017), while volatile ITCs are known to attract predators and parasitoids (Gols *et al.*, 2009; Bruce, 2014). The volatile allyl-ITC present in the frass of *P. xylostella* larvae attracts the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae) and the parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) (Reddy *et al.*, 2002). The attracted predators and parasitoids may reduce the density of herbivores and thus in turn protect the plants from too much damage.

Herbivore predators and parasitoids may contact plant chemical defenses when they consume herbivore tisues (Hartmann, 2004; Ode, 2006; Gols *et al.*, 2008; Harvey *et al.*, 2011). At these higher trophic levels, plant defenses could act either by directly affecting the growth and development of parasitoids and predators, or indirectly by reducing the quality of herbivores available as prey or hosts. In addition to these upward negative effects of plant defensive chemicals on herbivore antagonists, some adapted herbivores are capable of sequestering or accumulating those plant defensive chemicals (Thorpe *et al.*, 1986; Kumar *et al.*, 2014; Robert *et al.*, 2017). Pyrrolizidine alkaloids, for instance, are sequestered by leaf beetles of the genus *Oreina* from Asteraceae plants and can be concentrated in herbivore defensive secretions to ward off antagonists (Hartmann, 2004). Also, as previously mentioned, glucosinolates of Brassicaceae plants can also be sequestered by some adapted herbivore insects for use against enemies.

1.3.1 The glucosinolate-myrosinase system in tri-trophic communication

Certain Brassicaceae-specialized herbivores co-opt the plant glucosinolate-myrosinase system of their host for their own defense against higher trophic levels (Müller *et al.*, 2002; Kazana *et al.*, 2007; Beran *et al.*, 2014). In addition to the cabbage aphid, larvae of the turnip sawfly *A. rosae*, for example, excrete (“bleed”) the glucosinolates sequestered in its hemolymph out of the integument upon assault, protecting this species from predators like

the ant *Myrmica rubra* (Hymenoptera: Formicidae) (Müller *et al.*, 2002) and the parasitoid wasp *Vespula vulgaris* (Hymenoptera: Vespidae) (Müller *et al.*, 2003). In addition to *B. brassicae*, the leaf beetle *P. striolata* also mimics the natural two-component defensive system by endogenously producing myrosinases which, in the presence of sequestered glucosinolates, release toxic ITCs upon predator assault (Kazana *et al.*, 2007; Beran *et al.*, 2014). Upon attack the ITCs formed by the aphids severely impact the survival of the predatory ladybug *Adalia bipunctata* (Coleoptera: Coccinellidae) (Kazana *et al.*, 2007). The predatory lacewing *C. carnea* takes longer to develop when feeding on glucosinolate-sequestering *B. brassicae* aphids, compared to feeding on non-sequestering *M. persicae* aphids (Kos *et al.*, 2011). In contrast to the effective repellence conferred by glucosinolate sequestration, however, it is not yet fully understood how predators and parasitoids are affected by herbivorous prey or hosts that contain glucosinolates or their metabolites only transiently.

Even insects that do not sequester plant glucosinolates can also transmit their effects to higher trophic levels, either directly by exposing predators to the ingested plant toxins, or indirectly through the influence of these toxins on herbivore fitness. One effective pest control strategy against the diamondback moth *P. xylostella* is the utilization of biological control agents such as the generalist predatory larvae of the common green lacewing *C. carnea* and the specialized endoparasitoid *Diadegma semiclausum* (Hymenoptera: Ichneumonidae) to reduce diamondback moth abundance **(Figure 6)** (Li *et al.*, 2016). In the course of predation and parasitization, the lacewing larvae and the developing parasitoid wasps inevitably encounter plant-derived glucosinolates or their metabolites in *P. xylostella,* raising a general question of whether plant metabolites exert effects on higher trophic level organisms beyond herbivores. Indeed, the performance of predatory *C. carnea* and the development of endoparasitoid *D. semiclausum* are correlated to the Brassicaceae plant species used by the herbivore for food (Kos *et al.*, 2011). Previous research has shown that the performance of predatory *C. carnea* varies according to the glucosinolate profiles of the host plants that its *B. brassicae* aphid prey feeds on (Kos *et al.*, 2011). *D. semiclausum* development is likewise influenced by the species on which its *P. xylostella* hosts were reared, possibly due to interspecific differences in glucosinolate profiles (Gols *et al.*, 2008; Gols *et al.*, 2009; Dosdall *et al.*, 2011). However, whether glucosinolates, ITCs or other glucosinolate metabolites are indeed responsible for influencing *C. carnea* and *D. semiclausum* development, and whether these compounds can benefit or harm herbivore enemies, is unknown. Moreover, to what extent these

antagonists alter glucosinolate metabolism in their prey or host is also not known.

Figure 6. *Chrysoperla carnea* preys on a *Plutella xylostella* larva (left), and *Diadegma semiclausum* parasitizes a *P. xylostella* larva (right).

This thesis aims at gaining a more profound understanding of the consequences of plant secondary metabolites, specifically the glucosinolates of Brassicaceae plants, at higher trophic levels. To this goal, I have traced the movement of glucosinolate metabolites from plants through the specialized herbivore *P. xylostella* into higher trophic level organisms such as the generalist predator *C. carnea* **(manuscript I)** and the specialized parasitoid *D. semiclausum* **(manuscript II)**, and concomitantly quantified how glucosinolate metabolism affects their performance and development. In parallel to studying how plant-derived metabolites that are only transiently present in a herbivore can affect predators, I have also analyzed the piercing-sucking, glucosinolate-sequestering aphid herbivore *B. brassicae* with respect to the transport and accumumation of these chemical defenses as well as their physiological significance to the generalist predator *C. carnea* **(manuscript III)**. I was therefore able to show that glucosinolate-derived compounds travel between trophic levels, and that experimentally manipulating the metabolic balance between these trophic levels can cause strong species-specific effects.

1.4 Scope of thesis

The ultimate goal of this thesis is to obtain an in-depth understanding of the seminal role of plant glucosinolates and their metabolism in herbivores and their natural enemies. This goal is addressed by three independent manuscripts which all focus on this theme.

In **Manuscript I,** a plant-mediated RNAi approach was used to selectively knock down the expression of the detoxification-related *gss* genes in *P. xylostella*. I demonstrated that suppression of glucosinolate desulfation increased larval ITC levels, which had a significant negative impact on *P. xylostella* fitness, establishing that this detoxification mechanism is beneficial to the herbivore. Although the increased ITC concentrations in *P.*

xylostella larvae impaired the growth of predatory *C. carnea* larvae, *C. carnea* detoxified ITCs via the general mercapturic acid pathway and excreted the metabolites in their larval anal secretion, resulting in no net effect on pupal mortality and adult reproduction. Therefore, my work showed that both the herbivore and its predator detoxify plant defensive chemicals with their own independent mechanisms with resulting benefits to their fitness.

In **Manuscript II**, the ecological ramifications caused by manipulating the expression of the *gss* gene family in *P. xylostella* were tested with the specialist endoparasitoid *D. semiclausum.* I observed that the increased ITC amounts present in *gss*-silenced *P. xylostella* larvae lowered endoparasitoid emergence. Unlike the generalist predator *C. carnea*, *D. semiclausum* alleviated ITC toxicity by increasing the excretion of the toxin by its herbivorous host. Thus, detoxification of plant glucosinolate defenses by the herbivore *P. xylostella* is also beneficial for its endoparasitic wasp *D. semiclausum*.

In **Manuscript III**, I clarified details of glucosinolate metabolism and its resulting ecological ramifications in the interaction of a glucosinolate-sequestering aphid and a predatory lacewing. I demonstrated that *B. brassicae* specifically accumulated aliphatic glucosinolates that were hydrolyzed by an endogenous myrosinase. The formation of high concentrations of ITCs caused severe impact on *C. carnea*. Although the lacewing possesses a mechanism to detoxify ITCs via conjugation, it was incapable of detoxifying all the toxic ITCs ingested from the aphid.

2. Overview of Manuscripts

This thesis is based on the following manuscripts.

2.1 Manuscript I

Tritrophic metabolism of plant chemical defenses and its effects on herbivore and predator performance

Ruo Sun, Xingcong Jiang, Michael Reichelt, Jonathan Gershenzon, Sagar Subhash Pandit, Daniel Giddings Vassão

Published in eLife (2019), Volume 8:e51029, doi:10.7554/eLife.51029.

Summary

In this study, we demonstrated that glucosinolate sulfatase is a genuine detoxification mechanism for the diamondback moth *Plutella xylostella* larvae by blocking the glucosinolate-disarming mechanism of this herbivore via a plant-mediated RNAi. Efficient suppression of glucosinolate sulfatase activity reduced *P. xylostella* development, survival, and reproduction due to the accumulation of ITCs as glucosinolate hydrolysis products. Nonetheless, this detoxification mechanism is costly. *P. xylostella* larvae produce glucosinolate sulfatase only when feeding on a plant containing glucosinolates, and perform better when they feed on plants without glucosinolates. The predatory lacewing *Chrysoperla carnea*, used in *P. xylostella* pest control, did not suffer long-term negative effects when feeding on *P. xylostella* whose glucosinolate detoxification mechanism was suppressed. *C. carnea* larvae detoxified the accumulated ITCs, which slightly slowed their larval development, but did not affect their reproduction or their preference for feeding on *P. xylostella* larvae. In this system, plant defenses and their detoxification strongly influenced herbivore fitness, but only subtly affected a generalist predator of the herbivore.

Author Contributions

Conceived project: RS (50 %), XJ, JG, SSP, DGV

Designed experiments: RS (60 %), SSP

Performed experiments: RS (90 %), XJ

Chemical analysis: RS (80 %), MR

Data analysis: RS (80 %), SSP, DGV

Manuscript writing: RS (70 %), JG, DGV

2.2 Manuscript II

Detoxification of plant defensive glucosinolates by an herbivorous caterpillar is beneficial to its endoparasitic wasp

Ruo Sun, Rieta Gols, Jeffrey A. Harvey, Michael Reichelt, Jonathan Gershenzon, Sagar Subhash Pandit, Daniel Giddings Vassão

Published in Molecular Ecology (2020), Volume 29(20): 4014-4031. doi:10.1111/mec.15613.

Summary

In this study, we showed that detoxification of plant glucosinolate defenses by the diamondback moth *Plutella xylostella* is also beneficial for its endoparasitic wasp *Diadegma semiclausum*. By genetically manipulating plants to produce an interference RNA construct that silenced the expression of a glucosinolate detoxification gene in the herbivore, we were able to test the effect of this detoxification process on the *P. xylostella* larvae and on its specialized endoparasitoid. Inhibition of herbivore glucosinolate detoxification slowed endoparasitoid development and reduced adult emergence by increased accumulation of ITCs, while simultaneously suppressing the expression of genes of a parasitoid-symbiotic polydnavirus that aids parasitism. Although, the parasitoid manipulated *P. xylostella* to increase its excretion ot ITCs, this did not alleviate the negative effects of blocking glucosinolate detoxification. These results indicate that the parasitoid, like the herbivore, enjoys improved performance from herbivore detoxification of glucosinolates.

Author Contributions

Conceived project: RS (50 %), RG, JAH, JG, SSP, DGV

Designed experiments: RS (60 %), RG, JAH, SSP

Performed experiments: RS (90 %), RG, JAH

Chemical analysis: RS (80 %), MR

Data analysis: RS (80 %), DGV

Manuscript writing: RS (70 %), RG, JAH, JG, DGV

2.3 Manuscript III

Adaptation of cabbage aphid to aliphatic glucosinolates overwhelms detoxification in a lacewing predator

Ruo Sun, Xingcong Jiang, Michael Reichelt, Jonathan Gershenzon, Daniel Giddings Vassão

Prepared for Journal of Pest Science

Summary

In this study, we demonstrated that the aliphatic glucosinolates accumulated by the cabbage aphid *Brevicoryne brassicae* negatively affect the development of a predator, the green lacewing *Chrysoperla carnea*. First, we showed that accumulation of distinct aliphatic glucosinolates by the aphid *B. brassicae* is selective and occurs at different rates compared to indolic glucosinolates. The catalytic activity of the endogenous *B. brassicae* myrosinase (BMY) correlates positively with the types of glucosinolates selectively sequestered. Second, by simulating predation, we found that *B. brassicae* hydrolyze their stored glucosinolates to isothiocyanates (ITCs) as well as to non-toxic glucosinolate metabolites. Third, the aliphatic ITCs formed impair the growth and development of *C. carnea*, in spite of the detoxification mechanism present. Thus, this predator has a limted capacity for ITC conjugation and only partially mitigates the toxicity of the aliphatic ITCs when it ingests its aphid prey.

Author Contributions

Conceived project: RS (60 %), XJ, JG, DGV

Designed experiments: RS (60 %), XJ, DGV

Performed experiments: RS (70 %), XJ

Chemical analysis: RS (90 %), MR

Data analysis: RS (80 %), XJ, DGV

Manuscript writing: RS (50 %), XJ, JG, DGV

3. Manuscript I

Tritrophic metabolism of plant chemical defenses and its effects on herbivore and predator performance

Ruo Sun, Xingcong Jiang, Michael Reichelt, Jonathan Gershenzon, Sagar Subhash Pandit, Daniel Giddings Vassão

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RESEARCH ARTICLE

Tritrophic metabolism of plant chemical defenses and its effects on herbivore and predator performance

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Abstract Insect herbivores are frequently reported to metabolize plant defense compounds, but the physiological and ecological consequences are not fully understood. It has rarely been studied whether such metabolism is genuinely beneficial to the insect, and whether there are any effects on higher trophic levels. Here, we manipulated the detoxification of plant defenses in the herbivorous pest diamondback moth (*Plutella xylostella*) to evaluate changes in fitness, and additionally examined the effects on a predatory lacewing (*Chrysoperla carnea*). Silencing glucosinolate sulfatase genes resulted in the systemic accumulation of toxic isothiocyanates in *P. xylostella* larvae, impairing larval development and adult reproduction. The predatory lacewing *C. carnea*, however, efficiently degraded ingested isothiocyanates via a general conjugation pathway, with no negative effects on survival, reproduction, or even prey preference. These results illustrate how plant defenses and their detoxification strongly influence herbivore fitness but might only subtly affect a third trophic level.

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Introduction

The chemical defenses of plants constitute a major obstacle to herbivore feeding. Many plant defenses are glycosylated pro-toxins, such as glucosinolates, benzoxazinoids, cyanogenic glucosides, and iridoid glucosides, that are non-toxic by themselves, but liberate toxins after activation by glucosidases (***Morant et al., 2008***; ***Pentzold et al., 2014***). Glucosinolates are activated specifically by β-thioglucosidases called myrosinases, which are stored separately from their substrates to avoid self-intoxication (***Bones and Rossiter, 1996***). Activation occurs upon plant damage, for example herbivore feeding, when compartments containing enzymes and the glucosylated pro-toxins are ruptured, leading to mixing of their contents and subsequent hydrolysis. The glucosinolate-derived aglucones are unstable and rearrange to form isothiocyanates and other products such as the generally less toxic nitriles, either spontaneously or by the action of so-called specifier proteins that help guide rearrangement in *A. thaliana* and other Brassicaceae plants (***Halkier and Gershenzon, 2006***; ***Wittstock et al., 2016***; ***Wittstock et al., 2004***). Glucosinolate side-chains can further dictate post-hydrolysis reactivities and occasionally lead to additional intramolecular reactions (e.g. cyclization to form oxazolidine-2-thione derivatives) or rapid loss of the –N=C=S group (e.g. to form the carbinol products of indolic glucosinolates) (***Wittstock et al., 2016***), which alter toxicity. Isothiocyanates, the most typical glucosinolate hydrolysis products, are considered toxic to a variety of herbivorous organisms due to their lipophilic properties that facilitate diffusion through membranes and the electrophilic –N=C=S functional core that reacts with intracellular nucleophiles (***Hanschen et al., 2012***;

Jeschke et al., 2016a). This liberation of toxic products from glucosinolates constitutes the characteristic 'mustard oil bomb' defense system of plants of the family Brassicaceae and related families.

Some herbivores possess mechanisms to potentially avoid the toxicity of the glucosinolate-myrosinase system (*Jeschke et al., 2016a*; *Winde and Wittstock, 2011*). For example, *Plutella xylostella* (the diamondback moth, Lepidoptera: Plutellidae), a notorious agricultural pest specializing on plants of the Brassicaceae, produces glucosinolate-specific sulfatases that rapidly desulfate glucosinolates to form desulfo-glucosinolates (*Ratzka et al., 2002*), which are no longer substrates for myrosinases and are therefore non-toxic. This process is controlled by a small gene family encoding glucosinolate sulfatases (GSSs) with varying specificity for different types of glucosinolates. GSS1 desulfated all glucosinolates tested in vitro except for 1MOI3M, while GSS3 only metabolized benzenic and indolic glucosinolates and GSS2 accepted only a few very long-chain aliphatic glucosinolates (*Heidel-Fischer et al., 2019*). GSSs may help to avoid the toxicity of the glucosinolate-myrosinase system, and indeed it was reported that *P. xylostella* contains sufficient GSS (based on an in vitro assay of purified protein) to readily desulfate all ingested glucosinolates (*Ratzka et al., 2002*). Moreover, when isotopically labeled glucosinolates were administered to *P. xylostella*, over 80% were converted to desulfo-glucosinolates (*Jeschke et al., 2017*). However, it cannot be automatically assumed that desulfation is an effective detoxification strategy without knowing if it is of net benefit to the performance and fitness of the herbivore, since desulfation may incur unanticipated physiological costs.

P. xylostella is currently the most devastating pest of cultivated Brassicaceae crops in the world, causing losses of US$ 4–5 billion per year (*Zalucki et al., 2012*). Its destructiveness can be attributed to its extremely short life cycle (14 days), which allows it to complete more than 20 generations per year, and its rapid development of resistance to pesticides (*Sarfraz et al., 2005*), making it one of the most difficult agricultural pests to control. 'Natural enemies' have therefore been explored as alternative *P. xylostella* control agents, including predators, parasitoids, entomopathogenic fungi, bacteria and viruses (*Sarfraz et al., 2005*). One natural enemy used in diamondback moth management is the predatory lacewing *Chrysoperla carnea* (the common green lacewing, Neuroptera: Chrysopidae) (*McEwen et al., 2001*; *Reddy et al., 2004*). When *C. carnea* preys on *P. xylostella* larvae feeding on Brassicaceae plants, the lacewing can be expected to encounter plant-derived glucosinolates or their metabolites. However, it is unclear whether such plant defense compounds or derivatives might harm *P. xylostella* predators. Plant chemicals can traverse trophic levels and affect predators, as when they are sequestered by a herbivore for its own defense (*Beran et al., 2014*; *Kumar et al., 2014*; *Müller et al., 2002*). The cabbage aphid, *Brevicoryne brassicae*, for example, accumulates high concentrations of glucosinolates from its host plants and uses these compounds in defense against predators (*Kos et al., 2011*). In this work, we therefore attempted to down-regulate *P. xylostella* GSS activity to determine if glucosinolate desulfation fundamentally benefits *P. xylostella*, and also whether or not it has consequences for a higher trophic level.

We chose plant-mediated RNAi to silence *gss* genes since this method is reported to have high success against lepidopteran targets (*Kumar et al., 2012*; *Kumar et al., 2014*; *Mao et al., 2007*; *Poreddy et al., 2017*). In leaves of the *Arabidopsis thaliana* Col-0 accession used here, aliphatic and indolic glucosinolates constitute around 85% and 15% of the total glucosinolate pool, respectively, with 4-methylsulfinylbutyl glucosinolate (4MSOB) representing over 70% of the aliphatic glucosinolates (*Brown et al., 2003*). Using *A. thaliana* plants with wild-type glucosinolates and *myb28myb29* mutant plants without aliphatic glucosinolates, both engineered to target *gss* gene expression, we achieved significant silencing of *gss* in *P. xylostella*. We demonstrated that suppression of glucosinolate desulfation increased larval isothiocyanate levels, which had significant negative impacts on *P. xylostella* growth, survival and reproduction, establishing that this detoxification mechanism is beneficial to the herbivore in spite of its observed cost. Although the increased isothiocyanate accumulation in *P. xylostella* larvae impaired the growth of predatory *C. carnea* larvae, *C. carnea* detoxified isothiocyanates via the general mercapturic acid pathway and excreted the metabolites in their larval anal secretion resulting in no net effect on pupal mortality and adult egg-laying capacity. Therefore, our work shows that both the herbivore and its predator detoxify plant defensive chemicals with their own independent mechanisms with resulting benefits to reproductive fitness.

Results

gss1 is abundantly expressed in the midgut of *P. xylostella* larvae

To determine the role of glucosinolate sulfatase (GSS) in *P. xylostella* in more detail, we explored the location of *gss* gene expression by qRT-PCR in dissected tissues of fourth-instar larvae. The *gss1* gene was highly expressed in the midgut epithelium, but had very low expression in hemolymph, integument and fat bodies (***Figure 1A***). After feeding on *A. thaliana* wild-type Col-0 plants containing natural levels of glucosinolates, *gss1* expression in larval midgut tissues was approximately 17-fold higher than after feeding on *myb28myb29* plants, which lack aliphatic glucosinolates (***Figure 1A***), suggesting that the expression of this gene is regulated by dietary glucosinolate ingestion. Similar patterns were shown by *gss2* and *gss3* (***Figure 1—figure supplement 1A,B***). However, glucosinolates did not induce the expression of any of several other potential detoxification genes analyzed in the midgut of *P. xylostella* fourth-instar larvae (***Figure 1—figure supplement 1C***).

To analyze the cell-level expression of *gss* within the larval midgut, we conducted fluorescent in situ hybridization (FISH) experiments utilizing antisense *gss*-specific riboprobes. The labeling of *gss1*-expressing cells suggested a broad distribution in cell types, as seen in a typical transverse section of *P. xylostella* midgut (***Figure 1B***). The counter staining of nuclei indicated that *gss1*-positive midgut cell types include the columnar cells bearing microvillar structures, and the basal midgut cells that differ in nuclear shape and size (***Figure 1C,D***). FISH labeling of *gss2* and *gss3* revealed that both have a similar expression pattern as *gss1* (***Figure 1—figure supplement 2A–F***). Moreover two-color FISH experiments using the antisense probe pairs, *gss1 and gss2* or *gss1 and gss3*, indicated that the three *gss* forms are most likely co-expressed in the same midgut cells, as inferred by the largely overlapping red and green labeling patterns (***Figure 1—figure supplement 2G–J***). The specificities of the labeling conferred by individual *gss* antisense probes were verified using the corresponding sense probes, which did not generate any labeling (***Figure 1—figure supplement 2K–M***).

Silencing of *P. xylostella gss1* expression reduces GSS enzyme activity

To determine the impact of GSS on *P. xylostella* larval performance and glucosinolate metabolism, we used plant-mediated RNAi to downregulate its expression. Transgenic *A. thaliana* lines were generated by infiltration with *Agrobacterium tumefaciens* transformed with a virus-based dsRNA production system, which consisted of the tobacco rattle virus-derived vector (pTRV2) with a 526 bp fragment of *gss1* (***Figure 1E***). Both *A. thaliana* Col-0 and *myb28myb29* plants, with and without aliphatic glucosinolates, respectively, were transformed. As a negative control, plants were also infiltrated with *A. tumefaciens* transformed with an empty vector pTRV2 construct. Plants infiltrated with the *gss1* RNAi construct were indistinguishable from both untreated and empty vector construct-infiltrated plants regarding growth, morphology, glucosinolate profile, and levels of flavonoids and phenylpropanoids involved in plant defense (***D'Auria and Gershenzon, 2005***) (***Figure 1—figure supplement 3***).

When *P. xylostella* larvae ingested Col-0 plants (with wild-type glucosinolate levels) infiltrated with the *gss1* RNAi construct (***Figure 1E***), *gss1* expression in the midgut epithelium was lowered to about 2% of that found in larvae fed on Col-0 plants infiltrated with the empty vector construct (***Figure 1F***). After feeding on *myb28myb29* plants (without aliphatic glucosinolates), larval *gss1* transcript levels were lower than after feeding on Col-0, but *gss1* silencing on *myb28myb29* plants also led to a substantial (63%) transcriptional reduction (***Figure 1F***). Subsequently, we measured the GSS activities in protein extracts from the midgut epithelium of *gss1*-silenced and non-silenced *P. xylostella* larvae using in vitro enzyme assays. Formation of desulfo-4-methylsulfinylbutyl glucosinolate (desulfo-4MSOB) from 4MSOB by midgut extracts of *gss1*-silenced larvae was reduced to less than 50% of that formed by extracts of non-silenced larvae, indicating *gss1* expression and GSS activity were both greatly suppressed by the RNAi treatment (***Figure 1G***). While *gss2* and *gss3*, like *gss1*, were co-silenced by the treatment, transcript levels of genes encoding other sulfatases and the *sulfatase modifying factor 1* (*sumf1*) were not influenced by *gss1* silencing (***Figure 1—figure supplement 4***).

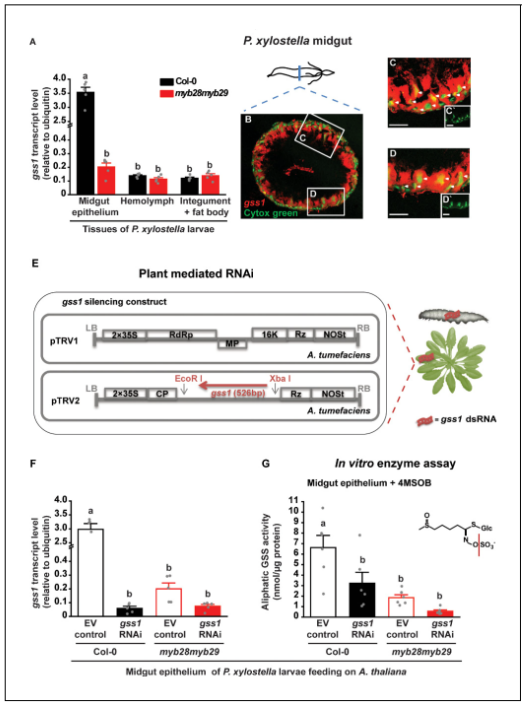

Figure 1. Localization and plant-mediated RNAi silencing of *gss1* gene expression in *P. xylostella* larvae. (**A**) Levels of *gss1* transcripts (expressed relative to ubiquitin) are higher in midgut epithelium than in hemolymph, integument and fat bodies of fourth-instar larvae feeding on *A. thaliana* Col-0 (wild-type glucosinolates) and *myb28myb29* (no aliphatic glucosinolates) plants (Plants, $F_{1,24}$ = 338.032, p≤0.0001; Tissues, $F_{2,24}$ = 367.522, p≤0.0001; Plants*Tissues, $F_{2,24}$ = 336.042, p≤0.0001; *n* = 5 for all bars). (**B–D**) Visualization of *gss1*-expressing cells in the midgut of *P. xylostella* as seen in a transverse section. Cells containing *gss1* transcripts were strongly stained by means of fluorescent in situ hybridization (FISH) using a *gss1*-specific antisense riboprobe labeled by digoxigenin (DIG) (in red). Counter staining of nuclei of midgut cells employed Cytox green (in green). Two selected areas in **B** (**C** and **D**) are magnified further. Both the columnar cells and the basal midgut cells are *gss1*-positive, with white arrows pointing to nuclei of different morphologies. **C'** and **D'** are lower magnification views of the nuclear staining presented in **C** and **D**, respectively. Scale bar, 50 µm. (**E**) Silencing strategy for *gss1* employed a virus-based dsRNA-producing system in the host plant *A. thaliana* created by infiltration of tobacco rattle virus (TRV, detail structure described in ***Ratcliff et al., 2001***) engineered to express a 526 bp fragment of *gss1* dsRNA. (**F**) Levels of *gss1* transcripts (expressed relative to ubiquitin) in larval midgut epithelium ($F_{3,16}$ = 185.508, p≤0.0001, *n* = 5 for all bars) and (**G**) levels of GSS activity measured in vitro in extracts of midgut epithelium ($F_{3,20}$ = 10.697, p≤0.0001, *n* = 6 for all bars) of fourth-instar larvae feeding on empty vector (EV) control and *gss1*-RNAi plants in backgrounds of Col-0 and *myb28myb29 A. thaliana*. The aliphatic glucosinolate 4MSOB (5 mM) was used as a substrate. Significant differences (p≤0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with a two-way ANOVA in **A** and with one-way ANOVA in **F** and **G**.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. *gss1* gene transcript levels and GSS in vitro enzyme assays with 4MSOB glucosinolate.
Figure supplement 1. Transcripts of *gss* genes are localized in the *P. xylostella* larval midgut epithelium and are induced by dietary ingestion of glucosinolates.
Figure supplement 1—source data 1. Transcript levels of *gss2, gss3* and other detoxification-related genes.
Figure supplement 2. Transcripts of *gss* genes are localized in midgut cell types of *P. xylostella* fourth-instar larvae.
Figure supplement 3. Infiltration of *A.thaliana* Col-0 and *myb28myb29* lines with *gss1* RNAi and empty vector (EV) control constructs does not alter their morphology and chemical phenotypes.

Figure 1 continued on next page

Figure 1 continued

Figure supplement 3—source data 1. Glucosinolate, flavonoid, and phenylpropanoid profiles in *A. thaliana* plants.

Figure supplement 4. Plant-mediated RNAi of *gss1* co-silences *gss2* and *gss3* due to high sequence similarity, and suppresses the desulfation of indolic glucosinolates as well.

Figure supplement 4—source data 1. *gss2* and *gss3* gene transcript levels, and GSS in vitro enzyme assays with I3M glucosinolate.

Silencing of *gss* decreases *P. xylostella* growth, survival and reproduction

To determine the impact of GSS on *P. xylostella* performance, we compared the phenotypes of larvae that were either *gss*-silenced or non-silenced and fed on *A. thaliana* plants with or without aliphatic glucosinolates. When fed continuously on Col-0 plants with aliphatic glucosinolates, *gss*-silenced larvae grew 33% less than non-silenced larvae at six dph (days post hatching), and the growth gap continued to widen until eight dph, when *gss*-silenced larvae were only 64% as heavy as non-silenced larvae before pupation (***Figure 2A***). Although pupal weights were not significantly different between treatments (***Figure 2—figure supplement 1***), the pupal mortality of *gss*-silenced insects was nearly 4-fold higher than non-silenced ones (***Figure 2B***).

We then separated the resulting adults by gender to examine the influence of *gss* silencing on fecundity. Female moths arising from the four different larval treatments, *gss*-silenced or non-silenced from either *A. thaliana* Col-0 or *myb28myb29*, were all mated with male moths arising from non-silenced larvae from Col-0, while male moths arising from the same four treatments were all mated with female moths arising from non-silenced larvae from Col-0. The number of eggs laid was counted two days after mating, and the number of eggs hatching successfully was recorded later. Egg laying and hatching were reduced only in treatments involving adults arising from *gss*-silenced larvae fed on aliphatic glucosinolate containing (Col-0) plants. Female moths from *gss*-silenced Col-0 larvae laid 56% less eggs than female moths on all other treatments (***Figure 2C***). However, these eggs did not hatch at a reduced rate (***Figure 2E,G***). Meanwhile, male moths arising from *gss*-silenced larvae fed on Col-0 did not affect the egg laying capacity of females arising from non-silenced larvae (***Figure 2D***), but did decrease egg hatching by 36% (***Figure 2F***). The distribution and lower slopes observed in the correlation between the numbers of eggs hatched and the numbers of eggs laid by this group suggest that the two factors were independent, with low-hatching batches spread among differently sized broods (***Figure 2H***). We confirmed these results by mating adults arising from *gss*-silenced or non-silenced larvae with adults from larvae fed on untreated Col-0 or *myb28myb29* plants to eliminate any influence from infiltration with the empty vector construct (***Figure 2—figure supplement 2***).

Comparing *P. xylostella* performance on the two plant lines, larvae fed on *myb28myb29* plants (without aliphatic glucosinolates) grew faster and had significantly higher mass than larvae fed on Col-0 plants (with aliphatic glucosinolates) (***Figure 2A***). This difference may result from the formation of toxic glucosinolate hydrolysis products despite the presence of GSS. Alternatively, better performance on *myb28myb29* might be ascribed to reduced amounts of glucosinolate sulfatase activity due to decreased expression of the gene (***Figure 1F,G***), indicating a trade-off between energy spent on glucosinolate detoxification and larval growth (***Jeschke et al., 2016b***; ***Jeschke et al., 2017***). However, considering only larvae fed on *myb28myb29* plants, *gss* silencing did not cause physiological changes (***Figure 2B–H***). Therefore, silencing of *gss* impacted *P. xylostella* larval growth and development only in the presence of aliphatic glucosinolates, causing pupal mortality and sex-specific effects on adults.

Silencing of *gss* increases the formation of isothiocyanates, toxic glucosinolate hydrolysis products, in *P. xylostella* larvae

To determine the influence of *gss* silencing on insect glucosinolate metabolism after ingestion, we quantified the previously described (***Jeschke et al., 2017***) metabolites of 4-methylsulfinylbutyl glucosinolate (4MSOB), which represents 75% of the total aliphatic glucosinolate content of *A. thaliana* Col-0 leaves (***Figure 1—figure supplement 3***). Non-silenced fourth-instar larvae with normal levels

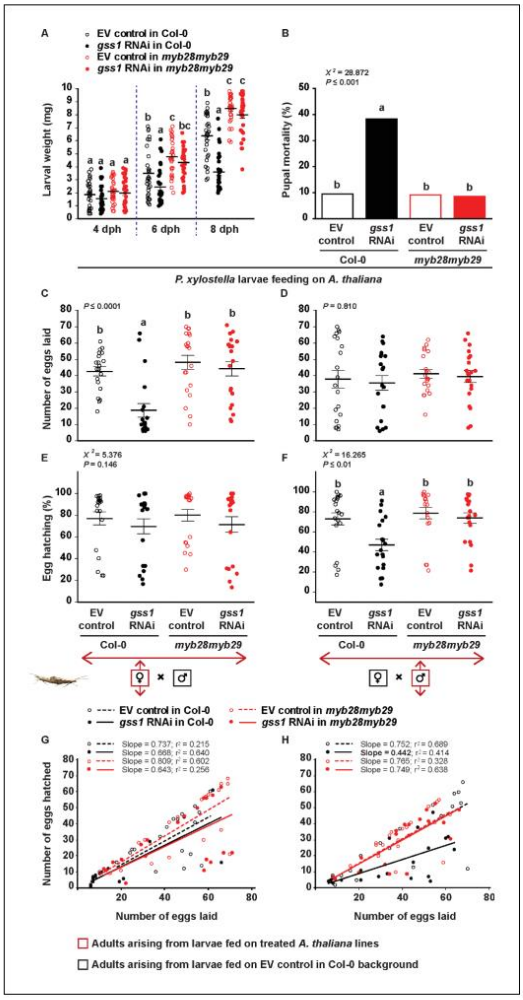

Figure 2. Silencing of *gss* affects *P. xylostella* growth and fitness in the presence of aliphatic glucosinolates. Larvae were fed on empty vector (EV) control and *gss1* RNAi plants from either Col-0 (wild-type glucosinolates) or *myb28myb29* (no aliphatic glucosinolates) backgrounds, and various parameters of larval performance were measured: (**A**) Weights of larvae after four dph (days post hatching), six dph, and eight dph were reduced by silencing but only in the presence of wild-type glucosinolates (six dph, Plants $F_{1,116}$ = 33.471, p≤0.0001; RNAi $F_{1,116}$ = 7.440, p≤0.01; Plants*RNAi $F_{1,116}$ = 1.322, p=0.253. eight dph, Plants $F_{1,116}$ = 145.33, p≤0.0001; RNAi $F_{1,116}$ = 37.083, p≤0.0001; Plants*RNAi $F_{1,116}$ = 18.340, p≤0.0001. n = 30 for all treatments); (**B**) Mortality (%) in pupae was highest in those pupae arising from silenced larvae fed on wild-type glucosinolates (χ^2 = 228.872, df = 3, p≤0.001; n = 63, 60, 66 and 59, respectively). The number of eggs laid by females was measured in crosses between (**C**) females arising from larvae raised on the four plant lines mated with males arising from larvae fed on EV control Col-0 plants and (**D**) males arising from larvae raised on the four plant lines mated with females arising from larvae fed on EV control Col-0 plants. Only crosses with females arising from *gss*-silenced larvae fed on Col-0 showed a decline in egg production ($F_{3,76}$ = 11.157, p≤0.0001, n = 20 for all treatments). Hatching (%) of eggs laid by females (**E, F**), and the correlation between the numbers of eggs hatched and the numbers of eggs laid (**G, H**) were recorded from crosses between (**E, G**) females arising from larvae raised on the four plant lines mated with

Figure 2 continued on next page

Figure 2 continued

males arising from larvae fed on EV control Col-0 plants, and (**F, H**) from males arising from larvae raised on the four plant lines mated with females arising from larvae fed on EV control Col-0 plants. Only crosses with males arising from *gss*-silenced larvae fed on Col-0 showed a decline in egg hatching rate (χ^2 = 216.265, *df* = 3, p≤0.01; *n* = 20 for all treatments). Significant differences (p≤0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with two-way ANOVA in **A**, a proportions test with pairwise comparisons in **B**, Tukey HSD tests in conjunction with one-way ANOVA in **C–D**, and Dunn's post hoc tests in conjunction with non-parametric Kruskal-Wallis tests in **E–F**.

The online version of this article includes the following source data and figure supplement(s) for figure 2:

Source data 1. *P. xylostella* larval weight, pupal mortality, and adult egg laying capacity.
Figure supplement 1. Silencing of *gss* has no effect on weights of *P. xylostella* pupae.
Figure supplement 1—source data 1. *P. xylostella* pupal weight.
Figure supplement 2. Silencing of *gss* reduces number of eggs and their hatching percentage.
Figure supplement 2—source data 1. *P. xylostella* adult egg laying capacity.

of GSS activity desulfated 4MSOB and formed the non-toxic desulfo-4MSOB in the larval midgut epithelium (***Figure 3B***). However, when GSS activity was lowered by plant-mediated RNAi, levels of desulfo-4MSOB decreased by more than 90%, while the concentrations of the toxic hydrolysis product 4MSOB-isothiocyanate (4MSOB-ITC) increased over 10-fold in the midgut of *gss*-silenced larvae, with a concomitant rise in the hemolymph (***Figure 3C***). Intact 4MSOB, present only in trace amounts in non-silenced midguts, became a very prominent peak in *gss*-silenced larvae (***Figure 3A***), suggesting that plant myrosinases in the *P. xylostella* midgut lumen are not very efficient, and so may be easily outcompeted by insect GSS in non-silenced insects. Larvae of *P. xylostella* excreted most of the 4MSOB and its metabolites into the frass. Active GSSs and myrosinase were also excreted that continued to react with the 4MSOB present (***Figure 3—figure supplement 1***), likely leading to the smaller differences in desulfo-4MSOB and 4MSOB-ITC concentrations between the frass of *gss*-silenced larvae and non-silenced insects as between the concentrations of these compounds in the midgut (***Figure 3B,C***). At the end of larval development, *P. xylostella* retained considerable amounts of 4MSOB-ITC in pupae and adults, 210 and 390 nmol/g, respectively (***Figure 3C***). However, the desulfo-4MSOB remaining was excreted at the prepupal stage (***Figure 3B***). Neither desulfo-4MSOB nor 4MSOB-ITC were detectable in *P. xylostella* eggs (***Figure 3B,C***).

The desulfation of indol-3-ylmethyl (I3M) glucosinolate was also inhibited in *gss*-silenced *P. xylostella* larvae. This glucosinolate, which represents 76% and 87% of the indolic glucosinolate pool in *A. thaliana* Col-0 and *myb28myb29*, respectively (***Figure 1—figure supplement 3B***), was shown to be less efficiently desulfated in *gss*-silenced than empty vector control lines according to in vitro enzyme assays (***Figure 1—figure supplement 4***) and metabolomic analyses (***Figure 3—figure supplement 2***). Nevertheless, the lack of indolic glucosinolate desulfation had no observable negative effects on the performance of *P. xylostella* larvae (***Figure 2***). There were no differences between silenced and non-silenced larvae feeding on *myb28myb29* plants, which lack aliphatic glucosinolates and contain slightly elevated indolic glucosinolate levels (***Figure 1—figure supplement 3B***). Thus the metabolites of I3M appear to be less toxic than those of 4MSOB to *P. xylostella*, as also observed for other herbivores (***Jeschke et al., 2017***). In addition to the lower amounts of I3M than 4MSOB in *A. thaliana*, the I3M-isothiocyanate (I3M-ITC) formed upon hydrolysis is unstable under physiological conditions and reacts largely with ascorbic acid (***Figure 3—figure supplement 2***).

4MSOB-ITC is responsible for the negative effects of *gss* silencing on *P. xylostella* performance

As isothiocyanates are thought to cause most of the toxic effects of glucosinolates (***Brown and Hampton, 2011***; ***Wittstock et al., 2003***), we hypothesized that the significantly higher concentrations of 4MSOB-ITC resulting from *gss* silencing would explain the lower larval growth, higher pupal mortality, and reduced reproduction. To examine this possibility, *myb28myb29* plants lacking 4MSOB and transformed with either the *gss1* RNAi or empty vector constructs were additionally infiltrated with a natural concentration of 4MSOB-ITC in a solvent of 0.4% aqueous ethanol (***Figure 4—figure supplement 1***). Solvent-infiltrated *myb28myb29* plants were used as negative controls. Feeding on 4MSOB-ITC-infiltrated plants strongly reduced larval growth (***Figure 4A***), and

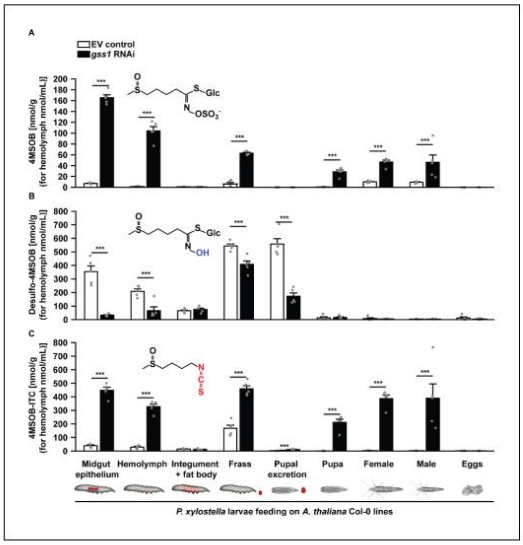

Figure 3. Silencing of *gss* decreases desulfo-4MSOB and increases 4MSOB-ITC concentrations in *P. xylostella*. (**A**) Intact 4MSOB (Midgut epithelium, $F_{3,16}$ = 931.79, p≤0.0001; Hemolymph, $F_{3,16}$ = 172.90, p≤0.0001; Frass, $F_{3,16}$ = 391.817, p≤0.0001; Pupa, $F_{3,16}$ = 68.59, p≤0.0001; Female, $F_{3,16}$ = 150.48, p≤0.0001; Male, $F_{3,16}$ = 9.64, p≤0.001; n = 5 for all bars), (**B**) desulfo-4MSOB (Midgut epithelium, $F_{3,16}$ = 66.94, p≤0.0001; Hemolymph, $F_{3,16}$ = 34.84, p≤0.0001; Frass, $F_{3,16}$ = 329.97, p≤0.0001; Pupal excretion, $F_{3,16}$ = 129.825, p≤0.0001; n = 5 for all bars), and (**C**) 4MSOB-ITC (Midgut epithelium, $F_{3,16}$ = 356.30, p≤0.0001; Hemolymph, $F_{3,16}$ = 269.44, p≤0.0001; Frass, $F_{3,16}$ = 182.96, p≤0.0001; Pupal excretion, $F_{3,16}$ = 81.248, p≤0.0001; Pupa, $F_{3,16}$ = 78.44, p≤0.0001; Female, $F_{3,16}$ = 211.63, p≤0.0001; Male, $F_{3,16}$ = 13.83, p≤0.0001; n = 5 for all bars) were measured in various larval tissues, excretions, pupae, and adult moths developed from *gss*-silenced and non-silenced larvae feeding on *A. thaliana* Col-0 (wild-type glucosinolates). The concentrations of 4MSOB and its metabolites were nearly undetectable in *P. xylostella* fed on *myb28myb29* (no aliphatic glucosinolates) plants, and these data are not shown in the figure, but given in ***Figure 3—source data 1***. Significant differences (p≤0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with one-way ANOVA.

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. The concentration of 4MSOB glucosinolate metabolites in *P. xylostella*.
Figure supplement 1. Enzymatic conversion of 4MSOB to desulfo-4MSOB and 4MSOB-ITC is enzymatic and occurs in fresh frass.
Figure supplement 1—source data 1. Enzyme assay of *P. xylostella* larval frass with 4MSOB glucosinolate.
Figure supplement 2. Silencing of *gss* decreases desulfo-indol-3-ylmethyl glucosinolate (desulfo-I3M) and increases a metabolite of indol-3-ylmethyl glucosinolate isothiocyanate (I3M-ITC) in *P. xylostella*.
Figure supplement 2—source data 1. The concentration of I3M glucosinolate metabolites in *P. xylostella*.

pupae of larvae fed on 4MSOB-ITC-infiltrated plants suffered 4-fold higher mortality than those feeding on solvent-infiltrated plants (***Figure 4B***). Furthermore, the female moths of larvae feeding on 4MSOB-ITC-infiltrated plants laid approximately 45% less eggs than those feeding on solvent-infiltrated plants (***Figure 4C***), and eggs fertilized by male moths of larvae feeding on 4MSOB-ITC-infiltrated plants had approximately 50% lower hatching success (***Figure 4F,H***). Therefore, the negative physiological effects suffered by *gss*-silenced *P. xylostella* larvae fed on *A. thaliana* Col-0 plants with aliphatic glucosinolates are likely caused by the exposure to 4MSOB-ITC resulting from hydrolysis of glucosinolates that were not efficiently desulfated as in non-silenced insects.

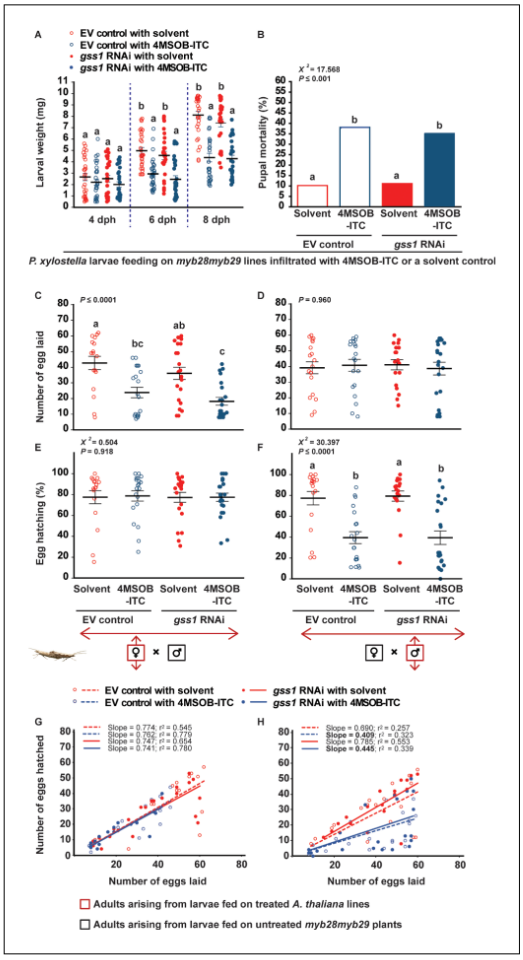

Figure 4. Negative effects on *P. xylostella* fitness after *gss* silencing are attributed to the increased 4MSOB-ITC concentrations in the insect body. Larvae that were *gss*-silenced and non-silenced were fed on *myb28myb29* leaves infiltrated with 4MSOB-ITC (dissolved in a 0.4% aqueous ethanol solvent) or solvent-infiltrated control leaves and the following parameters were measured: (**A**) weights of larvae four dph (days post hatching), six dph and eight dph, (**B**) mortality (%) in pupae formed from treated larvae, (**C**) number of eggs laid by females arising from treated larvae mated with males developed from control larvae feeding on untreated *myb28myb29* plants, (**D**) number of eggs laid by female control larvae mated with males arising from treated larvae, (**E**) hatching (%) of eggs laid by females arising from treated larvae mated with males developed from control larvae, (**F**) hatching (%) of eggs laid by females arising from control larvae mated with males developed from treated larvae, and the correlations between the numbers of eggs hatched and the numbers of eggs laid (**G**) by females arising from treated larvae mated with males arising from control larvae, and (**H**) by females arising from control larvae mated with males arising from treated larvae. Feeding on 4MSOB-ITC caused a significant decline in larval weight (six dph, RNAi $F_{1,116}$ = 2.130, p=0.147; Infiltration $F_{1,116}$ = 46.287, p≤0.0001; RNAi*Infiltration $F_{1,116}$ = 0.012, p=0.913. eight dph, RNAi $F_{1,116}$ = 1.394, p=0.240; Infiltration $F_{1,116}$ = 103.860, p≤0.0001; RNAi*Infiltration $F_{1,116}$ = 0.765, p=0.384. *n* = 30 for all treatments), a significant increase in pupal mortality (χ^2 = 217.568, *df* = 3, p≤0.001; *n* = 49, 42, 45 and 51, respectively), a significant decline in egg laying in crosses when females arise from larvae fed on 4MSOB-ITC ($F_{3,76}$ = 10.046, p≤0.0001, *n* = 17, 20, 21 and 22, respectively), and a significant decline in egg

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Figure 4 continued

hatching rate when males arose from larvae fed on 4MSOB-ITC (χ^2 = 230.397, *df* = 3, p≤0.0001; *n* = 19, 20, 18 and 22, respectively). Significant differences (p≤0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with two-way ANOVA in **A**, a proportions test with pairwise comparisons in **B**, Tukey HSD tests in conjunction with one-way ANOVA in **C–D**, and Dunn's post hoc tests in conjunction with non-parametric Kruskal-Wallis tests in **E–F**.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. *P. xylostella* larval weight, pupal mortality, and adult egg laying capacity in complementation experiment.

