



Hijacking the Mustard-Oil Bomb: How a Glucosinolate-Sequestering Flea Beetle Copes With Plant Myrosinases

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Myrosinase enzymes play a key role in the chemical defense of plants of the order Brassicales. Upon herbivory, myrosinases hydrolyze the β -S-linked glucose moiety of glucosinolates, the characteristic secondary metabolites of brassicaceous plants, which leads to the formation of different toxic hydrolysis products. The specialist flea beetle, *Phyllotreta armoraciae*, is capable of accumulating high levels of glucosinolates in the body and can thus at least partially avoid plant myrosinase activity. In feeding experiments with the myrosinase-deficient *Arabidopsis thaliana* *tgg1* \times *tgg2* (*tgg*) mutant and the corresponding *Arabidopsis* Col-0 wild type, we investigated the influence of plant myrosinase activity on the metabolic fate of ingested glucosinolates in adult *P. armoraciae* beetles. *Arabidopsis* myrosinases hydrolyzed a fraction of ingested glucosinolates and thereby reduced the glucosinolate sequestration rate by up to 50% in adult beetles. These results show that *P. armoraciae* cannot fully prevent glucosinolate hydrolysis; however, the exposure of adult beetles to glucosinolate hydrolysis products had no impact on the beetle's energy budget under our experimental conditions. To understand how *P. armoraciae* can partially prevent glucosinolate hydrolysis, we analyzed the short-term fate of ingested glucosinolates and found them to be rapidly absorbed from the gut. In addition, we determined the fate of ingested *Arabidopsis* myrosinase enzymes in *P. armoraciae*. Although we detected *Arabidopsis* myrosinase protein in the feces, we found only traces of myrosinase activity, suggesting that *P. armoraciae* can inactivate plant myrosinases in the gut. Based on our findings, we propose that the ability to tolerate plant myrosinase activity and a fast glucosinolate uptake mechanism represent key adaptations of *P. armoraciae* to their brassicaceous host plants.

Keywords: plant-insect interaction, two-component defense, herbivore adaptation, isothiocyanate, detoxification, *Phyllotreta*

INTRODUCTION

Many plants deter herbivores with a chemical defense that is activated upon tissue damage (Morant et al., 2008). The chemical defense compound is usually stored as inactive glucose conjugate in the vacuole of plant cells. When plant tissue is damaged, the glucose moiety is hydrolyzed by a defensive β -glucosidase, originally localized separately from the glucose conjugate, which liberates toxic and deterrent compounds (Morant et al., 2008; Pentzold et al., 2014b). Activated chemical defenses are usually more efficient in deterring chewing herbivores that cause extensive tissue damage than in deterring insects with less invasive feeding modes, such as sap suckers (Pentzold et al., 2014b).

A number of herbivorous insects evolved resistance against this plant defense strategy (Pentzold et al., 2014b) and some highly adapted species even accumulate (sequester) plant glucosides in their bodies and deploy them for defense against predators (Kazana et al., 2007; Opitz and Müller, 2009; Beran et al., 2019). However, it is currently not well understood how chewing insects prevent the hydrolysis of ingested plant glucosides by plant β -glucosidases. A study with turnip sawfly larvae (*Athalia rosae*) suggests that a rapid absorption of ingested glucosides (glucosinolates) across the gut epithelium prevents their hydrolysis in the gut; this is possibly facilitated by low plant β -glucosidase activity in the anterior gut (Abdalsamee et al., 2014). A rapid uptake mechanism was also proposed to enable western corn rootworm larvae (*Diabrotica virgifera virgifera*), to sequester benzoxazinoid glucosides; however, there was no evidence for reduced β -glucosidase activity in the larval gut (Robert et al., 2017). Burnet moth larvae (*Zygaena filipendulae*) which sequester cyanogenic glucosides avoid extensive plant β -glucosidase activity by a leaf-snipping feeding mode causing only minor tissue damage. Moreover, the alkaline pH in the midgut lumen of burnet moth larvae inhibits plant β -glucosidase activity (Pentzold et al., 2014a).