Figure supplement 1. Concentrations of 4MSOB-ITC and conjugates formed in crushed leaves of Col-0 *A. thaliana* plants, and in *myb28myb29* leaves infiltrated with 4MSOB-ITC in aqueous ethanol or with pure aqueous ethanol as a control.

Figure supplement 1—source data 1. The concentration of 4MSOB-ITC derivates in *A. thaliana* plants.

Lacewing larvae predating upon *gss*-silenced *P. xylostella* detoxify and mobilize activated glucosinolate hydrolysis products

When consuming *P. xylostella* larvae or other herbivores feeding on glucosinolate-containing plants, predators such as the lacewing *C. carnea* almost inevitably encounter glucosinolates or their metabolic products. To determine the effect of these compounds on a herbivore predator, we therefore explored the consequences of altering *P. xylostella* larval glucosinolate metabolism on *C. carnea*. When larvae of this lacewing preyed on *gss*-silenced *P. xylostella* larvae fed on plants containing aliphatic glucosinolates, significantly greater amounts of 4MSOB-ITC (and significantly lesser amounts of desulfo-4MSOB) deriving from *P. xylostella* larvae were present in the gut, hemolymph and Malpighian tubules compared to *C. carnea* preying on non-silenced *P. xylostella* larvae (***Figure 5B,C***). The lacewing metabolized a large portion of the 4MSOB-ITC ingested from *gss*-silenced *P. xylostella* larvae via the general mercapturic acid pathway (***Figure 5A***), an isothiocyanate detoxification pathway used by many organisms (***Gloss et al., 2014***; ***Schramm et al., 2012***), resulting in 44 nmol/mL and 67 nmol/g of the *N*-acetylcysteine conjugate of 4MSOB-ITC (4MSOB-ITC-NAC) in the hemolymph and Malpighian tubules, respectively (***Figure 5D*** and ***Figure 5—figure supplement 1***). Due to the lack of connection between midguts and hindguts of *C. carnea* larvae (***Figure 5E***) (***McEwen et al., 2001***), the soluble contents of the midgut, including glucosinolates and their metabolites, can only be excreted after being taken up into the hemolymph, secreted into the Malpighian tubules, transported to the silk-separating reservoir, and deposited in the anal secretion (***Figure 5E***) (***McEwen et al., 2001***). For *C. carnea* preying on *gss*-silenced *P. xylostella*, 327 nmol/mL 4MSOB-ITC and 116 nmol/mL of the detoxification product, 4MSOB-ITC-NAC, were detected in the anal secretion (***Figure 5C,D***). The low amount of 4MSOB-ITC remaining in *C. carnea* larvae was excreted in the pupal pellet during pupation, resulting in undetectable 4MSOB-ITC residues in adults (***Figure 5C***). Lacewing larvae preying on non-silenced *P. xylostella* larvae feeding on Col-0 plants with aliphatic glucosinolates excreted approximately 320 nmol/g of desulfo-4MSOB in the pupal pellet, resulting in virtually no detectable desulfo-glucosinolate being retained in adults and the meconium (***Figure 5B***).

Ingestion of prey-derived isothiocyanates slows lacewing development, but has no effects on adult fitness and prey choice

The metabolism of toxic glucosinolate hydrolysis products by *C. carnea* incurs costs that are visible as a slight delay in its larval development. Lacewing larval growth was reduced when feeding on *gss*-silenced isothiocyanate-containing *P. xylostella* larvae fed on Col-0 plants (with wild-type glucosinolates) compared to non-silenced *P. xylostella* larvae or *P. xylostella* larvae fed on *myb28myb29* plants (without aliphatic glucosinolates) (***Figure 6A***). Additionally, pupation was delayed by about two days for *C. carnea* larvae fed on the *gss*-silenced *P. xylostella* larvae raised on Col-0 plants, but without affecting the final proportion of larvae successfully reaching the pupal stage (***Figure 6B*** and ***Figure 6—source data 1***). Similarly, the exposure to isothiocyanates from *gss*-silenced prey also did not affect *C. carnea* pupal mortality and adult egg-laying capacity (***Figure 6C,D***). Thus, ingestion of *gss*-silenced *P. xylostella* larvae with higher 4MSOB-ITC concentrations only caused a slight delay in

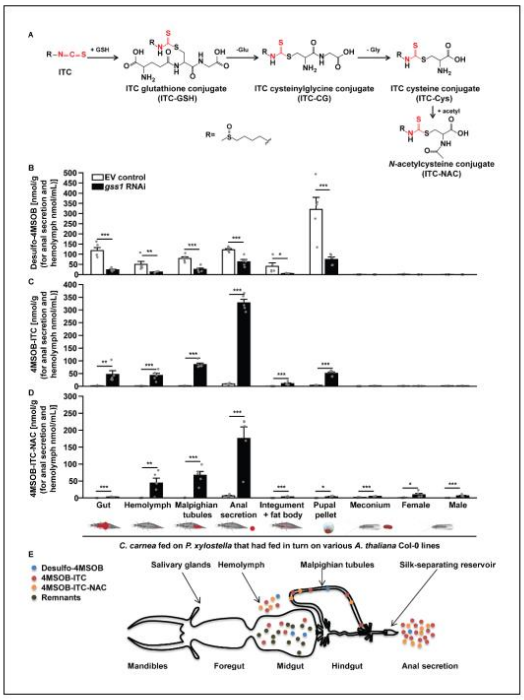

Figure 5. The lacewing *C. carnea* circumvents the toxicity of glucosinolate hydrolysis products by conjugation and mobilization. (**A**) General mercapturic acid pathway for detoxification of 4MSOB-ITC in various insects: ingested 4MSOB-ITC is detoxified by conjugation with glutathione (GSH), followed by cleavage to recover glutamate (Glu) and glycine (Gly) and further modification of cysteine (Cys) to form the final *N*-acetylcysteine conjugate (4MSOB-ITC-NAC). Distribution of (**B**) desulfo-4MSOB, (**C**) 4MSOB-ITC, and (**D**) 4MSOB-ITC-NAC conjugate in various tissues and excretions of *C. carnea* larvae and adults arising from larvae that predated upon *gss*-silenced and non-silenced *P. xylostella* larvae feeding on *A. thaliana* Col-0 (wild-type glucosinolates) and *myb28myb29* (no aliphatic glucosinolates) plants. Predation on *gss*-silenced *P. xylostella* larvae caused a significant reduction in desulfated 4MSOB (Gut, $F_{3,16}$ = 32.378, p≤0.0001; Hemolymph, $F_{3,16}$ = 10.23, p≤0.001; Malpighian tubules, $F_{3,16}$ = 50.855, p≤0.0001; Anal secretion, $F_{3,16}$ = 199.006, p≤0.0001; Integument and fat body, $F_{3,16}$ = 5.959, p≤0.01; Pupal pellet, $F_{3,16}$ = 24.907, p≤0.001; n = 5 for all bars), a significant increase in the toxic hydrolysis product, 4MSOB-ITC (Gut, $F_{3,16}$ = 9.895, p≤0.001; Hemolymph, $F_{3,16}$ = 22.967, p≤0.0001; Malpighian tubules, $F_{3,16}$ = 180.333, p≤0.0001; Anal secretion, $F_{3,16}$ = 110.697, p≤0.0001; Integument and fat body, $F_{3,16}$ = 13.919, p≤0.001; Pupal pellet, $F_{3,16}$ = 14.571, p≤0.0001; n = 5 for all bars), and a significant increase in the detoxification product 4MSOB-ITC-NAC (Gut, $F_{3,16}$ = 29.098, p≤0.0001; Hemolymph, $F_{3,16}$ = 9.242, p≤0.001; Malpighian tubules, $F_{3,16}$ = 33.688, p≤0.0001; Anal secretion, $F_{3,16}$ = 32.659, p≤0.0001; Integument and fat body, $F_{3,16}$ = 14.981, p≤0.0001; Pupal pellet, $F_{3,16}$ = 6.544, p≤0.01; Meconium, $F_{3,16}$ = 18.232, p≤0.0001; Female, $F_{3,16}$ = 5.547, p≤0.01; Male, $F_{3,16}$ = 16.777, p≤0.0001; n = 5 for all bars). Since the concentrations of 4MSOB and its metabolites were nearly undetectable in *C. carnea* preying on *P. xylostella* whose larvae fed on *myb28myb29* plants, these data are not shown in the figure, but given in ***Figure 5—source data 1***. (**E**) A schematic representation of the alimentary tract of a *C. carnea* larva preying on *P. xylostella gss*-silenced larvae feeding on Col-0 (redrawn from ***McEwen et al., 2001***) showing storage of the majority of free and conjugated isothiocyanates and derivatives in the anal secretion reservoir. Significant differences (p≤0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with one-way ANOVA in **B–D**).

The online version of this article includes the following source data and figure supplement(s) for figure 5:

Source data 1. The concentration of 4MSOB glucosinolate metabolites in *C. carnea*.
Figure supplement 1. *C. carnea* detoxifies 4MSOB-ITC by forming the 4MSOB-ITC-NAC conjugate.
Figure supplement 1—source data 1. The concentration of 4MSOB-ITC derivates in third-trophic level.

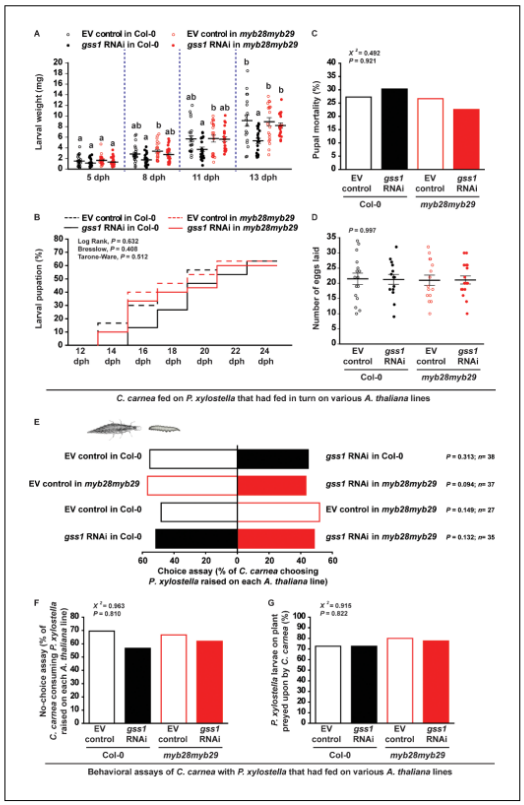

Figure 6. *C. carnea* larval development is slowed by glucosinolate metabolites in its prey, but behavior and adult fitness are not affected. (**A**) *C. carnea* larval weights (eight dph, days post hatching, $F_{3,90}$ = 5.164, p≤0.05, *n* = 24, 23, 23 and 24, respectively; eleven dph, $F_{3,82}$ = 3.235, p≤0.05, *n* = 22, 21, 22 and 21, respectively; thirteen dph, $F_{3,73}$ = 5.767, p≤0.01, *n* = 18, 20, 20 and 19, respectively) were decreased when predating on *gss*-silenced *P. xylostella* larvae feeding on *A. thaliana* Col-0 with wild-type glucosinolate levels in comparison to predation on non-silenced larvae or larvae feeding on plants without aliphatic glucosinolates. (**B**) Although the duration of the pupal stage (EV control in Col-0: 18.3 day; *gss1* RNAi in Col-0: 20.9 day; EV control in *myb28myb29*: 18.6 day; *gss1* RNAi in *myb28myb29*: 19.1 day; see ***Figure 6—source data 1***) was influenced by the prey, the final percentages of pupation between 12 to 24 dph (Log Rank, *df* = 3, p=0.632; Breslow, *df* = 3, p=0.408; Tarone-Ware, *df* = 3, p=0.512; *n* = 30 for all treatments) were not affected. (**C**) *C. carnea* pupal mortality (%) (χ^2 = 20.492, *df* = 3, p=0.921; *n* = 33, 33, 30 and 31, respectively) and (**D**) adult egg-laying capacity ($F_{3,58}$ = 0.018, p=0.997, *n* = 16, 14, 15, 17, respectively) were not significantly affected by predation upon *gss*-silenced and non-silenced *P. xylostella* larvae feeding on Col-0 (wild-type glucosinolates) or *myb28myb29* (no aliphatic glucosinolates) plants. (**E**) Choice assays, (**F**) no-choice assays (χ^2 = 20.963, *df* = 3, p=0.810; *n* = 23, 23, 21 and 21, respectively), and (**G**) predation trials on plants (χ^2 = 20.915, *df* = 3, p=0.822; *n* = 40 in all bars) showed that *C. carnea* did not avoid *gss*-silenced *P. xylostella* larvae, in spite of their higher 4MSOB-ITC concentrations. Significant differences (p≤0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with one-way ANOVA in **A**,**D**, Kaplan-Meier survival analysis test in **B**, two-sided proportions test in C,F,G, and *C. carnea* prey choices were analyzed with a two-sided binomial test (between two *P. xylostella* larvae) and GLM with a binomial distribution and a logit link function (between each treatment) in **E**.

The online version of this article includes the following source data for figure 6:

Source data 1. *C. carnea* larval weight, the percentages of pupation and pupal mortality, adult egg laying capacity, and behavior assays.

larval growth, but did not reduce pupal or adult performance compared to consumption of non-silenced *P. xylostella* larvae or *gss*-silenced *P. xylostella* fed on *myb28myb29* plants (**Figure 6A–D**). The ability of *C. carnea* to detoxify isothiocyanates is also reflected in the fact that its behavior is not much influenced by the glucosinolate processing capacity of its prey. This lacewing displayed no particular feeding preference towards either *gss*-silenced or non-silenced larvae fed on either Col-0 or *myb28myb29* plants (**Figure 6E,F**). Similarly, the percentages of *P. xylostella* larvae from each treatment preyed upon by *C. carnea* were nearly identical (**Figure 6G**).

Discussion

The GSS system is induced by glucosinolate ingestion: trade-off between development and detoxification

Herbivorous insects have developed a variety of mechanisms to overcome plant defense compounds (**Després et al., 2007**), among which detoxification involving functionally diverse enzymes is most often studied (**Heidel-Fischer and Vogel, 2015**). In several cases, expression of detoxification-related genes and resulting enzyme activities are induced in the herbivore midgut in response to plant defense compounds (**Adesanya et al., 2017**; **Kumar et al., 2014**; **Schweizer et al., 2017**). We show that this scenario also holds true for the diamondback moth (*Plutella xylostella*), a herbivore specialized on plants of the Brassicaceae that employs a glucosinolate sulfatase (GSS) activity to disarm the glucosinolate-myrosinase defense system of its host plants (**Ratzka et al., 2002**). Although GSS had been assumed to be constitutively present in *P. xylostella* (**Winde and Wittstock, 2011**), by comparing larvae feeding on *A. thaliana* plants differing in glucosinolate contents, we and others (**Heidel-Fischer et al., 2019**) have observed the induction of *gss* transcript levels and GSS enzyme activity in the midgut upon dietary glucosinolate ingestion (**Figure 1A,G**). The GSS detoxification system of the specialized herbivore *P. xylostella* is therefore also inducible, similarly to the GSS activity previously identified in the generalist-feeding desert locust (*Schistocerca gregaria*) (**Falk and Gershenzon, 2007**).

The inducibility of GSS can be seen as a way for the herbivore to minimize the physiological costs of detoxification. By producing this enzyme only when needed and predominantly in the gut epithelial tissues (**Figure 1B**) that are the first cells to take up glucosinolates from the gut, the insect may minimize the resources needed for GSS synthesis and maintenance. However, induced GSS detoxification still imposes costs, as inferred from slower larval development when feeding on glucosinolate-containing wild-type plants relative to aliphatic glucosinolate-depleted mutant plants (**Figure 2A**). A trade-off between growth and detoxification capacity has also been noted for other insect herbivores feeding on glucosinolate-containing plants. Several generalist lepidopteran caterpillars resist glucosinolate poisoning by metabolizing isothiocyanates, the chief toxic products of glucosinolate hydrolysis, to their corresponding glutathione (GSH) conjugates. However, the cost of biosynthesizing the GSH necessary for isothiocyanate detoxification leads to delayed larval development (**Jeschke et al., 2016b**; **Jeschke et al., 2017**). Our findings on the plasticity of *gss* gene transcription in *P. xylostella* larvae and its trade-off with growth are consistent with these results. Further research is needed to understand the regulatory machinery behind growth-detoxification trade-offs.

Plant-mediated RNAi efficiently silences *P. xylostella gss* with severe physiological and fitness consequences

In order to investigate the importance of GSS on *P. xylostella* performance and glucosinolate metabolism, we attempted to reduce GSS activity by *gss* gene silencing. Silencing the expression of target genes by RNAi is often used to clarify their functions in vivo (**Poreddy et al., 2015**; **Wei et al., 2019**; **Zhang et al., 2015**), and is gaining increasing interest as a tool for crop protection against insect pests (**Zhang et al., 2017**; **Zotti et al., 2018**). Nevertheless, it has been documented that RNAi is less efficient in lepidopterans than in other insect orders and gives rise to varying effects in different species (**Shukla et al., 2016**; **Terenius et al., 2011**). Previous studies on *Heliothis virescens* revealed that exogenous dsRNA is not only readily degraded, but also lacks intracellular transport in larvae, reducing the efficiency of RNAi (**Shukla et al., 2016**). *P. xylostella*, on the other hand, has been successfully silenced by feeding droplets of dsRNA (**Bautista et al., 2009**) and bacterially produced dsRNA (**Israni and Rajam, 2017**). However, as a result of the absence of RNA-dependent RNA

polymerases in insects, continuous feeding is required to maintain gene silencing (**Scott et al., 2013**). Plant-mediated dsRNA delivery has been shown to be an effective way to silence target genes in lepidopterans (**Kumar et al., 2014**; **Mao et al., 2007**; **Poreddy et al., 2017**; **Poreddy et al., 2015**; **Zhang et al., 2017**), for which dsRNA can be constantly incorporated by dietary ingestion. In our research, qPCR and in vitro enzyme assays showed that all three *gss* genes were significantly silenced by plant-mediated RNAi in *P. xylostella* (**Figure 1F** and **Figure 1—figure supplement 4A**), while other sulfatases were not affected (**Figure 1—figure supplement 4C**).

Silencing of *gss* in *P. xylostella* larvae severely reduced the enzymatic efficiency of glucosinolate desulfation as measured by both in vitro assays of midgut protein extracts (**Figure 1G**) and glucosinolate metabolite profiles (**Figure 3**). This was accompanied by a large increase in isothiocyanates (**Figure 3C**), the typical toxic products arising from hydrolysis of aliphatic glucosinolates. These metabolic changes resulted in a dramatic decline in larval growth (50%) and an increase in pupal mortality (4-fold) (**Figure 2A,B**), as well as a decline in both egg laying (over 50%) and in egg hatching (about 30%) in different mating combinations (**Figure 2C,F**). The changes observed in egg laying and hatching indicate a sex-specific effect of ITCs, wherein ITC-exposed females laid fewer eggs, and mating with ITC-exposed males decreased the success of egg hatching (**Figure 2C,F**). Interestingly, egg clutches seem to gather into two separate clusters of high and low hatching success. Hatching success was not linked to the numbers of eggs laid by each female, with low-hatching egg batches distributed among clutches of different sizes (**Figure 2G,H**). We can also discard effects from parental ages as a source of this distribution, as only newly emerged moths were paired; however, the frequency and duration of copulation were not recorded. Bimodal egg hatching patterns were also observed when larvae fed on infiltrated ITC (**Figure 4**) and on *myb28myb29* plants (**Figure 2—figure supplement 2**) instead of Col-0 EV controls (**Figure 2**), indicating this effect was consistent among experiments and suggesting it is linked to mating under these conditions. For instance, the isolation of mating pairs into one couple per mating chamber, preventing females from being able to choose among mates, could have led to incompatible matings. It has been documented that repeated mating and multiple partners increase insect egg fertility, with females able to invest less in incompatible males or entirely avoiding them, while incompatible pairings can lead to less viable or unfertilized eggs being laid (**Arnqvist and Nilsson, 2000**; **Saheb et al., 2009**; **Tregenza and Wedell, 2002**). However, this bimodality and the sex-specific effects of ITCs on *P. xylostella* reproduction deserve further investigation.

The negative effects of *gss* silencing on *P. xylostella* growth and reproduction were also observed when larvae fed on leaves from glucosinolate-lacking mutant plants that were complemented with natural concentrations of the major isothiocyanate formed by *A. thaliana* Col-0 leaves (**Figure 4**). These results support the involvement of this isothiocyanate in the negative consequences of *A. thaliana* aliphatic glucosinolates on silenced *P. xylostella*. Infiltration of ITCs into detached leaves only approximates the natural larval feeding conditions, and could for example lead to unnatural concentrations of ITC-derived products or alter larval feeding behavior. Nevertheless, the combined amounts of 4MSOB-ITC and its known conjugates in larvae after ingestion (**Figure 4—figure supplement 1**) very closely matched not only the natural concentrations of those compounds but also the total 4MSOB-ITC dose administered to leaves, suggesting that little additional metabolism had taken place. Additionally, the relative differences in larval weights between *gss*-silenced and control larvae feeding on wild-type *A. thaliana* leaves (approximately 40% lower at eight dph, **Figure 2A**) and between larvae fed on 4MSOB-ITC-infiltrated and non-infiltrated leaves (approximately 40–50% lower at eight dph, **Figure 4A**) are also similar, supporting an equivalence of the treatments.

At the time of its initial discovery in *P. xylostella* (**Ratzka et al., 2002**), desulfation of glucosinolates was recognized as a way for insect herbivores to avoid glucosinolate toxicity by preventing myrosinase catalysis of glucosinolate hydrolysis since desulfo-glucosinolates are known not to be substrates for myrosinases (**Matile, 1980**). The original report calculated that the GSS activity in each larva was sufficient to readily desulfate all ingested glucosinolates (**Ratzka et al., 2002**), and more recent quantitative isotopic tracer experiments revealed that over 80% of the glucosinolates ingested by *P. xylostella* are indeed desulfated (**Jeschke et al., 2017**). Here we show that desulfation in *P. xylostella* is a detoxification reaction that increases performance and reproductive fitness, in spite of the energy investment required. This conclusion is consistent with a recent evolutionary study (**Heidel-Fischer et al., 2019**) demonstrating that the *gss* genes of *P. xylostella* acquired their

present function encoding GSSs under positive selection pressure. Thus it seems likely that the glucosinolate desulfation reactions reported in the desert locust *S. gregaria* (**Falk and Gershenzon, 2007**), the turnip sawfly *Athalia rosae* (**Opitz et al., 2011**), the silverleaf whitefly *Bemisia tabaci* (**Malka et al., 2016**) and the flea beetle *Psylliodes chrysocephala* (**Beran et al., 2018**) also function as genuine detoxification measures.

Nevertheless, the desulfation process may be not equally effective for all types of glucosinolates. The 132 documented natural glucosinolates are classified as aliphatic, indolic and benzenic depending upon their side-chain structures, with further diversification from added double bonds, hydroxyl or carbonyl groups, and sulfur oxidation (**Agerbirk and Olsen, 2012**). A recent characterization of *P. xylostella* GSS enzymes indicates that the three individual GSS enzymes desulfate specific subsets of glucosinolate substrates, with GSS1 being particularly important for desulfation of aliphatic glucosinolates (**Heidel-Fischer et al., 2019**). Accordingly, the transcripts of *gss1* are higher than *gss2* and *gss3* in the midgut of *P. xylostella* larvae, consistent with aliphatic glucosinolates being the most abundant class in *A. thaliana* Col-0 plants (**Figure 1A** and **Figure 1—figure supplement 1**). Selective knock-out of *gss* genes may be a promising approach to more precisely determine the substrate range and efficiency of the GSS enzymes in vivo and provide more information about the relative toxicities of different glucosinolate types.

Herbivores feeding on glucosinolate-containing plants possess other mechanisms to avoid glucosinolate toxicity, which could complement GSS (**Jeschke et al., 2016a**; **Winde and Wittstock, 2011**). In this study, we found no evidence that *P. xylostella* naturally makes significant use of sequestration or formation of nitriles in addition to desulfation for processing glucosinolates. However, there are strong hints that *P. xylostella* larvae do possess further adaptations to avoid glucosinolate toxicity. After *gss* silencing, we could still detect substantial quantities of intact glucosinolates in the larval midgut and hemolymph (**Figure 3A**), demonstrating that myrosinase activity is not fully efficient in the *P. xylostella* gut. Myrosinase inhibition was also indicated in frass where intact glucosinolates were also found, and spiking with 4MSOB resulted in the formation of 10-fold higher concentrations of desulfo-4MSOB than 4MSOB-ITC (**Figure 3—figure supplement 1**). Although inhibition of myrosinase activity was proposed as a way to prevent glucosinolate intoxication (**Jeschke et al., 2016a**), until now there has been no concrete evidence to support it. GSS itself may inhibit myrosinase action not only by diversion of the substrate for hydrolysis, but by the inhibitory effect of the sulfate moiety released from the desulfation process on myrosinase activity (**Shikita et al., 1999**).

Predatory lacewing larvae that metabolize the isothiocyanates in their *gss*-silenced larval prey incur a cost in reduced growth, but avoid long-term effects on fitness

Certain species of insect herbivores are well known to actively accumulate plant glucosinolates as defenses against their enemies (**Beran et al., 2014**; **Francis et al., 2001**; **Kazana et al., 2007**; **Winde and Wittstock, 2011**). Glucosinolate-sequestering insects typically contain high concentrations of these compounds, with *B. brassicae* wingless aphids and *Phyllotreta striolata* beetles both sequestering upwards of 30 µmol glucosinolates/g (**Kazana et al., 2007**; **Beran et al., 2014**), and the sawfly *Athalia rosae* hemolymph having 10–30 µmol glucosinolates/g hemolymph (**Müller et al., 2001**). Both *B. brassicae* and *P. striolata* not only sequester glucosinolates but also produce their own glucosinolate-activating myrosinases (**Pontoppidan et al., 2001**; **Jones et al., 2001**; **Beran et al., 2014**), and glucosinolate hydrolysis has strong negative effects on their predators (**Francis et al., 2001**; **Kos et al., 2011**).

Even insect herbivores that do not sequester glucosinolates may pose risks for predators and parasitoids because of the transient presence of these compounds or their hydrolysis products in their bodies. In this study, *gss*-silenced *P. xylostella* larvae fed on plants containing wild-type glucosinolate levels retained approximately 450 nmol/g fresh weight 4MSOB-ITC in larval midgut cells and ~300 nmol/mL in hemolymph (**Figure 3**), at least a 10-fold increase over non-silenced larvae. The predatory larvae of the common green lacewing *C. carnea* then detoxified these compounds via the general mercapturic acid pathway (**Figure 5A**), a process used by many generalist herbivores (**Schramm et al., 2012**) with the remaining isothiocyanates being deposited in the anal secretion (**Figure 5C**). However, *C. carnea* larval development was reduced on diets of *gss*-silenced *P. xylostella* (**Figure 6A**) likely reflecting the metabolic cost of detoxification and transport. In a previous study, the generalist herbivore *S. littoralis* was shown to suffer up to a 50% decline in growth rate on

ITC-containing diet, due to the diversion of cysteine towards biosynthesis of the glutathione required for isothiocyanate detoxification (**Figure 5A**), instead of towards protein synthesis (**Jeschke et al., 2016b**). However, *C. carnea* ingested lower levels of ITCs in its *P. xylostella* prey than those found in the guts of herbivores feeding on glucosinolate-containing plants, and so in spite of slower larval growth, no negative long-term effects on survival and fitness were observed (**Figure 6A–D**).

Besides functioning in the excretion of uric acid and production of prepupal silk (**Craig and Phillip, 1987**), the anal secretion of *C. carnea* also plays a role as a defense droplet which is transferred to the head or antennae of attacking enemies (**McEwen et al., 2001**). The presence of glucosinolate-derived isothiocyanates in the anal secretion might contribute to its defensive properties since isothiocyanates are repellent to several animals (**Newman et al., 1992**), but this topic needs further investigation. The detoxification ability of herbivore enemies like lacewings may also benefit the plant, since predatory insects can contribute to significant reductions in insect herbivore damage (**Price et al., 1980**). Interestingly, the isothiocyanate hydrolysis products of plant glucosinolates are reported to attract other herbivore enemies, parasitoids of insect herbivores that feed on glucosinolate-containing plants (**Blande et al., 2007**; **Gols and Harvey, 2009**).

Conclusion and perspectives

The enzymatic desulfation of glucosinolates by *P. xylostella* has been long thought to allow these insects to suppress the activation of glucosinolate hydrolysis and thus circumvent the glucosinolate-myrosinase defense system of their host plants. By silencing the genes encoding GSSs in this insect, we demonstrated that desulfation significantly increases *P. xylostella* larval growth, survival and reproductive fitness. Given the metabolic costs of desulfation noted above and the possibility that this process is not the only mechanism present to avoid glucosinolate toxicity in *P. xylostella*, silencing or other genetic manipulation was necessary to unequivocally demonstrate its benefits to the organism. Our ability to effectively target the *gss* genes of this insect by plant-mediated RNAi suggests that application of RNAi via crop plants or by direct spraying (**Zhang et al., 2017**; **Zotti et al., 2018**), possibly in combination with increased glucosinolate levels in plant tissue, would significantly reduce *P. xylostella* damage by negatively impacting growth, survival and reproduction, as we observed in the laboratory. Additionally, we observed that a generalist larval predator was well-equipped to deal with the increased toxin content of its silenced prey. The lacewing efficiently detoxified and stored the ingested toxins with potential benefits for its own defense. It will be interesting to determine whether more specialized *P. xylostella* predators, which are not normally exposed to glucosinolate hydrolysis products, are also able to tolerate these plant-derived toxins.

Materials and methods

Plants

Arabidopsis thaliana Columbia-0 (Col-0) accession (with wild-type glucosinolates) and transgenic *myb28myb29* (without aliphatic glucosinolates) knockout mutant plants (**Sønderby et al., 2007**) were used. Plants were grown in a controlled short-day environment chamber at 21˚C, 60% relative humidity, and a 14:10 hr light:dark photoperiod. *Brassica napus* for maintenance of insect cultures was grown in a controlled environment greenhouse under the same conditions.

Insects

Plutella xylostella colonies, obtained from Bayer (Monheim am Rhein, Germany), were fed on *B. napus* leaves and maintained in a controlled short-day environment chamber. Eggs of *P. xylostella* were collected for experiments by inducing females to lay on a sheet of parafilm placed above *B. napus* leaves in colonies for two days. After hatching, *P. xylostella* larvae to be used in experiments were fed on *A. thaliana* plants growing under controlled short-day conditions, as described above. *Chrysoperla carnea* eggs were purchased from Katz Biotech AG (Baruth, Germany) and maintained in a controlled long-day environment chamber at 21˚C, 70% relative humidity, and a 16:8 hr light: dark photoperiod.

RNA isolation and cDNA synthesis

Midguts of *P. xylostella* fourth-instar larvae, which abundantly express *gss* (**Ratzka et al., 2002**), were dissected and pooled into TRIzol reagent (Invitrogen, Waltham, MA, USA), and then kept at 4˚C before use. Total RNA was isolated from stored midguts according to the manufacturer's protocol and was subjected to DNaseI (Thermo Fisher Scientific, Waltham, MA, USA) treatment to eliminate genomic DNA contamination. cDNA was synthesized from this RNA by SuperScript III Reverse transcriptase kits (Invitrogen).

gss1 silencing construct for plant-mediated RNAi

The complete coding sequences of *P. xylostella gss* genes were retrieved from **Heidel-Fischer et al. (2019)**. A 526 bp stretch of *gss1* was selected and cloned from *gss1* in a synthesized cDNA pool obtained from primer pairs *gss1*F and *gss1*R (**Supplementary file 1**). *Xba*I and *Eco*RI restriction enzyme cutting sites were added to the ends of the selected *gss1* fragment using the primer pairs *gss1*VF and *gss1*VR (**Supplementary file 1**), and the fragment was further digested by *Xba*I and *Eco*RI (Thermo Fisher Scientific). The pTRV1 (YL192) and pTRV2 (YL156) vectors, which have been described previously (**Burch-Smith et al., 2006**), were used to establish a virus-based dsRNA producing system for plant mediated RNAi. The restriction-digested 526 bp *gss1* fragment was inserted into the *Xba*I-*Eco*RI-cut pTRV2 multiple cloning site by T4 DNA ligase (Invitrogen). pTRV2 containing the 526 bp *gss1* fragment was then used for the *gss1* RNAi construct and the empty vector without insert was used for construction of the empty vector control construct. Simultaneously, a pTRV2 (YL154) vector carrying a *phytoene desaturase* (*pds*, which leads to albino patches when silenced and can therefore serve as a positive control of transformation) insert, was prepared as a visible positive control of virus induced gene silencing.

Plant transformation

pTRV1 and pTRV2 containing *gss1*, empty vector, and *pds* constructs were transformed into *Agrobacterium tumefaciens* strain GV3101. Cultures of *A. tumefaciens* were grown overnight at 28˚C in LB medium containing 50 mg/L kanamycin and 50 mg/L gentamycin. The next day, *A. tumefaciens* cells were harvested by centrifugation (Avanti J-20XP, Beckman Coulter, Krefeld, Germany) at 4000 x g for 30 min. The collected cells were resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, and 150 µM acetosyringone in milli-Q water), adjusted to an OD_{600} of 1.5 with a UV/Vis spectrophotometer (Ultrospec 2100 pro, Biochrom US, Holliston, MA, USA), and incubated at room temperature for 4 hr. *A. tumefaciens* harboring pTRV1 and pTRV2 containing *gss1*, empty vector, or *pds* constructs were mixed in equal amounts before infiltration in plants. Infiltration was performed with a needleless 1 mL syringe into three leaves of the four to five leaf stage (approximately 15 days post seed germination) of *A. thaliana* Col-0 and *myb28myb29* plants. Four weeks after infiltration, the albino patches caused by photobleaching due to reduced *pds* levels in *pds*-construct infiltrated plants were employed as a silencing marker.

Plant metabolite extraction and HPLC analysis

Fifty-day old leaves of untreated, empty vector, and *gss1* RNAi infiltrated *A. thaliana* Col-0 and *myb28myb29* plants were collected in Falcon tubes and frozen in liquid nitrogen immediately. Leaves were freeze-dried (LPHA 1–4 LDplus freeze dryer, Martin Christ, Osterode am Harz, Germany) for 36 hr, and then homogenized by shaking with 3–4 metal balls (3 mm) in each tube. Glucosinolates, flavonoids, and phenylpropanoids were extracted from approximately 12 mg samples using 1 mL extraction solvent (80% methanol) with 50 µM sinalbin as an internal glucosinolate standard. After 5 min incubation on a horizontal shaker (230 rpm) with solvent, supernatants were collected by centrifugation (18,000 x g). Sequentially, 800 µL of each supernatant were loaded on DEAE-Sephadex A-25 columns (Sigma-Aldrich, Munich, Germany). The collected flow-through was diluted (1:3) with milliQ water for further flavonoid and phenylpropanoid measurements. The glucosinolates bound to DEAE-Sephadex, and were desulfated by 30 µL sulfatase treatment (for preparation of sulfatase solution see **Graser et al., 2000**) overnight at room temperature. The next day, desulfo-glucosinolates were eluted with 500 µL milliQ water. All collected fractions were stored at −20˚C until analysis.

Desulfo-glucosinolates, flavonoids, and phenylpropanoids were analyzed on an Agilent Technologies 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a diode-array detector using a Nucleodur Sphinx RP column (250 × 4.6 mm×5 µm, Macherey-Nagel, Düren, Germany). Desulfo-glucosinolates were detected at 229 nm and quantified according to **Burow et al. (2006)**. Water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0–1 min, 1.5% B; 1–6 min, 5% B; 6–8 min, 7% B; 8–18 min, 21% B; 18–23 min, 29% B; and 23.1–24 min, 100% B; 24.1–28 min, 1.5% B, at a flow rate of 1.0 mL/min. Flavonoids and phenylpropanoids were detected at 330 nm according to the method described by **Onkokesung et al. (2014)** with 0.2% formic acid and acetonitrile employed as mobile phases A and B, respectively. The elution profile was: 0–20 min, 0% B; 20–20.1 min, 45% B; 20.1–22.1 min, 100% B; 22.1–26 min, 0% B, at a flow rate of 1.0 mL/min. All compounds were identified by comparison of retention times with those of authentic standards.

Collection of *P. xylostella* tissues

Larvae of *P. xylostella* were continuously fed on untreated, empty vector, and *gss1* RNAi infiltrated *A. thaliana* Col-0 and *myb28myb29* plants in transparent boxes in a controlled environment chamber under conditions as described above. Each box contained one plant and up to 100 larvae, with fresh plants provided as necessary to ensure feeding ad libitum. Tissues from ten larvae were pooled to produce one sample. Midgut epithelium, hemolymph, integument and fat body, and frass were collected from fourth-instar larvae, totaling approximately 5 mg, 5 µL, 10 mg and 10 mg respectively (fresh weights). Hemolymph was collected by a 2 µL pipette through a small wound scratched by a 5 mm needle. Midguts were dissected in TE buffer (Tris- EDTA buffer, pH 8.0) under a dissecting microscope and the content of the midgut was carefully removed. Dissected midgut epithelium, integument and fat body were carefully washed in TE buffer to remove any adhering plant material and hemolymph. Pupae were collected on the second day after pupation and kept individually in 1.5 mL tubes. The pupal excretion (approximately 5 mg from five pupae combined) was collected simultaneously. Adult moths were collected immediately after emergence, divided by sex, and paired for mating (pairs of female and male moths from the same treatments). Subsequently, eggs were collected on the second day after mating (around 5 mg fresh weight per sample). All collected larval tissues, pupae and adults were weighed and immediately frozen in liquid nitrogen, and then stored at −80˚C until further use.

Quantitative real-time PCR (qPCR)

To conduct tissue-specific *gss* transcript profiling and quantify *gss* silencing efficiency, *gss* transcripts were quantified in *P. xylostella* fourth-instar larvae. RNA isolation and cDNA syntheses were performed as mentioned above. qPCR was performed to measure *gss* transcripts in the cDNAs, as reported (**Senthil-Kumar and Mysore, 2014**) by using qRT-PCR SYBR Green kit (Agilent Technologies). Meanwhile, transcripts of detoxification related genes (belonging to the P450, GST, CoE, UGT, sulfatase and SUMF families) were measured in the midgut epithelium of *P. xylostella* fourth-instar larvae by qPCR. The ubiquitin gene was used as an internal control to normalize the abundance of the other gene transcripts. All gene accession numbers and primer pairs were designed via Primer 3 software version 4.0 and listed in (**Supplementary file 1**).

In situ hybridization

PCR products of *P. xylostella gss1*, *gss2* and *gss3* coding sequences were sequenced and then cloned into pGEM-T vectors (Promega, Wisconsin, USA), which were subsequently subjected to in vitro transcription. The linearized pGEM-T vectors consisting of *P. xylostella gss* coding sequences were utilized to synthesize both sense and antisense riboprobes labeled with digoxigenin (Dig) or biotin (Bio) using the T7/SP6 RNA transcription system (Roche, Mannheim, Germany).

Fourth-instar *P. xylostella* larvae were isolated and starved for 1 hr prior to the tissue preparation. Freshly dissected *P. xylostella* midguts were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Europe, The Netherlands). Transverse cryosections with the thickness of 16 µm were thaw mounted on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) at −21˚C (Jung CM300 cryostat). RNA In situ hybridization was performed as previously reported (**Jiang et al., 2018**) with slight modifications. In brief, the cryosections were first fixed (4% paraformaldehyde in 0.1 M

$NaHCO_3$, pH 9.5) at 4˚C for 25 min, then were subjected to a series of treatments at room temperature: a wash for 1 min in PBS (phosphate buffered saline: 0.85% NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.1), an incubation for 10 min in 0.2 M HCl, another two washes for 1 min in PBS, an incubation for 10 min in acetylation solution (0.25% acetic anhydride freshly added to 0.1 M triethanolamine) and three washes in PBS (3 min each). Afterwards, the sections were pre-hybridized for 15 min at 4˚C bathed in hybridization buffer (50% formamide, 5x SSC, 50 µg/mL heparin, and 0.1% Tween-20). A volume of 100 µL hybridization solution containing assayed sense or antisense riboprobes in hybridization buffer was evenly applied onto the tissue section. A coverslip was placed on top and slides were incubated in a moisture box at 60˚C overnight (18–20 hr). On the second day, slides were washed twice for 30 min in 0.1x SSC at 60˚C. Then each slide was treated with 1 mL 1% blocking reagent (Roche) for 40 min at room temperature.

Visualization of hybridized riboprobes was achieved by using an anti-Dig AP-conjugated antibody in combination with HNPP/Fast Red (Roche) for Dig-labeled probes and an anti-biotin streptavidin horse radish peroxidase-conjugate together with fluorescein-tyramides as substrate (TSA kit, Perkin Elmer, MA, USA) for biotin-labeled probes. Cytox green was diluted to 1:30000 in PBS buffer for nuclei counter staining, and each slide was covered with a 100 µL mixture of these components and incubated for 3 min at room temperature. Fluorescence signals were analyzed with a Zeiss LSM510 Meta laser scanning microscope (Zeiss, Oberkochen, Germany), and the acquired confocal image stacks were processed by ZEN 2009 software. The images presented in this paper were rendered by a projection of several optical planes selected from a range of confocal image stacks. For clearer presentation, images were slightly adjusted in brightness and contrast.

In vitro enzyme assay

Midguts of *P. xylostella* larvae feeding on empty vector and *gss1* RNAi infiltrated Col-0 and *myb28-myb29* plants were homogenized in Tris buffer (100 mM, pH 7.5). Concentrations of protein were measured using the BCA Protein Assay Macro Kit (Serva Electrophoresis, Heidelberg, Germany). Protein (1 µg amounts) from each sample in 50 µL Tris buffer was incubated with 50 µL 10 mM 4MSOB (Carl Roth, Karlsruhe, Germany) for 3 min at 25˚C. The reaction was stopped by adding 500 µL methanol. Subsequently, the concentration of desulfo-4MSOB formed was determined by LC-MS/MS (Agilent 1200 series-API3200). In vitro enzyme activity of I3M (Carl Roth), used as a representative of indolic glucosinolates, was measured in the same way.

Growth, development and reproduction of *P. xylostella*

To determine the impact of downregulated *gss* transcripts on *P. xylostella* performance, we monitored the weight of larvae, the mortality of pupae, and the egg-laying capacity and egg-hatching percentage of adults raised from treated larvae fed continuously on empty vector and *gss1* RNAi infiltrated Col-0 and *myb28myb29* plants prepared as described above. Firstly, weights of treated larvae were measured at 4, 6 and 8 dph (days post hatching). After pupation, around 50 pupae from each treatment were collected to monitor pupal mortality. The experiment was repeated independently three times (see ***Figure 2—source data 1***). To inspect the reproduction of *P. xylostella*, male and female fourth-instar larvae were separated (based on the light yellow spot caused by the testicle on the fifth abdominal segment of male larvae), and resulting pupae were paired in individual 35 mL plastic vials. Pupae usually emerged on the same day. If not, according to the their genitals, the newly emerged moth was paired with another newly emerged moth (if one was available) in a new vial, or discarded. Specifically, female moths from each treatment were separated and paired with male moths from larvae feeding on empty vector infiltrated Col-0 plants; in parallel male moths from each treatment were paired with female moths from larvae feeding on empty vector infiltrated Col-0 plants (***Supplementary file 2***). Paired moths were kept in 35 mL plastic vials with 6% sugar solution. Thirty replicates (pairings) were performed for each treatment, and successful mating in the first day post emergence was recorded. Two days post mating, the numbers of eggs laid by 20 of the successfully mated couples from each treatment were counted. The hatching percentage of those eggs was recorded 4 days later. As an additional control, treated moths mated with moths from larvae feeding on untreated Col-0 or *myb28myb29* plants were also studied (***Supplementary file 2***).

Metabolite extraction and LC-MS/MS analysis

Samples of *P. xylostella* tissue for targeted analyses of metabolites collected as described above (subsection 'Collection of *P. xylostella* tissues') were homogenized in 200 µL extraction solvent (60% methanol, pH 3.0) in 1.5 mL Eppendorf tubes with ceramic beads (Sigmund Lindner, Warmensteinach, Germany) by a Skandex S-7 homogenizer (Grootec GmbH, Kirchheim, Germany) for 3 min. Homogenized samples were centrifuged at 13,000 x g for 20 min at room temperature to separate undissolved particles. Clear supernatants were transferred to 2 mL amber glass vials with 0.3 mL glass inserts and further analysed by LC-MS/MS to determine glucosinolate, desulfo-glucosinolate and isothiocyanate concentrations. Analyses were performed on an Agilent Technologies 1200 Series HPLC (Agilent Technologies) coupled to an API 3200 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, Darmstadt, Germany). 4MSOB and desulfo-4MSOB were analyzed by loading samples onto a Nucleodur Sphinx RP column (250 × 4.6 mm×5 µm, Macherey-Nagel, Düren, Germany) with mobile phase A (0.2% formic acid in milliQ water) and mobile phase B (acetonitrile). The elution profile was: 0–1 min, 1.5% B; 1–6 min, 5% B; 6–8 min, 7% B; 8–9 min, 8.4% B; 9.1–10 min, 100% B; and 10.1–14 min, 1.5% B, at a flow rate of 1.0 mL/min. 4MSOB–ITC and its conjugates were analyzed by loading samples onto a Agilent Zorbax Eclipse XDB-C18 column (50 × 4.6 mm×1.8 µm, Agilent Technologies, Wilmington, DE, USA) with mobile phase A (0.05% formic acid in milliQ water) and mobile phase B (acetonitrile). The elution profile was: 0–0.5 min, 15% B; 0.5–2.5 min, 85% B; 2.5–3.5 min, 100% B; 3.5–6.0 min, 3.0% B, at a flow rate of 1.0 mL/min. I3M and desulfo-I3M were analyzed by loading samples onto the same column with the same mobile phases with an elution profile of: 0–0.5 min, 5% B; 0.5–4.0 min, 60% B; 4.1–6.0 min, 100% B; 6.1–8.5 min, 5% B, at a flow rate of 1.0 mL/min. I3C and its derivatives were analyzed by loading samples onto the same column with mobile phase A (10 mM ammonium formate in milliQ water) and mobile phase B (acetonitrile). The elution profile was: 0–0.5 min, 10% B; 0.5–6 min, 90% B; 6.1–7.5 min, 100% B; 7.6–10.0 min, 10% B, at a flow rate of 1.0 mL/min. Quantification of each compound was achieved by multiple reaction monitoring (MRM) of specific parent to product ion conversions for each compound. Parameters for 4MSOB, desulfo-4MSOB (***Malka et al., 2016***), 4MSOB–ITC and its conjugates (***Gloss et al., 2014***); and for I3M and desulfo-I3M (***Malka et al., 2016***) were previously determined, while those for I3C and its derivatives are given in ***Supplementary file 3***. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Quantification of individual compounds was achieved by external calibration curves, the origin of the external standards are given in ***Supplementary file 4***.