Sequestering herbivores must avoid the hydrolysis of the ingested plant glucosides they sequester, but the extent to which plant β -glucosidase activity influences glucoside sequestration has rarely been assessed. The brassicaceous model plant *Arabidopsis thaliana* offers an ideal system to address this question. The activated defense of *Arabidopsis* and other plants of the order Brassicales is the glucosinolate-myrosinase system (Halkier and Gershenzon, 2006; Blažević et al., 2020). Glucosinolates are a structurally diverse group of amino acid-derived thioglucosides that are hydrolyzed by β -thioglucosidases called myrosinases. The resulting aglucone is unstable and can give rise to different hydrolysis products including isothiocyanates, nitriles and epithionitriles (Figure 1). Which hydrolysis products are formed depends on various factors such as the structure of the glucosinolate side chain, pH conditions, and the presence of so-called plant specifier proteins (Wittstock et al., 2016). Among the possible hydrolysis products, isothiocyanates are the most reactive and thus toxic for small herbivores (Jeschke et al., 2016a). The *Arabidopsis tgg1* \times *tgg2* double knock-out mutant (*tgg*) is devoid of myrosinase activity in leaves (Barth and Jander, 2006) and thus can be

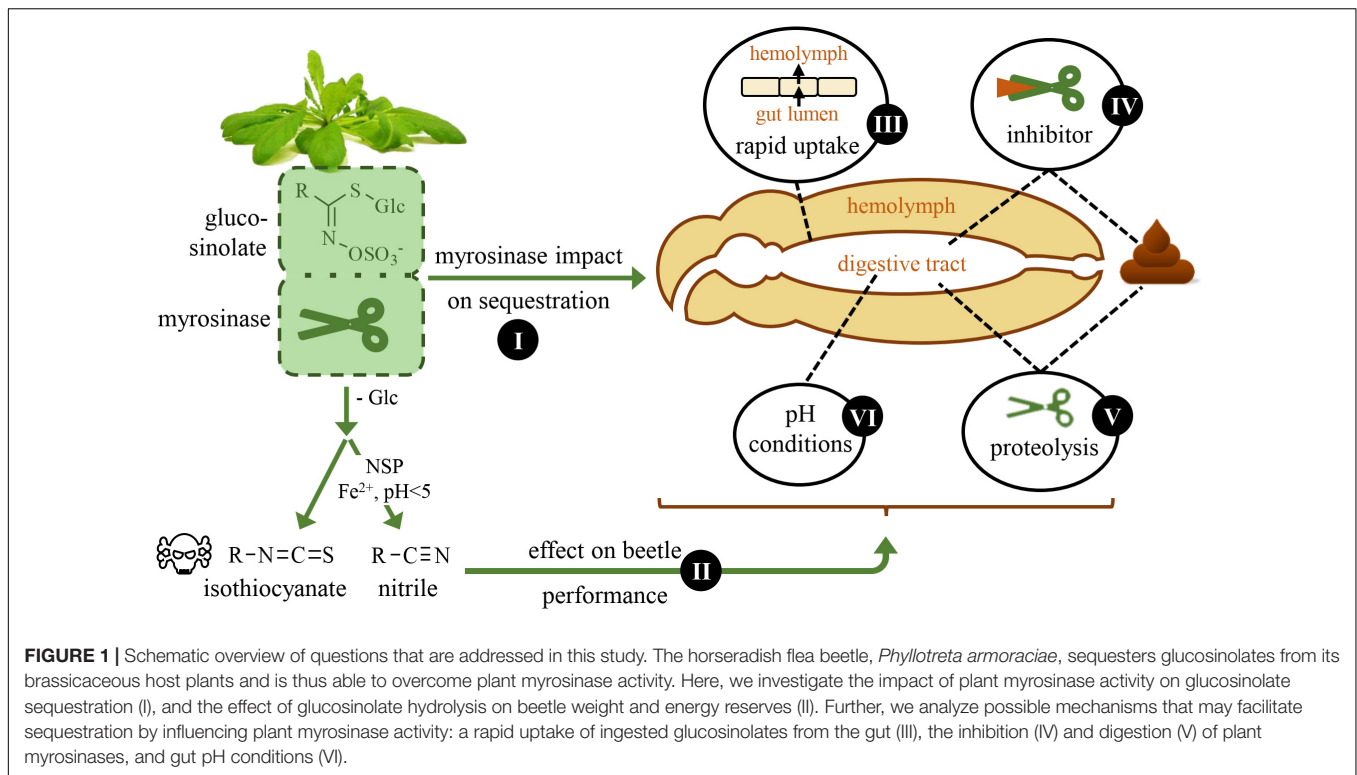
used for comparative feeding studies with the corresponding *Arabidopsis* Col-0 wild type (wild type). For example, in feeding experiments performed with the cabbage stem flea beetle, *Psylliodes chrysocephala*, adult beetles sequestered six times more glucosinolates from the myrosinase-deficient *Arabidopsis tgg* mutant than from the wild type (Beran et al., 2018). In a similar feeding experiment performed with larvae of the horseradish flea beetle, *Phyllotreta armoraciae*, only traces of sequestered glucosinolates were detected in wild type-fed larvae, whereas comparatively high glucosinolate levels were found in *tgg*-fed larvae (Sporer et al., 2020). In contrast to *P. armoraciae* larvae, adult beetles were able to sequester glucosinolates from wild type leaves (Yang et al., 2020), which suggests that plant myrosinase activity has a stronger influence on glucosinolate sequestration in larvae compared to adults. Plant myrosinase activity influences not only sequestration, but also the feeding behavior of *Phyllotreta* flea beetles. In field experiments, the crucifer flea beetle, *Phyllotreta cruciferae*, caused less feeding damage on *Brassica rapa* plants selected for high myrosinase activity than on plants selected for low myrosinase activity (Siemens and Mitchell-Olds, 1996).

Here, we investigated the influence of plant myrosinase activity on glucosinolate sequestration, the effect of glucosinolate hydrolysis on beetle performance and potential mechanisms that facilitate the sequestration of intact glucosinolates in the adult life stage of *P. armoraciae* (Figure 1). In a previous feeding study, we recovered about 35% of the total ingested glucosinolates from wild type leaves in the beetle body and feces, whereas the metabolic fate of more than 60% of the total ingested glucosinolates remained unknown (Yang et al., 2020). One possible explanation is that the unrecovered glucosinolates were hydrolyzed by plant myrosinases. To investigate this possibility, we performed a series of comparative feeding experiments with myrosinase-deficient and wild type *Arabidopsis* plants. In nature, *P. armoraciae* is closely associated with horseradish, *Armoracia rusticana*, a plant species that is characterized by high levels of allyl glucosinolate (Li and Kushad, 2004; Ciska et al., 2017). Therefore, we additionally investigated the influence of plant myrosinase activity on the sequestration of allyl glucosinolate by spiking the intact glucosinolate into *Arabidopsis* leaves. We observed a negative influence of plant myrosinase activity on glucosinolate sequestration and confirmed that a fraction of ingested glucosinolates is hydrolyzed in *P. armoraciae*. We thus asked whether the metabolism of glucosinolate hydrolysis products incurs a metabolic cost in *P. armoraciae*. Finally, we explored possible mechanisms that allow *P. armoraciae* to partially prevent the hydrolysis of ingested glucosinolates by plant myrosinase.

MATERIALS AND METHODS

Plants and Insects

Food plants, *Brassica juncea* cv. “Bau Sin” and *Brassica rapa* cv. “Yu-Tsai-Sum” (Known-You Seed Co., Ltd., Taiwan) were cultivated in a controlled environment chamber (24°C, 55% relative humidity, 14 h light/10 h dark period).



Arabidopsis thaliana plants were cultivated under short day conditions in a controlled environment chamber (21°C, 55% relative humidity, 10-h light/14-h dark period). Experiments were performed with *A. thaliana* Col-0 (wild type) and two mutants in the *A. thaliana* Col-0 background: the myrosinase deficient *A. thaliana* *tgg1* × *tgg2* (*tgg*) double knockout mutant (Barth and Jander, 2006), and the *A. thaliana* *myb28* × *myb29* (*myb*) double knockout mutant which does not produce aliphatic glucosinolates (Sønderby et al., 2007).