Frass spiking assay

Larvae of *P. xylostella* were continuously fed on *myb28myb29* mutant plants (without aliphatic glucosinolates) in transparent boxes (n = 6 boxes), each box containing one plant and around 50 larvae. Fresh plants were provided every day until larvae reached the fourth-instar stage. Then, fresh larval frass was collected (one separate sample per box) and immediately frozen in liquid nitrogen for further use. Two frass aliquots (approximately 0.5 mg each) were weighed from each frass stock for enzyme assays. One aliquot was heated at 100˚C for 30 min as a negative control. Both heat-inactivated and fresh frass sets were spiked with 4MSOB (10 µL of 2.5 mM) and incubated for 15 min at room temperature. The reaction was stopped by adding 100 µL methanol. Subsequently, concentrations of 4MSOB, desulfo-4MSOB and 4MSOB-ITC were measured by LC-MS/MS to determine *P. xylostella* GSS and plant myrosinase activities.

Complementation experiments

To determine whether formation of 4MSOB-ITC in *P. xylostella* caused the phenotypes attributed to *gss* silencing, complementation experiments were conducted by infiltrating 4MSOB-ITC (BIOZOL Diagnostica Vertrieb, Eching, Germany) into empty vector and *gss1* RNAi infiltrated *myb28myb29* leaves. Natural concentrations of 4MSOB-ITC were employed as would result from hydrolysis of typical *A. thaliana* Col-0 foliage: 250 nmol/g fresh weight leaf, as determined by LC-MS/MS (***Figure 4—figure supplement 1***). Thus 0.3 µL of 800 µM 4MSOB-ITC per mg fresh leaf was infiltrated in a solvent of 0.4% aqueous ethanol. Leaves infiltrated with solvent alone served as negative controls (***Figure 4—figure supplement 1***). Larvae of *P. xylostella* were continuously fed on these leaves from

hatching. Larval weight, pupal mortality, egg-laying capacity and egg-hatching percentage were recorded as previously mentioned.

Collection of *C. carnea* tissues

Larvae of *C. carnea* were fed continuously with *P. xylostella* larvae that in turn fed on empty vector or *gss1* RNAi infiltrated Col-0 or *myb28myb29* plants. Each *C. carnea* larva was separately fed on *P. xylostella* larvae from the corresponding groups in a 35 mL vial. Numbers and developmental stages of the prey were chosen according to the predator developmental stage to ensure *C. carnea* always had sufficient food. First- and second-instar *C. carnea* larvae were fed on second- and fourth-instar *P. xylostella* larvae, respectively. When *C. carnea* larvae reached third-instar, guts with gut lumen, hemolymph, Malpighian tubules, anal secretion, and integument and fat bodies were collected, totaling approximately 3 mg, 1 µL, 0.5 mg, 3 µL and 5 mg respectively (fresh weights). Tissues of three larvae were pooled to produce one sample. The anal secretion of *C. carnea* larvae was collected with a 10 µL pipette. Larvae transfer the droplet to the pipette tip as a defense reaction when touched by the tip on the dorsal abdomen. Collected anal secretion was washed into 200 µL extraction solvent (60% methanol, pH 3.0) immediately and kept at −20˚C until further analyses. Hemolymph of each larva was collected by a 10 µL pipette through a small wound in the posterior abdominal segment scratched by a 5 mm needle. Tissues were dissected under a dissecting microscope and washed in TE buffer carefully to remove adhered hemolymph. Pupal pellet left in the cocoon was collected after adult emergence (approximately 1 mg from three pupae combined). Meconium (approximately 5 mg from three adults combined) excreted by the adults in the first few hours after emergence was collected together with the adults. All collected larval, pupal and adult tissues and excretions were weighed and immediately frozen in liquid nitrogen, and then stored at −80˚C until further analyses.

Performance of *C. carnea*

To determine the influence of 4MSOB-ITC on *C. carnea*, the performance of this lacewing was assessed when continuously preying on *P. xylostella* fed on empty vector or *gss1* RNAi infiltrated plants from either Col-0 or *myb28myb29* backgrounds. Newly hatched *C. carnea* (0 dph, days post hatching) were fed with second-instar *P. xylostella* larvae from each treatment in individual 35 mL plastic vials ($n \geq 60$ per treatment). To examine *C. carnea* larval growth (***Figure 6A***) and pupation (***Figure 6B***), a subset of *C. carnea* larvae ('development subset', $n = 30$ in individual vials for each treatment) was separated five dph and observed until pupation while continuously feeding on fourth-instar *P. xylostella* larvae from each treatment. *C. carnea* larval weights were recorded 5, 8, 11 and 13 dph, and the number of pupae was recorded from 13 dph (day when the first larva pupated) until 24 dph (last larva pupated). The *C. carnea* larvae not included in the development subset were also fed continuously on *P. xylostella* larvae from the respective treatments, and surviving insects from the two *C. carnea* subsets were combined per treatment upon pupation to record pupal mortality (***Figure 6C***, $n = 30–33$). Pupal mortality measurements were repeated two additional times with independent groups of pupae (see ***Figure 6—source data 1***) fed as above, but without recording of larval development. Numbers of eggs laid (***Figure 6D***) were recorded from an independent batch of insects raised for this purpose alone: freshly emerged adults were sexed, and couples (females and males originating from the same treatment and hatched on the same day) were paired in 500 mL plastic boxes with 6% honey solution ($n = 14–17$ couples per treatment). The number of eggs laid was counted on the fourth day post mating.

Predation bioassay for *C. carnea*

Choice and no-choice assays were conducted in 35 mL plastic vials. Third-instar larvae of the lacewing *C. carnea* were used that had been feeding on *P. xylostella* larvae fed in turn on empty vector infiltrated Col-0 plants. Fourth-instar larvae of *P. xylostella* fed on empty vector or *gss1* RNAi infiltrated plants from either Col-0 or *myb28myb29* backgrounds were used as prey. Lacewing larvae were starved for 12 hr before the assay and not reused after testing. In choice assays (***Figure 6E***, 27-44 replicates for each treatment, with three independent experiment repetitions, see ***Figure 6—source data 1***), each *C. carnea* larva was allowed to choose between two test *P. xylostella* larvae from different treatments. Each assay consisted of 20 min of observation, and data were not

collected from *C. carnea* that did not make a choice after 20 min; that is, only data from *C. carnea* larvae that captured and killed a *P. xylostella* larva within 20 min were recorded. In no-choice assays (***Figure 6F***, 21–23 replicates for each treatment, with four independent experiment repetitions, seen in ***Figure 6—source data 1***), one *P. xylostella* larva was offered to a *C. carnea* larva in each assay container, and the *P. xylostella* larvae captured and killed within 20 min were counted. In a larger scale bioassay (***Figure 6G***), the percentage of *P. xylostella* larvae on a plant that were preyed upon by the lacewing *C. carnea* was determined. *P. xylostella* fourth-instar larvae (40 larvae for each treatment, with two independent experiment repetitions, see ***Figure 6—source data 1***) fed since hatching on empty vector- or *gss1* RNAi-infiltrated plants from either Col-0 or *myb28myb29* backgrounds were transferred to intact *A. thaliana* plants of the corresponding genotypes a few hours before the assay. Five third-instar *C. carnea* larvae were then placed on each plant, and the numbers of remaining *P. xylostella* larvae were counted after 24 hr.

Statistical analyses

Data were analyzed using the SPSS statistics package version 17.0 and R version 3.6.1. Figures were created using Origin 2019. All data were checked for statistical prerequisites such as homogeneity of variances and normality. Quantitative data (gene transcripts in the larval midgut, in vitro enzyme assays, metabolites in leaf and larval tissues, *C. carnea* larval weights, and egg numbers) were analyzed by one-way ANOVA; multiple comparisons (*gss* gene transcripts in different larval tissues, enzyme activities in the frass spiking assay, and *P. xylostella* larval weights) were analyzed by two-way ANOVA; and statistically significant differences ($p \leq 0.05$) between means (±s.e.) were determined by Tukey HSD tests. Significance ($p \leq 0.05$) of the binary results of mortality and predation assays was evaluated using a two-sided proportions test. *P. xylostella* egg hatching data were analyzed by Dunn's post hoc tests in conjunction with non-parametric Kruskal-Wallis tests. Significance ($p \leq 0.05$) of *C. carnea* larval pupation was determined by Kaplan-Meier survival analysis. *C. carnea* choice assays between caterpillar sources were analyzed with a two-sided binomial test, and effects between each treatment were analyzed by a generalized linear model (GLM) with a binomial distribution and a logit link function. Statistical tests and numbers of replicates are provided in the figure legends and Figure – Source Data files. Letters in graphs represent $p \leq 0.05$; asterisks represent *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; and data groups in each panel that are not labeled with asterisks or letters are not statistically different from each other.

Data and materials availability

All the data needed to understand and assess the conclusions of this research are available in the manuscript; additional data and materials related to this paper may be requested from the authors.

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Additional files

Supplementary files

- Supplementary file 1. Primer sets for gene cloning and qRT-PCR validation.
- Supplementary file 2. The sources of *Plutella xylostella* moths for adult fecundity experiments.
- Supplementary file 3. LC-MS/MS parameters used for the multiple reaction monitoring (MRM) analyses of I3C derivatives. Q1, quadrupole one voltage; Q3, quadrupole three voltage; DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.
- Supplementary file 4. External standards used for quantification.
- Transparent reporting form

Data availability

All data generated or analysed during this study are included in the manuscript and supporting files. Source data files have been provided for figures and figure supplements.

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4. Manuscript II

Note: the formatting of the manuscript that was not yet published was partially modified in order to better match the formatting of the rest of the thesis.

Detoxification of plant defensive glucosinolates by an herbivorous caterpillar is beneficial to its endoparasitic wasp

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Detoxification of plant defensive glucosinolates by an herbivorous caterpillar is beneficial to its endoparasitic wasp

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Abstract

Plant chemical defences impact not only herbivores, but also organisms in higher trophic levels that prey on or parasitize herbivores. While herbivorous insects can often detoxify plant chemicals ingested from suitable host plants, how such detoxification affects endoparasitoids that use these herbivores as hosts is largely unknown. Here, we used transformed plants to experimentally manipulate the major detoxification reaction used by *Plutella xylostella* (diamondback moth) to deactivate the glucosinolate defences of its Brassicaceae host plants. We then assessed the developmental, metabolic, immune, and reproductive consequences of this genetic manipulation on the herbivore as well as its hymenopteran endoparasitoid *Diadegma semiclausum*. Inhibition of *P. xylostella* glucosinolate metabolism by plant-mediated RNA interference increased the accumulation of the principal glucosinolate activation products, the toxic isothiocyanates, in the herbivore, with negative effects on its growth. Although the endoparasitoid manipulated the excretion of toxins by its insect host to its own advantage, the inhibition of herbivore glucosinolate detoxification slowed endoparasitoid development, impaired its reproduction, and suppressed the expression of genes of a parasitoid-symbiotic polydnavirus that aids parasitism. Therefore, the detoxification of plant glucosinolates by an herbivore lowers its toxicity as a host and benefits the parasitoid *D. semiclausum* at multiple levels.

KEYWORDS
detoxification, glucosinolate-myrosinase system, isothiocyanate, multitrophic interaction, parasitism

1 | INTRODUCTION

Multitrophic interactions involving plants, insect herbivores, and their antagonists are ubiquitous in terrestrial ecosystems and underpin our understanding of the structure and function of ecological communities (Stam et al., 2014). Most plants in nature are attacked by insect herbivores, and high infestations can severely damage plant tissues and thus reduce plant fitness (Johnson, Lajeunesse, & Agrawal, 2006). To prevent or reduce attack, plants employ an array of strategies to reduce herbivory, including the production of a wide assortment of toxic, repellent, antidigestive, and antinutritive chemical defences (Mithöfer & Boland, 2012). Plant chemical defences can also traverse trophic levels, moving up the food chain to affect not only the consuming herbivores but subsequently also herbivore predators (Hartmann, 2004;

Nishida, 2002; Petschenka & Agrawal, 2016) and parasitoids (Gols & Harvey, 2009; Harvey, 2005; Harvey, Van Dam, & Gols, 2003; Ode, 2019), and even the fourth trophic level (Harvey et al., 2003; Orr & Boethel, 1986). Nearly three decades ago, Gauld, Gaston, and Janzen (1992) formulated the "nasty host hypothesis", suggesting that herbivore feeding on toxic plants would be detrimental to endoparasitoid development and survival. In apparent support of this hypothesis, the fitness of the predatory wasp *Copidosoma sosares* was shown to be negatively influenced by the presence of toxic furanocoumarins in the haemolymph of its host, the specialized herbivore parsnip webworm *Depressaria pastinacella* (Lampert, Zangerl, Berenbaum, & Ode, 2008; McGovern, Zangerl, Ode, & Berenbaum, 2006; Ode, Berenbaum, Zangerl, & Hardy, 2004), while the nicotine content in the diet of the herbivore *Manduca sexta* affected not only the endoparasitoid *Cotesia congregata*, but also its hyperparasitoid *Lysibia nana* (Barbosa, Gross, & Kemper, 1991; Harvey, Van Dam, Witjes, Soler, & Gols, 2007; Thorpe & Barbosa, 1986). While specialized herbivores can sometimes detoxify ingested plant defensive chemicals efficiently, how these processes affect higher trophic levels and how these defences are in turn metabolized by predators and parasitoids is poorly understood.

Plants in the Brassicaceae, which include oilseed and vegetable crops, produce glucosinolates as their characteristic chemical defences. Glucosinolates are accompanied in plants by enzymes called myrosinases that hydrolyse the parent glucosinolates upon plant damage, forming isothiocyanates (ITCs) and other products (Halkier & Gershenzon, 2006). ITCs are toxic to a broad range of organisms, including the larvae of lepidopteran herbivores (Wittstock, Kliebenstein, Lambrix, Reichelt, & Gershenzon, 2003). Because of their amphiphilic properties and high reactivity (Hanschen et al., 2012), ITCs can directly modify proteins and interfere with intracellular redox homeostasis (Brown & Hampton, 2011). ITCs also typically react quickly with the intracellular nucleophile glutathione (GSH) leading to its depletion (Jeschke, Gershenzon, & Vassão, 2016a). The presence of glucosinolates in plants has fueled selection for a suite of mechanisms in herbivores that mitigate or circumvent exposure to toxic glucosinolate hydrolysis products (Jeschke et al., 2016b), including behavioural adjustments, detoxification, rapid excretion processes and sequestration (Winde & Wittstock, 2011). Larvae of *Plutella xylostella* (the diamondback moth, Lepidoptera: Plutellidae), a specialized herbivore that is a devastating pest of brassicaceous crops (Furlong, Wright, & Dosdall, 2013; Zalucki et al., 2012), can feed without ill effects on glucosinolate-containing plants due to the activity of glucosinolate sulphatases (GSS). These enzymes are abundant in the digestive system of this insect, and desulphate glucosinolates preventing myrosinase-catalysed hydrolysis and ITC formation (Ratzka, Vogel, Kliebenstein, Mitchell-Olds, & Kroymann, 2002). Sun et al. (2019) showed that genetic disruption of *P. xylostella* desulphation led to the increased formation of ITCs, which caused steep declines in larval growth, survival and reproduction, demonstrating desulphation to be a genuine detoxification strategy in this herbivore.

At higher trophic levels, plant defences can act indirectly by reducing the quality of herbivores available as prey or hosts. Alternatively, direct exposure to defensive chemicals ingested by the prey or host can negatively affect the growth and development of parasitoids and predators (Gols & Harvey, 2009; Kaplan, Carrillo, Garvey, & Ode, 2016; Lampert, Zangerl, Berenbaum, & Ode, 2011; Ode, 2019). When the common green lacewing *Chrysoperla carnea* fed on *P. xylostella* in which glucosinolate desulphation was blocked by RNA interference, its development was slowed by consuming these ITC-containing prey (Sun et al., 2019). Nevertheless, *C. carnea* suffered no reproductive effects, perhaps due to its ability to metabolize ITCs via a general detoxification pathway. However, it is not clear how other predators or parasitoids cope with plant defences such as ITCs in their prey or host.

The solitary endoparasitoid *Diadegma semiclausum* (Hymenoptera: Ichneumonidae) is an important natural enemy of *P. xylostella*, and is frequently used in biocontrol programmes against this pest (Furlong et al., 2013; Li, Eigenbrode, Stringam, & Thiagarajah, 2000). Young larvae of this parasitoid develop by feeding on the haemolymph of the caterpillar host until the parasitoid larva reaches its final instar. At this point, it starts to feed on all tissues indiscriminately and eats the host completely, until it pupates in the puparium made by the host caterpillar before death. During all phases of parasitoid growth, the host caterpillar keeps feeding on the plant, thereby exposing the parasitoid larvae continuously to glucosinolates and their metabolites. Previous research has shown that *D. semiclausum* development is influenced by the brassicaceous species on which their *P. xylostella* hosts were reared, possibly due to interspecific differences in glucosinolate profiles (Dosdall, Zalucki, Tansey, & Furlong, 2011; Gols & Harvey, 2009; Gols et al., 2008). However, whether glucosinolates, ITCs or other glucosinolate metabolites benefit or harm endoparasitoids of *P. xylostella* such as *D. semiclausum*, is unknown. Moreover, how parasitism by *D. semiclausum* influences *P. xylostella* glucosinolate metabolism and development is also not known.

Here, we manipulated the detoxification capacity of *P. xylostella* larvae in order to examine the effects of plant glucosinolate defences on the interactions between *P. xylostella* and *D. semiclausum*. We hypothesized that blocking the desulphation activity responsible for glucosinolate detoxification would lead to the accumulation of ITCs in host tissues, affecting in turn the development of the endoparasitoid. Additionally, we also examined the effects of blocking glucosinolate desulphation on the immune responses of the herbivore against parasitism.

2 | MATERIALS AND METHODS

2.1 | Experimental overview

Plant-mediated RNAi was used to silence detoxification-related genes in the herbivore *P. xylostella*. *Arabidopsis thaliana* Columbia-0 (Col-0) plants, which naturally contain glucosinolates, were engineered to produce dsRNA targeting the *Pxgss* genes encoding glucosinolate sulphatases (GSS) responsible for glucosinolate detoxification in the herbivore. While it is unclear whether *P. xylostella* is a natural herbivore of wild *A. thaliana*, this plant has been shown to serve as a suitable host for *P. xylostella*, supporting

similar growth as on cultivated *Brassica* crops (Barker, Poppy, & Payne, 2007). *Arabidopsis thaliana* mutants deficient in the production of glucosinolates were also engineered for RNAi and were used as toxin-free controls. Through the combination of these treatments (presence or absence of glucosinolate defences in the plant and presence or absence of glucosinolate detoxification in the herbivore), we investigated the effects of glucosinolate ingestion and its detoxification by the specialist herbivore both on the herbivore and on the parasitoid *D. semiclausum*. Insect parameters measured included growth and development, survival, protein and lipid contents, and herbivore immune phenoloxidase (PO) activity, while the movement of glucosinolate metabolites among trophic levels was quantified by targeted HPLC-MS/MS analyses. Finally, quantitative real-time PCR (qPCR) was conducted to examine the effects of glucosinolate-derived ITCs on the expression of selected herbivore and parasitoid genes. These procedures are described in detail in the following subsections.

2.2 | Plants and insects

Arabidopsis thaliana Col-0 accession plants with wild type-glucosinolate levels (obtained from the Arabidopsis stock center) and transgenic *myb28myb29* knockout mutants (without aliphatic glucosinolates, obtained from Daniel J. Kliebenstein, University of California Davis) (Sønderby et al., 2007) were used. *Arabidopsis thaliana* plants were grown in a climate-controlled short-day environmental chamber at 21°C, 60% relative humidity, and a 14:10 hr, light:dark photoperiod in a soil mixture (80% Fruhstorfer "Nullerde", 10% vermiculite, and 10% sand). *Brassica napus* used for rearing of insect cultures was grown in a greenhouse under the same conditions as for *A. thaliana*. Colonies of *Plutella xylostella*, obtained from Bayer (Monheim am Rhein, Germany), were fed on *B. napus* leaves and maintained in a controlled short-day environment chamber. Eggs of *P. xylostella* were collected for experiments by allowing females to lay eggs on a sheet of Parafilm placed on top of *B. napus* leaves for 2 days. *Diadegma semiclausum* were reared on *B. napus* infested with *P. xylostella* caterpillars in a climate-controlled long-day environmental chamber (21°C, 70% relative humidity, and a 16:8 hr light:dark photoperiod), in cages containing cotton balls imbibed with a 20% (w/v) sucrose solution in distilled water. Larvae of *P. xylostella* used for experiments were fed on *A. thaliana* plants in a similarly controlled long-day environmental chamber after hatching.

2.3 | *Plutella xylostella Pxgss* gene silencing by plant-mediated RNAi

Plant mediated RNAi was used to silence the expression of *Pxgss* genes in the midgut of *P. xylostella* larvae, as described in Sun et al. (2019). In short, we constructed a tobacco rattle virus-based dsRNA producing system (*Pxgss*-RNAi construct) targeting *Pxgss* in *P. xylostella* larvae. An empty vector construct was used as a negative control. Both the *Pxgss*-RNAi construct and empty vector construct were transformed into *A. thaliana* Col-0 wild-type and *myb28myb29* double knockout mutant plants via *Agrobacterium tumefaciens* (strain GV3101). We had previously determined that plant transformation with these constructs had no effects on plant morphology, glucosinolate profile, and levels of other defensive secondary metabolites (Sun et al., 2019).

2.4 | Measurements of *P. xylostella* growth and development

Plutella xylostella larvae were fed ad libitum on either empty vector-transformed or *Pxgss* RNAi-transformed *A. thaliana* plants (from both Col-0 and *myb28myb29* backgrounds) after hatching in a controlled environmental chamber under the conditions described above. The percentage of larval pupation, which occurred from 7–10 days post hatching, was recorded in the four treatment groups relative to the initial numbers of hatched larvae (approximately 120 larvae per treatment). In the late fourth-instar stage, *P. xylostella* larvae were collected in 1.5 ml Eppendorf tubes to measure larval weights (20 replicates per treatment) as well as soluble protein (five replicates per treatment, three larvae were pooled in one sample) and lipid content (five replicates per treatment, three larvae pooled) after freeze-drying (ALPHA 1–4 LD plus freeze dryer, Martin Christ, Osterode am Harz, Germany). Protein and lipid content were measured to assess the nutritional status of the insects after *Pxgss*-silencing and glucosinolate ingestion. Fresh and dry bodyweights were measured using a microbalance (XP26, Mettler Toledo, Gießen, Germany). Soluble protein and lipid content were measured as described below in 2.8.

2.5 | *Diadegma semiclausum* parasitism

Approximately three hundred *P. xylostella* second-instar larvae of each of the four treatments, *Pxgss*-silenced or unsilenced feeding on either *A. thaliana* Col-0 or *myb28myb29* plants, were individually exposed to *D. semiclausum* to be parasitized once. Female and male *D. semiclausum* adults were sexually mature and maintained together for a few days to promote mating before being exposed to *P. xylostella*. After parasitism, *P. xylostella* larvae were returned to their original food plant until they developed into prepupae. Before the parasitoid larva enters pupation, it excretes a meconium containing waste products, which is present as a black pellet inside its cocoon. The cocoons were kept individually in 5 ml amber glass vials with a cotton cover until emergence. Subsequently, newly emerged adults of *D. semiclausum* were sexed and transferred to 1.5 ml Eppendorf tubes, and immediately frozen in liquid nitrogen for further analyses. As part of these experiments, unparasitized *P. xylostella* fed on either *A. thaliana* Col-0 or *myb28myb29* plants were raised under the same conditions (21°C, 70% relative humidity, and a 16:8 hr light:dark photoperiod). The detailed experimental time line for *D. semiclausum* parasitism of *P. xylostella* is shown in Figure S1.

2.6 | *Diadegma semiclausum* larval development duration, adult emergence and adult bodyweight

To determine the physiological effects of glucosinolates ingested by host larvae on the parasitoid in the absence of RNAi (Figure 1), second-instar *P. xylostella* larvae were parasitized by female *D. semiclausum* as described above and allowed to feed ad libitum on either *A. thaliana* Col-0 or *myb28myb29* plants (one plant per treatment, approximately 50–100 larvae for each of the two treatments). Plants were changed every day to ensure that sufficient food was available. This experiment was repeated three times independently (see Supporting Information file). Then, the percentages of successful *D. semiclausum* adult emergence were calculated relative to the number of *P. xylostella* larvae parasitized. Emerged *D. semiclausum* adults were sexed, freeze-dried and weighed (approximately 20 males and 10 females were obtained per treatment).

To determine the physiological effects of silencing *Pxgss* on *D. semiclausum* (Figure 2g–j), a second experimental setup was used. Silenced and nonsilenced second-instar *P. xylostella* larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* plants (approximately 600 larvae per treatment, 2,400 larvae in total, with 100 larvae per plant in a single cage, plants exchanged daily to ensure food availability) were parasitized by female *D. semiclausum* adults. First, we recorded the emergence of *D. semiclausum* adults (12–16 days post parasitism) from only a subset of the insects (two cages per group, i.e., around 120–180 surviving parasitized *P. xylostella* per treatment, see Supporting Information file). Next, *D. semiclausum* adults emerging from all groups (all six cages per treatment) were sexed, immediately frozen in liquid nitrogen, freeze-dried and weighed (40 male replicates and 10 female replicates were collected per treatment). Soluble protein and lipid contents in these adults were measured as described below in 2.8. Only males were used for this chemical analysis, as the number of female wasps was insufficient for reliable measurement.

2.7 | 4MSOB-ITC complementation experiment

In leaves of *A. thaliana* Col-0 plants grown for these experiments, 4-methylsulphinylbutyl glucosinolate (4MSOB) represents over 70% of the aliphatic glucosinolates (Brown, Tokuhisa, Reichelt, & Gershenzon, 2003). To determine whether the elevated 4-methylsulphinylbutyl isothiocyanate (4MSOB-ITC) concentrations in *Pxgss*-silenced *P. xylostella* caused the *D. semiclausum* phenotypes observed, complementation experiments were conducted by infiltrating 4MSOB-ITC into leaves of *myb28myb29* plants lacking aliphatic glucosinolates as described. A 0.3 μl quantity of 800 μM 4MSOB-ITC per mg fresh leaf was injected using a needleless syringe (Katagiri, Thilmony, & He, 2002) to mimic the natural content of damaged leaves (Sun et al., 2019). Leaves infiltrated with solvent (0.4% aqueous ethanol) served as negative controls. *Diadegma semiclausum* female adults were allowed to parasitize *P. xylostella* larvae (approximately 600 per treatment) continuously feeding on these leaves, and *D. semiclausum* adult emergence percentage, body dry weight, soluble protein and lipid contents were measured. First, the percentage of *D. semiclausum* adult emergence (13–16 days post parasitization) from approximately 200 successfully parasitized *P. xylostella* per treatment was determined. Then, emerged *D. semiclausum* adults were sexed, immediately frozen in liquid nitrogen, freeze-dried and weighed (30 male replicates and 10 female replicates per treatment). Third, soluble protein and lipid content in adults was measured as described below in 2.8.

2.8 | Protein and lipid content

Soluble protein content was determined by the Bradford assay (Bradford, 1976). Samples were homogenized in 200 μl Tris-HCl buffer (100 mM, pH 7.5) with ceramic beads (Sigmund Lindner, Warmensteinach, Germany) in 1.5 ml Eppendorf tubes using a Skandex S-7 homogenizer (Grootec GmbH, Kirchheim, Germany) for 3 min. Homogenized samples were centrifuged at 13,000 ***g*** for 20 min at 4°C to separate undissolved particles. Clear supernatants were transferred to 1.5 ml Eppendorf tubes, and 20 μl of each sample were used to measure protein concentration (Serva Electrophoresis, Heidelberg, Germany) according to the manufacturer's instructions. Soluble protein content was normalized by insect dry weight.

Lipid content was determined following a previously published protocol (Jeschke et al., 2016a). Weighed and pulverized dried body samples (approximately 5 mg for *P. xylostella* larvae, and approximately 2.5 mg for *D. semiclausum* adults, five replicates per treatment) were extracted two times with 1 ml of 2:1 chloroform:methanol by vortexing for 30 s. After centrifugation at 13,000 ***g*** for 20 min at 4°C and careful removal of the lipid-containing solvent, the remaining powder was dried at 80°C for 48 hr and weighed to calculate the proportion of lipid present.

2.9 | Immune phenoloxidase (PO) activity

PO activity is part of a critical host immune defence reaction that promotes melanization during the encapsulation response against parasitoids (Strand & Pech, 1995). To compare PO activity in the haemolymph of nonsilenced and *Pxgss*-silenced *P. xylostella* fourth-instar larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* mutant plants, "spontaneous PO" activity assays were performed as described by Barthel et al. (2016), without a protease pretreatment to activate pro-PO in the samples. Haemolymph of eight larvae (1 μl from each larva) was pooled into one sample and 10 samples per treatment were assessed for their PO activities. Haemolymph was extracted by puncturing the larvae with a sterile hypodermic needle and immediately pipetting the "bleeding" haemolymph into 1.5 ml amber safe-lock Eppendorf tubes with 200 μl ice-cold sodium cacodylate solution (0.01 M Na-cacodylate, 0.005 M $CaCl_2$ in Milli-Q water). The haemolymph mixture was directly frozen in liquid nitrogen and stored at −80°C until measurement. To measure PO activity, frozen samples were thawed on ice then centrifuged at 4°C and 2,800 ***g*** for 15 min to remove cell debris. An aliquot (100 μl) of the resulting supernatant (10 replicates per treatment, one measurement per replicate) was transferred to a 96 well polystyrene plate (VWR International, Darmstadt, Germany) and subsequently mixed with 200 μl

of 3 mM L-DOPA (Sigma-Aldrich, Munich, Germany, freshly prepared and covered with silver foil) per single well, and 100 µl sodium cacodylate solution treated in the same way was measured as a negative control (eight replicates). Absorbance at 490 nm was measured once per minute for 45 min at 30°C in a Multiskan Spectrum multiplate reader (Thermo-Electron, Waltham, MA, USA). The changes in absorbance from 15–26 min of the 45 min measurements were confirmed to be linear and were used to calculate the PO activity (1U = 0.001 AU/min, V_{max} expressed as mU). Data were obtained with SkanIt Software for Multiskan Spectrum version 2.1 (Thermo-Electron).

2.10 | Metabolic analyses

To analyse how glucosinolates were metabolized by *D. semiclausum*, third-instar *D. semiclausum* larvae at 7–8 days post parasitism (three larvae pooled per sample) were collected from *P. xylostella* using a dissecting microscope and washed in TE buffer (Tris-EDTA buffer, pH 8) to remove residual *P. xylostella* haemolymph. The remaining carcass of *P. xylostella* was also collected (approximately 3 mg per sample). Meconium pellets (approximately 1.5 mg pooled per sample) from *D. semiclausum* cocoons were collected immediately after adult emergence. Eclosed adults of *D. semiclausum* (approximately 1.5 mg per sample) were sexed, weighed and frozen in liquid nitrogen for subsequent analyses. To compare how glucosinolates were metabolized by nonparasitized and parasitized *P. xylostella*, samples of approximately 5 mg of frass and 5 µl of haemolymph of *P. xylostella* fourth-instar larvae were collected as described by Sun et al. (2019). In addition, pupae of nonparasitized *P. xylostella* and synchronous prepupae of parasitized *P. xylostella* (approximately 4 mg each) were collected. Before tissues were collected from parasitized *P. xylostella*, the presence of a second-instar parasitoid was confirmed under the microscope. Prepupae parasitized by *D. semiclausum* or nonparasitized pupae were collected on the second day after *P. xylostella* stopped eating and moving. All tissues (five replicates per treatment) were collected (Figure S1), weighed, immediately frozen in liquid nitrogen, and stored at −80°C until analysis.

Diadegma semiclausum and *P. xylostella* tissues collected for quantification of 4MSOB metabolites were homogenized in 150 µl and 200 µl respectively of extraction solvent (60% aqueous methanol, pH 3.0), in 1.5 ml Eppendorf tubes with ceramic beads for 3 min in a Skandex S-7 homogenizer. Homogenized samples were centrifuged at 13,000 ***g*** for 20 min at room temperature to separate undissolved particles. Clear supernatants were transferred to 2 ml amber glass vials with 0.3 ml glass inserts and analysed by LC-MS/MS to determine the concentrations of the major glucosinolate in *A. thaliana* Col-0, 4MSOB, and its derivatives desulpho-4MSOB, 4MSOB-ITC, and ITC conjugates. Analyses were performed on an Agilent Technologies 1,200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 3,200 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, Darmstadt, Germany). 4MSOB and desulpho-4MSOB were analysed by loading samples onto a Nucleodur Sphinx RP column (250 × 4.6 mm × 5 µm, Macherey-Nagel, Düren, Germany) with mobile phase A (0.2% formic acid in milliQ water) and mobile phase B (acetonitrile). 4MSOB–ITC and its conjugates were analysed by loading samples onto a Agilent XDB-C18 column (50 × 4.6 mm × 1.8 µm, Agilent Technologies, Waldbronn, Germany) with mobile phase A (0.05% formic acid in milliQ water) and mobile phase B (acetonitrile). The elution gradient profiles were previously described in Sun et al. (2019); MS parameters for 4MSOB, desulpho-4MSOB, 4MSOB–ITC and its conjugates were also described before (Gloss et al., 2014; Malka et al., 2016); Analyst 1.5 software (Applied Biosystems Sciex, Germany) was used for data acquisition and processing. Quantification of individual compounds was achieved by external calibration curves (the external standards are given in Table S1).

2.11 | RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR)

We quantified *ecdysone receptor* (*EcR*) gene transcripts in nonparasitized and parasitized *P. xylostella* larvae (five replicates per treatment), and *D. semiclausum* symbiotic polydnavirus (PDV)-related gene transcripts, namely *vankyrin1*, *vankyrin2* and *viral innexin1*, in parasitized *P. xylostella* larvae (10 replicates per treatment). *EcR* is induced by the insect hormone ecdysone to control larval development and pupation (Israni & Rajam, 2017), while *vankyrin1*, *vankyrin2* and *viral innexin1* are three of the best-studied PDV-related gene transcripts (Etebari et al., 2011). For these measurements, fourth-instar larvae were individually pooled into TRIzol reagent (Invitrogen, Waltham, MA, USA) in 1.5 ml Eppendorf tubes, and then kept at 4°C before RNA isolation. Total RNA was isolated from stored larvae according to the manufacturer's protocol and then subjected to DNaseI (Thermo Fisher Scientific, Waltham, MA, USA) treatment to eliminate genomic DNA contamination. cDNA was synthesized from this RNA by SuperScript III Reverse transcriptase kits (Invitrogen). qPCR was performed to measure gene transcripts in the cDNA samples using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). The *P. xylostella* ubiquitin gene was used as an internal control to normalize the abundance of gene transcripts. All gene accession numbers and primer pairs (designed via Primer3 software version 4.0) are listed in Table S2.

2.12 | Statistical analyses

Data were analysed using the R statistics package version 3.6.1, including the "agricolae" (de Mendiburu, 2019), "car" (Fox & Weisberg, 2019), "dunn.test" (Dinno, 2017), "FSA" (Ogle, Wheeler, & Dinno, 2019), and "survival" (Therneau, 2015; Therneau & Grambsch, 2000) packages. Figures were created using Origin 2019 and Adobe Illustrator CS5. All data were checked for statistical prerequisites such as homogeneity of variances and normality. The statistical significance ($p \leq .05$) of differences in *P. xylostella* larval weights, *D. semiclausum* adult weights, soluble protein and lipid content, PO activity in the haemolymph of *P. xylostella*, metabolic analyses in comparisons between nonparasitized and parasitized *P. xylostella*, and *P. xylostella ecdysone receptor* gene transcripts in nonparasitized and parasitized *P. xylostella* larvae were all analysed with two-way or multifactor ANOVAs followed by Tukey HSD tests for post

hoc comparisons. The significance ($p \leq .05$) of differences in metabolic analyses among *D. semiclausum* tissues was analysed by nonparametric Mann-Whitney-Wilcoxon tests. The significance ($p \leq .05$) of the difference in the percentage of *D. semiclausum* adult emergence whose larvae parasitized on *P. xylostella* larvae feeding on *A. thaliana* plants containing or lacking aliphatic glucosinolates was evaluated using a two-sided proportions test. *D. semiclausum* adult protein and lipid content in ITC complementation experiments were expressed as independent means (±SE) and significant differences determined by two-tailed *t* tests. Significant differences ($p \leq .05$) in the results of *P. xylostella* pupation and *D. semiclausum* emergence percentages post hatching (parasitism) were evaluated using Cox-regression survival analyses, and differences in *D. semiclausum* emergence percentage in ITC complementation experiments were determined by Kaplan-Meier survival analysis. *D. semiclausum* symbiotic PDVs-related gene transcripts in *P. xylostella* larvae were analysed by Kruskal-Wallis tests with Dunn's post hoc tests. Letters in the graphs represent $p \leq .05$, and asterisks represent: n.s, $p \geq .05$; *, $p \leq .05$; **, $p \leq .01$; ***, $p \leq .001$.

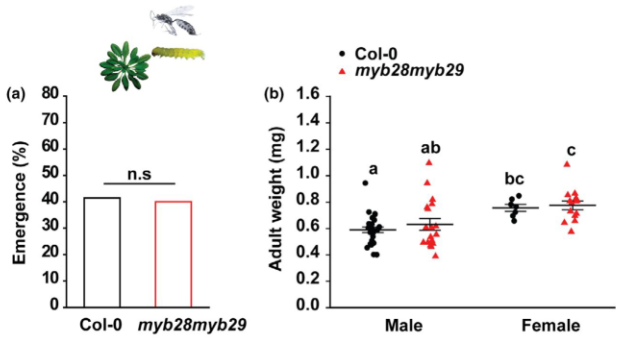
3 | RESULTS

3.1 | *Arabidopsis* glucosinolate content does not influence the development of the parasitoid *Diadegma semiclausum* in *Plutella xylostella* hosts with nonsilenced detoxification

To examine how the development of *D. semiclausum* is affected by the glucosinolate content in the diet of its *P. xylostella* host, *D. semiclausum* was allowed to parasitize *P. xylostella* second-instar larvae (Figure S1) that were fed ad libitum on *Arabidopsis thaliana* plants either containing (wild-type Col-0) or lacking (*myb28myb29* mutant) aliphatic glucosinolates. Aliphatic glucosinolates comprise 70%–80% of the total foliar glucosinolates in *A. thaliana* Col-0 plants (Brown et al., 2003), and are the only glucosinolate class in this plant that forms stable ITCs after hydrolysis by plant myrosinases. Adult emergence of *D. semiclausum* was similar irrespective of the glucosinolate content in the diet of its host, with approximately 40% emergence success in both groups (Figure 1a). The body masses of *D. semiclausum* male and female adults were also not affected by the glucosinolate content in the diet of their host (Figure 1b).

3.2 | Blocking glucosinolate detoxification affects the development and physiology of *Plutella xylostella* caterpillars

In order to explore how *D. semiclausum* is affected by plant toxins in its herbivorous host *P. xylostella*, we used RNAi targeting the *Pxgss* genes that encode glucosinolate sulphatases (GSSs) in the herbivore, to block glucosinolate detoxification. GSSs desulphate plant glucosinolates in the larval midgut, forming nontoxic desulpho-glucosinolates that are not capable of being activated by plant myrosinases to form toxic glucosinolate hydrolysis products (Ratzka et al., 2002). Suppression of *Pxgss* expression had previously been shown to cause reduced GSS activity and increased concentrations of the toxic ITCs resulting from hydrolysis of glucosinolates in *P. xylostella* larvae, causing negative



***D. semiclausum* developing in *P. xylostella* hosts feeding on *A. thaliana* plants with (Col-0) or without (*myb28myb29*) aliphatic glucosinolates**

FIGURE 1 Plant glucosinolate content has little impact on the development of *Diadegma semiclausum* in *Plutella xylostella* hosts. *D. semiclausum* females were allowed to parasitize *P. xylostella* larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* plants (with or without aliphatic glucosinolates, respectively), and the following variables were measured. (a) *Diadegma semiclausum* adult emergence percentage ($\chi^2 = 0.001$, $p = .976$, $n = 82$ and 80, respectively); and (b) *D. semiclausum* adult dry bodyweight (sex, $F_{1,62} = 20.356$, $p \leq .0001$; plant, $F_{1,62} = 1.020$, $p = .317$; sex × plant, $F_{1,62} = 0.084$, $p = .772$; $n = 27$, 18, 7 and 14 represent the respective numbers of replicates in each of the treatments presented in the graph, from left to right) were not affected by aliphatic glucosinolate content. Bars denote means and the interval is the SE. Significant differences ($p \leq .05$) were determined by a two proportions *z*-test in (a). and Tukey HSD tests in conjunction with two-way ANOVA in (b)

fitness effects (Sun et al., 2019). Here, we examined the effect of *Pxgss* silencing on *P. xylostella* growth, development and chemical composition in more detail. The pupation of *Pxgss*-silenced *P. xylostella* larvae was delayed in comparison to nonsilenced controls, but only on food plants containing aliphatic glucosinolates (Figure 2b). Silencing of *Pxgss* did not affect the growth of caterpillars in terms of late fourth-instar larval biomass, irrespective of whether the food plant contained glucosinolates or not (Figure 2c). Glucosinolates in leaf tissues did affect larval metabolism since feeding on glucosinolate-containing plants led to an approximately 40% reduction in soluble protein levels (Figure 2d), while lipid levels were not affected (Figure 2e). In order to assess how *Pxgss* silencing and exposure to ITCs affect general larval immune responses, we measured the activity of phenoloxidase (PO) in the larval haemolymph. PO activity is part of a critical host immune defence reaction that promotes melanization during the encapsulation response against parasitoids (Strand & Pech, 1995). However, PO activity was not affected by *Pxgss* silencing or dietary glucosinolate ingestion (Figure 2f), suggesting that exposure to ITCs does not impair this aspect of *P. xylostella* immunity.

3.3 | Blocking glucosinolate detoxification in the host caterpillar negatively affects development of the endoparasitoid *D. semiclausum*

We determined whether development of the endoparasitoid *D. semiclausum* was affected by the metabolism of glucosinolates in its herbivorous host *P. xylostella*. The emergence of *D. semiclausum* parasitizing *gss*-silenced *P. xylostella* larvae that had been fed on glucosinolate-containing Col-0 plants was delayed and less successful than the emergence of *D. semiclausum* parasitizing nonsilenced larvae or parasitizing larvae feeding on plants without aliphatic glucosinolates (Figure 2g). However, *D. semiclausum* adults from *Pxgss*-silenced hosts feeding on glucosinolate-containing plants were approximately 10% heavier than those from the other treatment groups (Figure 2h). Development of the parasitoid in *P. xylostella* larvae (both silenced and control) that had been fed on glucosinolate-containing Col-0 plants led to 40%–70% lower concentrations of soluble proteins in adults compared to conspecifics developing in hosts feeding on plants without aliphatic glucosinolates (Figure 2i). On the other hand, *D. semiclausum* parasitizing *Pxgss*-silenced *P. xylostella* larvae feeding on Col-0 plants contained approximately 25% more lipids than those in the other treatment groups (Figure 2j).

To confirm that the developmental effects described for *D. semiclausum* were indeed the result of glucosinolates and their derived ITCs in *Pxgss*-silenced *P. xylostella* larvae, a 4-methylsulphinylbutyl-ITC (4MSOB-ITC) complementation experiment was performed using *myb28myb29* leaves (lacking aliphatic glucosinolates) that were infiltrated with a natural ITC concentration mimicking ITCs found in damaged *A. thaliana* Col-0 leaves. 4MSOB, the glucosinolate precursor of 4MSOB-ITC, is the major glucosinolate in *A. thaliana* Col-0 aerial parts, comprising about 70% of the total aliphatic glucosinolate pool (Brown et al., 2003). *D. semiclausum* developing from *P. xylostella* larvae that had fed on 4MSOB-ITC-infiltrated plants had delayed development (Figure S2a), lower adult emergence success (Figure S2a), higher adult weights (Figure S2b) and higher adult lipid content (Figure S2d) compared to *D. semiclausum* parasitizing larvae that had fed on *myb28myb29* leaves infiltrated only with solvent (negative control).

3.4 | Glucosinolate metabolites are transferred from *P. xylostella* hosts to *D. semiclausum* larvae

Younger larvae of *D. semiclausum* primarily feed on the haemolymph of their hosts, but later instars consume almost all tissues just before they complete larval development and pupate (Figure S1). Therefore, these larvae will inevitably encounter glucosinolates or their metabolites while developing in a *P. xylostella* host that feeds on glucosinolate-containing plant tissues. *D. semiclausum* larvae developing in *gss*-silenced *P. xylostella* feeding on glucosinolate-containing plants encountered higher amounts of 4MSOB-ITC and reduced amounts of desulpho-4-methylsulphinylbutyl glucosinolate (desulpho-4MSOB) in host tissues than those developing on nonsilenced *P. xylostella* larvae (Figure 3a,b). In addition, higher amounts of the known 4MSOB-ITC mercapturic acid pathway conjugates (ITC-GSH, ITC-CG and ITC-Cys; Figure S3a,b) were detected in *D. semiclausum* larvae developing in *Pxgss*-silenced *P. xylostella* larvae feeding on Col-0 plants, compared to nonsilenced larvae (Figure 3c). Before pupation, *D. semiclausum* excreted nearly all of the 4MSOB-derived metabolites ingested from *P. xylostella* larvae into the meconium pellet, resulting in nearly no glucosinolate metabolites remaining in the bodies of adult parasitoids (Figure 3).

3.5 | Parasitism by *D. semiclausum* alters glucosinolate metabolism and excretion in the host *P. xylostella*

The increases in levels of 4MSOB-ITC and its conjugates in *Pxgss*-silenced *P. xylostella* carcasses after *D. semiclausum* parasitism were much lower in magnitude than the decreases in desulpho-4MSOB (Figure 3). To learn more about the efflux of 4MSOB metabolites, we directly compared the quantities of 4MSOB metabolites present in haemolymph, frass, and pupae of parasitized and nonparasitized *P. xylostella* larvae. Although the concentrations of 4MSOB-ITC were significantly higher in the haemolymph of *gss*-silenced than in nonsilenced *P. xylostella* larvae, parasitism reduced 4MSOB-ITC haemolymph levels in *Pxgss*-silenced larvae by 87% (Figure 4a). Conversely, parasitism significantly increased 4MSOB-ITC levels in the frass of *Pxgss*-silenced larvae compared to unparasitized silenced controls (Figure 4b). At the prepupal stage, parasitism decreased 4MSOB-ITC in *P. xylostella* to nearly undetectable levels, while the pupae of unparasitized larvae still had measurable 4MSOB-ITC content (Figure 4c). Accordingly, 4MSOB-ITC conjugates were more abundant in the frass and prepupae of parasitized

P. xylostella, compared to nonparasitized larvae, although these conjugates were found in lower amounts than 4MSOB-ITC itself (Figure S3).

Although *Pxgss* silencing successfully reduced the formation of the 4MSOB detoxification product desulpho-4MSOB in the host, the haemolymph of parasitized *P. xylostella* larvae contained approximately 75% less desulpho-4MSOB than that of nonparasitized larvae, for both *Pxgss*-silenced and nonsilenced larvae (Figure 4d). Similarly, the frass from parasitized *Pxgss*-silenced *P. xylostella* larvae contained about 85% less desulpho-4MSOB than frass from nonparasitized silenced larvae, whereas desulpho-4MSOB in nonsilenced larvae was not affected by parasitism (Figure 4e). However, the prepupae of both *Pxgss*-silenced and unsilenced larvae parasitized by *D. semiclausum* contained 17.3- and 47.9-fold higher concentrations of desulpho-4MSOB, respectively, than nonparasitized *P. xylostella* pupae (Figure 4f). Therefore, larvae of *D. semiclausum* appear to influence the excretion of 4MSOB-ITC by the host and to absorb desulpho-glucosinolates when parasitizing *P. xylostella*, and these compounds are retained in the prepupae (Figure 4g).