The laboratory rearing of *Phyllotreta armoraciae* was established in October 2012 with beetles collected from horseradish plants in Laasdorf, Thuringia, Germany. The beetles are relatively small with an approximate adult body length of 3 mm and weight of about 2–3 mg per beetle. Beetles were reared on potted 3–4 week old *B. juncea* or *B. rapa* plants in a controlled environment chamber (24°C, 60% relative humidity, 14 h light/10 h dark period). Adult beetles were provided with new plants every week and plants with eggs were kept separately for larval development. After 3 weeks, any remaining plant material was removed and the soil containing pupae was kept in plastic containers (9 L volume, Lock&Lock, Seoul, South Korea). Newly emerged adults were collected every 2–3 days. Unless stated otherwise, experiments were performed with newly emerged beetles that had been reared on *B. juncea* plants. We did not determine the sex of the beetles used in experiments unless stated otherwise.

Sequestration Experiments

To analyze whether plant myrosinase activity influences the sequestration of glucosinolates in *P. armoraciae* beetles, we

performed sequestration experiments with the myrosinase-deficient *Arabidopsis* *tgg* mutant and the corresponding wild type Col-0.

In *Experiment 1*, we fed newly emerged beetles for 1 day with detached leaves of *Arabidopsis* wild type or *tgg* plants ($n = 28$ per genotype, two beetles per replicate). On the next day, each remaining leaf was weighed, frozen in liquid nitrogen, and stored at -20°C until freeze drying. To allow for digestion of ingested plant material, we fed the beetles one additional day on *Arabidopsis* *myb* leaves before beetles were weighed, frozen in liquid nitrogen, and stored at -20°C until extraction. The extraction and analysis of glucosinolates by high performance liquid chromatography coupled with diode array detection (HPLC-DAD) was performed as described in Beran et al. (2014). Adult *P. armoraciae* beetles convert ingested 4-methylsulfinylbutyl (4MSOB) glucosinolate, the major aliphatic glucosinolate in *Arabidopsis* wild type and *tgg* plants, into 4-methylthiobutyl (4MTB) glucosinolate (Yang et al., 2020). Therefore, we summed up the concentrations of 4MSOB and 4MTB glucosinolates per mg fresh weight in each beetle sample and expressed this concentration relative to the concentration of both glucosinolates per mg fresh weight in the corresponding leaf sample, which was set to 1. To establish whether beetles feed equal amounts from both *Arabidopsis* lines, we quantified the beetle feeding damage (2 beetles per leaf disc with 16 mm diameter) over 1 day using the software Fiji (Schindelin et al., 2012) ($n = 8$ per genotype).

In *Experiment 2*, we fed newly emerged beetles with *Arabidopsis* wild type, *tgg*, or *myb* leaves for 1 day ($n = 5$, 5 beetles per replicate). Afterwards, feces was collected in 50 μL

ultrapure water containing 0.1% (v/v) formic acid, mixed with 50 μ L pure methanol and stored at -20°C . Beetles were frozen in liquid nitrogen and stored at -20°C until extraction. Remaining leaves were weighed, frozen in liquid nitrogen and freeze-dried. Feces samples were homogenized for 2 min at 25 Hz in a TissueLyzerII (Qiagen, Hilden, Germany) using metal beads. Beetles were homogenized in 500 μ L 50% (v/v) methanol using plastic pestles. Freeze-dried leaves were homogenized to powder as described for feces samples and extracted with 800 μ L of 50% (v/v) methanol. Samples were centrifuged at 4°C for 10 min at $16,000 \times g$ and supernatants were stored at -20°C until analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). We only quantified 4MSOB glucosinolate and 4MSOB glucosinolate-derived hydrolysis products in this experiment because currently no LC-MS/MS method for the quantification of 4MTB glucosinolate-derived hydrolysis products is available. Identification and quantification of 4MSOB glucosinolate and 4MSOB glucosinolate-derived metabolites in samples was performed on an Agilent 1200 HPLC system (Agilent, Santa Clara, CA, United States) connected to an API3200 tandem mass spectrometer (AB Sciex Germany GmbH, Darmstadt, Germany) using external standard curves prepared from authentic standards of each compound as described in Beran et al. (2018). After quantification, we subtracted the average amounts of each metabolite in bodies or feces of *myb*-fed control beetles (background control) from those detected in wild type- or *tgg*-fed beetles.