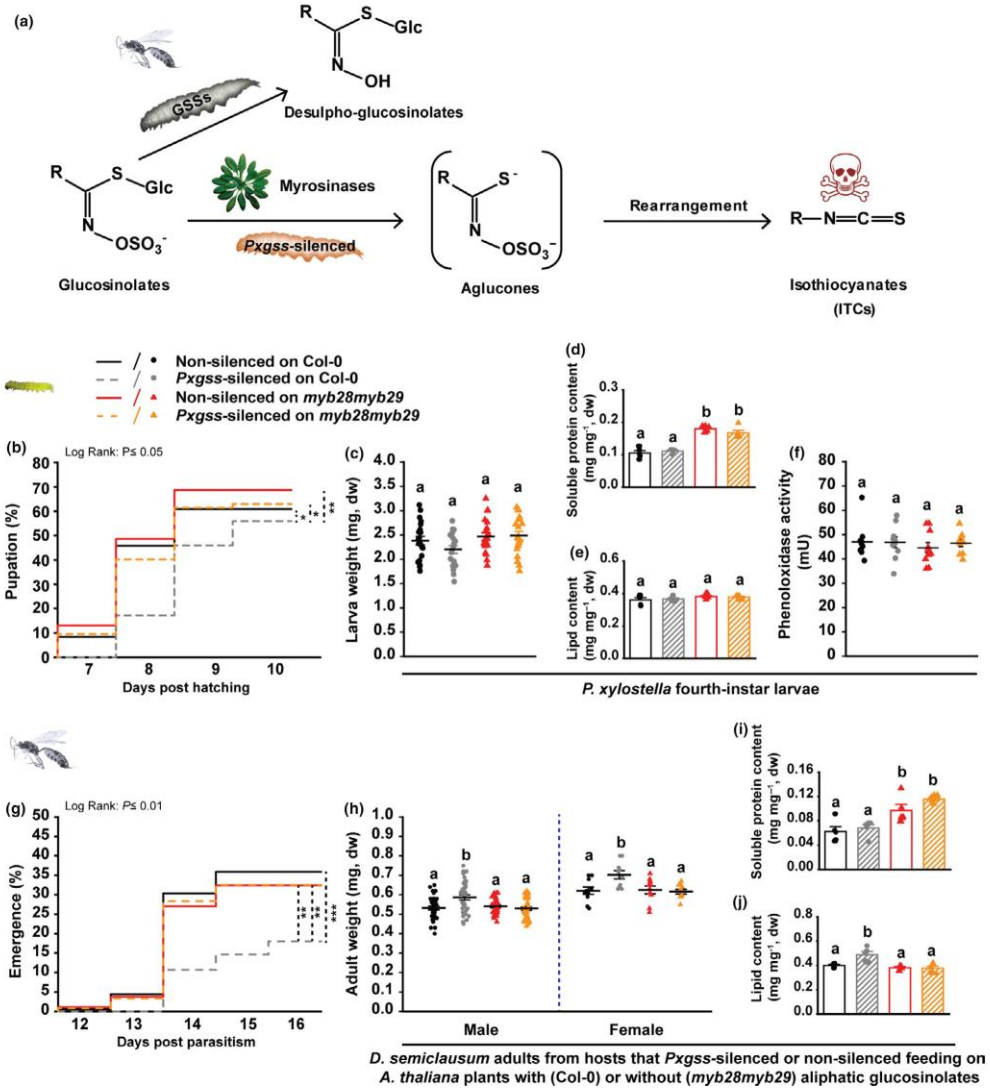


FIGURE 2 The endoparasitoid *Diadegma semiclausum* and its host *Plutella xylostella* are negatively affected by silencing of *Pxgss* in the presence of plant glucosinolates. (a) Plant glucosinolates are normally rapidly desulphated by *P. xylostella* glucosinolate sulphatases (GSSs) to form nontoxic desulpho-glucosinolates. Silencing of *Pxgss* increases glucosinolate hydrolysis by plant myrosinases, resulting in formation of toxic isothiocyanates (ITCs). (b–f) Nonsilenced and *Pxgss*-silenced *P. xylostella* larvae fed on *A. thaliana* Col-0 (with aliphatic glucosinolates) and *myb28myb29* (without aliphatic glucosinolates) plants and the following variables were measured: (b) *Plutella xylostella* egg-to-pupation percentage between 7 and 10 d post hatching; Log rank, $df = 3$, $p \leq .05$; *Pxgss*-silenced on Col-0 compared with nonsilenced on Col-0, $Z = 2.025$, $p \leq .05$, with nonsilenced on *myb28myb29*, $Z = 3.252$, $p \leq .01$, with *Pxgss*-silenced on *myb28myb29*, $Z = 2.089$, $p \leq .05$; egg numbers were 120, 111, 115 and 127, respectively; (c) larval dry weight (plant, $F_{1,76} = 4.834$, $p \leq .05$; *gss*-silencing, $F_{1,76} = 0.936$, $p = .336$; plant × *gss*-silencing, $F_{1,76} = 1.380$, $p = .244$; $n = 20$ in all treatments); (d) soluble protein content (plant, $F_{1,16} = 125.027$, $p \leq .0001$; *gss*-silencing, $F_{1,16} = 0.267$, $p = .612$; plant × *gss*-silencing, $F_{1,16} = 2.539$, $p = .131$; $n = 5$ in all treatments); (e) lipid content (plant, $F_{1,16} = 3.299$, $p = .088$; *gss*-silencing, $F_{1,16} = 0.008$, $p = .930$; plant × *gss*-silencing, $F_{1,16} = 0.222$, $p = .643$; $n = 5$ in all treatments) of *P. xylostella* fourth-instar larvae; and (f) immune phenoloxidase (PO) activity in fourth-instar *P. xylostella* larval haemolymph (plant, $F_{1,36} = 0.479$, $p = .493$; *gss*-silencing, $F_{1,36} = 0.185$, $p = .670$; plant × *gss*-silencing, $F_{1,36} = 0.280$, $p = .600$; $n = 10$ in all treatments). (g–j) *Diadegma semiclausum* larvae were allowed to parasitize nonsilenced and *Pxgss*-silenced *P. xylostella* larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* plants, and the following parameters were measured: (g) cumulative *D. semiclausum* adult emergence percentage between 12 and 16 d post parasitism; Log Rank, $df = 3$, $p \leq .01$; *Pxgss*-silenced on Col-0 compared with nonsilenced on Col-0, $Z = 3.685$, $p \leq .001$, with nonsilenced on *myb28myb29*, $Z = 3.086$, $p \leq .01$, with *Pxgss*-silenced on *myb28myb29*, $Z = 2.845$, $p \leq .01$; parasitized numbers were 181, 150, 185 and 120, respectively; (h) *D. semiclausum* adult dry bodyweight (male, plant, $F_{1,156} = 6.284$, $p \leq .05$; *gss*-silencing, $F_{1,156} = 5.067$, $p \leq .05$; plant × *gss*-silencing, $F_{1,156} = 11.229$, $p \leq .01$; $n = 40$; female, plant, $F_{1,36} = 5.133$, $p \leq .05$; *gss*-silencing, $F_{1,36} = 3.971$, $p = .054$; plant × *gss*-silencing, $F_{1,36} = 6.170$, $p \leq .05$; $n = 10$); and *D. semiclausum* male adult; (i) soluble protein content (plant, $F_{1,16} = 33.186$, $p \leq .0001$; *gss*-silencing, $F_{1,16} = 2.852$, $p = .111$; plant × *gss*-silencing, $F_{1,16} = 0.820$, $p = .378$; $n = 5$ in all bars); and (j) lipid content (plant, $F_{1,16} = 15.192$, $p \leq .01$; *gss*-silencing, $F_{1,16} = 6.785$, $p \leq .05$; plant × *gss*-silencing, $F_{1,16} = 7.886$, $p \leq .05$; $n = 5$ in all bars). Significant differences ($p \leq .05$) were determined by Cox regression survival analysis tests in (b) and (g), significant differences between means (±SE) were determined by Tukey HSD tests in conjunction with two-way ANOVA in (c–f) and (h–j).

3.6 | Blocking glucosinolate detoxification in the host caterpillar reduces expression of immune suppression genes by a symbiotic virus of the parasitoid

Symbiotic polydnaviruses (PDVs), a family of dsDNA viruses, are injected by female parasitoids in several subfamilies of the Ichneumonidae and Braconidae (e.g., Campopleginae, Microgastrinae) into the haemolymph of their hosts and cause immune suppression, resulting in lower rates of encapsulation of the developing parasitoids (Beckage, 2011; Webb et al., 2006) as well as changes in the phenology of the host (Harvey, 2005). PDVs associated with ichneumonid parasitoids like *D. semiclausum* are called ichnoviruses, and produce proteins important in infection, such as vankyrins and viral annexins (Tanaka et al., 2007), to reduce the rates of encapsulation of the developing parasitoids in host haemolymph. To determine if higher ITC levels might affect the expression of symbiotic PDV-related genes upon parasitism, the expression of three well-studied viral-related gene transcripts (Etebari et al., 2011), *vankyrin1*, *vankyrin2* and *viral innexin1*, was measured by qRT-PCR. Expression of these three genes was reduced by 80%, 70% and 62%, respectively, upon *D. semiclausum* parasitism in *Pxgss*-silenced hosts feeding on Col-0 plants (with aliphatic glucosinolates) compared to nonsilenced hosts feeding on Col-0 plants or either silenced or nonsilenced hosts feeding on *myb28myb29* plants (without aliphatic glucosinolates) (Figure 5).

3.7 | Parasitism by *D. semiclausum* reduces *P. xylostella ecdysone receptor* (*EcR*) expression

To further explore how *gss* silencing influences parasitism of *P. xylostella* by *D. semiclausum*, we measured the expression of a *P. xylostella* ecdysone-related gene (*EcR*), which is induced by ecdysone to control larval development and pupation (Israni & Rajam, 2017). *EcR* transcripts were reduced 30%–70% in *P. xylostella* fourth-instar larvae upon successful parasitism by *D. semiclausum* (Figure S4). However, *Pxgss* silencing and the glucosinolate content of the *P. xylostella* diet had no significant effect on expression.

4 | DISCUSSION

4.1 | Detoxification of plant defences by an herbivore improves the performance of an endoparasitoid

Plant defensive chemicals consumed by herbivores can affect higher trophic levels, impacting predators and parasitoids both directly and indirectly (Gols & Harvey, 2009; Harvey et al., 2003; Nishida, 2002; Ode, 2019; Petschenka & Agrawal, 2016). As a corollary of the "nasty host hypothesis" (Gauld et al., 1992), herbivores with efficient mechanisms to detoxify plant defences might be expected to serve as more suitable hosts or prey for their enemies. Our results agree with this assertion demonstrating that the glucosinolate detoxification pathway in the crucifer-feeding host *P. xylostella* benefits not only the herbivore, but also maximizes the performance of *D. semiclausum* parasitoids developing inside this herbivore. When the gene encoding glucosinolate-detoxifying sulphatase in *P. xylostella* was silenced, this herbivore suffered a 50% decline in growth and a 4-fold increase in pupal mortality (Sun et al., 2019). Moreover, the emergence of adult *D. semiclausum* from silenced hosts decreased by about 50% (Figure 2g). These effects could be directly attributed to the accumulation in the parasitoid of ITCs, the toxic hydrolysis products of glucosinolates (Figure 3a). When *D. semiclausum* was

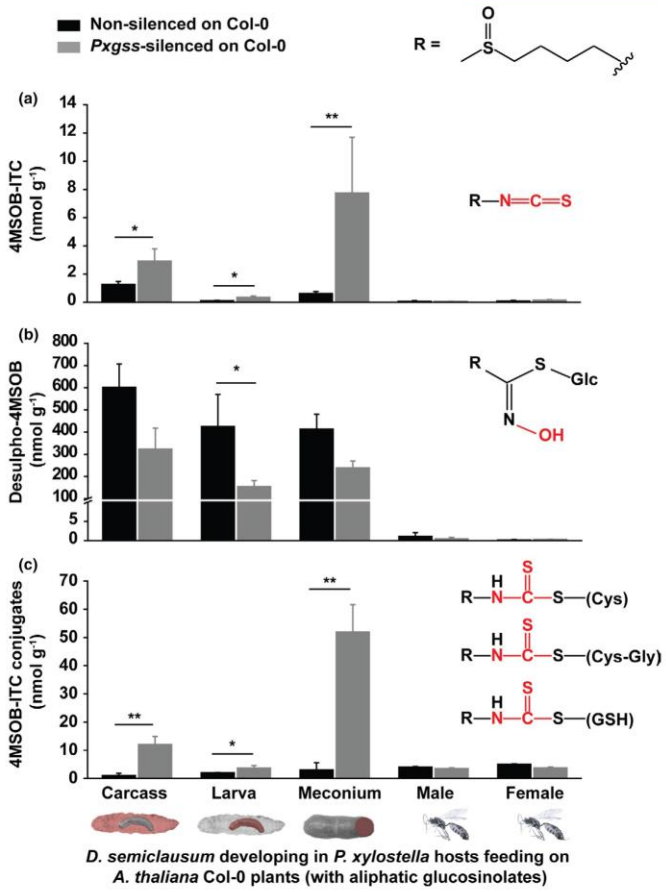


FIGURE 3 Metabolites of 4MSOB are present in *Diadegma semiclausum* parasitizing *P. xylostella* larvae fed on *A. thaliana* Col-0 plants. *D. semiclausum* was allowed to parasitize nonsilenced and *Pxgss*-silenced *P. xylostella* larvae feeding on either *A. thaliana* Col-0 (with aliphatic glucosinolates) or *myb28myb29* (without aliphatic glucosinolates) plants. (a) 4MSOB-ITC (host carcass, $p \leq .05$; parasitoid larva, $p \leq .05$; meconium, $p \leq .01$; $n = 5$); (b) Desulpho-4MSOB (host carcass, $p = .151$; parasitoid larva, $p \leq .05$; meconium, $p = .056$; $n = 5$ in all bars): and (c) 4MSOB-ITC conjugates (host carcass, $p \leq .01$; parasitoid larva, $p \leq .05$; meconium, $p \leq .01$; $n = 5$) were quantified in the carcass of *P. xylostella* prepupae, third-instar larvae of *D. semiclausum*, meconium left in the cocoon and adults of *D. semiclausum*, in which *D. semiclausum* parasitized either nonsilenced (black bars) or *Pxgss*-silenced (grey bars) *P. xylostella*. The general mercapturic acid pathway is shown in Figure S3a. 4MSOB-ITC-GSH: 4MSOB-ITC-glutathione conjugate; 4MSOB-ITC-CG: 4MSOB-ITC-cysteinylglycine conjugate; and 4MSOB-ITC-Cys: 4MSOB-ITC-cysteine conjugate; concentrations are shown in Figure S3b as stacked bars. 4MSOB and its metabolites were nearly undetectable in *P. xylostella* larvae fed on *myb28myb29* plants and are not shown in the graphs. Coloured objects depict the parts being analysed. Significant differences ($p \leq .05$) between means (±SE) were determined by Mann-Whitney Wilcoxon tests in a–c. separately conducted for each tissue

reared on *P. xylostella* larvae developing on ITC-infiltrated foliage, the same declines in parasitoid emergence were noted (Figure S2a). In untransformed *P. xylostella* where the glucosinolate detoxification system was functional, *D. semiclausum* tolerated a range of glucosinolate content in the herbivore diet (Figure 1). This suggests that previous reports on changes in *D. semiclausum* developmental variables, such as cocoon and adult weight and duration of development (Dosdall et al., 2011; Kahuthia-Gathu, Löhr, & Poehling, 2008), are probably not caused by alterations in the glucosinolate content of the food plant of the host herbivore. Although we employed genetically

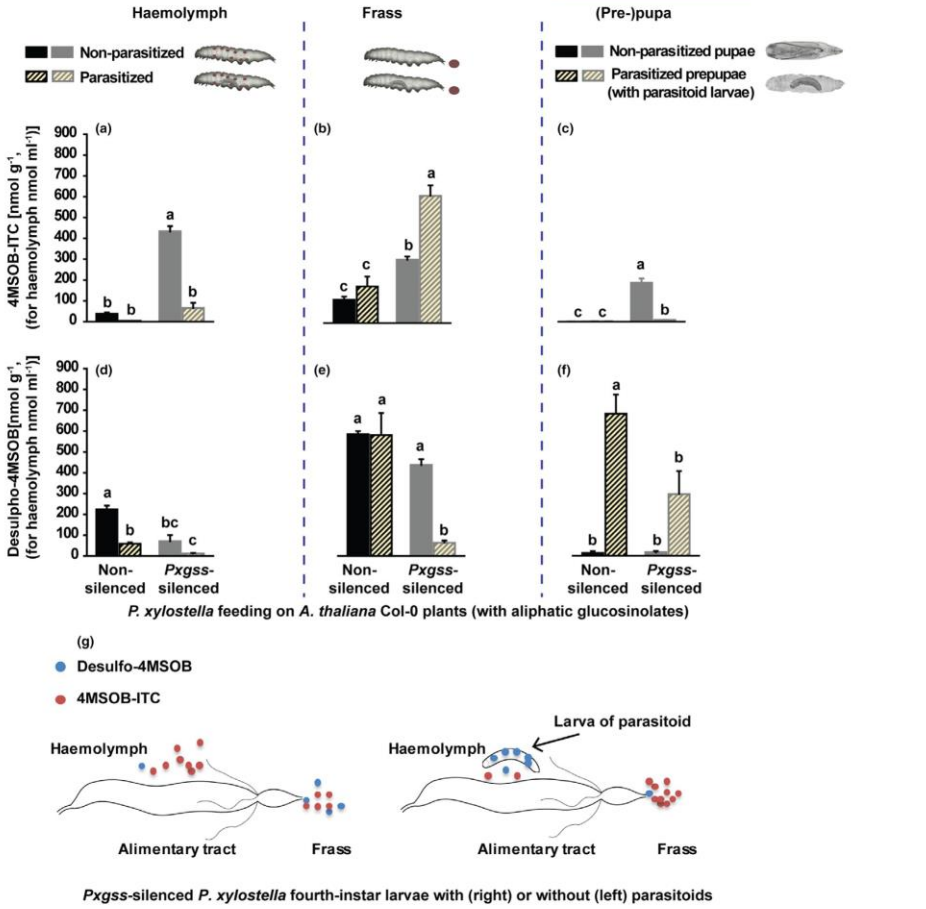
modified plant lines in this study to block a specialized detoxification reaction of an herbivore, which increased the accumulation of toxic isothiocyanate products in its body, this is also reflective of natural situations. Brassicaceae plants are also subject to herbivory by numerous species of generalist herbivores without specialized detoxification pathways. Several lepidopteran species are known to produce and accumulate large quantities of isothiocyanates when feeding on glucosinolate-containing plants (Jeschke et al., 2017), and so infesting parasitoids would encounter a similar situation to that in sulphatase-silenced *P. xylostella*.

How plant defences affect endoparasitoid performance is poorly understood despite the fact that endoparasitoids constitute a very abundant group of enemies of insect herbivores (Gols & Harvey, 2009; Harvey, van Dam, Raaijmakers, Bullock, & Gols, 2011; Ode, 2006). Since the physiology of endoparasitoids is very tightly coupled with that of their hosts, these insects could be very susceptible to plant toxin content in the host diet and the extent of detoxification by the host. Accordingly, a few studies have shown that increased toxin levels in the host diet can actually lead to reduced parasitoid performance (Barbosa et al., 1991; Garvey, Creighton, & Kaplan, 2020). The tolerance of natural enemies of insect herbivores to allelochemicals in host or prey tissues can also depend on the degree of specialization of the natural enemy on their host or prey. For example, while low concentrations of the furanocoumarin xanthotoxin did not affect the specialist parasitoid *Copidosoma sosares* in its interaction with its specialist herbivore host *Depressaria pastinacella*, exposure of the parasitoid *Copidosoma floridanum* (which has a much broader host range) to this compound in the haemolymph of one of its polyphagous hosts reduced survival and offspring production (Lampert et al., 2011). Plant chemical defences can also affect predators. For instance, the wolf spider *Camptocosa parallela* shows lower preference for tobacco hornworm (*Manduca sexta*) prey containing higher nicotine levels (Kumar, Pandit, Steppuhn, & Baldwin, 2014), and this predator prefers prey that detoxify nicotine (Kumar, Rathi, Schöttner, Baldwin, & Pandit, 2014). Likewise, predators such as the ladybug *Adalia bipunctata* are deterred by the glucosinolate-sequestering cabbage aphid *Brevicoryne brassicae* (Kazana et al., 2007). The larval development of the lacewing *C. carnea* is reduced when preying on *Pxgss*-silenced *P. xylostella* (Sun et al., 2019). The greater physiological intimacy between parasitoids and their hosts compared to predators and their prey may have resulted in parasitoids of insect herbivores being better adapted to the presence of plant defence metabolites in their hosts than predators. Furthermore, parasitoids developing in hosts that contain plants toxins can themselves coopt these plant defences against their own antagonists such as hyperparasitoids (Bowers, 2003; van Nouhuys, Reudler, Biere, & Harvey, 2012) offering them the opportunity to exploit enemy free space, a concept that is usually restricted to insect herbivores (Murphy, Lill, Bowers, & Singer, 2014). Thus, the presence of toxins in their host may not only help protect parasitoids indirectly (avoiding predation of their host), but also directly against their own enemies in the fourth-trophic level (Murphy et al., 2014).

4.2 | Herbivore detoxification can modulate the immune response to endoparasitoids

Numerous factors can influence the outcome of insect herbivore-endoparasitoid interactions. We hypothesized that plant defences could influence the immune response of the herbivore host against the parasitoid. Adult female parasitoids in several subfamilies of the Ichneumonidae and Braconidae (e.g., Campopleginae, Microgastrinae) inject symbiotic polydnaviruses (PDVs) into the host haemolymph, which produce proteins that alter host growth (Harvey, 2005) and disrupt cellular and humoral immune responses leading to overall immune suppression (Hasegawa, Erickson, Hersh, & Turnbull, 2017). PDV vankyrins are homologues of the *Drosophila melanogaster* NF-κβ transcription factor inhibitor Iκβ (Kroemer & Webb, 2005, 2006). These proteins are thought to protect parasitoids from the cellular immune system of the host by suppressing NF-κβ signaling cascades (Tian, Zhang, & Wang, 2007), which blocks blood cell formation and the cellular encapsulation response against parasitoids (Gueguen, Kalamarz, Ramroop, Uribe, & Govind, 2013). PDV innexins are homologues of insect innexins, which form gap junctions between the cytoplasm of insect cells and so play crucial roles in cellular immune responses (Hasegawa & Turnbull, 2014; Turnbull, Volkoff, Webb, & Phelan, 2005). Viral innexins can perturb the physiological functions of native insect innexins (Hasegawa et al., 2017). Here, we observed that the expression of the PDV genes *vankyrin1*, *vankyrin2* and *viral innexin1* was suppressed when glucosinolate detoxification was blocked in *P. xylostella* hosts feeding on glucosinolate-containing plants (Figure 5). This may have contributed to the lower emergence rate of *D. semiclausum* adults from these hosts (Figure 2g). Interestingly, *D. semiclausum* adults that emerged successfully (but belatedly; Figure 2g) from *Pxgss*-silenced *P. xylostella* larvae feeding on Col-0 plants were slightly heavier than the *D. semiclausum* adults from the other treatments (Figure 2h). These wasps also had a higher lipid content (Figure 2j) than those developing from *P. xylostella* control hosts, but did not differ in protein content (Figure 2i). At the herbivore level, dietary ITCs are known to increase larval lipid content while decreasing protein content, due to the imbalance in amino acid metabolism caused by ITC detoxification, which depletes cysteine levels (Jeschke et al., 2016a).

Plant defence compounds could also act directly on the herbivore's immune response against parasitism (Kaplan et al., 2016). The enzyme phenoloxidase (PO) has an important function in melanization against foreign agents by producing quinone groups that are polymerized to promote encapsulation (González-Santoyo & Córdoba-Aguilar, 2011). PO activity in the haemolymph of *Heliothis subflexa* was shown to benefit from the withanolide defences of its *Physalis* host plant (Barthel et al., 2016). However, here we found that PO enzyme activity in the haemolymph of *P. xylostella* was not influenced by manipulating its glucosinolate metabolism and the resulting exposure to ITCs (Figure 2f). Karimzadeh and Wright (2008) compared the



***Pxgss*-silenced *P. xylostella* fourth-instar larvae with (right) or without (left) parasitoids**

FIGURE 4 Parasitism by *D. semiclausum* affects the concentrations and distribution of 4MSOB metabolites in *gss*-silenced and nonsilenced *P. xylostella* larvae feeding on *A. thaliana* Col-0 plants. Comparison of 4msob-ITC in (a) haemolymph (*gss*-silencing, $F_{1,16} = 162.62$, $p \leq .0001$; parasitism, $F_{1,16} = 125.73$, $p \leq .0001$; *gss*-silencing × parasitism, $F_{1,16} = 86.04$, $p \leq .0001$; $n = 5$ in all bars); and (b) frass (*gss*-silencing, $F_{1,16} = 74.87$, $p \leq .0001$; parasitism, $F_{1,16} = 26.06$, $p \leq .001$; *gss*-silencing × parasitism, $F_{1,16} = 11.18$, $p \leq .01$; $n = 5$ in all bars) of nonparasitized and parasitized *P. xylostella* fourth-instar larvae. Comparison of 4MSOB-ITC concentrations in (c) pupae of nonparasitized *P. xylostella* and prepupae of parasitized *P. xylostella* with parasitoids inside (*gss*-silencing, $F_{1,16} = 125.623$, $p \leq .0001$; parasitism, $F_{1,16} = 22.565$, $p \leq .001$; *gss*-silencing × parasitism, $F_{1,16} = 6.882$, $p \leq .05$; $n = 5$ in all bars). Comparison of desulpho-4MSOB concentrations in (d) haemolymph (*gss*-silencing, $F_{1,16} = 115.72$, $p \leq .0001$; parasitism, $F_{1,16} = 92.58$, $p \leq .0001$; *gss*-silencing × parasitism, $F_{1,16} = 39.05$, $p \leq .0001$; $n = 5$ in all bars); and (e) frass (*gss*-silencing, $F_{1,16} = 35.62$, $p \leq .0001$; parasitism, $F_{1,16} = 11.80$, $p \leq .01$; *gss*-silencing × parasitism, $F_{1,16} = 10.96$, $p \leq .01$; $n = 5$ in all bars) of nonparasitized and parasitized *P. xylostella* fourth-instar larvae. Comparison of desulpho-4MSOB concentrations in (f) pupae of nonparasitized *P. xylostella* and prepupae of parasitized *P. xylostella* with parasitoids inside (*gss*-silencing, $F_{1,16} = 7.034$, $p \leq .05$; parasitism, $F_{1,16} = 42.909$, $p \leq .0001$; *gss*-silencing × parasitism, $F_{1,16} = 7.245$, $p \leq .05$; $n = 5$ in all bars). (g) A schematic representation of the alimentary tract of nonparasitized (left) and parasitized (right) *Pxgss*-silenced *P. xylostella* larvae feeding on *A. thaliana* Col-0 (with aliphatic glucosinolates) plants, with coloured dots representing relative quantities of desulpho-4MSOB and 4MSOB-ITC and where they might accumulate. Significant differences ($p \leq .05$) between means (±SE) were determined by Tukey's HSD tests in conjunction with two-way ANOVA in (a–f)

effect of variation in plant quality on the immune response in parasitized and unparasitized *P. xylostella* hosts and found that these responses were only transient or were negated by the effect of parasitism by *Cotesia plutellae* itself. In contrast, Bukovinszky et al. (2009) reported that the induction of plant defences can impair immune functionality of the host suggesting a detoxification-immunity tradeoff. Thus, the effect of plant toxins on the host immune response depends on the players involved in the tritrophic interaction.

4.3 | Endoparasitoid performance is enhanced by modification of host gene expression and alteration of ingestion and distribution of plant defences

Parasitism can influence the physiology and behaviour of herbivores in a multitude of ways. We demonstrated here that *D. semiclausum* parasitism alters *P. xylostella* ecdysone receptor (*EcR*) expression in a way that inhibits host pupation. However, this inhibition happened independently of the glucosinolate content of the host diet (Figure S4). Such manipulation of host development is crucial for completion of the endoparasitoid life cycle. Another example involving *EcR* concerns a symbiotic bracovirus of the endoparasitoid *Cotesia vestalis*, which produces a miRNA that arrests host growth by altering the expression of the host *EcR* gene (Wang et al., 2018).

Parasitism can sometimes induce herbivores to ingest plant toxins to improve survival of the host (Bruce, 2014) or that of the parasitoid (Pashalidou et al., 2015). For instance, parasitized *Grammia incorrupta* (woolly bear caterpillars) engage in self-medication, increasing their ingestion of plant pyrrolizidine alkaloid toxins in response to endoparasitic tachinid flies resulting in improved herbivore survival (Singer, Mace, & Bernays, 2009). Parasitization can also affect the feeding habits of *Pieris rapae* larvae (Van Der Meijden & Klinkhamer, 2000), and *M. sexta* larvae parasitized by *Cotesia congregata* decrease their feeding in the last stages of parasitoid development by induced anorexia, which actually seems to benefit the parasitoid (Adamo, Linn, & Beckage, 1997). Moreover, parasitism can also affect the metabolism of plant toxins by the herbivorous host. For example, parasitism of webworm larvae (*D. pastinacella*) by *C. sosares* lowers furanocoumarin detoxification rates (per unit of larval weight) in the host, potentially increasing the haemolymph concentrations of these toxins (McGovern et al., 2006). In the present study, parasitism by *D. semiclausum* did not increase glucosinolate ingestion by its host, but did result in increased excretion of ITCs. When desulphation was blocked by *Pxgss* gene silencing, the hydrolysis of glucosinolates by plant myrosinases resulted in increased concentrations of toxic ITCs in haemolymph and frass (Figure 4a,b), but these were lower in parasitized hosts than in nonparasitized ones. Therefore, *D. semiclausum* appears to alter the distribution of toxic plant defence metabolites in *P. xylostella* to limit its exposure while it feeds on herbivore tissues. The mechanisms used by this parasitoid to manipulate ITC distribution remain to be determined.

4.4 | Herbivores that detoxify defences and parasitoids that benefit from detoxification: Consequences for plant protection

The benefits of *P. xylostella* glucosinolate detoxification for both the herbivore and its parasitoid *D. semiclausum* are of considerable relevance to the plant as well. Plants that are attacked by herbivores with the ability to detoxify their major defence compounds face a dilemma, since producing increased concentrations of defences will probably not be an effective countermeasure. One strategy is to switch resources to the production of greater amounts of other defence compounds in its arsenal (Koricheva, Nykanen, & Gianoli, 2004), a tactic employed by *A. thaliana* (Burow et al., 2009) and other Brassicaceae (Kuchernig, Burow, & Wittstock, 2012), especially when fed upon by *Pieris rapae*, a herbivore that also

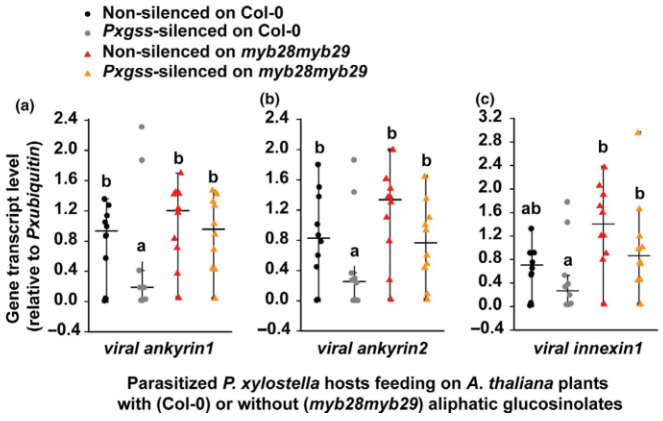


FIGURE 5 Expression of *D. semiclausum* symbiotic polydnavirus (PDV)-related genes is suppressed in *Pxgss*-silenced *P. xylostella* larvae. (a) *vankyrin1* ($X^2 = 13.704$, $df = 3$, $p \leq .01$; $n = 10, 8, 10$ and 10, respectively); (b) *vankyrin2* ($X^2 = 13.299$, $df = 3$, $p \leq .01$; $n = 10, 8, 10$ and 10, respectively); and (c) *viral innexin1* ($X^2 = 16.105$, $df = 3$, $p \leq .01$; $n = 10, 8, 10$ and 10, respectively) gene transcripts in *D. semiclausum*-parasitized *P. xylostella* fourth-instar larvae feeding on either *A. thaliana* Col-0 or *myb28myb29* plants. Significant differences ($p \leq .05$) between medians were determined by Kruskal-Wallis tests with Dunn's post hoc tests in a–c

detoxifies glucosinolates (Wittstock et al., 2004). Herbivory by *P. rapae* alters the major route of glucosinolate activation in *A. thaliana* so that instead of isothiocyanates, nitriles are formed (Burow et al., 2009), with these compounds deterring *P. rapae* oviposition (Mumm et al., 2008). Such diversion of defensive resources to other products of the same pathway, or even the activation of separate biosynthetic pathways can therefore help plants protect themselves from attack by a well-adapted herbivore.

Another plant strategy to combat an herbivore with a strong capacity to detoxify chemical defences is to recruit natural enemies of herbivores (Gols et al., 2015). Plant volatiles released in response to herbivore damage have been demonstrated to attract herbivore enemies (Clavijo McCormick, Unsicker, & Gershenzon, 2012; Hare, 2011). While this can increase the fitness of plants attacked by herbivores (Gols et al., 2015; van Loon, de Boer, & Dicke, 2000), evidence showing that this happens under natural conditions is scarce (Clavijo McCormick et al., 2012). In the Brassicaceae, *A. thaliana* and other species emit volatile glucosinolate hydrolysis products and other volatile metabolites that attract herbivore predators and parasitoids (Bruce, 2014; Gols & Harvey, 2009; Hopkins, van Dam, & van Loon, 2009; Mumm et al., 2008). For example, the predator *C. carnea* and the parasitoid *C. plutellae* are both significantly attracted by allyl isothiocyanate present in the frass of *P. xylostella* larvae feeding on cabbage (Reddy, Holopainen, & Guerrero, 2002). 3-Butenyl-ITC is attractive to *Diaeretiella rapae*, a parasitoid that predominantly attacks Brassicaceae-feeding aphids (Blande, Pickett, & Poppy, 2007). As one of the world's most destructive pests of Brassicaceae plants (Talekar & Shelton, 1993), *P. xylostella* has developed significant resistance to most synthetic pesticides, as well as to modern biological pesticides like *Bacillus thuringiensis* (Bt) toxins (Li, Feng, Liu, You, & Furlong, 2016). Thus increased use of natural enemies is being explored to reduce *P. xylostella* damage (Furlong et al., 2013; Sarfraz, Keddie, & Dosdall, 2005). Our current results suggest that the manipulation of herbivore metabolism could be useful in such an effort. On the one hand, plants that inhibit herbivore detoxification should suffer less damage due to the decreased performance of herbivores. On the other hand, in the case of the *P. xylostella* – crucifer interaction, the higher ITC levels in the frass of silenced *P. xylostella* caterpillars may attract more *P. xylostella* (Pivnick, Jarvis, & Slater, 1994; Renwick, Haribal, Gouinguené, & Städler, 2006) as well as predators and parasitoids, potentially making these plants useful as "dead-end trap crops". However, the effects of such modifications on the populations of herbivore enemies are nuanced and species-specific, and the ecological ramifications of such an approach in both natural and agricultural settings need further research. In this study, we demonstrated that *P. xylostella* detoxification of glucosinolates enhances the performance of *D. semiclausum*. Enhanced parasitoid performance may positively impact parasitoid population dynamics and enhance their recruitment by plants, which may ultimately have a positive effect on plant fitness.

In conclusion, the desulphation of plant glucosinolates by the specialist herbivore *P. xylostella* prevents the formation of toxic ITCs and thus increases insect growth, survival and reproductive success. Here we show that this detoxification reaction also benefits a representative of the next trophic level, *D. semiclausum*, a widespread endoparasitoid of *P. xylostella* caterpillars, by increasing adult emergence and decreasing development time. Future research on other endoparasitoids is needed to determine if the overall susceptibility of *D. semiclausum* to plant defences in its herbivore host is a general trait of this group. Glucosinolate desulphation also appears to facilitate the action of symbiotic polydnaviruses of *D. semiclausum* that suppress host immunity, but more work is needed to understand the mechanisms responsible for improved parasitoid performance.

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AUTHOR CONTRIBUTIONS

R.S., D.G.V., J.G., S.S.P., R.G., and J.A.H. planned and designed the research; R.S. performed experiments and collected data; R.S., and M.R. analysed the data; and R.S., D.G.V., R.G., J.A.H., and J.G. drafted the manuscript with input from all coauthors.

DATA AVAILABILITY STATEMENT

Supporting Information figures and tables are available in the Supporting Information document. Raw data and statistical analyses are available as a separate Supporting Information file.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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5. Manuscript III

Note: the formatting of the manuscript that was not yet published was partially modified in order to better match the formatting of the rest of the thesis.

Adaptation of cabbage aphid to aliphatic glucosinolates overwhelms detoxification in a lacewing predator

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Prepared for Journal of Pest Science

Adaptation of cabbage aphid to aliphatic glucosinolates overwhelms detoxification in a lacewing predator

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Abstract

The cabbage aphid *Brevicoryne brassicae* is a notorious agricultural pest that specializes on Brassicaceae plants. By sequestering glucosinolates from its host plants and producing its own myrosinase, this aphid has evolved a defense system paralleling that in plants, which affords a basic deterrence against enemies. However, we know little about the metabolic fate of individual glucosinolates during aphid sequestration and activation, nor about the effects of this defense on aphid enemies. In this paper, we probed these questions focusing on *B. brassicae* and a generalist predator, the common green lacewing *Chrysoperla carnea*. We found that the accumulation of distinct glucosinolates by *B. brassicae* occurred at differential rates, with accumulation of aliphatic glucosinolates occurring at a faster rate than accumulation of indolic glucosinolates. Among the aliphatic glucosinolates, compounds with shorter side chains accumulated more rapidly. Characterization of recombinant *B. brassicae* myrosinase revealed a strong positive correlation between enzymatic activities towards different glucosinolates and their rate of accumulation. After simulated predation, the amount of toxic isothiocyanates (ITCs) was quantitatively outweighed by the amounts of other less toxic hydrolysis products or conjugates, e.g. nitriles and ITC-conjugates. Nevertheless, the formation of aliphatic ITCs in aphids significantly impaired development in *C. carnea.* Tissue-specific metabolite quantification demonstrated that the lacewings employed both conjugation and mobilization to reduce the toxicity of aliphatic ITCs, but these strategies were only partially effective. These results illustrate the metabolic fates of glucosinolates after sequestration by an aphid herbivore and further after lacewing predation on the aphid, and the consequences for lacewing survival and development. They might be instructive for integrative pest management approaches targeting the cabbage aphid.

Keywords

accumulation, detoxification, glucosinolates, isothiocyanates, multi-trophic interaction, sequestration

1. Introduction

Plants of the order Brassicales, such as those in the Brassicaceae and Capparaceae families, produce glucosinolates and myrosinases as an effective two-component defense against non-adapted herbivores and pathogens. Glucosinolates are constitutively and abundantly accumulated in sulfur-rich *S*-cells (Koroleva *et al.*, 2011), whereas myrosinases (β-thioglucoside glucohydrolases) are present in protein-enriched idioblasts, the myrosin cells (Rask *et al.*, 2000; Wittstock *et al.*, 2002; Andréasson *et al.*, 2003). Such a compartmentation avoids the self-intoxication that could result from the unintended activation of glucosinolates by myrosinases, so that activation occurs only on tissue damage, e.g. during herbivore feeding. The aglucones thus produced are unstable and rearrange to form an array of downstream metabolites including isothiocyanates (ITCs), simple nitriles (CNs), thiocyanates, and epithionitriles. The outcome of hydrolysis is dependent on the glucosinolate side chains, the presence of protein modulators (specifier proteins), as well as the reaction conditions and cofactor present (Eisenschmidt-Bönn *et al.*, 2019). Among the products of glucosinolate hydrolysis, ITCs (containing an –N=C=S functional group) play major roles in plant-herbivore interactions. Other hydrolysis products, such as CNs, are thought to be less toxic than ITCs, and can have a role in indirect defense (Burow *et al.*, 2006).

Glucosinolates themselves are amino acid-derived, glucosylated specialized metabolites (Blažević *et al.*, 2020). The basic structure of glucosinolates comprises three building blocks: a *β*-thioglucose moiety, a sulfonated oxime moiety, and a structurally diverse side chain that allows the characterization of glucosinolates into three major categories, namely aliphatic, indolic and benzenic glucosinolates (Agerbirk *et al.*, 2012). In general, the toxicity of glucosinolates-derived ITCs is conferred by the electrophilic –N=C=S functional group that reacts with intracellular nucleophiles (Hanschen *et al.*, 2012; Jeschke *et al.*, 2016). However, the side chain structure can affect the toxicity too. ITC side chains help dictate post-hydrolysis reactivities, and toxicity is altered for example by a rapid loss of the –N=C=S group (e.g. to form the carbinol products of indolic glucosinolates) (Wittstock *et al.*, 2016). Additionally, the lipophilicity of the side chain can facilitate the diffusion of ITCs through the cellular lipid bilayer membranes to reach the intracellular environment. While the electron-withdrawing groups on the side chain can increase overall electrophilicity and reactivity, electrostatic attraction or steric hindrance between the side chain and target proteins will also promote selective reactivity (Brown *et al.*, 2011). Hence, in addition to the unique properties of the electrophilic –N=C=S functional group, the structural diversity of various types of ITC side chains expands their potency to a broader range of targets.

The cabbage aphid *Brevicoryne brassicae* (Hemiptera: Aphididae) is a piercing-sucking herbivore, and as such can largely suppress the glucosinolate-myrosinase defense system by minimizing tissue damage (Louis *et al.*, 2012). *B. brassicae* has been found to selectively accumulate aliphatic glucosinolates from host plants (Kos *et al.*, 2011). However, how distinct glucosinolates are dynamically accumulated has not been clarified. In addition to sequestering certain ingested glucosinolates in its hemolymph, *B. brassicae*

concomitantly produces its own endogenous myrosinase (BMY, β-thioglucoside glucohydrolase of *B. brassicae*) in its head and thoracic muscles (Kazana *et al.*, 2007). It is yet unknown that to what extent the catalytic activity of endogenous BMY corresponds to the glucosinolates actually sequestered by the insect. As in the plant, once insect tissues have been disrupted by predators, sequestered glucosinolates and BMY meet resulting in the formation of toxic ITCs, giving *B. brassicae* the moniker “walking mustard oil bomb” (Kazana *et al.*, 2007). However, the factors influencing glucosinolate hydrolysis in *B. brassicae* are not fully understood despite their ecological importance.

The glucosinolates sequestered by *B. brassicae* and the resulting ITCs formed may move up the food chain, and cause negative effects on higher-trophic levels (Kazana *et al.*, 2007; Kos *et al.*, 2011). As an important biocontrol organism, the common green lacewing *Chrysoperla carnea* has received increasing research attention for its ability to handle prey like aphids and soft caterpillars. It has recently been shown that *C. carnea* larvae can tolerate dietary 4-methylsulfinylbutyl glucosinolate (4MSOB-Gls), by detoxifying the hydrolysis product 4MSOB-ITC via the general mercapturic acid pathway, and storing some of this compound into its anal defensive secretion. In spite of 4MSOB-ITC causing a slightly impaired larval development, this had no impact on pupal mortality and adult egg-laying capacity (Sun *et al.*, 2019). However, *B. brassicae* differs markedly from the *P. xylostella* used in this previous study in its content of toxic glucosinolate metabolites. Thus it is hard to predict the physiological and metabolic response of *C. carnea* to preying on *B. brassicae*.

In this paper, we aim to examine in detail the metabolism of glucosinolates in this aphid-lacewing interaction and its ecological effects. First, we quantified the accumulation of *Arabidopsis thaliana* glucosinolates by *B. brassicae.* Second, we characterized the catalytic specificity of aphid BMY towards a repertoire of *A. thaliana* glucosinolates. Third, we analyzed the activation products of the sequestered glucosinolates in *B. brassicae* in response to a predation-like mechanical stimulus. Subsequently, we measured the physiological impact caused by toxic glucosinolate metabolites produced by *B. brassicae* on the predatory lacewing *C. carnea.* Together, this study advances our understanding of how the cabbage aphid successfully co-opts a plant defense, and the fate of a lacewing predator that encounters these defenses in its prey.

2. Materials and Methods

Plants and insects

Arabidopsis thaliana land race Columbia-0 (Col-0) accession wide-type plants (with wild-type glucosinolates), transgenic *myb28myb29* knockout mutant plants (without aliphatic glucosinolates) (Sønderby *et al.*, 2007), and *myb28myb29cyp79b2b3* knockout mutant plants (without any glucosinolates) (Mikkelsen *et al.*, 2003) were used for experiments. Brussels sprouts (*Brassica oleraceae var. gemmifera*) used for rearing of insect cultures. Plants were grown in climate-controlled short-day environmental chambers at 21°C, 60% relative humidity, and a 14:10 h light:dark photoperiod. *Brevicoryne brassicae* colonies,

generously provided by Dr. Rieta Gols (Wageningen University & Research, Wageningen, Netherlands), were fed on Brussels sprouts plants and maintained in a climate-controlled long-day environmental chamber at 21°C, 60% relative humidity, and a 16:8 h light:dark photoperiod. *Chrysoperla carnea* purchased from Katz Biotech AG (Baruth, Germany) was maintained in the same controlled long-day environmental chamber. Experiments were conducted in a similarly controlled long-day environmental chamber.

Sequestration of glucosinolates from host plants by *B. brassicae*

To measure the accumulation of various host plant glucosinolates in *B. brassicae* aphids, we analyzed the glucosinolate content of *B. brassicae* and plants of their host *A. thaliana* Col-0, at the time points of 1 h, 3 h, 6 h, 9 h, 1 day, 2 days, 3 days, 4 days, and 6 days post infestation. First, we purged the glucosinolate content of *B. brassicae* by rearing aphids on *A. thaliana myb28myb29cyp79b2b3 plants* (without any glucosinolates) over 10 days. Then, we transferred 40 adults to each *A. thaliana* Col-0 plant, with 56 plants being infested in total. At each time point, 10 adults from each plant were pooled into a 1.5 mL Eppendorf tube as one sample (4 replicates per time point). Simultaneously, the corresponding host plant leaves were collected in 5 mL tubes (4 replicates per time point). Samples were immediately frozen in liquid nitrogen. Leaf material was ground using a tissue-grinding pestle. The samples were kept under -80 °C and then weighed (FW, fresh weight) before metabolite extraction and detection.

Metabolite extraction

The weighed *B. brassicae* aphids (approximately 6 mg per sample) and *A. thaliana* Col-0 plants (approximately 50 mg per sample) were further homogenized in 200 µL and 500 µL extraction solvent (60% methanol, pH 3.0), respectively, with ceramic beads (Sigmund Lindner, Warmensteinach, Germany) using a Skandex S-7 homogenizer (Grootec GmbH, Kirchheim, Germany) for 3 min. Homogenized samples were centrifuged at 13,000 x *g* for 20 min, at room temperature to separate undissolved particles. Clear supernatants were transferred to 2 mL amber glass vials with 0.3 mL glass inserts and analysed by LC-MS/MS (Agilent 1200 series HPLC – API 5000 mass spectrometer).

RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR)

Brevicoryne brassicae endogenous myrosinase (BMY) gene transcripts were quantified at different aphid life stages and for different diets (with or without glucosinolates). *Brevicoryne brassicae* 2nd- and 4th-instar nymphs and adults were fed on *A. thaliana* Col-0 plants (with wild-type glucosinolates), and 4th instar nymphs were fed on *A. thaliana myb28myb29* (without aliphatic glucosinolates) and *myb28myb29cyp79b2b3* (without any glucosinolates) plants. *B. brassicae* from each treatment were pooled into TRIzol reagent (Invitrogen, Waltham, MA, USA) in 1.5 mL Eppendorf tubes (5 replicates per treatment, each replicate tube containing around 10 aphids) and then kept at 4 °C before use. Total RNA was isolated from stored aphids according to the manufacturer's protocol and was subjected to DNaseI (Thermo Fisher Scientific, Waltham, MA, USA) treatment to eliminate genomic DNA contamination. cDNA was synthesized from this RNA using a SuperScript III Reverse

transcriptase kit (Invitrogen). The number of gene transcripts in these cDNA samples were measured using qPCR performed with Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA). Elongation factor 1 alpha (*EF-1α)* gene was used as an internal control to normalize the abundance of gene transcripts. All the gene accession numbers and primer pairs, which were designed via Primer3 software version 4.0, are listed in Table S1.

Myrosinase activity of *B. brassicae* protein extract

To determine the myrosinase activity *B. brassicae* with glucosinolates at different aphid life stages and while feeding on different food sources (with or without glucosinolates), protein extracts were prepared from *B. brassicae* 2nd- and 4th-instar nymphs and adults fed with *A. thaliana* Col-0 plants (with wild-type glucosinolates), and 4th-instar nymphs fed with *A. thaliana myb28myb29* (without aliphatic glucosinolates) and *myb28myb29cyp79b2b3* (without any glucosinolates) plants. Approximately 6 mg of aphids were pooled into 1.5 mL Eppendorf tubes as one sample (4 replicates per treatment). Samples were homogenized in ice-cold citric acid buffer (200 µL, 50 mM, 10% glycerol; pH 4.1) with ceramic beads using a homogenizer for 3 min. Homogenized samples were centrifuged at 13,000 x *g* for 20 min at 4 °C to separate undissolved particles. Clear supernatants were transferred to 1.5 mL Eppendorf tubes, and 20 µL of each sample were used to measure protein concentration using the BCA Protein Assay Macro Kit (Serva Electrophoresis, Heidelberg, Germany) according to the manufacturer's instructions. A 0.5 µg quantity of extracted protein for each sample was dissolved in citric acid buffer (50 µL, 50 mM, pH 4.1) and reacted with 1 mM glucosinolate at 28 °C for 10 min. An aliquot of 10 µL of the reaction solution was taken out at 0 min and 10 min time points and added to 90 µL of pure methanol to immediately stop the reaction. The concentration of glucosinolates was determined by LC-MS/MS (Agilent 1200 series HPLC – API 5000 mass spectrometer) and the amount of glucosinolate reacted was calculated based on the concentration at the 0 min point minus the concentration at the 10 min point. Aliphatic 4MSOB-GIs (4-methylsulfinylbutyl glucosinolate) and indolic I3M-GIs (Indolyl-3-methyl glucosinolate) were used for the assays.

Recombinant BMY expression and purification

To determine the specific activity of *B. brassicae* endogenous BMY with glucosinolates, BMY protein expressed in *Escherichia coli* cells. The complete coding sequence of the *bmy* gene was retrieved from Jones *et al.* (2002). The full length *bmy* mRNA sequence was cloned from the synthesized cDNA pool obtained from the primer pair *bmy*FLF and *bmy*FLR. XhoI and BamHI restriction enzyme cutting sites were added to the ends of the full-length *bmy* using the primer pairs *bmy*VF and *bmy*VR, and the fragment was further digested by XhoI and BamHI (Thermo Fisher Scientific). The pET28a vector used to express the target protein was also digested by XhoI and BamHI. The restriction enzyme -digested *bmy* was inserted into the XhoI-BamHI-cut pET28a cloning site by T4 DNA ligase (Invitrogen). BL21 (DE3) *E. coli* cells (Invitrogen) were transformed by the vector containing the *bmy* insertion by chemical transfection. Colonies were selected and incubated in 5 mL LB medium with 80 µg/mL kanamycin to amplify overnight at 37°C. 1 mL of culture was added to 100 mL of

LB medium containing 80 µg/mL of kanamycin and incubated at 37°C for 3 h until an OD_{600} value of 0.5 was achieved. IPTG (Isopropyl-β-D-thiogalactopyranoside) was added to trigger protein expression at a final concentration of 1 mM. The culture was incubated overnight at 18°C with 200 rpm shaking. Simultaneously, *E. coli* BL21 (DE3) cells containing pET28a empty vector were used as negative control.

The histidine-tagged recombinant BMY was affinity purified over Ni-NTA agrose resin (Qiagen, Hilden, Germany). *E. coli* cells were collected in 50 mL Falcon tubes and centrifuged (13,000 x *g* at 4 °C for 30 min). The supernatant was discarded and the pellet was resuspended in lysis buffer (1.5 mL, 50 mM Tris, 20 mM imidazole, 500 mM NaCl, 10% glycerol, and 0.5% Tween 20; pH 7.5) with protease inhibitor mix HP (1:100 v/v) (Serva Electrophoresis) and Benzonase (2 µL/10 mL lysis buffer) (Merck KGaA, Darmstadt, Germany). The cells were incubated on ice for 30 min before being lysed by an ultrasonic homogenizer (Sonoplus HD 2070, Bandelin, Berlin, Germany). The supernatant of the lysed cells was collected after centrifugation (13,000 x *g* at 4 °C for 30 min) and transferred to equilibrated Ni-NTA agrose resin in 2 mL Eppendorf tubes. The binding of the histidine tagged protein to Ni-NTA agrose resin was accomplished by mixing under circular rotation at 4 °C for 1 hour. The collected Ni-NTA agrose resin was washed twice using wash buffer (50 mM Tris, 20 mM imidazole, 500 mM NaCl, and 10% glycerol; pH 7.5). Then the protein was eluted from Ni-NTA agarose resin using elution buffer (50 mM Tris, 250 mM imidazole, 500 mM NaCl, and 10% glycerol; pH 7.5). The buffer was exchanged to a citric acid buffer (50 mM, and 10% glycerol; pH 4.1) using Amicon ultra-30 K centrifugal filter units (Merck KGaA). Purity of the eluted recombinant proteins was analyzed by SDS-PAGE (BioRad, Hercules, California, USA).

Glucosinolate extraction from *A. thaliana* Col-0 wild-type plants

To measure BMY activity with *A. thaliana* wild-type glucosinolates, six-week old *A. thaliana* Col-0 plants were harvested and immediately frozen in liquid nitrogen to avoid activation of glucosinolates by plant myrosinases. Subsequently, plants were thoroughly freeze-dried using an ALPHA 1-4 LDplus freeze dryer (Martin Christ, Osterode am Harz, Germany) for 2 days and homogenized by shaking with 5-6 metal balls (3 mm) per tube. The crude extraction of glucosinolates from the plants was accomplished by incubating with 10 mL of 80% methanol per gram of dry weight and shaking for 5 min. The supernatant collected after centrifugation (4000 x *g* at 4 °C for 30 min) was passed through Amicon Ultra centrifugal filters of molecular weight cut-off 10 K (Merck KGaA) to remove the plant proteins including plant myrosinases. The flow-through was then evaporated to remove the solvent on a rotatory evaporator (BÜCHI Rotavapor R-114, BÜCHI Labortechnik AG, Essen, Germany) and resuspended in 1 mL miliQ water for further use.

Myrosinase assay with *A. thaliana* wild-type glucosinolates

To measure the glucosinolate substrate preference of recombinant BMY and a protein extract of *B. brassicae* 4th-instar nymphs, enzyme activities were determined with extracted *A. thaliana* glucosinolates. Soluble protein of *B. brassicae* fed on *A. thaliana*

myb28myb29cyp79b2b3 plants (without any glucosinolates) was extracted by homogenizing in citric acid buffer (50 mM, and 10% glycerol; pH 4.1). Recombinant BMY protein expressed in *E. coli* cells was purified as described above. Protein concentration was measured using the BCA Protein Assay Macro Kit (Serva Electrophoresis). A 2 µg quantity of protein from each sample in 100 µL citric acid buffer (50 mM, pH 4.1) was reacted with 10 µL crude glucosinolates extracted from *A. thaliana* Col-0 plants at 28 °C. 10 µL aliquots of the reaction solution were taken out at 0 min, 5 min, 10 min, 15 min, 30 min and 60 min reaction time points, and added to 90 µL pure methanol to immediately stop the reaction. Meanwhile, denatured protein of *B. brassicae*, which was heated at 100 °C for 1 h, and protein of *E. coli* cells was processed as controls to look for non-enzymatic and non-BMY protein degradation of glucosinolates. Subsequently, the concentration of remaining intact glucosinolates was determined by LC-MS/MS (Agilent 1200 series HPLC – API 5000 mass spectrometer) to calculate the percentage of glucosinolate hydrolysis.

Myrosinase assay with pure glucosinolates

To determine the substrate specificity of recombinant BMY toward pure glucosinolates, 1 µg of purified recombinant BMY was individually incubated with 2 mM final concentration of glucosinolate in citric acid buffer (50 µL, 50 mM, pH 4.1) for 60 min at 28 °C. The reaction was terminated by boiling for 2 min at 100 °C and the formation of glucose removed from glucosinolates was determined by the glucose oxidase procedure using the Glucose Assay Kit (Sigma, St. Louis, Missouri, USA) according to the manufacturer's instructions. The absorbance of colored products used for glucose concentration quantification was measured at 540 nm using an Infinite M200 Luminescence Microplate Reader (Tecan, Männedorf, Switzerland). Glucosinolates used for assay are listed in (Table S2).

Myrosinase kinetic assays

Kinetic assays were conducted to measure recombinant BMY activity with aliphatic glucosinolates: 3MSOP-Gls (3-methylsulfinylpropyl glucosinolate), 4MSOB-Gls, 5MSOP-Gls (5-methylsulfinylpentyl glucosinolate) and 8MSOO-Gls (8-methylsulfinyloctyl glucosinolate); the indolic glucosinolates I3M-Gls and 1MOI3M-Gls (1-methoxyindolyl-3-methyl glucosinolate); and the benzenic glucosinolates benzyl-Gls (benzyl glucosinolate) and sinalbin (p-hydroxybenzyl glucosinolate). 0.3 µg of purified recombinant BMY protein (53.74 kDa) was reacted with 1 µM to 1500 µM of glucosinolates in citric acid buffer (50 µL, 50 mM, pH 4.1) for 10 min at 28°C. The reaction was terminated by adding 450 µL of pure ice-cold methanol. The concentration of remaining glucosinolates was measure by LC-MS/MS (Agilent 1200 series HPLC – API 5000 mass spectrometer) to calculate the percentage of hydrolyzed glucosinolates.

Glucosinolate-derived metabolites in *B. brassicae* damaged tissues

To detect the formation of 4MSOB-Gls- and I3M-Gls-derived metabolites *in vivo,* the tissues of *B. brassicae* 4th instar nymphs were ruptured with a brush to mimic damage caused by an enemy attack. 40 aphids on each of 5 *A. thaliana* Col-0 plants were injured and allowed to continue feeding on the host plants. At the time points of 0 min, 5 min, and 20 min post

tissue damage, 10 aphids from each plant were pooled into a 1.5 mL Eppendorf tube as one sample. All of the samples were immediately frozen in liquid nitrogen. Metabolites extracted from these samples were processed as described in the "*metabolite extraction*" section. 4MSOB-Gls- and I3M-Gls-derived metabolites were measured using LC-MS/MS (Agilent 1200 series HPLC – API 5000 mass spectrometer).

The conversion of glucosinolate to simple nitrile in the presence of Fe (II)

To determine the correlation of the Fe (II) concentration with simple nitrile (CN) formation in glucosinolate hydrolysis catalyzed by *B. brassicae* myrosinase, 4MSOB-Gls was hydrolyzed by recombinant BMY or extracted *B. brassicae* proteins in the presence of different concentrations of Fe (II). A 2 µg portion of purified recombinant BMY or proteins extracted from *B. brassicae* 4th-instar nymphs were assayed with 1 mM 4MSOB-Gls in citric acid buffer (100 µL, 50 mM, pH 4.1) with EDTA (50 mM, conjugation agent of Fe(II)); or 0 mM, 0.01 mM, 0.05 mM, 0.1 mM, or 0.5 mM $(NH_4)Fe(SO_4)^2$ at 28 °C for 30 min. The reaction was stopped by adding 400 µL of pure methanol. As a negative control, the proteins were incubated in 100 µL of citric acid buffer without a glucosinolate substrate under the same conditions. Subsequently, the formation from 4MSOB-Gls of the simple nitrile (4MSOB-CN), and isothiocyanate (4MSOB-ITC) were determined by LC-MS/MS (Agilent 1200 series HPLC – API 5000 mass spectrometer).

Fe (II) concentration measurement

To compare the Fe (II) content in *B. brassicae* aphids with their host plants, 4th-instar aphids feeding on *A. thaliana* Col-0 (with wild-type glucosinolates) or *myb28myb29* (without aliphatic glucosinolates) plants and samples of the corresponding host plants were collected. Around 10 aphids from each plant were pooled as one samples (6 replicates per treatment). Simultaneously, the host plant leaves were collected in 5 mL tubes (6 replicates per treatment). Samples were immediately frozen in liquid nitrogen. Leaf materials was ground using a tissue-grinding pestle. The weighed (FW, fresh weight) *B. brassicae* aphids (approximately 5 mg per sample) and *A. thaliana* plant materials (approximately 50 mg per sample) were further homogenized in 200 µL and 500 µL of 5% TCA solution, respectively, with ceramic beads using a homogenizer for 3 min. Homogenized samples were centrifuged at 13,000 x *g* for 20 min at room temperature to separate undissolved particles. 40 µL of clear supernatant from each sample were used for a Fe (II) assay using a ferrozine chromogenic method with an iron assay kit (Japan Institute for the Control of Aging, Shizuoka, Japan) according to the manufacturer's instructions. The absorbance of colored products was measured at 560 nm using an Infinite M200 Luminescence Microplate Reader.

***C. carnea* larval development, pupation and adult weights**

To determine the physiological impact of glucosinolate accumulation in *B. brassicae* on its predatory *C. carnea*, larvae of *C. carnea* were continuously given *B. brassicae* fed on either *A. thaliana* Col-0 or *myb28myb29* plants since hatching. The larval development, larval mortality, pupation percentage and adult emergence of *C. carnea* were recorded. The weight of 30 *C. carnea* larvae from each group were determined at 5, 7, 9, 11, 13, 15, and

17 days post-hatching. Meanwhile, the incidence of mortality for 60 *C. carnea* larvae were recorded during 7-21 days post-hatching, and larval pupation in each group was recorded during 13-25 days post-hatching. The mortality and pupation percentages of *C. carnea* larvae were then calculated. Moreover, the number of emergences (from 30 pupae) in each group were recorded during 11-17 days post-pupation, and the percentage of emergence and the duration until emergence were calculated. The adults were sexed and weighed (14 male adults and 6 female adults).

Glucosinolate-derived metabolites in *C. carnea*

C. carnea preying on *B. brassicae* on *A. thaliana* Col-0 plants (with wild-type glucosinolates) were collected for metabolite analysis. Third-instar *C. carnea* larvae were collected (one larva as one sample). The anal secretions of *C. carnea* larvae (from three larvae pooled as one sample) were collected with a 10 µL pipette. Larvae transfered the secretion droplet to the pipette tip as a defense reaction when touched by the tip on their dorsal abdomen. Collected anal secretions were washed in extraction solvent (200 µL, 60% methanol, pH 3.0) immediately and kept at -20°C until further analysis. Pupal pellets left in the cocoons (from three cocoons pooled as one sample) were collected after adult emergence. Meconium excreted by the adults (from three adults pooled as one sample) in the first few hours after emergence was collected simultaneously with adults (one adult as one sample). All collected larvae, pupal pellets and adults, and excretions (5 replicates for each tissue) were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. The weighed tissues were homogenized in extraction solvent (200 µL, 60% methanol, pH 3.0) with ceramic beads. Samples were centrifuged to separate undissolved particles. Clear supernatants were analyzed by LC-MS/MS (Agilent 1200 series HPLC – API 5000 mass spectrometer) to detect the concentration of 4MSOB-Gls and its metabolites.

LC-MS/MS analysis

Intact glucosinolates, and metabolites of 4MSOB-Gls and I3M-Gls were detected on an Agilent Technologies 1200 Series HPLC (Agilent Technologies) coupled to an API 5000 triple-quadrupole mass spectrometer (Applied Biosystems Sciex Germany, Darmstadt, Germany). Intact glucosinolates were analyzed by loading samples on to a Nucleodur Sphinx RP column (250 × 4.6 mm × 5 µm, Macherey-Nagel, Düren, Germany) with mobile phase A (0.2% formic acid in milliQ water) and mobile phase B (acetonitrile). The elution profile was: 0-1 min, 1.5% B; 1-6 min, 1.5-5% B; 6-8 min, 5-7% B; 8-18 min, 7-21% B; 18-23 min, 21-29% B; 23-23.1 min, 29-100% B; 23.1-24min 100% B and 24.1-28 min 1.5% B; at a flow rate of 1.0 mL/min. 4MSOB-CN, 4MSOB–ITC and its conjugates were analyzed by loading samples onto an Agilent Zorbax Eclipse XDB-C18 column (50 × 4.6 mm× 1.8 µm, Agilent Technologies, Waldbronn, Germany) with mobile phase A (0.05% formic acid in milliQ water) and mobile phase B (acetonitrile). The elution profile was: 0-0.5 min, 3-15% B; 0.5-2.5 min, 15-85% B; 2.5-2.6 min, 85-100% B; 2.6-3.5 min 100% B and 3.5-6.0 min 3% B; at a flow rate of 1.1 mL/min. I3C-CN, I3C and its conjugates were analyzed by loading samples onto the same column with mobile phase A (10 mM ammonium formate in milliQ water) and mobile phase B (acetonitrile). The elution profile was: 0-0.5 min, 10% B; 0.5-6.0

min, 10-90% B; 6.0-6.1 min, 90-100% B; 6.1-7.5 min 100% B and 7.5-10.0 min 10% B; at a flow rate of 1.0 mL/min. Quantification of each compound was achieved by multiple reaction monitoring (MRM) of specific parent to product ion conversions for each compound. Detailed parameters are described in Table S3. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Quantification of individual compounds was achieved by external calibration curves; the origin of the external standards is listed in Table S2.

Statistical analyses

Data were analyzed using the R statistics package version 3.6.1, and figures were created using Origin 2019 and Adobe Illustrator CS5. All data were checked for statistical prerequisites such as homogeneity of variances and normality. Significant differences between means (± s.e.) of metabolites and enzyme assays were determined by Tukey HSD tests in conjugation with one/two-way ANOVA. Significance of *C. carnea* weight differences between medians was determined by two-tailed Mann-Whitney *U* Test. The percentages of *C. carnea* larval mortality, larval pupation and adult emergence were analyzed by Kaplan-Meier survival tests. Letters in the graphs represent $P \leq 0.05$, and asterisks represent: n.s, $P \geq 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

3. Results

Accumulation of glucosinolates in *B. brassicae* occurs at different rates

The cabbage aphid *B. brassicae* sequesters certain glucosinolates from its cruciferous host plants, but the relative accumulation of individual glucosinolates has not been studied. To this end, glucosinolate-free aphid adults were generated by rearing on a glucosinolate-deficient knock-out plant, and then transferred to *A. thaliana* Col-0 wild-type plants. The glucosinolates accumulated by the aphids were then quantified at given time points during a 6-day experiment (Figure S1). The glucosinolate content sequestered by the aphids was further compared to the content of the host plant. Overall, the relative accumulation rates revealed that sequestration of aliphatic glucosinolates occurred more rapidly than that of indolic glucosinolates: the concentration of aliphatic glucosinolates in *B. brassicae* exceeded that of aliphatic glucosinolates in *A. thaliana* within one day, while it required at least 2 days for indolic glucosinolates to reach the same concentration as in the host plant (Figure 1a). Interestingly, the four aliphatic glucosinolates with a methylsulfinyl group (with a MeS=O functional group on the side chain) had different accumulation rates; compounds with longer side chains were remarkably more highly accumulated since day 1 (Figure 1b). Quantitatively, by day 6 the accumulation was more pronounced for three aliphatic glucosinolates, namely 4MSOB-Gls (4-methylsulfinylbutyl glucosinolate), 4MTB-Gls (4-methylthiobutyl glucosinolate), and 8MSOO-Gls (8-methylsulfinyloctyl glucosinolate) (Figure 1c). Of note, 4MSOB-Gls, which dominates the total glucosinolate pool of *A. thaliana* Col-0 plants (around 55%), was also highly accumulated in *B. brassicae*. Although the indolic glucosinolate I3M-Gls (indolyl-3-methyl glucosinolate) was also noted to accumulate

to some extent by day 6, its concentration was much lower than that of its aliphatic counterparts. The two other indolic glucosinolates remained at very low levels (Figure 1c). Glucosinolate accumulation was also quantified in 2nd- and 4th-instar *B. brassicae* in a similar way to adults, and the selectivity did not differ substantially from that in adults (Figure 1c). Taken together, these results show that different glucosinolates are accumulated at different rates, and the length of side chain determines the selective accumulation of aliphatic glucosinolates.

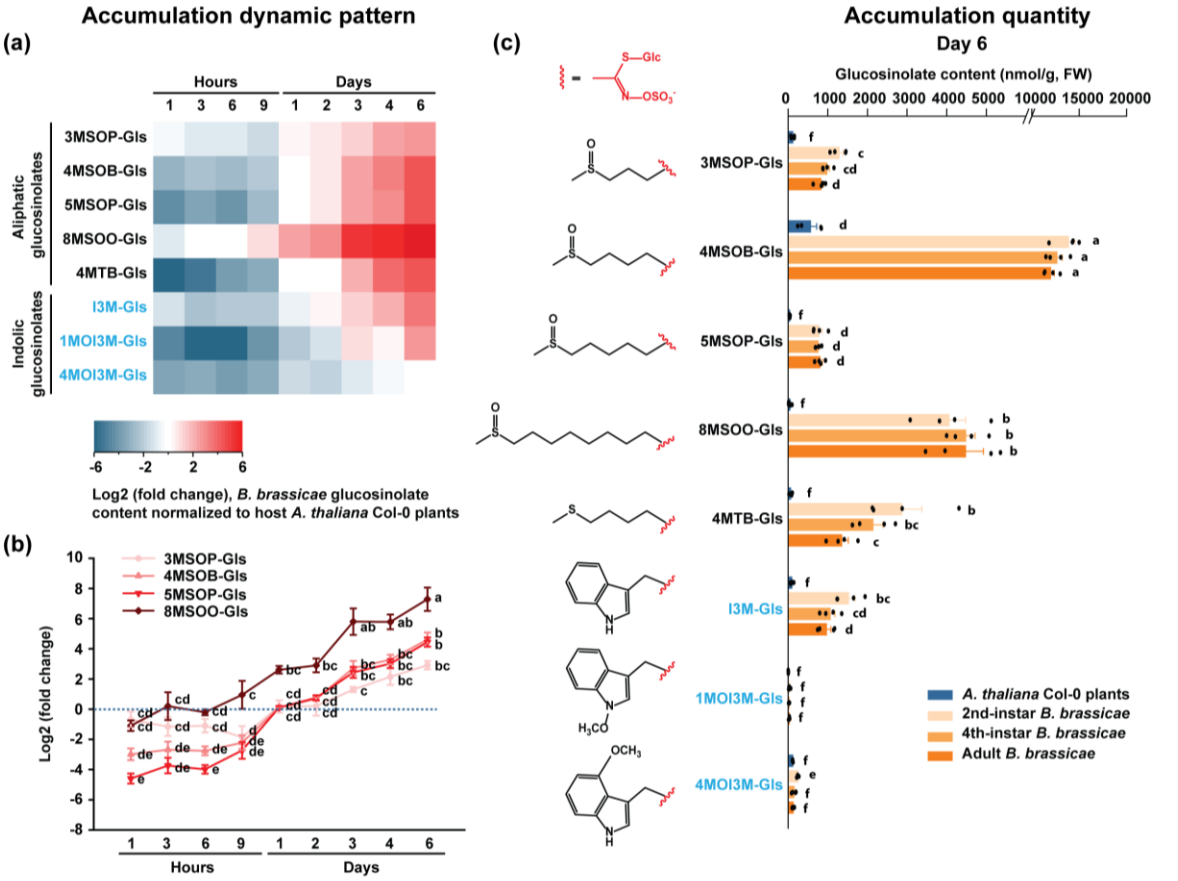

Figure 1. Dynamic accumulation of distinct glucosinolates in the cabbage aphid *B. brassicae*. **(a)** A heatmap shows the dynamic patterns of glucosinolate accumulation in *B. brassicae* adults fed with *A. thaliana* Col-0 plants. Color legend indicates the log2 (fold change) of the content of accumulated glucosinolate in the body of *B. brassicae* normalized to that of the host plants measured at each time post aphid infestation. The raw data of glucosinolate concentration in both *B. brassicae* and the host plant are shown in Figure S1. **(b)** The relative accumulation of distinct sulfinyl aliphatic glucosinolates occurred at differential rates increasing with side chain length (time: $F_{8,108}$ = 123.7, P≤ 0.001; metabolites: $F_{3,108}$ = 75.30, P≤ 0.001; time×metabolites: $F_{24,108}$ = 3.37, P≤ 0.001; n= 4 in all points). **(c)** The patterns of accumulation of aliphatic glucosinolates in *B. brassicae* did not differ among developmental stages. Accumulation quantity was tested at day 6 post infestation (organisms: $F_{3,96}$ = 247.2, P≤ 0.001; metabolites: $F_{7,96}$ = 708.2, P≤ 0.001; organisms×metabolites: $F_{21,96}$ = 72.85, P≤ 0.001; n= 4 in all bars). 3MSOP-Gls, 3-methylsulfinylpropyl glucosinolate; 4MSOB-Gls, 4-methylsulfinylbutyl glucosinolate; 5MSOP-Gls, 5-methylsulfinylpentyl glucosinolate; 8MSOO-Gls, 8-methylsulfinyloctyl glucosinolate; 4MTB-Gls, 4-methylthiobutyl glucosinolate; I3M-Gls, Indolyl-3-

methyl glucosinolate; 1MOI3M-Gls, 1-methoxyindol-3-ylmethyl glucosinolate; 4MOI3M-Gls, 4-methoxyindol-3-ylmethyl glucosinolate. Significant differences ($P \leq 0.05$) between means (±s.e.) were determined by Tukey HSD tests in conjunction with a two-way ANOVA.

The catalytic activity of *B. brassicae* correlates with glucosinolate sequestration patterns

Given that aliphatic glucosinolates were selectively accumulated, we sought to determine if this is reflected in the activity of the endogenous aphid myrosinase BMY. In a first step, we quantified the transcript level of the *bmy* gene in aphids when feeding on wide-type *A. thaliana* plants. We found that the *bmy* gene was constitutively expressed in both nymph and adult stages (Figure S2a). Moreover, we did not detect any significant alterations in *bmy* expression induced by feeding on three genotypes of host plants differing in glucosinolate content, as exemplified by testing 4th-instar nymphs (Figure S2b). Furthermore, the myrosinase activities of crude protein extracts towards two substrates (the aliphatic glucosinolate 4MSOB-Gls and the indolic glucosinolate I3M-Gls) were similar among aphid life stages and after feeding on plants containing distinct glucosinolate content (Figure S2c-f). These results suggest the constitutive expression of *bmy* in *B. brassicae* and that BMY activity is independent of aphid development and host glucosinolate content.

Next, we sought to correlate the catalytic activity of BMY to the mixture of glucosinolates in *A. thaliana* Col-0 plants. We therefore heterologously expressed *bmy* in *E. coli* and purified the recombinant BMY protein (Figure S3). When mixed with a plant glucosinolate extract, BMY had higher activity towards aliphatic glucosinolates than indolic glucosinolates (Figure 2a), in agreement with a parallel test using a crude protein extract from 4th-instar *B. brassicae* (Figure 2b). BMY hydrolyzed 74%-91% of aliphatic glucosinolates within 60 minutes, but only 39%-62% of indolic glucosinolates (Figure 2b). Irrespective of its native or recombinant form, BMY did not use the indolic glucosinolate 4MOI3M-Gls as a substrate (Figure 2a,b). We additionally quantified the glucose released during hydrolysis of 10 selected pure glucosinolates (Figure 2c). Consistent with the results above, BMY had higher activities towards pure aliphatic glucosinolates than towards benzenic or indolic glucosinolates (Figure 2c). Among the tested substrates, sinigrin and 3MSOP-Gls, two shorter-chain aliphatic substrates, supported the highest rate of glucose release (Figure 2c). This observed catalytic preference was further confirmed by the enzyme kinetic parameters of BMY for selected substrates, with the shorter-chain aliphatic glucosinolates 3MSOP-Gls and 4MSOB-Gls serving as the best substrates (Table 1). Note that the observed catalytic specificity is due to BMY per se, because both denatured *B. brassicae* proteins and proteins of null-vector *E. coli* system failed to degrade any substrates (data not show). Together, these results demonstrate that the catalytic activity of BMY is highest towards aliphatic glucosinolates, in particular those with the shorter side chains.

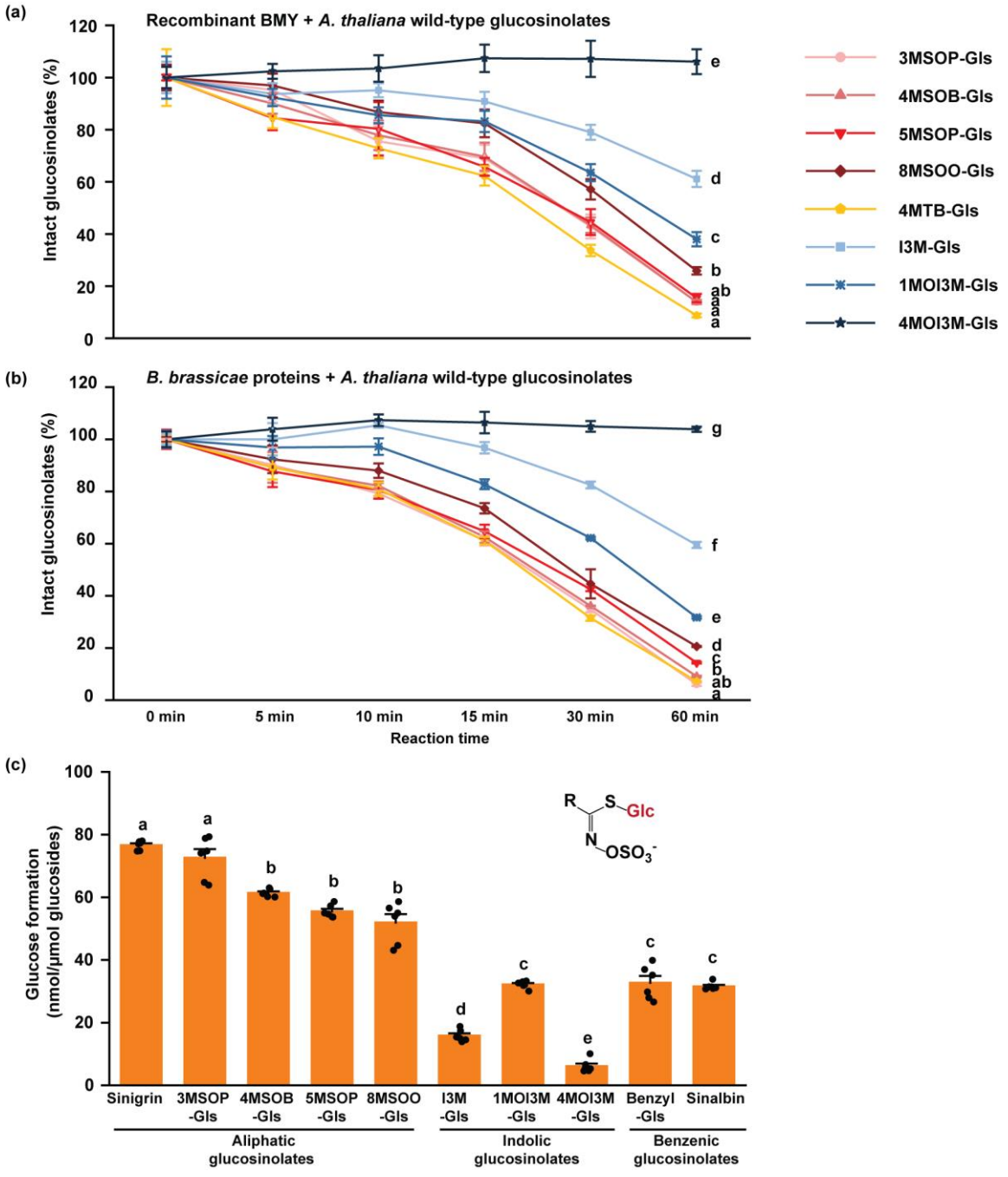

Figure 2. The enzymatic activity of *B. brassicae* myrosinase (BMY) is biased to aliphatic glucosinolates. Degradation of aliphatic glucosinolates occurred much faster than indolic glucosinolates using either a crude protein extract from *B. brassicae* **(a)** or purified recombinant BMY **(b).** Protein extract was from *B. brassicae* 3rd-instar nymphs fed on *A. thaliana myb28myb29cyp79b2b3* (no glucosinolates) plants (*B. brassicae* protein: $F_{7,16}$ = 3086, $P \leq 0.001$; recombinant BMY: $F_{7,16}$ = 192.2, $P \leq 0.001$; n= 3 in all points). **(c)** Quantification of glucose formation from three types of glucosinolates induced by BMY catalysis ($F_{9,50}$ = 249.1, $P \leq 0.001$; n= 6 in all bars). Sinigrin: 2-propenyl glucosinolate; Benzyl-Gls: benzyl glucosinolate; Sinalbin: p-hydroxybenzyl glucosinolate. Significant differences ($P \leq 0.05$) between means (±s.e.) were

determined by Tukey HSD tests in conjunction with one-way ANOVA.

Table 1. Michaelis-Menten kinetic assays of recombinant BMY with glucosinolates. 0.112 µM purified recombinant BMY acted on glucosinolates with the concentration ranging from 1 µM to 1500 µM (10 min at 28 °C).

Glucosinolates	V_{max}	K_M	k_{cat}	k_{cat}/K_M
	µmol mg^{-1} min^{-1}	mM	s^{-1}	mM^{-1} s^{-1}
3MSOP-GIs	10.51	0.37	6.28	16.84
4MSOB-GIs	10.40	0.59	9.32	15.79
5MSOP-GIs	13.57	0.72	8.10	11.19
8MSOO-GIs	11.73	0.75	7.01	9.29
1MOI3M-GIs	4.95	0.45	4.43	9.84
I3M-GIs	2.75	0.52	2.46	4.74
Benzyl-GIs	8.78	2.40	7.86	3.28
Sinalbin	6.99	1.33	6.26	4.71

Glucosinolate metabolites found in *B. brassicae* after simulated predation

The rupture of aphid tissues triggers the activation of sequestered glucosinolates, but what metabolites are formed is not known, in spite of their important physiological implications. Therefore, we used a brush to gently stroke *B. brassicae*, mimicking predator attack, and then analyzed the products resulting from hydrolysis of 4MSOB-GIs, the major aliphatic glucosinolate present in both *A. thaliana* plants and aphids that had fed upon it (Figure 1c). 4MSOB-GIs hydrolysis by the aphids led to both 4MSOB-ITC and the simple nitrile (4MSOB-CN) as primary products (Figure 3a). A significant drop (25%) of 4MSOB-GIs occurred at 5 minutes after external mechanical stimulation, paired with a concomitant generation of three types of 4MSOB-metabolites, namely, 4MSOB-CN, 4MSOB-ITC and 4MSOB-ITC-GSH (glutathione conjugates) (Figure 3b). These three 4MSOB-metabolites were abundant, with the formation of 4MSOB-ITC (6.3-fold increase after external mechanical damage) being faster than the other two metabolites (2.1- and 3.5-fold increase for 4MSOB-CN and 4MSOB-ITC-GSH, respectively). Therefore, 4MSOB-ITC represents only a portion of 4MSOB-GIs metabolites produced upon a stimulus mimicking predation. Twenty minutes after mechanical damage, the concentration of two downstream ITC-conjugates (4MSOB-ITC-CG (cysteine-glycine) and 4MSOB-ITC-Cys (cysteine)) wasalso

significantly elevated; the presumed final product of the mercapturic acid pathway, 4MSOB-ITC-NAC, was undetectable at all time points (Figure 3b). We additionally quantified products of the activation of I3M-Gls, the major indolic glucosinolate accumulated in *B. brassicae*. In stark contrast to 4MSOB-Gls, the direct hydrolysis product (indole-3-acetonitrile) was too scarce for detection; instead, several likely nontoxic metabolites downstream of I3M-ITC, namely, I3C and I3M-ascorbate, were detected (Figure S4). Overall, these results suggest that in *B. brassicae* the formation of toxic ITC hydrolysis products is specific for aliphatic glucosinolates, and that toxic ITCs are partially converted to non-toxic conjugates.

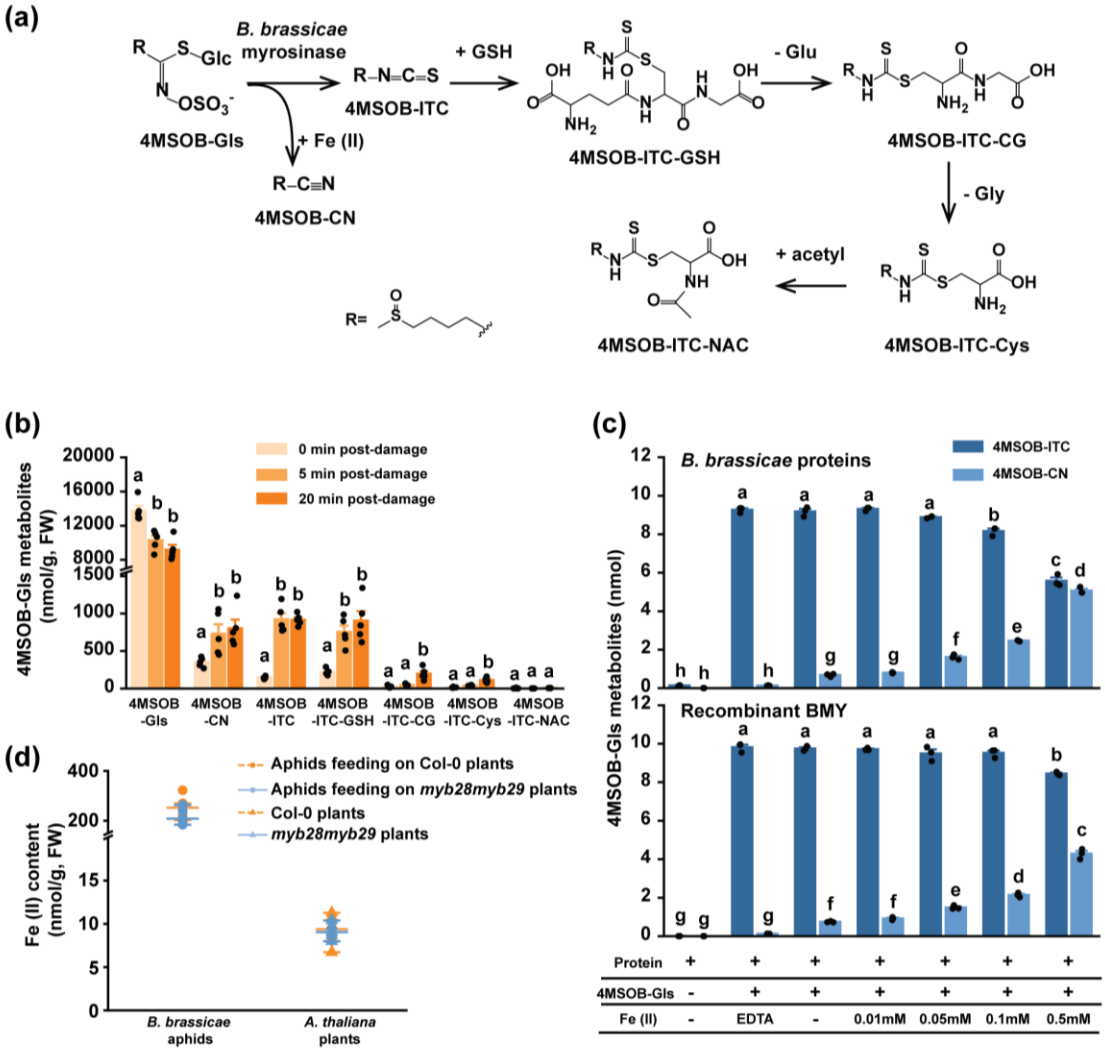

Figure 3. 4MSOB-Gls metabolic pathway in *B. brassicae* induced by simulated predation. (a) An overview of the general pathway for 4MSOB-Gls hydrolysis and ITC conjugation. Note that the substrate 4MSOB-Gls is diverted into two distinct directions forming nitrile and ITC derivatives, respectively. **(b)** Quantification of 4MSOB-Gls metabolites induced bysimulated predation. 4th-Instar stage nymphs *B. brassicae* were gently "attacked" by brush stroking. n= 5 in all bars. 4MSOB-Gls, $F_{2,12}$= 19.22, $P \leq 0.001$; 4MSOB-CN, $F_{2,12}$= 5.891, $P \leq 0.05$; 4MSOB-ITC, $F_{2,12}$= 78.22, $P \leq 0.001$; 4MSOB-ITC-GSH, $F_{2,12}$= 16.71, $P \leq 0.001$; 4MSOB-ITC-CG, $F_{2,12}$= 20.07, $P \leq 0.001$; 4MSOB-ITC-

Cys, $F_{2,12}$= 33.16, $P \leq 0.001$; 4MSOB-ITC-NAC, $F_{2,12}$= 2.395, $P \geq 0.05$. **(c)** Conversion of 4MSOB-Gls to 4MSOB-CN requires the co-presence of Fe (II) and BMY. The extracted *B. brassicae* protein (treatments: $F_{6,28}$ = 1077, $P \leq 0.001$; metabolites: $F_{1,28}$ = 16625, $P \leq 0.001$; treatments×metabolites: $F_{6,28}$ = 1079, $P \leq 0.001$; n= 3 in all bars) and recombinant BMY (treatments: $F_{6,28}$ = 1052, $P \leq 0.001$; metabolites: $F_{1,28}$ = 18164, $P \leq 0.001$; treatments×metabolites: $F_{6,28}$ = 690.2, $P \leq 0.001$; n= 3 in all bars) were spiked with 4MSOB-Gls in the presence of other co-factors. 4MSOB-CN formation is positively correlated with the addition of $(NH_4)Fe(SO_4)^2$ in the reaction system, but is blocked by adding the Fe (II) antagonist EDTA. **(d)** Fe (II) content present in 4th stage *B. brassicae* and the host plant the aphid fed on. Significant differences ($P \leq 0.05$) between means (±s.e.) were determined by Tukey HSD tests in conjunction with one-way ANOVA in **b**, and two-way ANOVA in **c.**

In plants of the Brassicales order, the formation of 4MSOB-CN from 4MSOB is mediated by the presence of specifier proteins and the cofactor Fe (II) (Eisenschmidt-Bönn *et al*., 2019). To examine the factors affecting glucosinolate hydrolysis in *B. brassicae* aphids, we conducted *in vitro* enzyme assays using either purified BMY or a crude *B. brassicae* protein extract, and determined that formation of 4MSOB-CN was in apparent competition with the production of 4MSOB-ITC and relied on Fe (II) in a concentration-dependent manner but not on a specifier protein (Figure 3c). We further compared Fe (II) content in *B. brassicae* and *A. thaliana*; the Fe (II) content in aphids exceeded that in the plant by about 30-fold, irrespective of whether the aphid had fed on plants containing or lacking aliphatic glucosinolates (Figure 3d).

Prey-derived aliphatic ITCs negatively affect growth and development of a lacewing predator

We next asked how interfering with glucosinolate accumulation would influence an aphid predator. We focused on the lacewing *C. carnea,* a generalist predator of *B. brassicae.* To manipulate levels of glucosinolates in aphids, we fed them on two genotypes of *A. thaliana* differing only in endogenous glucosinolate content: Col-0 wild-type plants with their natural glucosinolate content, and *myb28myb29* plants without aliphatic glucosinolates. We then quantified the growth and development of *C. carnea* fed with aphids from either of those groups. A striking reduction of body weight occurred in lacewing larvae fed with Col-0-reared aphids relative to *myb28myb29*-reared aphids; a statistical difference in gross weight emerged as early as 5 days post hatching lasting and lasted until the end of our experimental period (17 days post hatching) (Figure 4a). This pattern did not differ between male and female aphids. The mortality rate of lacewing larvae reached approximately 70% after 20 days feeding on Col-0-reared aphids; however, the mortality rate was only 15% for lacewings fed with *myb28myb29*-reared aphids over the same time span (Figure 4b). *C. carnea* larvae fed with Col-0-reared aphids also had much lower pupation success (less than 30%) than counterparts fed with *myb28myb29*-reared aphids (around 80%) at the end of the experiment (25 days post hatching) (Figure 4c). Further, we found that *C. carnea* fed with *myb28myb29*-reared aphids exhibited a shortened duration of the pupal stage, followed by a much higher emergence success compared to lacewings fed with Col-0-reared aphids (Figure 4d).

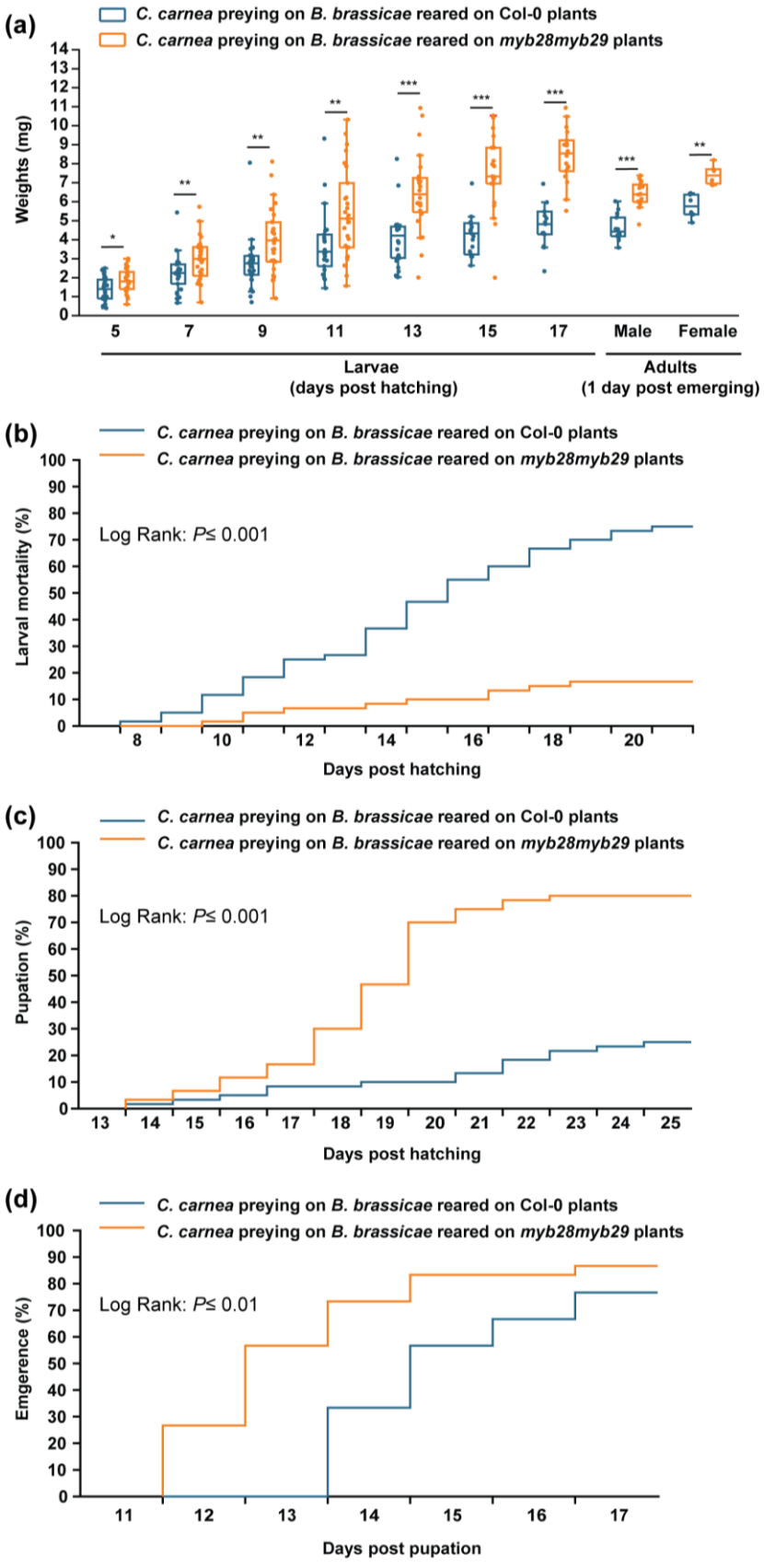
(a)
C. carnea preying on B. brassicae reared on Col-0 plants
C. carnea preying on B. brassicae reared on myb28myb29 plants
Weights (mg)
5
7
9
11
13
15
17
Male
Female
Larvae
(days post hatching)
Adults
(1 day post emerging)
(b)
C. carnea preying on B. brassicae reared on Col-0 plants
C. carnea preying on B. brassicae reared on myb28myb29 plants
Larval mortality (%)
Log Rank: P≤ 0.001
Days post hatching
(c)
C. carnea preying on B. brassicae reared on Col-0 plants
C. carnea preying on B. brassicae reared on myb28myb29 plants
Pupation (%)
Log Rank: P≤ 0.001
Days post hatching
(d)
C. carnea preying on B. brassicae reared on Col-0 plants
C. carnea preying on B. brassicae reared on myb28myb29 plants
Emgerence (%)
Log Rank: P≤ 0.01
Days post pupation

Figure 4. Ingestion of glucosinolate-sequestering cabbage aphids severely reduced survival and development of larvae of a predatory lacewing *C. carnea*. (a) Gross weight of *C. carnea* larvae fed on *B. brassicae* that were reared on either Col-0 plants (wild-type glucosinolates) or *myb28myb29* plants (no aliphatic glucosinolates) (5 day, Z= 2.536, P≤ 0.05, n= 30 for both; 7 day, Z= 2.868, P≤ 0.01, n= 30 for both; 9 day, Z= 2.964, P≤ 0.01, n= 28 and 30 respectively; 11 day, Z= 2.764, P≤ 0.01, n= 23 and 27 respectively; 13 day, Z= 3.925, P≤ 0.001, n= 22 and 24 respectively; 15 day, Z= 4.564, P≤ 0.001, n= 19 and 21 respectively; 17 day, Z= 4.754, P≤ 0.001, n= 15 and 18 respectively; male, Z= 4.112, P≤ 0.001, n= 14 for both; female, Z= 2.802, P≤ 0.01, n= 6 for both). **(b)** The larval mortality of *C. carnea* preying on aphids reared on either Col-0 or *myb28myb29* plants as food sources (Log Rank, X^2= 39.909, P≤ 0.001; n= 60 for all treatments). **(c)** Pupation rate of *C. carnea* larvae (Log Rank, X^2= 44.185, P≤ 0.001; n= 60 for all treatments) and **(d)** subsequent adult emergence rate after feeding with aphids reared on either Col-0 or *myb28myb29* plants (Log Rank, X^2= 8.965, P≤ 0.01; n= 30 for all treatments). Significant differences (P≤ 0.05, two-tailed assay) between medians were determined by Mann-Whitney U Test in **a**, and significant differences (P≤ 0.05) were determined by Kaplan-Meier survival analyses in **b-d.**

Ingestion of high amounts of 4MSOB-ITC overwhelms the detoxification capability of *C. carnea*

The severely impaired growth of the predatory lacewing *C. carnea* exposed to ITCs (Figure 4) suggested that its intrinsic detoxification capacity was insufficient against the high levels of ITCs ingested from its *B. brassicae* prey. To examine the efficiency of ITC detoxification in *C. carnea*, we provided *C. carnea* larvae with a constant diet of aphids fed on wild-type Col-0 *A. thaliana* starting from hatching. We then profiled the 4MSOB-metabolites in these lacewing larvae 15 days post-hatching (3rd-instar larval stage). Strikingly, large quantities of the non-toxic ITC metabolite 4MSOB-ITC-NAC were detected in both the anal secretion and larval body (Figure 5). While 4MSOB-ITC-NAC was absent in aphids (Figure 3), the concentration of this metabolite in lacewing larvae was higher than other glucosinolate derivatives. The toxic 4MSOB-ITC was also abundant in the anal secretion, together with ITC-conjugates, while comparatively less 4MSOB-ITC remained in the lacewing larval body (Figure 5). The simple nitrile 4MSOB-CN, another metabolite produced by *B. brassicae*, was present in high concentrations both in the anal secretion and larval bodies. Of note, the concentration of 4MSOB-CN was about 2-fold higher than 4MSOB-ITC in lacewing larval bodies, in stark contrast to aphids (Figure 3). In pupae and adult lacewings, significantly less 4MSOB-metabolites were detected, with only the exception of 4MSOB-ITC-NAC in the meconium, eventually resulting in adults free of any 4MSOB-metabolites (Figure 5). In all, these results show that *C. carnea* larvae expend a large amount of energy dealing with the large amounts of toxic 4MSOB-ITC, but surviving insects can excrete 4MSOB-ITC and its derivatives in spite of the metabolism being insufficient to deactivate all the ingested toxin.