In *Experiment 3*, we fed newly emerged beetles for 1 day with *Arabidopsis* wild type or *tgg* leaves containing 300 nmol allyl glucosinolate that was introduced by placing the petiole into an aqueous allyl glucosinolate solution (Carl Roth, Mannheim, Germany) as described in Schramm et al. (2012). This experiment was performed with adult beetles that had been reared on *B. rapa* plants, which do not produce allyl glucosinolate (Beran et al., 2018). The glucosinolate profile of unfed beetles reared on *B. rapa* was analyzed as described in Beran et al. (2014) ($n = 20$, with 5 beetles per replicate) to confirm that allyl glucosinolate is not present in beetles. To facilitate the distribution of spiked allyl glucosinolate within the leaf, we placed the petiole in a reaction tube filled with water. Newly emerged beetles were fed with spiked *Arabidopsis* leaves for 1 day ($n = 10$ per genotype, 5 beetles per replicate) and allyl glucosinolate-spiked leaves without beetles were kept under the same conditions as a recovery control ($n = 8$ – 10 per genotype). Each leaf was sampled separately, frozen in liquid nitrogen, freeze-dried, and homogenized with metal beads for 2 min at 25 Hz in a TissueLyzerII (Qiagen). Beetles were weighed and frozen in liquid nitrogen. Feces was collected in 100 μ L ultrapure water and mixed with 100 μ L pure methanol. Leaf and beetle samples were homogenized in 1 mL and 800 μ L 50% (v/v) methanol, respectively. After centrifugation for 10 min at $16,000 \times g$, supernatants were collected. Feces samples were homogenized as described for leaves and centrifuged at 4°C for 10 min at $16,000 \times g$. The supernatant was collected, the solvent evaporated using nitrogen and extracts were re-dissolved in 80 μ L 50% methanol. Samples were stored at -20°C until LC-MS/MS as described in Malka et al. (2016) using a modified elution gradient. The gradient consisted of formic acid (0.2%) in

water (solvent A) and acetonitrile (solvent B) and was carried out as follows: 1.5% (v/v) B (1 min), 1.5–5% (v/v) B (5 min), 5–7% (v/v) B (2 min), 7–12.6% (v/v) B (4 min), 12.6–100% (v/v) B (0.1 min), 100% (v/v) B (0.9 min), 100 to 1.5% (v/v) B (0.1 min), and 1.5% (v/v) B (3.85 min). Allyl glucosinolate was quantified using an external calibration curve. We recovered 97.8 ± 6.5 and $109.5 \pm 4.4\%$ (mean \pm SD) of the spiked glucosinolate from undamaged (control) wild type and *tgg* leaves, respectively, showing that only small amounts of spiked allyl glucosinolate were metabolized in *Arabidopsis* leaves under our assay conditions. To determine how much allyl glucosinolate beetles had ingested, we subtracted the allyl glucosinolate amount detected in each fed leaf from the average allyl glucosinolate amounts recovered from corresponding unfed control leaves. The amounts of allyl glucosinolate that were recovered in beetles and feces were expressed relative to the total ingested amount, which was set to 100%.