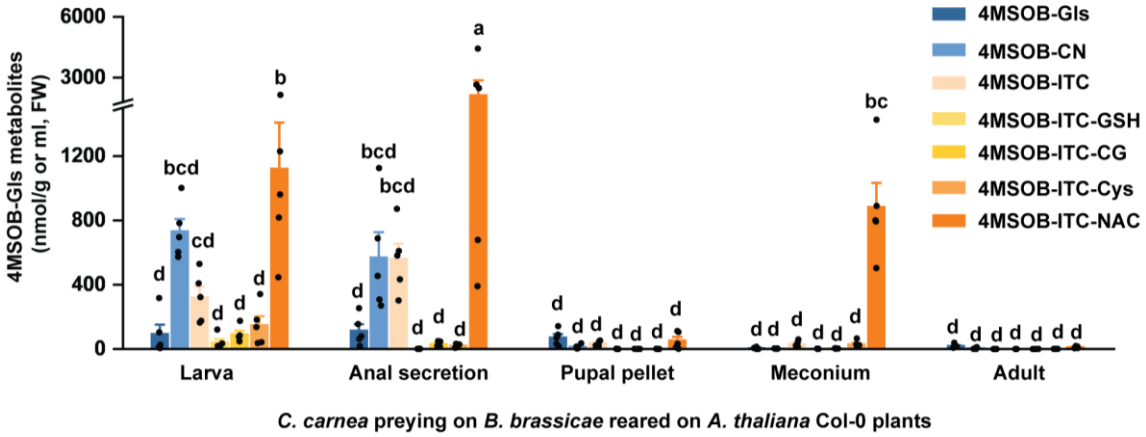

Figure 5. *C. carnea* alleviates the toxicity of the 4MSOB-ITC from ingested aphids by conjugation and mobilization. Detoxification of ingested 4MSOB-ITC includes metabolism into detoxified product 4MSOB-ITC-NAC via the general mercapturic acid pathway and mobilizing to anal secretion. Emerged lacewing adults excreted remaining 4MSOB-ITC-NAC in the meconium. Significant differences (tissues: $F_{4,140}$= 15.99, $P \leq 0.001$; metabolites: $F_{6,140}$= 21.82, $P \leq 0.001$; tissue×metabolites: $F_{24,140}$= 5.295, $P \leq 0.001$; n= 5 in all bars) between means (± s.e.) were determined by Tukey HSD test in conjunction with a two-way ANOVA.

4. Discussion

The cabbage aphid, a destructive agricultural pest, specializes on plants of the order Brassicales. Although Brassicales species produce a wide variety of glucosinolates, our results clearly show that the cabbage aphid accumulates predominantly aliphatic glucosinolates (Figure 1), in line with other studies (Kos *et al.*, 2011). Moreover, the accumulation of aliphatic glucosinolates occurs at different rates, dependent on the side chain structure (Figure 1b). Although the mechanism dictating the selective sequestration of particular glucosinolates is not understood, it likely involves specific transporters such as the ABC transporters that allow translocation of plant-derived materials across cellular membranes (Strauss *et al.*, 2013; Petschenka *et al.*, 2016).

Sequestration of plant defense compounds is more typical for specialist than generalist herbivores (Müller *et al.*, 2001; Kazana *et al.*, 2007; Beran *et al.*, 2014; Bramer *et al.*, 2017). It is one of a range of adaptations of Brassicales herbivores to allow feeding on glucosinolate-containing host plants (Cole, 1997; Louis *et al.*, 2012; Cao *et al.*, 2018). Sequestration may remove intact glucosinolates from the gut before they have a chance to become activated by the plant myrosinase. When later activated by the insect, these same glucosinolates can serve as defenses against predators and parasitoids (Chaplin-Kramer *et al.*, 2011; Kos *et al.*, 2011). The selectivity of glucosinolate sequestration in the cabbage aphid may result from a preference for compounds that are more effective in defense. Selectivity, such as the preference against indolic glucosinolates, could also help reduce the risk of autotoxicity. Certain indolic glucosinolates and their derivatives cause negative effects on the performance and physiology of some aphids (Kim *et al.*, 2007; Kim *et al.*,

2008). Aphids might also break down indolic glucosinolates during feeding to limit their uptake (Figure 1) (Kos *et al.*, 2011).

The endogenous cabbage aphid myrosinase (BMY) enables the cabbage aphid to produce defensive ITCs from the sequestered glucosinolates (Jones *et al.*, 2002; Kazana *et al.*, 2007) as long as the enzyme is able to accept the sequestered glucosinolates as substrates. We report here for the first time that the preference of BMY towards different glucosinolate substrates in vitro corresponds well to the pattern of glucosinolates sequestered by the aphid in vivo (Figure 1 and 2). Aliphatic glucosinolates may be preferred as substrates because of more favourable interactions with enzymatic binding sites (Husebye *et al.*, 2005). Preference for shorter chain aliphatic glucosinolates may be due to the specific active site architecture, and could have been selected for since shorter chain ITCs have greater volatility than longer chain ITCs and thus serve as better signals in aphid colonies to warn of predation. The hydrolysis products of some shorter chain aliphatic glucosinolates enhance neuronal response to the alarm pheromone of aphids (Dawson *et al.*, 1987; Kazana *et al.*, 2007), and impair the performance and physiology of aphid predators (Kos *et al.*, 2011). The weak activity of BMY with indolic glucosinolates corresponds to their low accumulation level. BMY is completely inactive with the substrate 4MOI3M, which serves as a feeding deterrent for maize aphid, and this glucosinolate is barely detected in the body of cabbage aphid (Figure 1).

In addition to ITCs, our results reveal that the cabbage aphid produces substantial amounts of nitriles as well as ITC conjugates upon glucosinolate activation (Figure 3). In the presence of BMY, the diversion of aliphatic glucosinolates into nitriles requires Fe (II) in a concentration-dependent manner (Figure 3), possibly in tandem with an NSP as in plants (Wittstock *et al.*, 2016). The simple nitriles formed are known to be less toxic than ITCs but benefit plants by attracting parasitoids or deterring herbivore oviposition (Mumm *et al.*, 2008). They may also have roles as defensive signals for aphids, but further research is needed. If nitriles do function in defense, the cabbage aphid's use of both Fe (II) and BMY in glucosinolate hydrolysis might help adjust the balance of glucosinolate metabolites.

Cabbage aphid predators, such as *C. carnea*, can suffer significantly from ingesting insects containing glucosinolates. While *C. carnea* possesses a metabolic mechanism to detoxify ITCs via conjugation (Sun *et al.*, 2019), this detoxification capacity appears to be limited. While the performance and fitness of *C. carnea* larvae were not negatively affected by the low levels of 4MSOB-ITC present in sulfatase-deficient *Plutella xylostella* (Sun *et al.*, 2019), they were significantly decreased by the much larger quantities of 4MSOB-ITC produced by the cabbage aphid (Figure 4 and 5). These findings suggest that generalist predators like the lacewing might possess only a limited capacity to overcome the toxicity of prey defenses, although certain specialist predators might display stronger tolerance (Pratt *et al.*, 2008). Research on a larger variety of cabbage aphid predators should give us a better picture of the effectiveness of glucosinolates in insect defense.

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Author Contribution

RS, XJ, and DGV conceived and designed the research. RS and XJ conducted the experiments and analyzed the data. RS, XJ, and DGV wrote the paper. RS, DGV, and MR developed the analytical methods. MR, XJ, DGV and JG provided comments and approved the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

This article does not describe any studies that involve human participants. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Supplemental Information

Figure S1. Absolute glucosinolate content in the body of adult *B. brassicae* and the host *A. thaliana* Col-0 plants during a 6-day aphid infestation.

Figure S2. The *bmy* gene expression and BMY enzymatic activity are independent of developmental stages and host glucosinolate content.

Figure S3. SDS-Page of the recombinant BMY protein expressed in a pET28a plasmid in *E. coli* cells.

Figure S4. Profiling metabolites of indolic glucosinolate I3M-Gls in response to tissue damage.

Table S1. Primer sets for gene clone and qRT-PCR validation.

Table S2. Substrates used for enzyme assay and external standards used for quantification.

Table S3. LC-MS/MS parameters used for the multiple reaction monitoring (MRM) analyses on the API 5000 triple-quadrupole mass spectrometer (Sciex).

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6. Discussion

To prevent attack by insect herbivores, plants synthesize a wide assortment of defensive chemicals that are either toxic, repellent, anti-digestive, or anti-nutritive to herbivores, or are attractive to carnivorous natural enemies that in turn reduce herbivore density. Yet many herbivorous insects feed on chemically well-defended plants without negative effects. It is important to understand the mechanisms that herbivorous insects utilize to avoid the effects of defensive chemicals as these provide new insights into the mode of action and effectiveness of plant defenses and help explain herbivore host range and adaptation. By focusing on crucifer-specialized herbivores such as *Plutella xylostella* **(manuscripts I & II)** and *Brevicoryne brassicae* **(manuscript III)**, my dissertation first highlights their distinct strategies to circumvent the plant glucosinolate-myrosinase defense system. After consumption by herbivores, plant chemical defenses may move up the food chain from plants to consuming herbivores to herbivore prdators and parasitoids of the third trophic level where they may also exert significant effects. My dissertation also explored the countermeasures adopted by the herbivore predator *Chrysoperla carnea* **(manuscript I & III)** and the herbivore endoparasitoid *Diadegma semiclausum* **(manuscript II)** when faced with plant chemical defenses in their prey or host *P. xylostella*, providing a comparative look at how different herbivore enemies respond to the manipulation of defenses by herbivores.

6.1 Glucosinolate desulfation: an effective but costly detoxification mechanism

Comprising over 350 genera and 3000 species, the Brassicaceae plant family has occupied an important position in plant science for decades (Fahey *et al.*, 2001). Among hundreds of cruciferous species studied, all were found to synthesize glucosinolates, a versatile class of plant compounds that consists of over a hundred reported natural examples with a wide variety of side chain structures (Blažević *et al.*, 2020). The broad distribution of crucifers has selected for many herbivores that developed efficient measures allowing them to feed on glucosinolate-containing plant tissue (Després *et al.*, 2007), among which enzyme-mediated detoxification is most often reported (Heidel-Fischer *et al.*, 2015; Ahn *et al.*, 2019). In many cases, expression of detoxification-relevant genes and the resulting enzyme activities mainly occur in the herbivore midgut and are induced in response to the ingestion of plant defense compounds (Kumar *et al.*, 2014; Adesanya *et al.*, 2017; Schweizer *et al.*, 2017). This scenario also holds true for *P. xylostella* **(manuscript I)**, an herbivore specialized on plants of the Brassicaceae that employs the activity of glucosinolate sulfatases (GSSs) to disarm the glucosinolate-myrosinase defense system of its host plants

(Figure 7) (Ratzka *et al.*, 2002). It was observed that GSS-encoding genes are predominantly expressed in gut epithelial cells, which are the first insect cells to contact the glucosinolates and isothiocyanates (ITCs) taken up from the gut lumen **(manuscript I)**. GSSs are highly active in the gut lumen and convert close to 90% of the ingested glucosinolates to non-toxic desulfo-glucosinolates (Ratzka *et al.*, 2002; Jeschke *et al.*, 2017). A recent characterization of *P. xylostella* GSS enzymes indicates that the three individual GSS enzymes (GSS1, GSS2, and GSS3) desulfate specific subsets of glucosinolate substrates (Heidel-Fischer *et al.*, 2019). The neofunctionalization (Heidel-Fischer *et al.*, 2019) and the co-expression of the *gss* genes in the midgut microvilli cells **(manuscript I)** allow *P. xylostella* larvae to effectively desulfate diverse glucosinolates and thus feed on a broad range of plants of the Brassicaceae family. In addition to its GSS enzyme activity, there are clues that *P. xylostella* also avoids glucosinolate activation by suppressing the activity of plant myrosinases in its alimentary canal system **(manuscript I)**. Although inhibition of myrosinase activity is often considered as a way to prevent glucosinolate intoxication (Jeschke *et al.*, 2016a), until now there is still a lack of concrete evidence to support it. Presumably, GSSs may inhibit myrosinase action not only by diversion of the substrate for hydrolysis, but also by the inhibitory effect of the sulfate moiety released from the desulfation process (Shikita *et al.*, 1999).

Given their important role in detoxification, GSSs were once assumed to be constitutively present in *P. xylostella* (Winde *et al.*, 2011). However, this notion has now been disproved by the finding of induction of *gss* transcript levels and GSS enzyme activity in the midgut upon dietary glucosinolate ingestion **(manuscript I)**, and similar observations from others (Heidel-Fischer *et al.*, 2019). Such an inducible detoxification system resembles those of certain generalist herbivores such as the desert locust *S. gregaria* (Falk *et al.*, 2007). *P. xylostella* seems to produce GSS enzymes only when necessary, and predominantly in the gut epithelial tissues, thus minimizing the resources needed for GSS synthesis and maintenance. Nevertheless, the development of *P. xylostella* larvae was slower when feeding on glucosinolate-containing wild-type plants relative to aliphatic glucosinolate-depleted mutant plants, therefore supporting a trade-off between growth and detoxification capacity; the inducibility of GSS-encoding genes may be a way for the herbivore to minimize the physiological costs of detoxification. A trade-off between growth and detoxification has also been observed for other insect herbivores feeding on Brassicaceae plants. Though several generalist lepidopteran caterpillars resist glucosinolate poisoning by metabolizing the major glucosinolate hydrolysis products, the

toxic ITCs, to their corresponding glutathione (GSH) conjugates, the cost of biosynthesizing the GSH necessary for ITC detoxification leads to delayed larval development (Jeschke *et al.*, 2016b; Jeschke *et al.*, 2017). A better understanding of the underlying machinery mediating growth-detoxification trade-offs may provide an important view into the molecular and cellular elements that potentially interplay with GSS in this insect.

In addition to desulfation and inhibition of myrosinase activity, the feeding of *P. xylostella* on plant tissue with high glucosinolate content gives rise to the question of whether there are other pathways for glucosinolate metabolism in *P. xylostella* larvae. The adoption of additional measures to avoid glucosinolate toxicity in addition to GSS catalysis is believed to increase the survival of other insect herbivores (Falk *et al.*, 2007; Malka *et al.*, 2016; Ahn *et al.*, 2019). A recent study of the substrate selectivity of GSSs from the beetle *P. chrysocephala* clearly showed that activities of GGS1 and GSS2 are limited to benzenic glucosinolates and do not include other types of glucosinolates, such as aliphatics and indolics (Ahn *et al.*, 2019), in contrast to the GSS1 from *P. xylostella* that is active towards a broad set of glucosinolates **(manuscript I)** (Heidel-Fischer *et al.*, 2019). The narrower substrate spectrum of GSS in *P. chrysocephala* suggests the existence of additional mechanisms for processing glucosinolates in this herbivore. Accordingly, glucosinolates in *P. chrysocephala* are concomitantly converted to simple nitriles at the expense of ITC formation, and ITCs are further degraded through a combination of the general mercapturic acid pathway and ITC conjugate hydrolysis (Beran *et al.*, 2018). As yet there is little evidence that *P. xylostella* naturally makes significant use of the formation of nitriles or ITC conjugation for processing glucosinolates in addition to desulfation **(manuscript I)**.

6.2 Substrate and location of an endogenous herbivore myrosinase may help minimize autotoxicity

β-Glucosidases, including β-thioglucosidases (myrosinases), are present in numerous organisms including insect herbivores, and can mimick the two-component defense systems of plants to trigger toxic hydrolysis reactions (Cairns *et al.*, 2010). Thus the modulation of endogenous β-glucosidase activity may help herbivores counter the effects of plant two-component defenses. Several herbivores, like *Callosobruchus maculatus* (bruchid beetle), *Epilachna varivestis* (Mexican bean beetle), and *Spilosoma virginica* (yellow woolly bear) are found to attenuate the formation of toxic hydrolysis products by either suppressing the activity or decreasing the transcript levels of β-glucosidases and their encoding genes during digestion of certain plants (Desroches *et al.*, 1997; Ballhorn *et al.*,

2010; Pankoke *et al.*, 2010). However, this notion may not hold true for the crucifer-specialized aphid *B. brassicae*, in which the expression of its endogenous myrosinase seems to be stable independently of the ingestion of glucosinolate-containing foods **(manuscript III)**. Instead, to avoid the undesirable self-intoxication by glucosinolate hydrolysis products, *B. brassicae* compartmentalizes its myrosinase into muscle tissues, avoiding direct contact with the hemolymph where glucosinolates are accumulated **(Figure 7)** (Kazana *et al.*, 2007). Self-protection is also promoted by the fact that not all types of glucosinolates are equivalently processed by the endogenous myrosinase: aliphatic glucosinolates apparently serve as much better substrates than indolic glucosinolates **(manuscript III)**. It is of particular interest to note that 4-methoxyindol-3-ylmethyl glucosinolate (4MOI3M), which is deterrent to another aphid, the green peach aphid *Myzus persicae* (Kim *et al.*, 2007; Pfalz *et al.*, 2009), was scarcely activated by *B. brassicae* myrosinase. The lack of 4MOI3M accumulation in *B. brassicae* (aliphatic glucosinolates are preferentially sequestered) coupled with the myrosinase selectivity pattern observed **(manuscript III)** keeps the aphids from sequestering glucosinolates that are poisonous to them. In addition to the formation of toxic ITCs upon tissue rupture, in the presence of Fe (II) *B. brassicae* can also direct glucosinolate hydrolysis towards simple nitriles **(Figure 7) (manuscript III)**, a less-toxic product of hydrolysis than ITCs without the electrophilic –N=C=S moiety. Nitriles are also the detoxification product of *Pieris*, sp. feeding on Brassicaceae plants (Wittstock *et al.*, 2004). Simple nitriles are known to benefit plants against *Pieris* herbivores by reducing the preference for adult oviposition and attracting parasitoids (Mumm *et al.*, 2008). However, the ecological roles of the simple nitriles formed by *B. brassicae* aphids await further study. Nevertheless, the myrosinase-catalyzed formation of ITCs by the herbivore caused substantial negative effects on the fitness and development of predatory *C. carnea* larvae **(manuscript III)**. This is in line with the prevailing notion that the sequestration of plant defensive chemicals enables herbivores to deter their natural enemies (Price *et al.*, 1980; Ode, 2006). Together, it is reasonable that the properties of the aphid myrosinase are the result of natural selection not only for its role against enemies, but also for minimizing autotoxicity.

6.3 Herbivore glucosinolate metabolism has consequences for other trophic levels

Ecological systems represent a typical closed-loop in which energy and materials continuously move along the food chain towards higher trophic levels. Such fluidity supports the extended impact of plant chemicals acting beyond herbivores (Hartmann, 2004; Gols *et*

al., 2008; Harvey *et al.*, 2011), but the metabolism of plant-derived glucosinolates in herbivore enemies is still obscure. Contact with glucosinolate metabolites generated in herbivores (mainly the ITC hydrolysis products) was thought to cause negative impacts on some enemies (Francis *et al.*, 2001; Müller *et al.*, 2002), but details have been lacking. The findings in this thesis provide new information on the effect of glucosinolates on herbivore enemies by correlating the concentrations of ITCs present in herbivores with the degree to which performance and development of the enemies are negatively affected **(manuscripts I & III)**. For example, the physiology of the predatory lacewing *C. carnea* was only subtly affected by the transiently increased level of ITCs in *gss*-silenced *P. xylostella,* but was greatly impacted by the high levels of ITCs in the aphid *B. brassicae.* These results suggest the existence of a critical threshold beyond which *C. carnea* suffers from prey ITCs, in spite of the ITC detoxification routes present in this insect **(Figure 7) (manuscript I)**. This proposal supports previous findings that glucosinolate content and concentrations in aphids dictate the varying development of its predator *C. carnea* (Kos *et al.*, 2011).

Manipulation of plant chemical metabolism by herbivores may trigger undesirable consequences for organisms from adjacent trophic levels. In this thesis, I observed that lowering the production of desulfated glucosinolates in *P. xylostella* intriguingly did not cause the same effects on the two enemy species tested, *C. carnea* and *D. semiclausum* **(manuscript I & II)**. This discrepancy in ITC tolerance between the two species can be attributed to the likelihood of encountering glucosinolate hydrolysis products. The generalist *C. carnea* feeds on many prey items, some of which may contain ITCs in varying concentrations. While the high concentrations of ITCs in *B. brassicae* reduced *C. carnea* performance, the lacewing tolerated the lower concentrations of ITCs in *P. xylostella* resulting from *gss*-silencing. In contrast, the endoparasitoid feeds exclusively on the tissues of its host *P. xylostella*, which do not normally contain ITCs and is thus more susceptible to the presence of ITCs in its diet **(manuscript II)**. Previous work gave a somewhat different pattern with the performance of the specialized parasitoid *Diaeretiella rapae* being positively correlated with the glucosinolate concentration of its host *B. brassicae,* while the generalist predator *Episyrphus balteatus* responded negatively to the defensive chemicals in its host (Kos *et al.*, 2012). But, here both parasitoid and predator were fed upon an insect that normally sequesters glucosinolates and produces hydrolysis products.

The utilization of plant toxins can enable herbivores to defend themselves against both predation and parasitization (Singer *et al.*, 2009; Lefèvre *et al.*, 2010; Kacsoh *et al.*,

2013). We asked whether endoparasitoids can also manipulate plant toxins derived from the diet of their hosts since it is well known that endoparasitoids manipulate host defenses to their own benefit. For example, a symbiotic bracovirus of the endoparasitoid *Cotesia vestalis* produces a miRNA that arrests host growth by modulating the expression of the host *ecdysone receptor* (*EcR*) gene (Wang *et al.*, 2018). This also applies to *D. semiclausum*, where parasitization altered *P. xylostella EcR* expression such that the host pupation was delayed **(manuscript II)**. Can such manipulation also extend to plant defense compounds? In our work, we found that the level of glucosinolate hydrolysis products in *P. xylostella* larvae was also affected by *D. semiclausum*, with excretion of 4MSOB-ITC being enhanced by parasitism **(Figure 7)**. How the endoparasitoid manipulates this process may involve molecular reprogramming of metabolism, transporters or the gut microbiota present. It was found in previous work that gut bacteria in mammalian alimentary canals metabolize glucosinolates to form simple nitriles, reducing the formation of ITCs (Luang-In *et al.*, 2016); similarly, the gut bacteria of a *Pseudomonas* strain in the coffee berry borer facilitate caffeine degradation (Ceja-Navarro *et al.*, 2015).

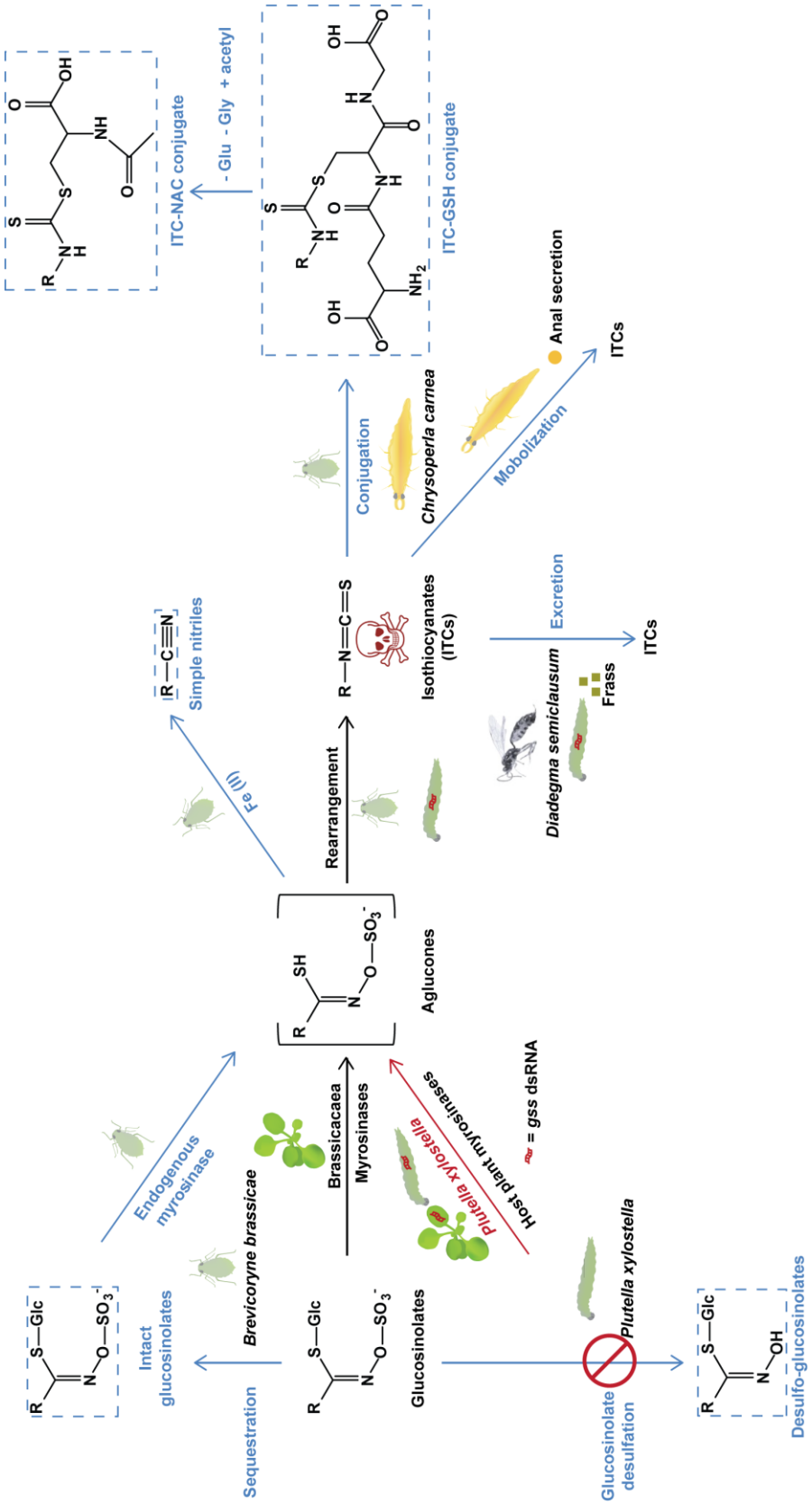

Figure 7. Glucosinolate metabolism in multi-trophic interactions. Glucosinolates are hydrolyzed by plant myrosinases forming aglucones that can rearrange to isothiocyanates and other metabolites (black arrows). Herbivores and their enemies counteract the plant glucosinolate-myrosinase defense system by detoxification or other countermeasures (blue arrows). *Plutella xylostella gss* gene silencing blocks desulfation and so triggers glucosinolate hydrolysis (red arrow).

6.4 Practical implications of the multi-trophic detoxification of plant defensive chemicals

P. xylostella is a specialist feeder on important Brassicaceae crops such as cauliflower, cabbage, garden cress, broccoli, brussels sprouts and rapeseed. In the last century, *P. xylostella* has emerged as one of the most devastating pests of economically important food crops, causing losses of US$ 4–5 billion per year (Zalucki *et al.*, 2012). Its devastation potential can be attributed to its very short (14 days) life cycle, which allows it to complete more than 20 generations per year. Additionally, *P. xylostella* has rapidly developed resistance to most insecticides (Sarfraz *et al.*, 2005a), including almost all classes of synthetic chemical insecticides and modern biological pesticides like *Bacillus thuringiensis* (Bt) toxins (Sarfraz *et al.*, 2005b). Thus, it has become one of the most difficult pests to control. In this thesis **(manuscript I)**, we demonstrate that the enzymatic desulfation of glucosinolates by *P. xylostella* is the major mechanism that allows these insects to suppress the activation of glucosinolate hydrolysis and thus circumvent the glucosinolate-myrosinase defense system of their host plants **(Figure 7)**. Hence, the manipulation of glucosinolate desulfation could provide a promising strategy to control this pest. We showed that silencing the genes encoding GSSs in *P. xylostella* larvae via plant-mediated RNAi **(Figure 7)** was effective in decreasing larval growth, survival and reproductive fitness **(manuscript I)**. The ability to effectively target the *gss* genes of this insect by plant-mediated RNAi suggests that application of RNAi via crop plants or by direct spraying (Zhang *et al.*, 2017; Zotti *et al.*, 2018), possibly in combination with increased glucosinolate levels, e.g. after methyl jasmonate treatment of plant tissue (Wiesner *et al.*, 2014), could considerably reduce *P. xylostella* damage to crops, as we observed in the laboratory.

Because of the remarkable resistance of *P. xylostella* to insecticides, natural enemies have been increasingly explored as alternative control agents (Talekar *et al.*, 1993). Among various enemies such as parasitoids, predators, entomopathogenic fungi, bacteria and viruses (Sarfraz *et al.*, 2005a), the predatory lacewing C. *carnea* and the specialized endoparasitoid *D. semiclausum* are the most frequently used biological control agents in the field to control *P. xylostella* outbreaks (Li *et al.*, 2016). The use of predators and parasitoids calls for an integration of various control measures, since no single method is likely to achieve complete control. However, natural enemies can also suffer from insecticides, via their prey or hosts, which serve as the actual targets. For example, certain commonly used pesticides belonging to oxadiazine, halogenated pyrrole, pyrethroid,

spinosoid and thiocarbamate classes cause mortality to various *P. xylostella* parasitoids (Sarfraz *et al.*, 2005b). A pest management approach that caused less harm to natural enemies might be more effective in controlling the herbivore. By showing that natural enemies can themselves mitigate the effects of plant-derived glucosinolate toxins either via efficient detoxification **(Figure 7) (manuscript I)** or increased excretion of toxic metabolites by the host **(Figure 7) (manuscript II)**, our findings provide first-hand laboratory evidence to support increased concentrations of glucosinolates in the crop as part of a pest management strategy.

6.5 Conclusion

Insect herbivores employ diverse strategies to cope with plant defensive chemicals in order to successfully feed on their host plants. The enzymatic desulfation of glucosinolates by *P. xylostella* larvae allows these insects to suppress the activation of glucosinolates and thus circumvent the glucosinolate-myrosinase defense system of their host plants, with the additional effect of blocking the effects of plant chemicals on natural enemies. Here, I describe the species-specific mechanisms by which a generalist herbivore predator and a specialist herbivore parasitoid respond to the presence of ITCs derived from plant glucosinolates. Additionally, I studied how a piercing-sucking insect, the aphid *B. brassicae*, sequesters glucosinolates and produces an activating glucosidase to construct its own glucosinolate-myrosinase system. In contrast to the transient presence of ITCs in *gss*-silenced *P. xylostella*, the high concentrations of ITCs produced by this aphid caused severe negative impacts on a predator. These results demonstrate that insect herbivores and their enemies both have metabolic strategies towards plant defense chemicals that influence the outcome of higher trophic level interactions.

7. Summary

As sessile organisms, plants synthesize a broad range of secondary metabolites as defenses to reduce herbivore damage. Yet many herbivores metabolize defenses and feed readily on chemically well-defended plants. However, it may be premature to call herbivore metabolism a detoxification without knowing whether it actually benefits herbivores. Once plant defenses are ingested by herbivores they may also be encountered by herbivore enemies. In contrast to the relatively well-studied pathways by which herbivores metabolize plant defensive chemicals, much less is known about how higher trophic levels minimize the threat of plant defensive chemicals in their herbivorous prey or hosts. In this study, chemical, biochemical and molecular biological approaches were combined with performance bioassays to discover how herbivore metabolism of Brassicaceae glucosinolates affected herbivores and herbivore enemies. I investigated two specialized herbivores, the diamondback moth (*Plutella xylostella*) and the cabbage aphid (*Brevicoryne brassicae*), examined the physiological roles of glucosinolate metabolism, and tested its ecological significance on two herbivore enemies, the predator *Chrysoperla carnea* and the endoparasitoid *Diadegma semiclausum*. Moreover, I determined how the enemies themselves employee metabolic countermeasures against plant chemical defenses ingested during consumption of herbivores as prey and hosts.

Plutella xylostella larvae manipulate the plant glucosinolate-myrosinase system using glucosinolate sulfatases (GSSs). GSSs modify glucosinolates to generate non-toxic and non-activatable desulfo-glucosinolates, thus inhibiting the formation of toxic glucosinolate hydrolysis products, i.e. isothiocyanates (ITCs). I first determined that *gss* genes were predominantly expressed in the larval midgut cells that are at the interface between insects and the plant metabolites present in the gut lumen. I then manipulated the expression of *gss* genes to uncover their role in the physiology and ecology of this herbivore. Silencing of *gss* in *P. xylostella* larvae severely reduced the efficiency of glucosinolate desulfation, and was accompanied by a large increase of ITCs, resulting in severely reduced *P. xylostella* fitness. Additionally, I confirmed that the toxicity of ITCs was responsible for the negative effects on herbivore fitness using ITC complementation, consistently with the previously observed toxicity of ITCs towards caterpillar herbivores. In addition to preemptively deactivating plant defensive chemicals, *P. xylostella* desulfation of glucosinolates impacts herbivore enemies at higher trophic levels. I showed that the larval development of the predator lacewing *C. carnea* was slightly slowed by preying on *gss*-

silenced *P. xylostella* larvae, which contained ITCs. Nevertheless, *C. carnea* suffered no reproductive effects, perhaps due to its ability to metabolize ITCs via a general detoxification pathway. On the other hand, the endoparasitoid *D. semiclausum*, which specially parasitizes *P. xylostella* and is used to the presence of desulfo-glucosinolates in the host hemolymph, suffered from severely reduced adult emergence when it unexpectedly encountered ITCs in its host after *gss* silencing.

Another crucifer-specialized herbivore, the cabbage aphid *B. brassicae* accumulates dietary glucosinolates from host plants. These compounds can be hydrolyzed by an endogenous insect myrosinase to produce ITCs when *B. brassicae* is attacked by predators, constituting an efficient defensive strategy. However, not all glucosinolates were accumulated by the aphid and activated by the aphid's myrosinase at the same rate. Aliphatic glucosinolates were accumulated by the cabbage aphid at a much higher rate compared to indolic glucosinolates, and served as much better substrates for its endogenous myrosinase. This might be a consequence of indolic glucosinolates having deterrent effects towards aphids. Besides the formation of toxic ITCs by the insect myrosinase, *B. brassicae* also hydrolyzed glucosinolates to yield simple nitriles, which are less-toxic products lacking the electrophilic –N=C=S moiety of ITCs, but the ecological significance of this product remains unknown. The amounts of ITCs produced by *B. brassicae* were much higher than those present in *gss*-silenced *P. xylostella*, and accordingly only the former significantly affected the growth of the predatory lacewing *C. carnea*. These results suggest the existence of a critical threshold beyond which *C. carnea* suffers from prey ITC content, in spite of the ITC detoxification routes present in this insect. In response to dietary exposure to ITCs, the predatory lacewing C*. carnea* and the endoparasitoid *D. semiclausum* both counteracted those toxins. *C. carnea* detoxified the ITC ingested from its prey via the general mercapturic acid pathway, and deposited both the ITC and its conjugates into its anal secretion, which plays a defensive role as a droplet that is transferred to the head or antennae of attacking enemies. On the other hand, the endoparasitoid *D. semiclausum* was able to increase the excretion of ITC in *gss*-silenced *P. xylostella* host larvae thus minimizing its own contact with the toxin.

In this thesis, I not only demonstrate the importance of herbivore mechanisms for processing plant-derived chemical defense in multi-trophic interactions, but also show that herbivore enemies also possess metabolic strategies that affect the movement of plant defense chemicals to higher trophic levels. I experimentally investigated the ecological

consequences of these metabolic strategies, and examined how their manipulation affects particular natural enemies, which are commonly used in pest control strategies. Thus, this study connects glucosinolates and their effects on organisms of different trophic levels, contributing to the knowledge of how plant activated defenses and insect adaptations mediate interactions in natural and agricultural systems.

8. Zusammenfassung

Da Pflanzen standortgebundene Organismen sind, synthetisieren sie eine große Vielfalt an strukturell sehr unterschiedlichen Planzensekundärstoffen, um sich gegen Fraßschädlinge zu verteidigen. Jedoch können viele herbivore Tiere diese Pflanzenabwehrstoffe abbauen und dadurch auch an chemisch gut verteidigten Pflanzen fressen. Von einer wirklichen Entgiftung kann bei diesem Abbau der Pflanzenstoffe durch die Herbivoren allerdings nur gesprochen werden, wenn die Tiere dadurch einen Vorteil erlangen. Die von den Herbivoren gebildeten Metabolite der Pflanzenabwehrstoffe werden wiederum von den Räubern oder Fraßfeinden der Herbivoren aufgenommen. Die Stoffwechselwege zum Abbau von Pflanzenabwehrstoffen in Herbivoren sind relativ gut untersucht, hingegen ist sehr wenig erforscht, wie Organismen auf den höheren trophischen Ebenen mit Pflanzenabwehrstoffen oder deren Metaboliten in ihrer Beute klar kommen. In dieser Studie wurden chemische, biochemische und molekularbiologische Methoden mit Fraßtests und Insektenentwicklungsstudien kombiniert, um zu untersuchen, wie sich der Metabolismus von Glucosinolaten (typische Abwehrstoffe in der Pflanzenfamilie der Brassicaceae) in Herbivoren auf diese und deren Räuber auswirkt. Für die Untersuchung wurden zwei auf Pflanzen der Brassicaceae spezialisierte Herbivoren ausgewählt: die Kohlmotte (*Plutella xylostella*) und die Kohlblattlaus (*Brevicoryne brassicae)*, an denen die physiologischen Auswirkungen des Glucosinolat-Abbaus exemplarisch studiert wurden. Darüber hinaus wurden die ökologischen Auswirkungen dieser zwei unterschiedlichen Glucosinolatabbauwege in Herbivoren auf die nächste trophische Ebene an dem räuberischen Insekt *Chrysoperla carnea* und dem Endoparasitoiden *Diadegma semiclausum* erforscht. Weiterhin wurde analysiert, ob und wie die Vertreter der höheren trophischen Ebene die Pflanzenabwehrstoffe und deren Metabolite, die sie beim Fressen der Herbivoren aufnehmen, verstoffwechseln und damit unschädlich machen können.

Plutella xylostella-Larven manipulieren das pflanzliche Glucosinolat-Myrosinase-System unter Verwendung von Glucosinolat-Sulfatasen (GSS). GSSs modifizieren Glucosinolate, um nicht toxische und nicht aktivierbare Desulfo-Glucosinolate zu erzeugen, wodurch die Bildung toxischer Glucosinolat-Hydrolyseprodukte, z.B.. Isothiocyanate (ITCs), gehemmt wird. Zunächst konnte gezeigt werden, dass *gss*-Gene überwiegend in den Mitteldarmzellen der Larven exprimiert werden, die sich an der Grenzfläche zwischen Insekten und den im Darmlumen vorhandenen Pflanzenmetaboliten befinden. Um die Rolle der GSS in der Physiologie und Ökologie dieses Pflanzenfressers aufzudecken, wurde die

Expression der *gss*-Gene genetisch manipuliert. Die Stummschaltung von *gss* in *P. xylostella*-Larven verringerte die Effizienz der Glucosinolat-Desulfatierung erheblich und ging mit einem starken Anstieg der Konzentration der ITCs im Insekt einher, was zu einer stark verringerten *P. xylostella*-Fitness führte. Mit der Fütterung von Isothiocyanaten an die im *gss*-Gene manipulierten Insekten konnte bestätigt werden, dass die Toxizität von ITCs für die negativen Auswirkungen auf die Fitness von Pflanzenfressern verantwortlich ist. Die Deaktivierung des Glucosinolat-Myrosinase-Systems durch die Desulfatierung in *P. xylostella* hat Auswirkungen auf die nächste trophische Ebene. Die Larvenentwicklung der Gemeinen Florfliege (*C. carnea*) wurde bei Fraß an *gss*-manipulierten *P. xylostella*-Larven, die dadurch ITCs enthielten, leicht verlangsamt. *C. carnea* wurde allerdings nicht in seinem Reproduktionserfolg beeinträchtigt, vermutlich durch seine Fähigkeit, ITCs über einen generellen Entgiftungsweg zu metabolisieren. Der Endoparasitoid *D. semiclausum* hingegen, der auf die Parasitierung von *P. xylostella* spezialisiert ist und an das Vorhandensein von Desulfo-Glucosinolaten in der Wirtshämolymphe gewöhnt ist, zeigte eine stark reduzierte Entwicklung der Adulten und verringerte Überlebensrate, wenn die Tiere ITCs ausgesetzt waren, wenn sie an *gss*-manipulierten Wirten gefressen hatten.

Ein anderer auf Kreuzblütler spezialisierter Herbivor, die Kohlblattlaus *B. brassicae*, reichert Glucosinolate aus Wirtspflanzen an. Diese Verbindungen können durch eine endogene Insekten-Myrosinase hydrolysiert werden, um ITCs zu produzieren, wenn *B. brassicae* von Räubern angegriffen wird, was eine effiziente Abwehrstrategie darstellt. Es werden jedoch nicht alle Glucosinolate gleichermassen akkumuliert und durch die Myrosinase der Blattlaus mit der gleichen Geschwindigkeit aktiviert. Aliphatische Glucosinolate werden von der Kohlblattlaus im Vergleich zu indolischen Glucosinolaten mit einer viel höheren Effizienz akkumuliert und sind bessere Substrate für die endogene Myrosinase. Das kann möglicherweise daran liegen, dass indolische Glucosinolate eine abschreckende Wirkung auf Blattläuse haben. Neben der Bildung toxischer ITCs durch die Insektenmyrosinase hydrolysiert *B. brassicae* Glucosinolate auch zu einfachen Nitrilen, bei denen es sich um weniger toxische Produkte handelt, welchen die elektrophile -N=C=S-Einheit von ITCs fehlt. Die ökologische Bedeutung der Nitrile ist jedoch unbekannt. Die Mengen an ITCs, die von *B. brassicae* produziert werden, sind viel höher als diejenigen, die in gss-stummgeschalteten *P. xylostella* vorhanden sind, und dementsprechend beeinflussten nur die ersteren das Wachstum der räuberischen Florfliege *C. carnea* signifikant. Diese Ergebnisse legen die Existenz einer kritischen Schwelle nahe, ab der *C. carnea* trotz der in diesem Insekt vorhandenen ITC-Entgiftungswege unter dem ITC-Gehalt

der Beute leidet. Bei Fütterung mit ITCs konterten sowohl die räuberische Florfliege *C. carnea* als auch der Endoparasit *D. semiclausum* diese Toxine. *C. carnea* entgiftete das von seiner Beute aufgenommene ITC über den allgemeinen Mercaptursäureweg und lagerte sowohl das ITC als auch seine Konjugate in seiner Analsekretion ab, welche zur Abwehr auf den Kopf oder die Antennen angreifender Feinde übertragen wird. Der Endoparasit *D. semiclausum* hingegen konnte die Ausscheidung der ITCs durch die *gss*-stummgeschalteten *P. xylostella*-Wirtslarven erhöhen und soweit den eigenen Kontakt mit den Toxinen minimieren.

In dieser Arbeit konnte nicht nur die Bedeutung von Mechanismen von Herbivoren für die Verstoffwechselung pflanzlicher chemischer Abwehr in multitrophischen Systemen gezeigt werden, sondern auch, dass Feinde von Pflanzenfressern ebenfalls Stoffwechselwege besitzen, die den Übergang von Pflanzenabwehrstoffen auf höhere trophische Ebenen beeinflussen. Es wurden experimentell die ökologischen Konsequenzen dieser Stoffwechselstrategien untersucht, und wie sich ihre Manipulation auf bestimmte natürliche Feinde auswirkt, die üblicherweise in Schädlingsbekämpfungsstrategien verwendet werden. Somit verbindet diese Studie Glucosinolate und deren Auswirkungen auf Organismen unterschiedlicher trophischer Niveaus und trägt dazu bei, herauszufinden, wie aktivierte pflanzliche Abwehrsysteme und Insektenanpassungen an diese SystemeWechselwirkungen in natürlichen und landwirtschaftlichen Systemen beeinflussen.

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10. Supplemental Information

10.1 Manuscript I – Supplemental Information

Tritrophic metabolism of plant chemical defenses and its effects on herbivore and predator performance

Ruo Sun, Xingcong Jiang, Michael Reichelt, Jonathan Gershenzon, Sagar Subhash Pandit, Daniel Giddings Vassão

Figure 1 – figure supplement 1. Transcripts of *gss* genes are localized in the *P. xylostella* larval midgut epithelium and are induced by dietary ingestion of glucosinolates.

Figure 1 – figure supplement 2. Transcripts of *gss* genes are localized in midgut cell types of *P. xylostella* fourth-instar larvae.

Figure 1 – figure supplement 3. Infiltration of *A. thaliana* Col-0 and *myb28myb29* lines with *gss1* RNAi and empty vector (EV) control constructs does not alter their morphology and chemical phenotypes.

Figure 1 – figure supplement 4. Plant-mediated RNAi of *gss1* co-silences *gss2* and *gss3* due to high sequence similarity, and suppresses the desulfation of indolic glucosinolates as well.

Figure 2 – figure supplement 1. Silencing of *gss* has no effect on weights of *P. xylostella* pupae.

Figure 2 – figure supplement 2. Silencing of *gss* reduces number of eggs and their hatching percentage.