In *Experiment 4*, we fed newly emerged beetles with allyl glucosinolate-spiked *Arabidopsis* wild type or *tgg* leaves (prepared as described in *Experiment 3*) for 1 day and simultaneously collected the headspace on Porapak-QTM volatile collection traps (25 mg; ARS, Inc., Gainesville, FL, United States) ($n = 6$ – 7 per genotype, 8 beetles per replicate). Leaves without beetles served as controls ($n = 4$ per genotype). The volatile collection and sample analysis by gas chromatography mass spectrometry (GC-MS) was performed as previously described in Sporer et al. (2020). Allyl isothiocyanate was quantified in headspace samples using an external calibration curve prepared from an authentic standard (Sigma-Aldrich, Steinheim, Germany). The glucosinolate amount per fed beetle was determined as described in *Experiment 1*.

Performance Experiment

To investigate whether the hydrolysis of ingested glucosinolates has a negative influence on beetle performance, we compared the fresh weight and energy reserves of beetles that had fed for 10 days on *Arabidopsis* wild type or *tgg* leaf discs. We separated newly emerged beetles into males and females and assigned them randomly to one of the two *Arabidopsis* genotypes ($n = 10$ females per genotype, $n = 8$ – 9 males per genotype). Each beetle was provided with a new leaf disc from an undamaged *Arabidopsis* plant every day for 10 consecutive days. After 10 days feeding, beetles were weighed, frozen in liquid nitrogen, and stored at -20°C until analysis of energy reserves. The contents of soluble protein, total lipids, glycogen, and soluble carbohydrates in individual beetles were determined as described in Foray et al. (2012) with minor modifications. Instead of a 96-well borosilicate microplate, we used a 96-well quartz glass microplate (Hellma Analytics, Müllheim, Germany) that was covered with MicroAmp clear adhesive film (Applied Biosystems, Waltham, MA, United States). The plate was heated using a ThermoMixer (Eppendorf, Hamburg, Germany) and for measurements we used a Tecan Infinite 200 Reader (Tecan, Crailsheim, Germany). As a control, we quantified the levels of soluble protein, amino acids, and sugars in rosette leaves of *Arabidopsis* wild type and *tgg* mutant plants (for details refer to **Supplementary Material**).

Distribution of Glucosinolates in *P. armoraciae* Shortly After Ingestion

To investigate whether ingested glucosinolates are rapidly absorbed across the gut epithelium in *P. armoraciae*, we allowed newly emerged beetles to feed for 1 min on wild type or *tgg* leaves. After 5 min, beetles were dissected into gut and remaining body (without head; $n = 3$, 3 beetles per replicate). Non-fed beetles were used as background control ($n = 2$ – 3 , 3 beetles per replicate). Dissected guts were washed twice in phosphate-buffered saline (PBS) pH 7.4 (Bio-Rad, Munich, Germany) before sampling. Samples were homogenized in 500 μ L 80% methanol containing 0.4 μ M 4-hydroxybenzyl glucosinolate as internal standard using plastic pestles and stored at -20°C until extraction and analysis by LC-MS/MS as described above in *Experiment 2*. We quantified 4MSOB glucosinolate in each sample using an external standard curve and expressed the glucosinolate distribution in the gut and rest of the body relative to the total amount detected in both samples (set to 100%).

Myrosinase Inhibition Assays

To determine whether *P. armoraciae* can inhibit ingested myrosinase activity, we performed myrosinase activity assays with gut content extracts of adult beetles. The gut content of adults was collected as follows: dissected guts were washed in extraction buffer [20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.2] containing protease inhibitors (cOmplete, EDTA-free, Roche, Mannheim, Germany) and cut open longitudinally to collect the gut content in 2.5 μ L of extraction buffer. For each sample, gut contents from 20 beetles were pooled, frozen in liquid nitrogen and stored at -20°C until extraction ($n = 4$). Samples were homogenized with metal beads for 2 min at 25 Hz in a TissueLyzer II, centrifuged at 4°C for 10 min at $16,000 \times g$ and the supernatant split into two subsamples of which one was boiled for 5 min at 99°C .