Figure 3 – figure supplement 1. Enzymatic conversion of 4MSOB to desulfo-4MSOB and 4MSOB-ITC is enzymatic and occurs in fresh frass.

Figure 3 – figure supplement 2. Silencing of *gss* decreases desulfo-indol-3-ylmethyl glucosinolate (desulfo-I3M) and increases a metabolite of indol-3-ylmethyl glucosinolate isothiocyanate (I3M-ITC) in *P. xylostella*.

Figure 4 – figure supplement 1. Concentrations of 4MSOB-ITC and conjugates formed in crushed leaves of Col-0 *A. thaliana* plants, and in *myb28myb29* leaves infiltrated with 4MSOB-ITC in aqueous ethanol or with pure aqueous ethanol as a control.

Figure 5 – figure supplement 1. *C. carnea* detoxifies 4MSOB-ITC by forming the 4MSOB-ITC-NAC conjugate.

Supplementary File 1. Primer sets for gene cloning and qRT-PCR validation.

Supplementary File 2. The sources of *Plutella xylostella* moths for adult fecundity experiments.

Supplementary File 3. LC-MS/MS parameters used for the multiple reaction monitoring (MRM) analyses of I3C derivatives.

Supplementary File 4. External standards used for quantification.

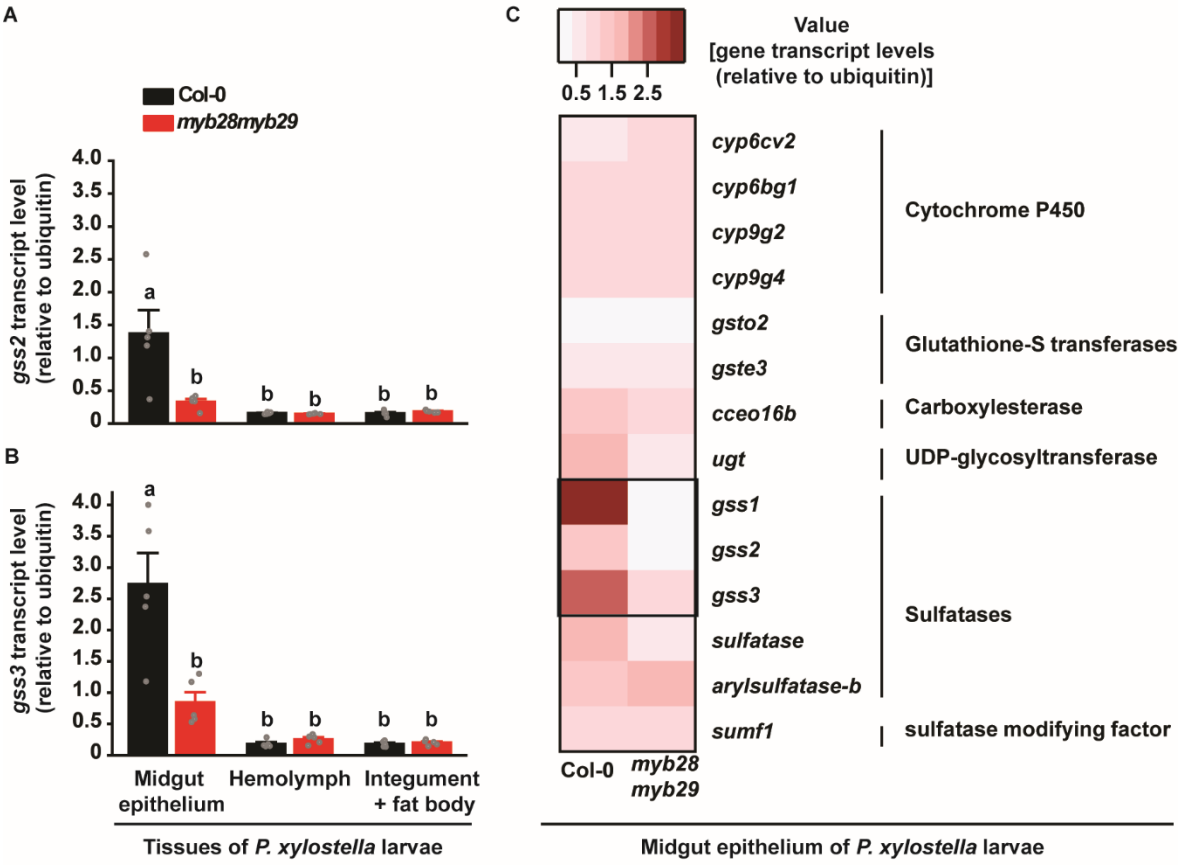


Figure 1 – figure supplement 1. Transcripts of *gss* genes are localized in the *P. xylostella* larval midgut epithelium and are induced by dietary ingestion of glucosinolates. Transcript levels of *gss2* **(A)** and *gss3* **(B)** (expressed relative to ubiquitin) in midgut epithelium, hemolymph, and integument and fat body of fourth-instar larvae (*gss2*: Plants, $F_{1,24}$= 8.244, $P \leq 0.01$; Tissues, $F_{2,24}$= 14.942, $P \leq 0.0001$; Plants*Tissues, $F_{2,24}$= 8.684, $P \leq 0.001$. *gss3*: Plants, $F_{1,24}$= 11.740, $P \leq 0.01$; Tissues, $F_{2,24}$= 36.609, $P \leq 0.0001$; Plants*Tissues, $F_{2,24}$= 13.685, $P \leq 0.001$. n= 5 in all bars). **(C)** Heat map of detoxification-related gene transcripts (selected members of P450, GST, CoE, UGT, sulfatase and SUMF families) in the midgut of *P. xylostella* fourth-instar larvae feeding on *A. thaliana* Col-0 and *myb28myb29* plants. Values represent gene transcripts (expressed relative to ubiquitin). Gene accession numbers are provided in (Supplementary File 1). See (Figure 1 – figure supplement 4A,C) for detailed expression of other sulfatase-related genes. Significant differences ($P \leq 0.05$) between means (±s.e.) were determined by Tukey HSD tests in conjunction with two-way ANOVA in **A** and **B**.

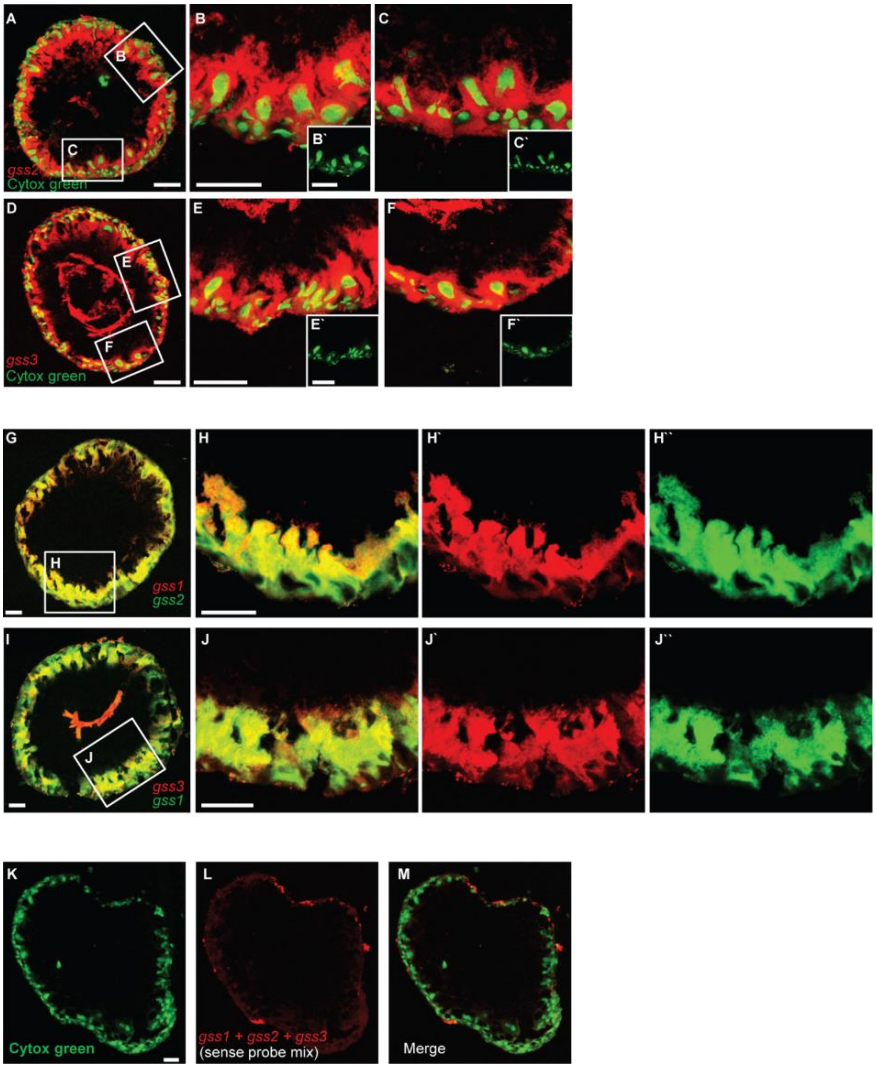


Figure 1 – figure supplement 2. Transcripts of *gss* genes are localized in midgut cell types of *P. xylostella* fourth-instar larvae. (A-F) Cells containing *gss2*- and *gss3* transcripts were visualized by means of FISH using *gss2*- and *gss3*-specific antisense riboprobes labeled by DIG (in red), respectively. Counter staining of nuclei of midgut cells employed Cytox green (in green). Selected areas in **A** and **D** are further magnified in **B**, **C**, **E** and **F**. **B`** to **F`** are lower magnification views of nuclear staining from **A** and **D**. **(G)** Transcripts of *gss1* and *gss2* were co-expressed in the same set of cells as visualized by means of two-color FISH using specific antisense riboprobes labeled by DIG (*gss1*, in red) and biotin (BIO) (*gss2*, in green). A selected area **(H)** in **G** is further magnified on the right to demonstrate the co-expression in more detail. **H`** and **H``**, represent the red and green fluorescent channels, respectively, of **H**. **(I)** Transcripts of *gss1* and *gss3* were largely co-expressed in the same set of cells as visualized by means of two-color FISH using specific antisense riboprobes labeled by DIG (*gss3*, in red) and BIO (*gss1*, in green). A selected area **(J)** in **I** is further magnified on the right to demonstrate the co-expression pattern in more detail. **J`** and **J``**, represent the red and green fluorescent channels, respectively, of **I**. **(K-M)** Demonstration of labeling specificity of individual *gss*-specific antisense riboprobes. No apparent red labeling was observed in a FISH experiment using a mixture of *gss1*, *gss2* and *gss3* sense probes labeled by DIG in conjunction with nuclear counter staining with Cytox green (green). Scale bar, 50 µm.

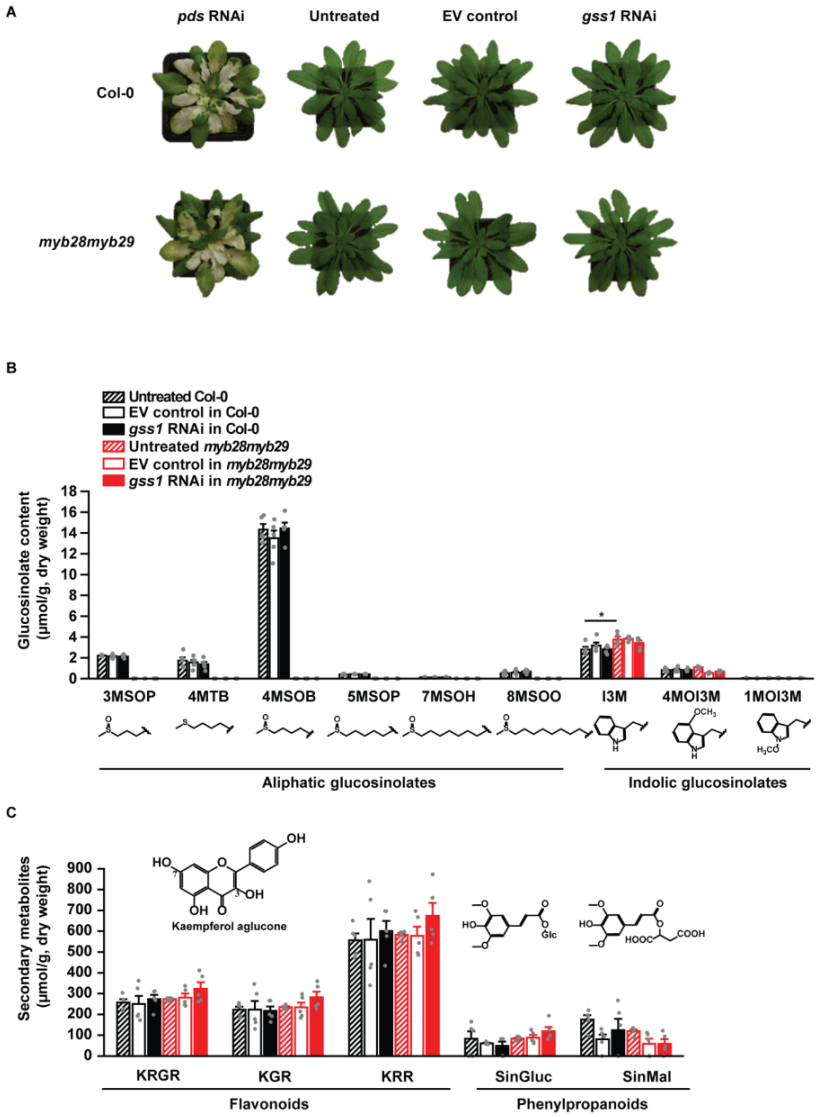


Figure 1 – figure supplement 3. Infiltration of *A. thaliana* Col-0 and *myb28myb29* lines with *gss1* RNAi and empty vector (EV) control constructs does not alter their morphology and chemical phenotypes. (A) Morphology of untreated, EV control and *gss1* RNAi plants in Col-0 and *myb28myb29* backgrounds. Phytoene desaturase (*pds*) RNAi constructs were used as a positive control of virus induced gene silencing. Photographs were taken 4 weeks after infiltration. **(B)** Glucosinolate profiles of untreated, EV control, and *gss1* RNAi plants in Col-0 and *myb28myb29* backgrounds (I3M, $F_{5,24}$= 4.031, $P \leq 0.01$, n= 5 in all bars). 3MSOP: 3-methylsulfinylpropyl; 4MSOB: 4-methylsulfinylbutyl; 5MSOP: 5-methylsulfinylpentyl; 7MSOH: 7-methylsulfinylheptyl; 4MTB: 4-methylthiobutyl; 8MSOO: 8-methylsulfinyloctyl; I3M: Indol-3-ylmethyl; 4MOI3M: 4-methoxyindol-3-ylmethyl; 1MOI3M: 1-methoxyindol-3-ylmethyl glucosinolate. **(C)** Concentrations of selected secondary metabolites (flavonoids and phenylpropanoids) in untreated, EV control, and *gss1* RNAi plants in Col-0 and *myb28myb29* backgrounds. KRGR, kaempferol 3-*O*-glucoside-2''-rhamnoside-7-*O*-rhamnoside; KGR, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside; KRR, kaempferol 3-*O*-rhamnoside-7-*O*-rhamnoside; SinGluc, 1-*O*-sinapoyl-*β*-D-glucose; SinMal, sinapoyl malate. Significant differences ($P \leq 0.05$) between means (±s.e.) were determined by Tukey HSD tests in conjunction with one-way ANOVA in **B** and **C**.

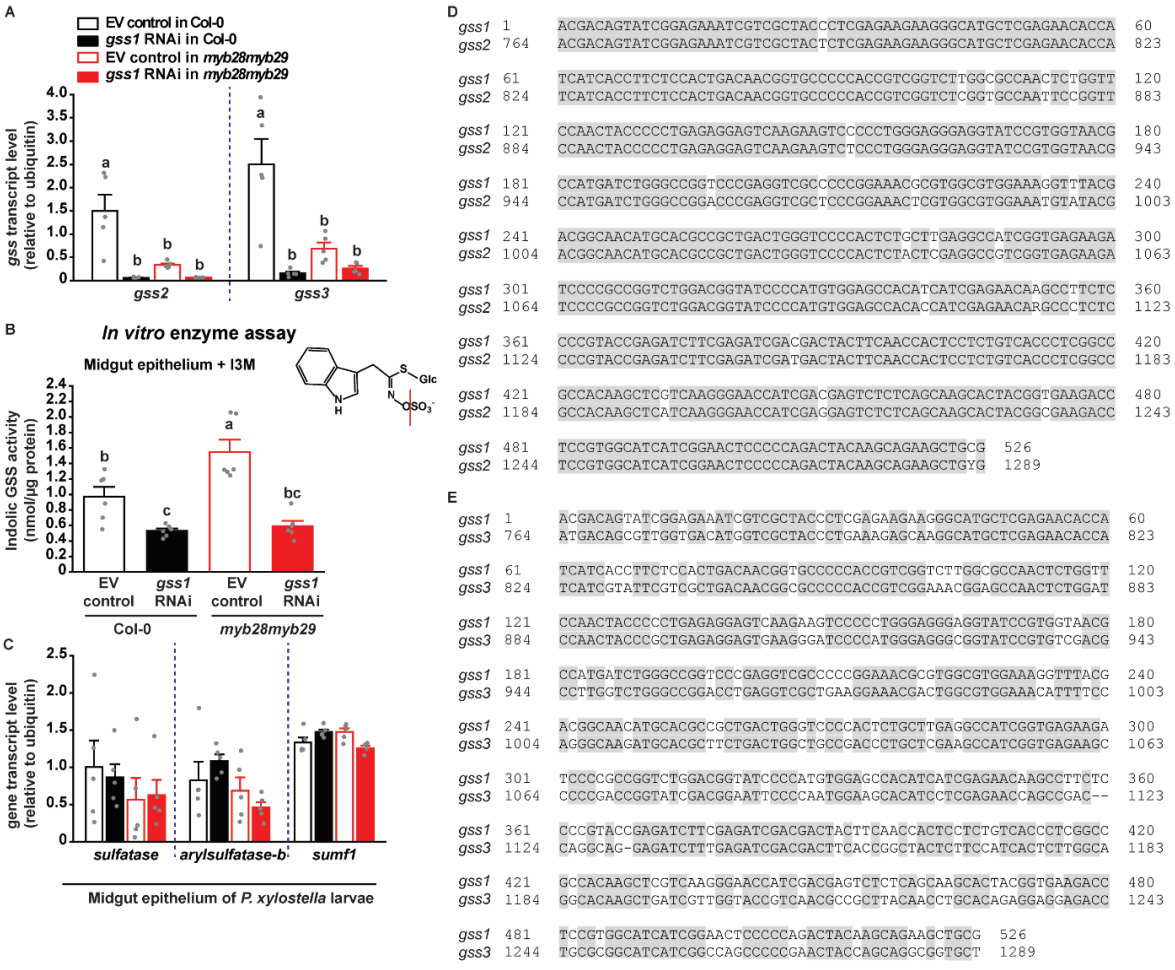


Figure 1 – figure supplement 4. Plant-mediated RNAi of *gss1* co-silences *gss2* and *gss3* due to high sequence similarity, and suppresses the desulfation of indolic glucosinolates as well. **(A)** Levels of *gss2* and *gss3* transcripts (expressed relative to ubiquitin) (*gss2*, $F_{3,16}$= 14.831, $P \leq$ 0.0001; *gss3*, $F_{3,16}$= 14.810, $P \leq$ 0.0001; n= 5 for all bars) in midgut epithelium of *P. xylostella* fourth-instar larvae show silencing after RNAi of *gss1*. **(B)** Levels of indolic glucosinolate sulfatase activity ($F_{3,20}$= 18.696, $P \leq$ 0.0001, n= 6 for all bars) measured *in vitro* in extracts of midgut epithelium of fourth-instar larvae using 5 mM I3M as substrate show suppression upon *gss1* silencing. **(C)** Levels of other sulfatase gene transcripts, including *sulfatase*, *arylsulfatase-b*, and *sulfatase modifying factor 1* (*sumf1*) (expressed relative to ubiquitin) were not silenced by *gss1* RNAi in midgut epithelium of fourth-instar larvae. All insects fed on empty vector (EV) control and *gss1* RNAi plants in backgrounds of Col-0 (wild-type glucosinolates) and *myb28myb29* (no aliphatic glucosinolates). Sequence alignments of *gss1* 526 bp cDNA fragment selected for RNAi silencing experiments are given with the homologous sequences of **(D)** *gss2* and **(E)** *gss3.* The sequence of *gss1* shows 95% identity with *gss2* and 75% with *gss3,* and possesses identical homologous regions of > 21 nt (similarities were determined using nucleotide BLAST). Significant differences ($P \leq$ 0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with one-way ANOVA in **A-C**.

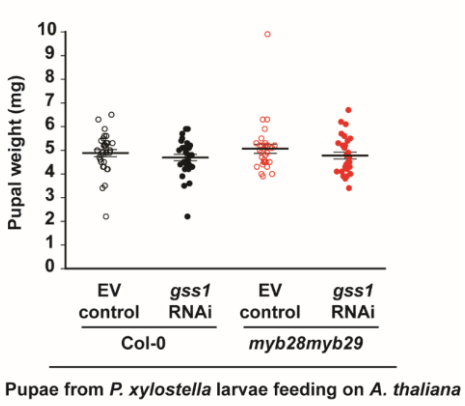


Figure 2 – figure supplement 1. Silencing of *gss* has no effect on weights of *P. xylostella* pupae. Weights of pupae were measured on the second day post pupation. Significant difference ($P \leq 0.05$) between means (±s.e.) was determined by Tukey HSD tests in conjunction with a one-way ANOVA.

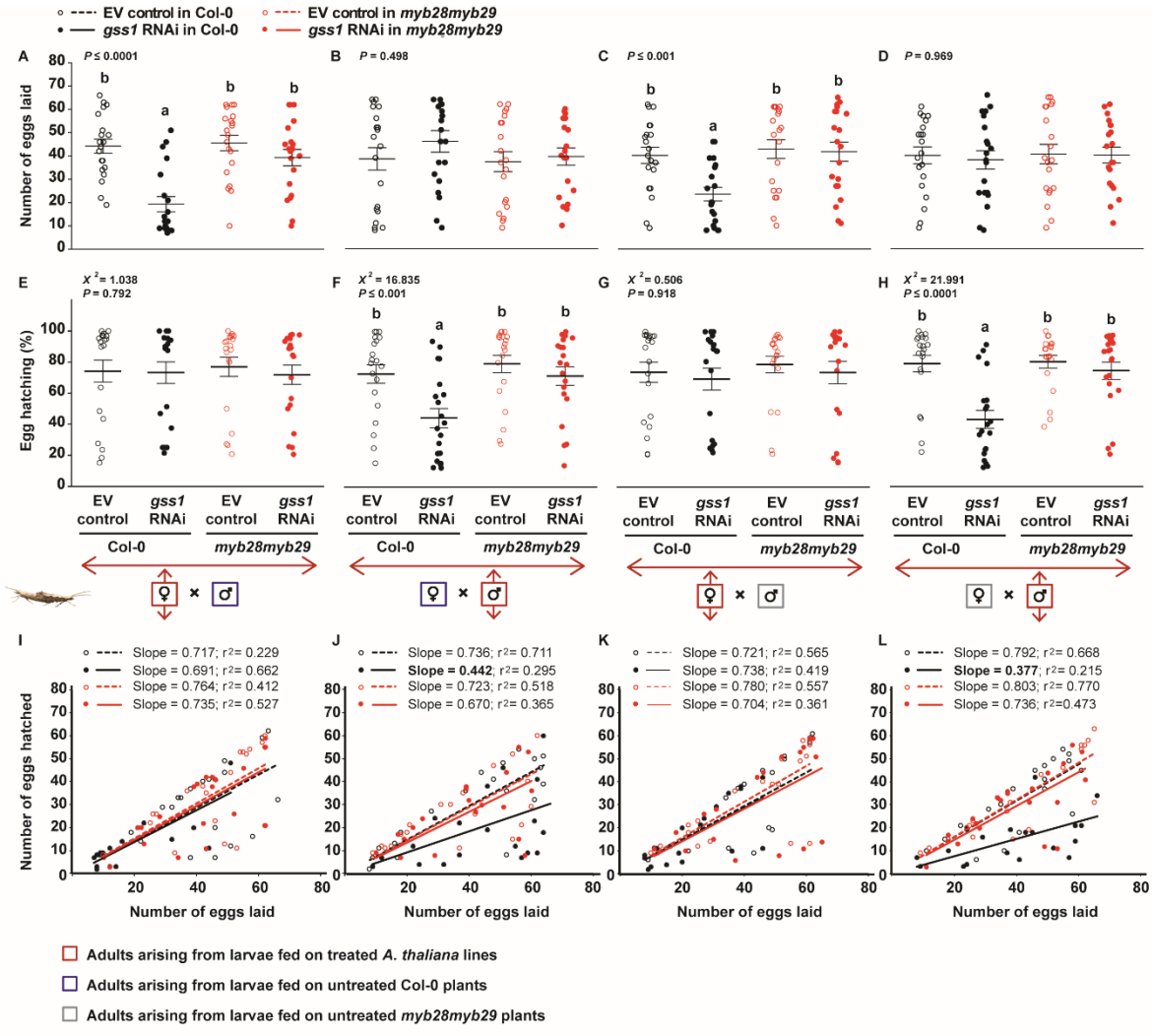


Figure 2 – figure supplement 2. Silencing of *gss* reduces number of eggs and their hatching percentage. Number of eggs laid by **(A)** *P. xylostella* females arising from larvae raised on the four plant lines (empty vector (EV) control and *gss1* RNAi in both Col-0 and *myb28myb29* backgrounds) mated with males arising from larvae feeding on untreated Col-0 plants ($F_{3,76}$= 13.560, P≤ 0.0001; n= 20), **(B)** females arising from larvae feeding on untreated Col-0 plants mated with males arising from larvae raised on the four plant lines, **(C)** females arising from larvae raised on the four plant lines mated with males arising from larvae feeding on untreated *myb28myb29* plants ($F_{3,76}$= 6.179, P≤ 0.001; n= 20), and **(D)** females arising from larvae feeding on untreated *myb28myb29* plants mated with males arising from larvae raised on the four plant lines. Hatching (%) of eggs laid by **(E)** females arising from larvae raised on the four plant lines mated with males arising from larvae feeding on untreated Col-0 plants, **(F)** females arising from larvae feeding on untreated Col-0 plants mated with males arising from larvae raised on the four plant lines (X^2= 16.835, df= 3, P≤ 0.001; n= 20), **(G)** females arising from larvae raised on the four plant lines mated with males arising from larvae feeding on untreated *myb28myb29* plants, and **(H)** females arising from larvae feeding on untreated *myb28myb29* plants mated with males arising from larvae raised on the four plant lines (X^2= 21.991, df= 3, P≤ 0.0001; n= 20). The correlations between the numbers of eggs hatched and the numbers of eggs laid were recorded from crosses between **(I)** females arising from larvae raised on the four plant lines mated with males arising from larvae feeding on untreated Col-0 plants, **(J)** females arising from larvae feeding on untreated Col-0 plants mated with males arising from larvae raised on the four plant lines, **(K)** females arising from larvae raised on the four plant lines mated with males arising from larvae feeding on untreated *myb28myb29* plants, and **(L)** females arising from larvae feeding on untreated *myb28myb29* plants mated with males arising from larvae raised on the four plant lines. The number of eggs laid was reduced only in matings involving females arising from *gss*-silenced larvae fed on plants with wild-type glucosinolate content (Col-0). The percentage of eggs hatching was reduced only in matings involving males arising from *gss*-silenced larvae fed on plants with wild-type glucosinolate content (Col-0). Significant differences (P≤ 0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with one-way ANOVA in **A-D**, and Dunn's post hoc tests in conjunction with non-parametric Kruskal-Wallis tests in **E-H**.

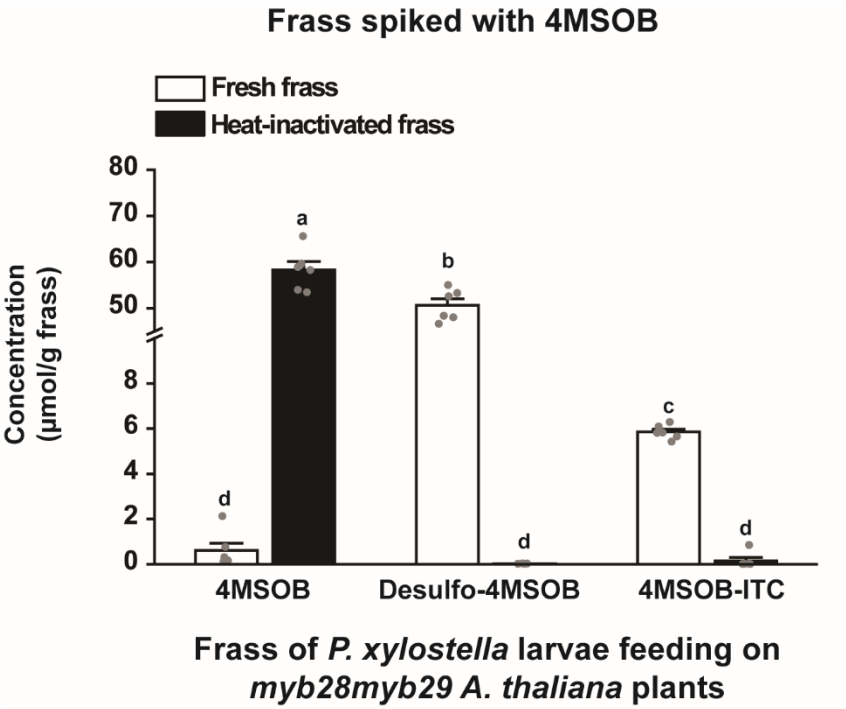


Figure 3 – figure supplement 1. Enzymatic conversion of 4MSOB to desulfo-4MSOB and 4MSOB-ITC is enzymatic and occurs in fresh frass. Depicted are the concentrations of 4MSOB, desulfo-4MSOB and 4MSOB-ITC in fresh and heat-inactivated larval frass spiked with 4MSOB (Metabolites, $F_{2,30}$= 456.246, P≤ 0.0001; Treatments, $F_{1,30}$= 0.359, P= 0.554; Metabolites*Treatments, $F_{2,30}$= 1667.024, P≤ 0.0001; n= 6 for all bars). Frass was collected from *P. xylostella* larvae feeding on *A. thaliana myb29myb29* leaves to ensure the absence of 4MSOB. Significant differences (P≤ 0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with a two-way ANOVA.

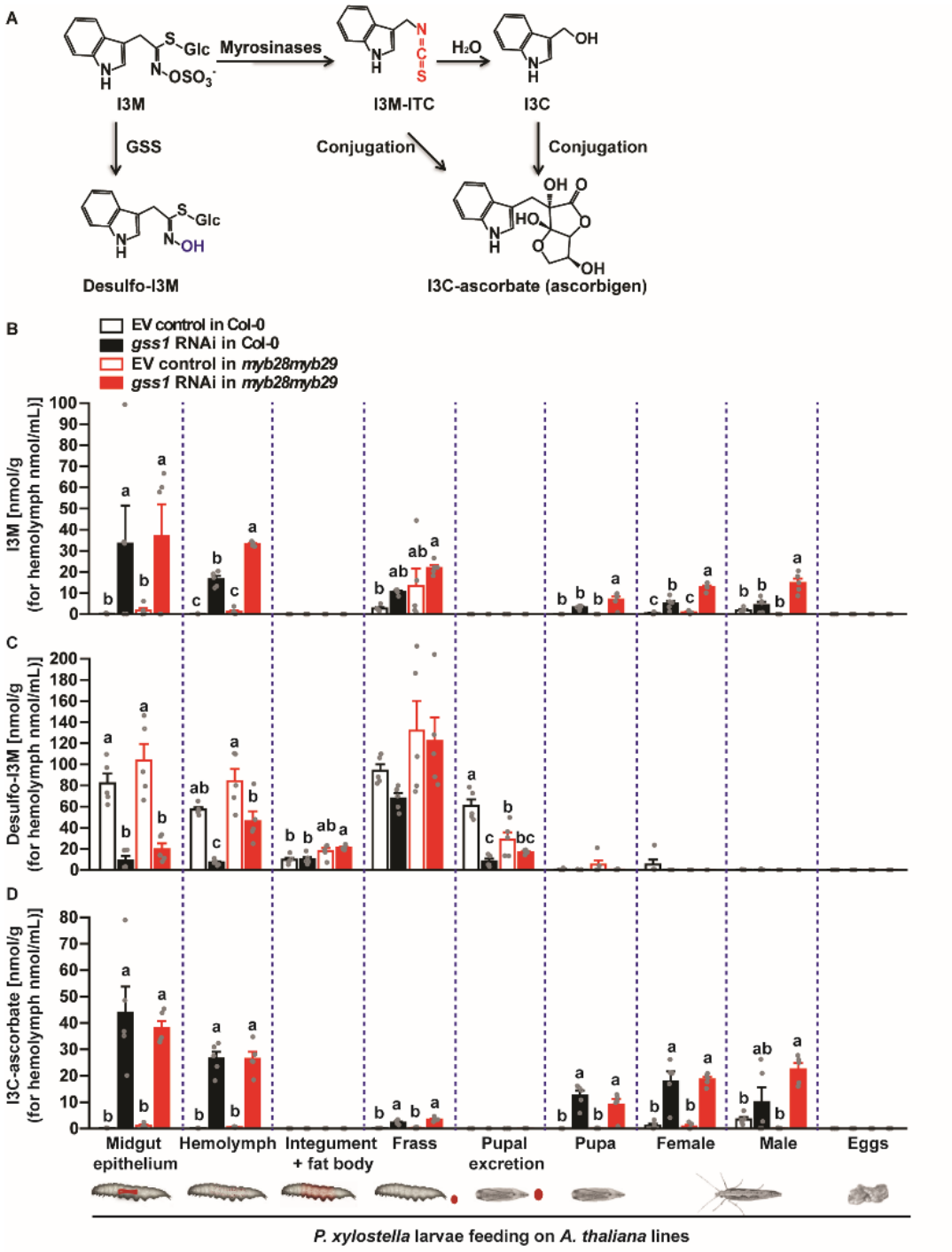


Figure 3 – figure supplement 2. Silencing of *gss* decreases desulfo-indol-3-ylmethyl glucosinolate (desulfo-I3M) and increases a metabolite of indol-3-ylmethyl glucosinolate isothiocyanate (I3M-ITC) in *P. xylostella*. (A) The pathways for formation of desulfo-I3M and indol-3-ylmethyl carbinol (I3C)-ascorbate (ascorbigen) in *P. xylostella*. When GSS activity in the midgut of *P. xylostella* is suppressed, plant myrosinases hydrolyze I3M to form I3M-ITC. However, I3M-ITC is unstable and is readily converted to I3C, which can conjugate with ascorbate to form I3C-ascorbate. In the presence of GSS, I3M is desulfated to form desulfo-I3M, reducing the substrate for

myrosinases. **(B)** I3M (Midgut epithelium, $F_{3,16}$= 17.095, P≤ 0.001; Hemolymph, $F_{3,16}$= 308.329, P≤ 0.0001; Pupa, $F_{3,16}$= 15.081, P≤ 0.001; Female, $F_{3,16}$= 53.596, P≤ 0.0001; Male, $F_{3,16}$= 24.462, P≤ 0.0001; n= 5 for all bars), **(C)** desulfo-I3M (Midgut epithelium, $F_{3,16}$= 23.338, P≤ 0.0001; Hemolymph, $F_{3,16}$= 17.750, P≤ 0.0001; Pupal excretion, $F_{3,16}$= 22.218, P≤ 0.0001; n= 5 for all bars), and **(D)** I3C-ascorbate (Midgut epithelium, $F_{3,16}$= 20.907, P≤ 0.0001; Hemolymph, $F_{3,16}$= 67.127, P≤ 0.0001; Frass, $F_{3,16}$= 34.41, P≤ 0.0001; Pupa, $F_{3,16}$= 19.899, P≤ 0.0001; Female, $F_{3,16}$= 25.107, P≤ 0.0001; Male, $F_{3,16}$= 10.13, P≤ 0.001; n= 5 for all bars) were measured in various larval tissues, excretions, pupae and adult moths developed from *gss*-silenced and non-silenced larvae feeding on Col-0 and *myb28myb29* plants (Data are shown in Figure 3 – figure supplement 2 – Source Data 1). Significant differences (P≤ 0.05) between means (±s.e.) were determined by Tukey HSD tests in conjunction with one-way ANOVA in **B-D**.

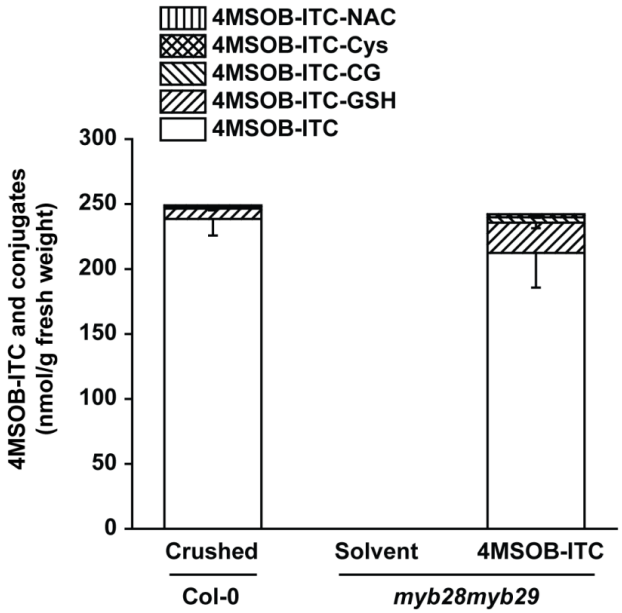


Figure 4 – figure supplement 1. Concentrations of 4MSOB-ITC and conjugates formed in crushed leaves of Col-0 *A. thaliana* plants, and in *myb28myb29* leaves infiltrated with 4MSOB-ITC in aqueous ethanol or with pure aqueous ethanol as a control. 4MSOB-ITC and conjugates were extracted with 60% aqueous methanol and analyzed by LC-MS/MS. NAC, *N*-acetylcysteine; CG, cysteinylglycine; Cys, cysteine; GSH, glutathione. (see Figure 5A for structures)

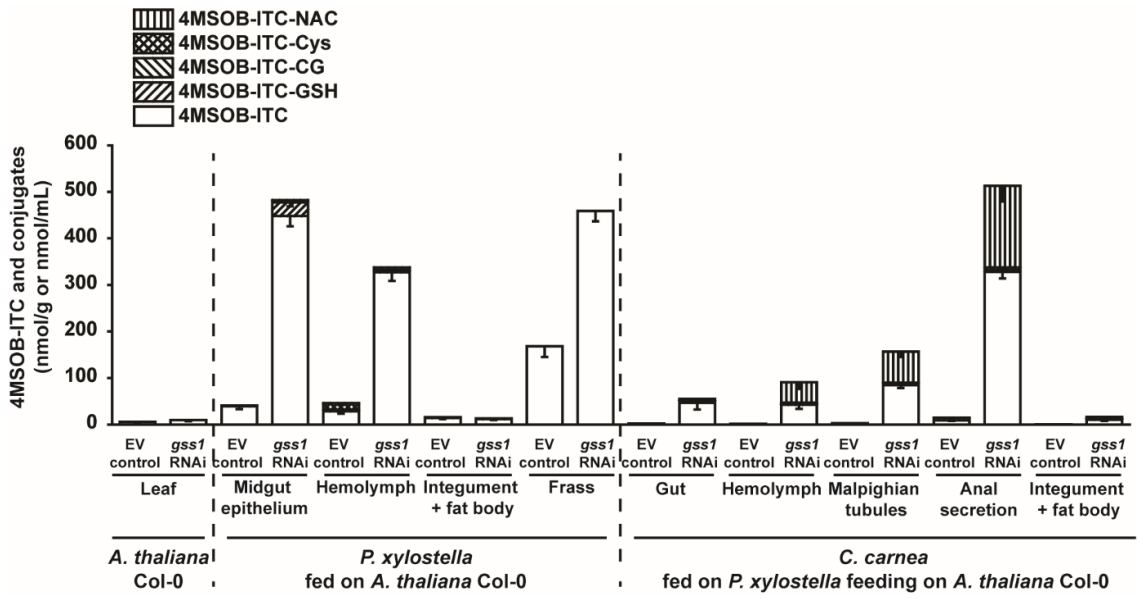


Figure 5 – figure supplement 1. *C. carnea* detoxifies 4MSOB-ITC by forming the 4MSOB-ITC-NAC conjugate. Depicted are levels of 4MSOB-ITC and its mercapturic acid pathway conjugates in *gss1* RNAi and empty vector (EV) control infiltrated Col-0 plants, in *P. xylostella* feeding on those plants, and in *C. carnea* after preying on *P. xylostella* larvae.

Supplementary File 1. Primer sets for gene cloning and qRT-PCR validation.

Name	Primer (5'à3')	Function	Gene accession
*gss1*F	ACGACAGTATCGGAGAAATCGT	526bp for silencing	MG022096
*gss1*R	CGCAGCTTCTGCTTGTAGTCTG		
*gss1*VF	GCGGCGGAATTCCGCAGCTTCTGCTTGTAGTCTG	Ligation to pTRV2	
*gss1*VR	GCGGCGTCTAGAACGACAGTATCGGAGAAATCGT		
*gss1*FLF	ATGGCGATTCTGCATCAAGCTGT	Full length	
*gss1*FLR	CTATTCGGAGTCGGAGTAGGGTT		
*gss1*QF	CATCAAGCTGTGGTGCTCCTC	QPCR/ riboprobe	
*gss1*QR	CTGCCGTCAAGCTGCTCC		
*gss2*QF	CTGCATCAAGTAGTGGTGATTCTG		MG022097
*gss2*QR	GCTTGCCGTCAAGCCTCGAT		
*gss3*QF	CTGAGGTCGCTGAAGGAAAC		MG022099

*gss3*QR	TGATGTCGTACAGGCACCAT		
*sulfatase*QF	CGCCTCTATGCTGGTATGGT	QPCR	XM011561926
*sulfatase*QR	CGACACGAATGCGATGAT		
*arylsulfatase-b*QF	ATGGCATGAGAGGAGGCTAT		XM011552133
*arylsulfatase-b*QR	TCTTGAGCCTTGAAGCCATC		
*sumf1*QF	AGACTAGTGTGACCAAACCCG		XM011551885
*sumf1*QR	AAATCTCATTGGGCCCTCGTTG		
*cyp6cv2*QF	CCTGGCACCCTGAATTTCCT		HQ829967
*cyp6cv2*QR	CTCTCGCCCACGATCTTGTT		
*cyp6bg1*QF	CCGTCCCTGTTCTACGCTTT		KX844829
*cyp6bg1*QR	TCAGGCTGCGCTCCTTAAAT		
*cyp9g2*QF	GGAATTGCATCGCATCTCGG		AB112959
*cyp9g2*QR	TTGCCGCACTTCTGGATCTC		
*cyp9g4*QF	GGGTTCCCGATGTTTGGAAAT		FJ023535
*cyp9g4*QR	ATCAGGTACAGCGGCACAAA		
*gsto2*QF	ACATGCGCTACTGTCCCTTT		KF929209
*gsto2*QR	TTCCCGAATGCACTCTTGCT		
*gste3*QF	CACCACGCCCGAGTATCTTA		U66342
*gste3*QR	CGCTCTTGCCGTATTTGTCC		
*cceo16b*QF	GAAGCGCACTAAAGCATGGG		KM008609
*cceo16b*QR	GGCTTGCTTGGCTTGATGTC		
*ugt*QF	TACGGGTACACGCCTTTCTG		XM011558530
*ugt*QR	TACAGGGCCGAGATGTGGTA		
UbiquitinQF	CGACTGATCTTCGCTGGTAAAC		NM001305519
UbiquitinQR	TCCTCTAAGCCTCAACACCAAG		

Supplementary File 2. The sources of *Plutella xylostella* moths for adult fecundity experiments.

Figure 2C,E,G		Figure 2D,F,H	
paired couples		paired couples	
Female	Male	Female	Male
EV control in Col-0	EV control in Col-0	EV control in Col-0	EV control in Col-0
gss1 RNAi in Col-0			*gss1* RNAi in Col-0
Ev control in *myb28myb29*			Ev control in *myb28myb29*
gss1 RNAi in *myb28myb29*			*gss1* RNAi in *myb28myb29*
Figure 2 - supplement figure 2A,E,I		Figure 2 - supplement figure 2B,F,J	
paired couples		paired couples	
Female	Male	Female	Male
EV control in Col-0	Col-0	Col-0	EV control in Col-0
gss1 RNAi in Col-0			*gss1* RNAi in Col-0
Ev control in *myb28myb29*			Ev control in *myb28myb29*
gss1 RNAi in *myb28myb29*			*gss1* RNAi in *myb28myb29*
Figure 2 - supplement figure 2C,G,K		Figure 2 - supplement figure 2D,H,L	
paired couples		paired couples	
Female	Male	Female	Male
EV control in Col-0	*myb28myb29*	*myb28myb29*	EV control in Col-0
gss1 RNAi in Col-0			*gss1* RNAi in Col-0
Ev control in *myb28myb29*			Ev control in *myb28myb29*
gss1 RNAi in *myb28myb29*			*gss1* RNAi in *myb28myb29*
Figure 4C,E,G		Figure 4D,F,H	
paired couples		paired couples	
Female	Male	Female	Male

EV control with 4MSOB-ITC in *myb28myb29*	*myb28myb29*	*myb28myb29*	EV control with 4MSOB-ITC in *myb28myb29*
EV control with solvent in *myb28myb29*			EV control with solvent in *myb28myb29*
gss1 RNAi with solvent in *myb28myb29*			*gss1* RNAi with solvent in *myb28myb29*
gss1 RNAi with 4MSOB-ITC in *myb28myb29*			*gss1* RNAi with 4MSOB-ITC in *myb28myb29*

Supplementary File 3. LC-MS/MS parameters used for the multiple reaction monitoring (MRM) analyses of I3C derivatives. Q1, quadrupole 1 voltage; Q3, quadrupole 3 voltage; DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.

GLS metabolites	Ionization mode	Q1 *m/z*	Q3 MRM fragment	Retention time (min)	Dp (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
I3C-Ascorbate-1	Positive mode	306.036	130.1	3.22	36	8	20	21	4
I3C-Ascorbate-2		306.036	76.8	3.22	36	8	20	87	4
I3C-GSH-1		437.03	308	2.62	41	3	18	19	12
I3C-GSH-2		437.03	130.1	2.62	41	3	18	47	4
I3C-CG-1		308.016	130	2.95	106	4.5	26	21	4
I3C-CG-2		308.016	76	2.95	106	4.5	26	35	4
I3C-Cys-1		251.067	130	2.88	21	2	14	25	4
I3C-Cys-2		251.067	77	2.88	21	2	14	77	4
I3C-NAC-1		293.029	130.1	4.6	21	8	14	17	4
I3C-NAC-2		293.029	76.9	4.6	21	8	14	83	4

Supplementary File 4. External standards used for quantification.

Compounds	Supplier
4MSOB	Carl Roth, Karlsruhe, Germany
Desulfo-4MSOB	Achieved by incubating 4MSOB with sulfatase (Graser *et al.* 2000) overnight
4MSOB-ITC	BIOZOL Diagnostica Vertrieb, Eching, Germany
4MSOB-ITC-GSH	Santa Cruz Biotechnology, Dallas, TX, United States
4MSOB-ITC-CG	Synthesized as described in (Schramm *et al.* 2012)
4MSOB-ITC-Cys	Santa Cruz Biotechnology, Dallas, TX, United States
4MSOB-ITC-NAC	Santa Cruz Biotechnology, Dallas, TX, United States
I3M	Carl Roth, Karlsruhe, Germany
Desulfo-I3M	Achieved by incubating I3M with sulfatase (Graser *et al*. 2000) overnight
I3C	Sigma-Aldrich, Munich, Germany
I3C-Ascorbate	Carbosynth Limited, Berkshire, UK

10.2 Manuscript II – Supplemental Information

Detoxification of plant defensive glucosinolates by an herbivorous caterpillar is beneficial to its endoparasitic wasp

Ruo Sun, Rieta Gols, Jeffrey A. Harvey, Michael Reichelt, Jonathan Gershenzon, Sagar Subhash Pandit, Daniel Giddings Vassão

Figure S1. Experimental time course for *Diadegma semiclausum* parasitism of *Plutella xylostella* and sampling points for metabolic analyses.

Figure S2. Exposure of *P. xylostella* larvae to 4MSOB-ITC causes the same effects on *D. semiclausum* as *Pxgss* silencing.

Figure S3. 4MSOB-ITC and its mercapturic acid pathway conjugates are present in non-parasitized and parasitized *P. xylostella* (*Pxgss*-silenced or non-silenced) fed upon *A. thaliana* Col-0 plants (containing aliphatic glucosinolates).

Figure S4. Expression of *P. xylostella ecdysone receptor* gene is strongly affected by parasitization by *D. semiclausum*, but not by *Pxgss* silencing or ingestion of glucosinolates.

Table S1. External standards used for quantification.

Table S2. Primer sets for qRT-PCR validation, and corresponding gene accession numbers.

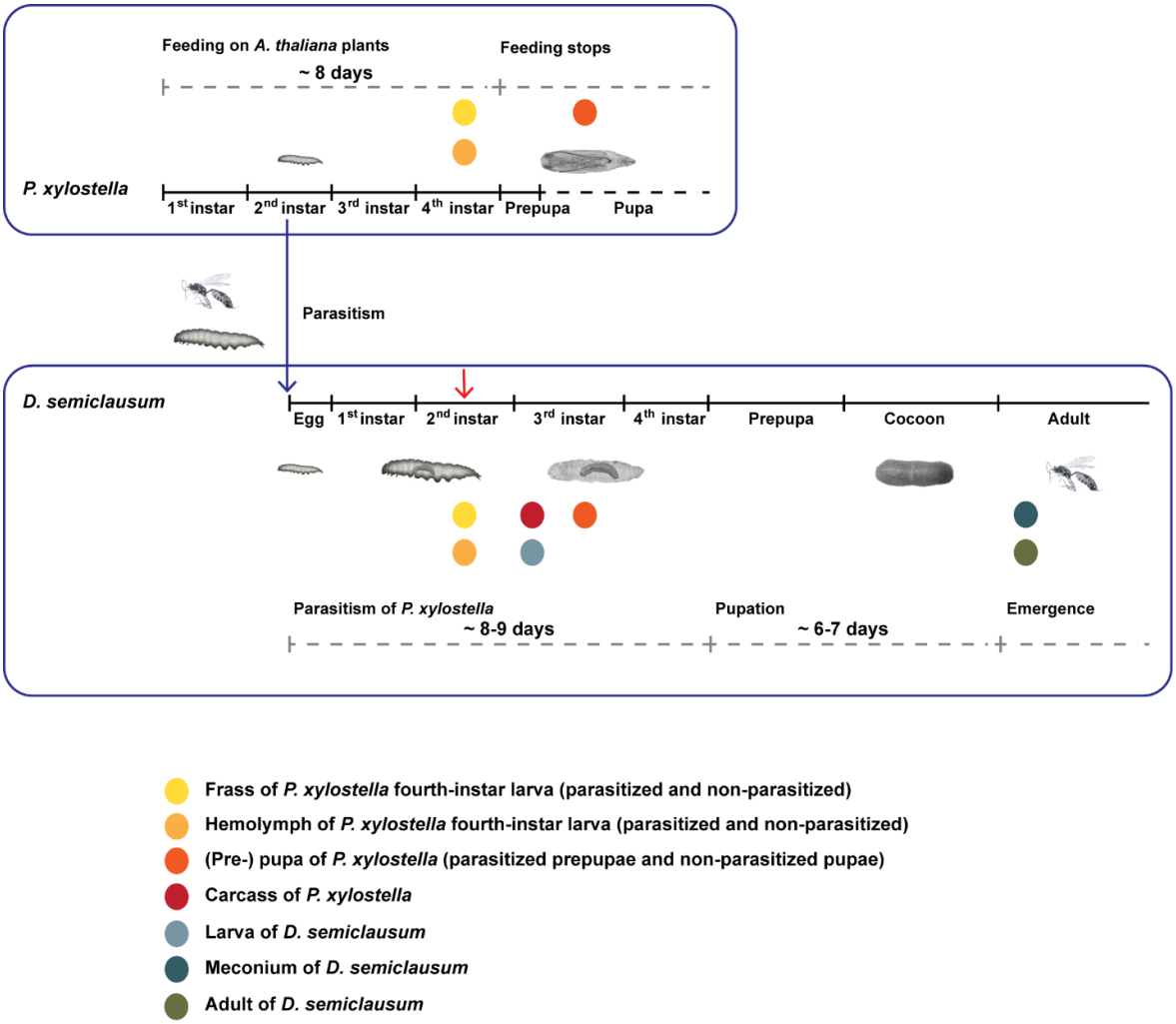


Figure S1 Experimental time course for *Diadegma semiclausum* parasitism of *Plutella xylostella* and sampling points for metabolic analyses. Non-silenced and *Pxgss*-silenced *P. xylostella* second-instar larvae were exposed to adult female *D. semiclausum* for parasitism, and then fed on either *A. thaliana* wild type Col-0 or *myb28myb29* plants until feeding stopped. Hemolymph and frass of fourth-instar non-parasitized and parasitized *P. xylostella* larvae, prepupae of parasitized *P. xylostella*, pupae of non-parasitized *P. xylostella*, third-instar *D. semiclausum* larva and corresponding *P. xylostella* carcass, meconium excreted by *D. semiclausum* when pupating, and adults of *D. semiclausum* were collected for analyses by LC-MS/MS. Parasitized *P. xylostella* fourth-instar larvae (time point is marked by red arrow in the graph) were collected for gene expression analyses by qRT-PCR.

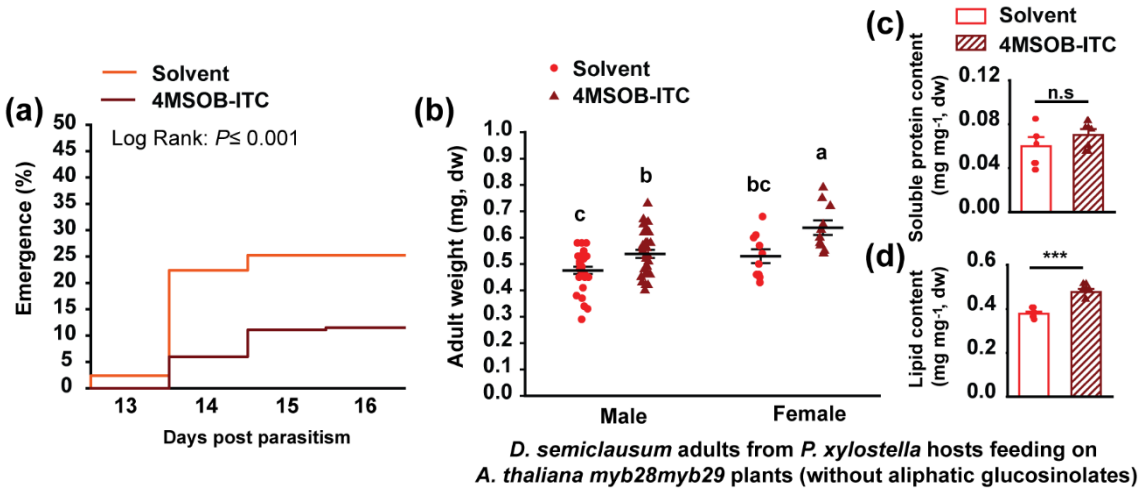


Figure S2 Exposure of *P. xylostella* larvae to 4MSOB-ITC causes the same effects on *D. semiclausum* as *Pxgss* silencing. *D. semiclausum*-parasitized *P. xylostella* larvae were fed on *A. thaliana myb28myb29* leaves infiltrated with either 4MSOB-ITC (in a solution of 0.4% aqueous ethanol) or solvent only as a control. The following variables were measured: **(a)** adult emergence percentage (Log Rank, *df*= 1, *P*≤ 0.001; *n*= 210 and 217, respectively for solvent and 4MSOB-ITC treatments), **(b)** adult dry weight (sex, $F_{1,76}$= 13.475, *P*≤ 0.001; treatment, $F_{1,76}$= 16.778, *P*≤ 0.001; sex×treatment, $F_{1,76}$= 1.156, *P*= 0.286; male, *n*= 30 in all treatments; female, *n*= 10 in all treatments), **(c)** soluble protein content (*t*= 1.044, *P*= 0.332, *n*= 5 in all bars) and **(d)** lipid content (*t*= 5.713, *P*≤ 0.001, *n*= 5 in all bars) in *D. semiclausum* male adults. Significant differences (*P*≤ 0.05) were determined by Kaplan-Meier survival analysis tests in **a**, and significant differences (*P*≤ 0.05) between means (± s.e.) were determined by Tukey HSD tests in conjunction with two-way ANOVA in **b**, and two-tailed *t*-tests for two independent means in **c,d**.

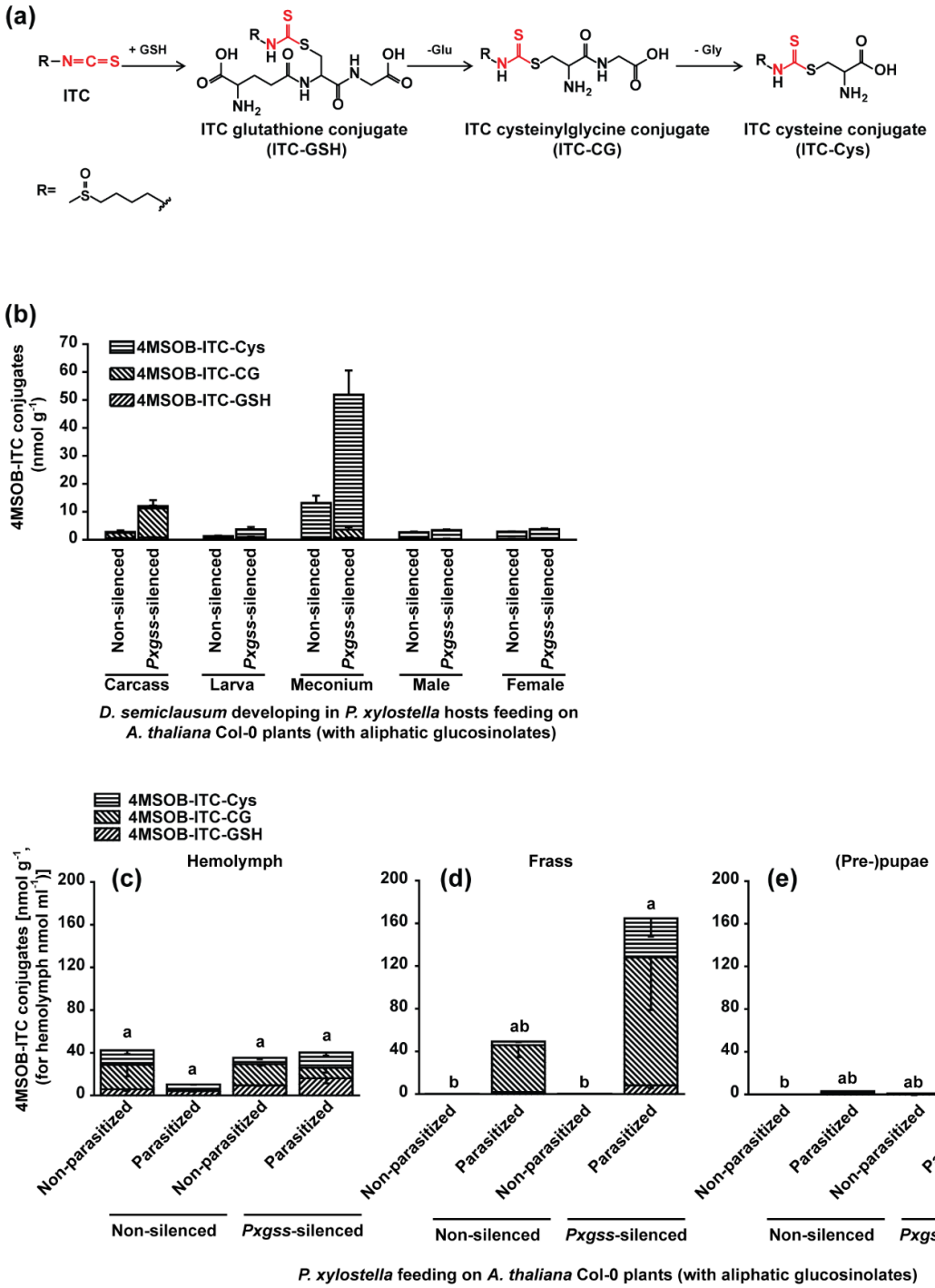


Figure S3 4MSOB-ITC and its mercapturic acid pathway conjugates are present in non-parasitized and parasitized *P. xylostella* (*Pxgss*-silenced or non-silenced) fed with *A. thaliana* Col-0 plants (containing aliphatic glucosinolates). (a) General mercapturic acid pathway used for detoxification of 4MSOB-ITC in various insects. Ingested 4MSOB-ITC is detoxified by conjugation with glutathione (4MSOB-ITC-GSH), followed by hydrolytic cleavages to form the 4MSOB-ITC-cysteinylglycine (4MSOB-ITC-CG) and 4MSOB-ITC-cysteine conjugates (4MSOB-ITC-Cys). **(b)** 4MSOB-ITC conjugates were quantified in the carcass of *P. xylostella* prepupae, third-instar larvae

of *D. semiclausum*, meconium left in the cocoon and adults of *D. semiclausum*, in which *D. semiclausum* parasitized either non-silenced or *Pxgss*-silenced *P. xylostella*. 4MSOB-ITC conjugates were present in **(c)** hemolymph (*gss*-silencing, $F_{1,16}$= 1.553, P= 0.231; parasitism, $F_{1,16}$= 2.164, P= 0.161; *gss*-silencing×parasitism, $F_{1,16}$= 4.009, P= 0.062; n= 5 in all bars) and **(d)** frass (*gss*-silencing, $F_{1,16}$= 2.758, P= 0.116; parasitism, $F_{1,16}$= 9.420, P≤ 0.01; *gss*-silencing×parasitism, $F_{1,16}$= 2.748, P= 0.117; n= 5 in all bars) of non-parasitized and parasitized *P. xylostella* fourth-instar larvae. These compounds were also present in **(e)** pupae of non-parasitized *P. xylostella* and prepupae of parasitized *P, xylostella* with parasitoid inside (*gss*-silencing, $F_{1,16}$= 1.904, P= 0.186; parasitism, $F_{1,16}$= 9.844, P≤ 0.01; *gss*-silencing×parasitism, $F_{1,16}$= 0.668, P= 0.426; n= 5 in all bars). In all cases, *P. xylostella* larvae hatched upon *A. thaliana* Col-0 plants (containing aliphatic glucosinolates). Significant differences (P≤ 0.05) between means (± s.e.) were determined by Tukey HSD tests in conjunction with two-way ANOVA in **c-e**.

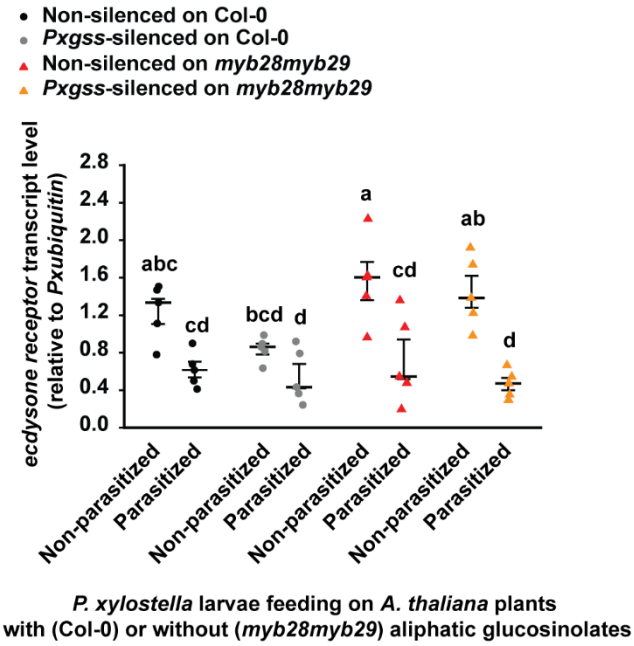


Figure S4 Expression of *P. xylostella ecdysone receptor* gene is strongly affected by parasitization by *D. semiclausum*, but not by *Pxgss* silencing or ingestion of glucosinolates. *D. semiclausum* parasitism reduced *EcR* gene expression (parasitism, $F_{1,32}$= 45.016, P≤ 0.0001; *gss*-silencing, $F_{1,32}$= 4.369, P≤ 0.05; plant, $F_{1,32}$= 5.560, P≤ 0.05; plant×parasitism, $F_{1,32}$= 5.011, P≤ 0.05; *gss*-silencing×plant×parasitism, $F_{1,32}$= 1.392, P= 0.247; n= 5 in all bars). Significant differences (P≤ 0.05) were determined by Tukey HSD tests in conjunction with multiple ANOVA.

Table S1. External standards used for quantification.

Compounds	Supplier
4MSOB	Carl Roth, Karlsruhe, Germany
Desulfo-4MSOB	Obtained by incubating 4MSOB with sulfatase (Graser *et al.* 2000) overnight
4MSOB-ITC	BIOZOL Diagnostica Vertrieb, Eching, Germany
4MSOB-ITC-GSH	Santa Cruz Biotechnology, Dallas, TX, United States
4MSOB-ITC-CG	Synthesized as described in (Schramm *et al.* 2012)
4MSOB-ITC-Cys	Santa Cruz Biotechnology, Dallas, TX, United States

Table S2. Primer sets for qRT-PCR validation, and corresponding gene accession numbers.

Name	Primer (5'-->3')	Gene accession
Pxecdysone receptor QF	TCAGTGCGCGATAAAGAGGA	NM001309151
Pxecdysone receptor QR	ACAGTCGAGAATCCTAGCGG	
Dsvankyrin1 QF	GTAGTACAGTGAAGCGCGTG	JI257593
Dsvankyrin1 QR	GCGATCTTTGCATCCACCTT	
Dsvankyrin2 QF	ACCGTACTACACATCGCAGT	JI257594
Dsvankyrin2 QR	CTTGAGCCAGTTGATACGCC	
Dsviral innexin1 QF	CTTGTGGCTCTGTATCGCAC	JI257597
Dsviral innexin1 QR	ACTGGCTATGGTCTCGTCAG	
Pxubiquitin QF	CGACTGATCTTCGCTGGTAAAC	NM001305519
Pxubiquitin QR	TCCTCTAAGCCTCAACACCAAG	

10.3 Manuscript III – Supplemental Information

Adaptation of cabbage aphid to aliphatic glucosinolates overwhelms detoxification in a lacewing predator

Ruo Sun, Xingcong Jiang, Michael Reichelt, Jonathan Gershenzon, Daniel Giddings Vassão

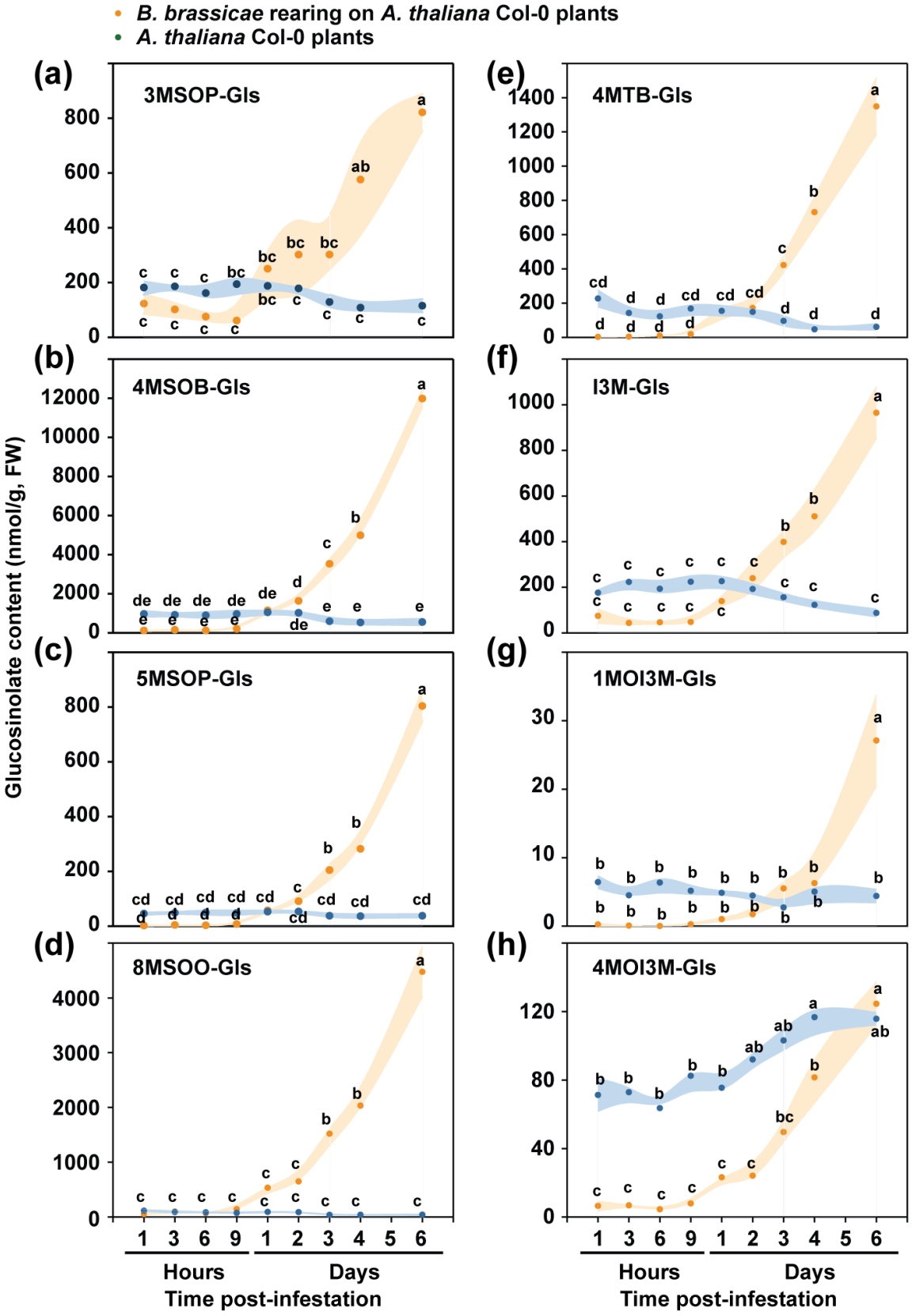
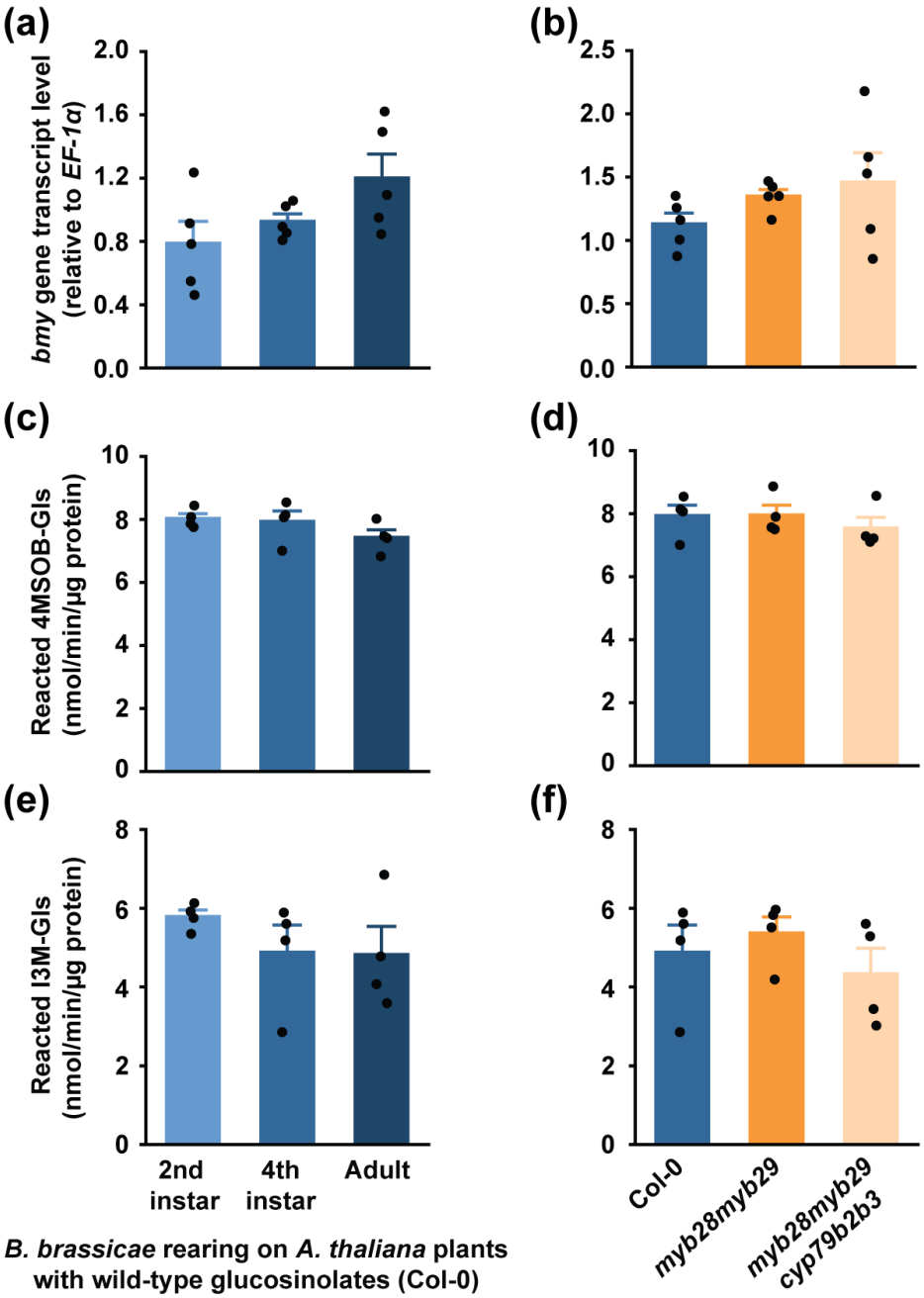

B. brassicae rearing on A. thaliana Col-0 plants
A. thaliana Col-0 plants
(a) 3MSOP-Gls
(b) 4MSOB-Gls
(c) 5MSOP-Gls
(d) 8MSOO-Gls
(e) 4MTB-Gls
(f) I3M-Gls
(g) 1MOI3M-Gls
(h) 4MOI3M-Gls
Glucosinolate content (nmol/g, FW)
1 3 6 9 1 2 3 4 5 6
Hours Days
Time post-infestation

Figure S1. Absolute glucosinolate content in the body of adult *B. brassicae* and the host *A. thaliana* Col-0 plants during a 6-day aphid infestation. (a) 3MSOP-Gls (organisms: $F_{1,54}$ = 13.51, $P \leq 0.001$; duration: $F_{8,54}$ = 4.700, $P \leq 0.001$; organisms×duration: $F_{8,54}$ = 7.193, $P \leq 0.001$; n= 4 in all points), **(b)** 4MSOB-Gls (organisms: $F_{1,54}$ = 477.8, $P \leq 0.001$; duration: $F_{8,54}$ = 224.0, $P \leq 0.001$; organisms×duration: $F_{8,54}$ = 264.8, $P \leq 0.001$; n= 4 in all points), **(c)** 5MSOP-Gls (organisms: $F_{1,54}$ = 222.0, $P \leq 0.001$; duration: $F_{8,54}$ = 120.0, $P \leq 0.001$; organisms×duration: $F_{8,54}$ = 128.5, $P \leq 0.001$; n= 4 in all points), **(d)** 8MSOO-Gls (organisms: $F_{1,54}$ = 290.8, $P \leq 0.001$; duration: $F_{8,54}$ = 68.18, $P \leq 0.001$; organisms×duration: $F_{8,54}$ = 72.23, $P \leq 0.001$; n= 4 in all points), **(e)** 4MTB-Gls (organisms: $F_{1,54}$ = 57.86, $P \leq 0.001$; duration: $F_{8,54}$ = 31.12, $P \leq 0.001$; organisms×duration: $F_{8,54}$ = 45.46, $P \leq 0.001$; n= 4 in all points), **(f)** I3M-Gls (organisms: $F_{1,54}$ = 18.97, $P \leq 0.001$; duration: $F_{8,54}$ = 16.17, $P \leq 0.001$; organisms×duration: $F_{8,54}$ = 28.71, $P \leq 0.001$; n= 4 in all points), **(g)** 1MOI3M-Gls (organisms: $F_{1,54}$ = 0.05, P= 0.824; duration: $F_{8,54}$ = 11.12, $P \leq 0.001$; organisms×duration: $F_{8,54}$ = 13.02, $P \leq 0.001$; n= 4 in all points), and **(h)** 4MOI3M-Gls (organisms: $F_{1,54}$ = 279.7, $P \leq 0.001$; duration: $F_{8,54}$ = 42.14, $P \leq 0.001$; organisms×duration: $F_{8,54}$ = 7.502, $P \leq 0.001$; n= 4 in all points). Significant differences ($P \leq 0.05$) between means (±s.e.) were determined by Tukey HSD tests in conjunction with two-way ANOVA.



***B. brassicae* rearing on *A. thaliana* plants with wild-type glucosinolates (Col-0)**

4th-Instar *B. brassicae* rearing on *A. thaliana* plants with wild-type glucosinolates (Col-0), or without aliphatic glucosinolates (*myb28myb29*) or without any glucosinolates (*myb28myb29cyp79b2b3*)

Figure S2. The *bmy* gene expression and BMY enzymatic activity are independent of developmental stages and host glucosinolate content. (a and **b)** qRT-PCR analysis of *bmy* gene expression for three stages ($F_{2,12}$ = 2.972, P= 0.089, n= 5 in all bars) and the 4th-instar nymphs fed on three *A. thaliana* genotypes ($F_{2,12}$ = 1.348, P= 0.296, n= 5 in all bars). Enzymatic activity against two selected glucosinolates using aphid protein extracts either from three stages **(c** and **e)** or from the 4th-instar nymphs fed on three different host **(d** and **f)**. **(c)**, $F_{2,9}$ = 1.674, P= 0.241, n= 4 in all bars;

(d), $F_{2,9}$ = 0.508 P= 0.618, n= 4 in all bars; **(e)**, $F_{2,9}$ = 0.859, P= 0.456, n= 4 in all bars; **(f)**, $F_{2,9}$ = 0.759, P= 0.496, n= 4 in all bars. Significant differences ($P \leq 0.05$) between means (± s.e.) were determined by Tukey HSD test in conjunction with one-way ANOVA.

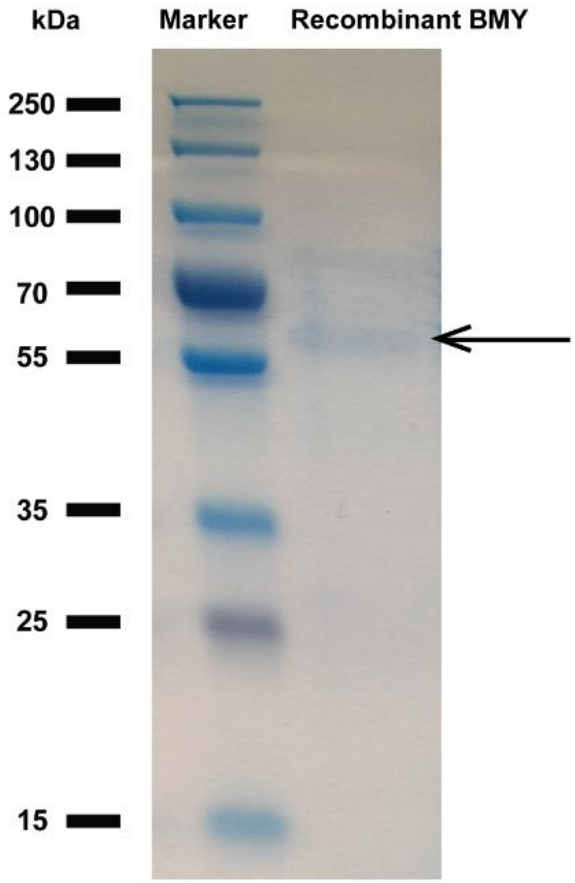


Figure S3. SDS-Page of the recombinant BMY protein expressed in a pET28a plasmid in *E. coli* cells. Molecular weight markers are indicated in kilodalton (kDa).

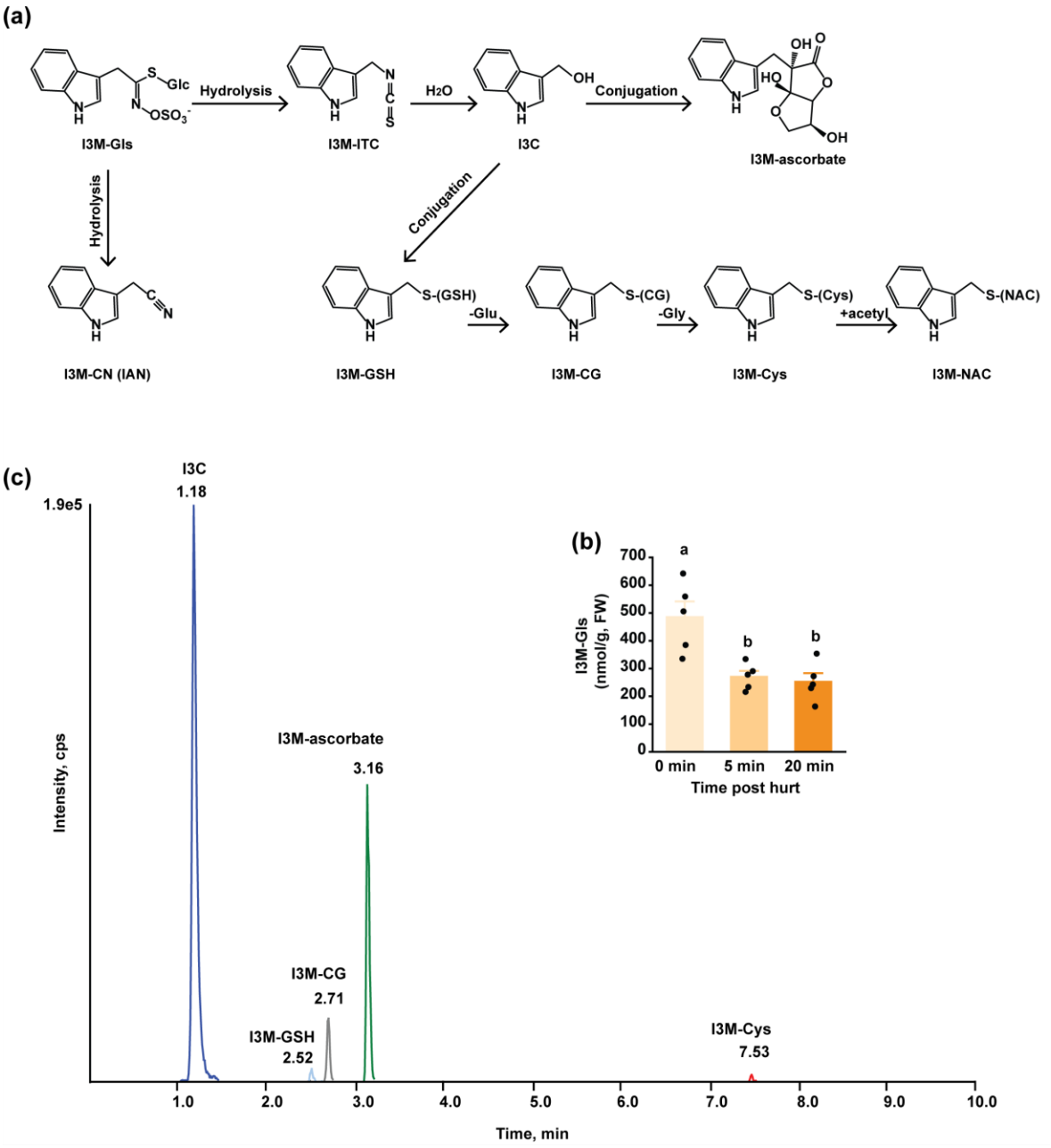


Figure S4. Profiling metabolites of indolic glucosinolate I3M-Gls in response to tissue damage. **(a)** An overview of the metabolic pathway of I3M. **(b)** Reduction of I3M-Gls induced by tissue damage identical to Figure 3 ($F_{2,12}$= 11.06, $P \leq 0.01$; n= 5 in all bars). **(c)** A chromatogram of LC/MS/MS shows detectable I3M-Gls-derivatives in *B. brassicae*. Note that I3M-ITC was not detected. Significant differences ($P \leq 0.05$) between means (± s.e.) were determined by Tukey HSD test in conjunction with a one-way ANOVA in **b**.

Table S1. Primer sets for gene clone and qRT-PCR validation.

Name	Primer (5'→3')	Function	Gene accession
bmyFLF	AATATGGATTATAAATTTCCAAAGG	Recombination BMY in *E. coli* cells	AF203780
bmyFLR	TGGTTTGCCAGTTGATACCAC		
bmyVF	GCGGCGGGATCCAATATGGATTATAAATTTCCAAAGG		
bmyVR	GCGGCGCTCGAGTGGTTTGCCAGTTGATACCAC		
bmyQF	AGGCTGGAATGAAGACGGAA	QPCR	
bmyQR	GGAATCACAGGCAATATCTCCA		
EF-1αF	ACCACCATACAGCGAAAGCC		AY219734
EF-1αR	GATGGGCACGAAAGCAACTG		

Table S2. Substrates used for enzyme assay and external standards used for quantification.

Compounds	Other names	Supplier	CAS#	CAT#
4MSOB-Gls	Glucoraphanin	Carl Roth, Karlsruhe, Germany	21414-41-5	2374.1
4MSOB-CN	4-(Methylsulfinyl)butanenitrile	Synthesized as described in (Beran *et al.*, 2018)	61121-65-1	
4MSOB-ITC	D,L-Sulforaphane	BIOZOL Diagnostica Vertrieb, Eching, Germany	142825-10-3	USB-289262
4MSOB-ITC-GSH	D,L-Sulforaphane Glutathione	Santa Cruz Biotechnology, Dallas, TX, United States	289711-21-3	sc-207496
4MSOB-ITC-CG	Sulforaphane-cysteine-glycine	Synthesized as described in (Schramm *et al.*, 2012)		
4MSOB-ITC-Cys	D,L-Sulforaphane-L-cysteine	Santa Cruz Biotechnology, Dallas, TX, United States	364083-21-6	sc-207499
4MSOB-ITC-NAC	D,L-Sulforaphane N-Acetyl-L-cysteine	Santa Cruz Biotechnology, Dallas, TX, United States	334829-66-2	sc-207497
Allyl glucosinolate	Sinigrin	Carl Roth, Karlsruhe, Germany	3952-98-5	5319.1
3MSOP-Gls	Glucoiberin	Phytoplan, Heidelberg, Germany	15592-34-4	3413.99
5MSOP-Gls	Glucoalyssin	Phytoplan, Heidelberg, Germany	499-37-6	3428.97
8MSOO-Gls	Glucohirsutin	Phytoplan, Heidelberg, Germany	21973-60-4	3438.97
Benzyl-Gls	Glucotropeolin	Santa Cruz Biotechnology, Dallas, TX, United States	5115-71-9	sc-285861
p-Hydroxybenzyl glucosinolate	Sinalbin	Carl Roth, Karlsruhe, Germany	16411-05-5	6694.1
I3M-Gls	Glucobrassicin	Carl Roth, Karlsruhe, Germany	4356-52-9	2279.1
1MOI3M-Gls	Neoglucobrassicin	Phytoplan, Heidelberg, Germany	5187-84-8	3434.97
4MOI3M-Gls	4-Methoxyglucobrassicin	Phytoplan, Heidelberg, Germany	83327-21-3	3433.94

Table S3. LC-MS/MS parameters used for the multiple reaction monitoring (MRM) analyses on the API 5000 triple-quadrupole mass spectrometer (Sciex). Q1, m/z of quadrupole one; Q3, m/z of quadrupole three; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

Metabolites	Ionization mode	Q1 m/z	Q3	Retention time (min)	Dp (V)	EP (V)	CE (V)	CXP (V)
3MSOP-Gls	Negative mode	421.80	95.90	5.80	-95.00	-4.50	-60.00	0.00
4MSOB-Gls		435.90	95.80	8.00	-95.00	-5.00	-60.00	0.00
8MSOO-Gls		492.10	95.80	19.00	-105.00	-4.50	-58.00	0.00
5MSOP-Gls		449.90	95.80	11.60	-95.00	-5.00	-60.00	0.00
7MSOH-Gls		477.90	95.80	16.50	-95.00	-5.00	-60.00	0.00
4MTB-Gls		419.90	95.90	18.00	-90.00	-11.00	-58.00	0.00
I3M-Gls		447.00	95.80	20.00	-95.00	-12.00	-50.00	0.00
1MOI3M-Gls		477.00	95.80	24.40	-95.00	-12.00	-50.00	0.00
4MOI3M-Gls		477.10	95.80	22.00	-95.00	-12.00	-50.00	0.00
4MSOB-CN	Positive mode	146.00	129.00	1.60	63.00	10.00	13.00	4.00
4MSOB-ITC		178.11	114.00	2.50	51.00	5.00	13.00	4.00
4MSOB-ITC-GSH		485.11	179.10	2.00	76.00	5.50	29.00	6.00
4MSOB-ITC-CG		356.07	136.10	1.90	46.00	11.00	15.00	4.00
4MSOB-ITC-Cys		299.06	136.10	1.70	51.00	3.00	15.00	4.00
4MSOB-ITC-Cyc		265.11	201.00	1.10	51.00	8.50	25.00	4.00
4MSOB-ITC-NAC		341.07	178.10	2.20	51.00	3.00	17.00	6.00
I3C	Positive mode	146.058	118.10	1.18	66.00	3.50	19.00	4.00
I3C-CN (IAN)		157.168	130.00	8.50	51.00	7.50	15.00	4.00
I3M-Ascorbate		306.036	130.10	3.16	36.00	8.00	21.00	4.00
I3M-GSH		437.03	308.00	2.52	41.00	3.00	19.00	12.00
I3M-CG		308.016	76.00	2.71	106.00	4.50	35.00	4.00

I3M-Cys		251.067	77.00	7.53	21.00	2.00	77.00	4.00
I3M-NAC		293.029	76.90	4.60	21.00	8.00	83.00	4.00

References

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11. Acknowledgements

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I want to thank my love, Dr. Xingcong Jiang, for your love, hugs, and company all these years. Thank you, my dear, for offering me valuable advice in life and scientific work and for lending me helping hands to deal with endless lab work and scientific writing. Thank you, my dear, for providing me so many delicious and creative meals during all these years. Last but not least, I am ever grateful to my best friends who live in China but give me a great

many spiritual supports and help me go through the darkest time in my life.

Upon being encoded in China, I resolved that I should receive post-transcriptional modifications in Germany. With these done, the migration back to China in a functional status is programmed as my fate.

Jena, 18 Jun, 2020

Ruo Sun

11. Acknowledgements

12. Eigenständigkeitserklärung

Hiermit erkläre ich, dass mir die geltende Promotionsordnung der der Fakultät für Biowissenschaften der Friedrich-Schiller-Universität Jena bekannt ist. Entsprechend § 5 Abs. 4 der Promotionsordnung bestätige ich, dass ich diese Dissertation selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe. Die vorliegende Arbeit wurde selbständig und ohne unzulässige Hilfesmittel oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt. Übernommene Inhalte aus anderen Quellen und von anderen Personen, die in dieser Arbeit Verwendung fanden oder auf welche direkt Bezug genommen wird, sind als solche eindeutig kenntlich gemacht.

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Jena, 18 Jun, 2020

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13. Curriculum vitae

Basic Information

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Education

- Doctoral candidate (since September 2015): Faculty of Biological Sciences, Friedrich Schiller University Jena, Germany.
- Master of Agriculture in Plant Protection (2010-2012): College of Agronomy and Biotechnology, China Agricultural University (CAU), China.
- Bachelor of Agriculture in Plant protection (2006-2010): College of Agronomy and Biotechnology, China Agricultural University (CAU), China.

Publications

- **Sun R.**, Jiang X., Reichelt M., Gershenzon J., Pandit S. S., Vassão D. G. (2019). Tritrophic metabolism of plant chemical defenses and its effects on herbivore and predator performance. eLife, 8: e51029. doi:10.7554/eLife.51029.

4. **Sun R.**, Gols R., Harvey J., Reichelt M., Gershenzon J., Pandit S.S., Vassão D. G. (2020). Detoxification of plant defensive glucosinolates by an herbivorous caterpillar is beneficial to its endoparasitic wasp. Molecular Ecology, 29(20): 4014-4031. doi:10.1111/mec.15613.

- **Sun R.**, Jiang X., Reichelt M., Gershenzon J., Vassão D. G. Adaptation of cabbage aphid to aliphatic glucosinolates overwhelms detoxification in a lacewing predator. Journal of Pest Science (in preparation).

Conference Presentations

Oral presentations

- **Sun R.** (2019). Detoxification of Brassicaceae glucosinolates in multi-trophic interactions. Talk presented at 10th Conference of Asia-Pacific Association of Chemical Ecologists, Hangzhou, CN
- **Sun R.** (2019). *Plutella xylostella* glucosinolate sulfatase, more than a counter-adaptation to the plant mustard oil bomb? Talk presented at 18th IMPRS Symposium, Max Planck Institute for Chemical Ecology, IMPRS, Dornburg, DE
- **Sun R.** (2018). Metabolomics of Brassicaceae plants and their herbivores help explain the costs and benefits of glucosinolate detoxification. Talk presented at 34th ISCE Meeting, International Society of Chemical Ecology, Budapest, HU

Poster presentations

- **Sun R.**, Gershenzon J., Pandit S.S., Giddings Vassão D. (2019). Glucosinolate metabolism in tritrophic interactions. Poster presented at Institute Symposium, Max Planck Institute for Chemical Ecology, Jena, DE
- **Sun R.**, Giddings Vassão D., Gershenzon J., Pandit S.S. (2018). Revealing the role of *Plutella xylostella*'s glucosinolate sulfatase in its interactions with the specialist parasitoid *Diadegma semiclausum*. Poster presented at 17th IMPRS Symposium, International Max Planck Research School, Dornburg, DE
- Jeschke V., Shekhov A., Easson M., Christoff Wouters F., Luck K., Seibel E., Secinti S., Manivannan A., **Sun R.**, Pandit S.S., Gershenzon J., Giddings Vassão D. (2016). Detoxification and mode of action of plant defenses in insect herbivores. Poster presented at SAB Meeting 2016, MPI for Chemical Ecology, Jena, DE

Academic and Professional Training

- Functional Assays are Not "Just-So" Stories (JSMC Course), speaker: Ian T. Baldwin, Dapeng Li, Rayko Halitschke, Rishav Ray, Suhua Li (Nov 2019)
- Plant Transformation Workshop, speaker: Dr. Axel Schmidt, Dr. Maricel Santoro, Dr.

Lorenzo Caputi (Nov 2019)

- Bioinformatics tools for mass spectrometry, speaker: Dr. Ales Svatos, Dr. Robert Winkler, Dr. Riya Christina Menezes, Dr. Natalie Wielsch (Feb 2019)
- Plant morphometry, speaker: Dr. Felipe Yon (Jan 2019)
- Data visualisation, speaker: Dr. Rick Scavetta (Dec 2018)
- NMR spectroscopy, speaker: Dr. Christian Paetz, Bettina Dudek, Dr. Renate Ellinger (Jun 2018)
- Advanced English course for scientists, speaker: Dr. Andrew Durso (Apr-Jul 2018)
- Academic writing: How to create good texts, speaker: Dr. Melanie Lynn Conrad (Mar 2018)
- The basics of light and fluorescence microscopy, speaker: Dr. Veit Grabe (Feb 2018)
- Fundamentals of Mass Spectrometry, speaker: Dr. Ales Svatos, Dr. Athula Attygalle (Jun 2017)
- Adobe Illustrator, speaker: Dr. Nico Überschaar (May 2017)
- SciFinder - Chemical Abstracts Service, speaker: Dr. Karin Färber (Oct 2016)
- Introduction to Basic Statistics and R, speaker: Dr. Grit Kunert (Aug-Sep 2016)

Scientific Activities

- Supervised at IMPRS Recruitment 2019: candidate parenting (Sep 2019)
- Supervised at IMPRS Recruitment 2017: candidate parenting (Nov 2017)
- Presented at visit (weiterführende Schule): The role of Glucosinolates in plant-herbivore relationships and multitrophic interactions; Schüler/innen der Biologie-Kurses am Otto-Schott-Gymnasium (May 2017)

Jena, 18 Jun, 2020

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