Assays (50 μ L total volume) consisted of 0.1 mM ascorbic acid (Fluka, Buchs, Switzerland), 0.2 mM 4MSOB glucosinolate (substrate), 0.5 ng/ μ L partially purified myrosinase from *Sinapis alba* (Sigma-Aldrich, details of protein purification are described in the **Supplementary Material**) and (a) gut content extract (corresponding to four beetles), (b) boiled gut content extract, or (c) extraction buffer (control). Assays with gut extracts but without myrosinase and assays containing only substrate were used as additional controls. Assays were incubated for 15 min at 30°C , the reaction was stopped by 5 min boiling at 99°C and extracted with 100 μ L 80% methanol containing 0.2 mM 4-hydroxybenzyl glucosinolate as an internal standard. Myrosinase activity was determined by quantifying the remaining 4MSOB glucosinolate amount in each assay as described in *Experiment 1*.

Detection of Myrosinase Enzyme and Activity in Beetle Feces

To determine whether *P. armoraciae* can degrade ingested plant myrosinase enzymes, we collected feces of 90 adults that had fed on *Arabidopsis* wild type leaves for 1 day in a total volume of 1 mL 20 mM MES buffer pH 6.5 containing protease inhibitors (cOmplete, EDTA-free). After homogenization with metal beads

for 3 min at 25 Hz in a TissueLyzer II and centrifugation at 4°C for 10 min at $16,000 \times g$, we precipitated soluble proteins in the supernatant using trichloroacetic acid and washed the pellet with acetone. The protein pellet was dissolved in Laemmli buffer (Bio-Rad), boiled for 15 min at 95°C , and separated on a 12.5% Criterion Tris-HCl precast gel (Bio-Rad). Protein bands were stained with colloidal Coomassie G250 (Carl Roth), excised from the gel, and digested with porcine trypsin (Promega, Madison, WI, United States) as described in Shevchenko et al. (2006). Samples were re-dissolved in 30 μ L 1% (v/v) formic acid and 2 μ L were analyzed by nano-UPLC-MS^E analysis as described in Vassão et al. (2018). Data were acquired using data-independent acquisition, referred to as enhanced MS^E. MS data were collected using MassLynx v4.1 software (Waters, Milford, MA, United States).

The processing of nano-UPLC-MS^E data and protein identification was performed as follows: the acquired continuum of LC-MS^E data were processed using the ProteinLynx Global Server (PLGS) version 2.5.2 (Waters) to generate product ion spectra for database searching according to the ion accounting algorithm described in Li et al. (2009). Processed data were searched against a reference sequence (Refseq) database containing *Arabidopsis thaliana* sequences (40785 sequences, downloaded from the Identical Protein Groups database at the National Center for Biotechnology Information (NCBI)¹ combined with a subdatabase containing common contaminants (O'Leary et al., 2016). Database searching was performed at a false discovery rate (FDR) of 2% with the following parameters: minimum numbers of fragments per peptide (3), peptides per protein (1), fragments per protein (7), and maximum number of missed tryptic cleavage sites (1). Searches were restricted to tryptic peptides with a fixed carbamidomethylation of cysteine residues along with variable oxidation of methionine. Proteins were classified according to the algorithm described for PAnalyzer software (Prieto et al., 2012) and divided into four groups: conclusive, indistinguishable, ambiguous, and non-conclusive. Conclusive and indistinguishable hits were considered as confident matches.

To determine whether *P. armoraciae* excretes active myrosinase enzyme, we analyzed myrosinase activity in feces homogenates and compared this activity with the corresponding ingested myrosinase activity in leaves. We used newly emerged *P. armoraciae* beetles that had been reared on *B. rapa*, and fed them for 1 day with *myb* leaves containing myrosinase activity but no 4MSOB glucosinolate ($n = 6$, 6 beetles per replicate). Leaves were weighed before and after feeding to determine the ingested plant fresh weight. Leaves were supplied with water during the experiment and the average proportional weight gain of intact leaves was used to correct the initial leaf weight ($n = 16$). Feces from each replicate were collected in 130 μ L extraction buffer [20 mM MES buffer, pH 6.5, containing protease inhibitors (cOmplete, EDTA-free)]. Feces and fed leaves were frozen in liquid nitrogen and stored at -80°C until extraction. Feces samples were homogenized with metal beads at 25 Hz for 2 min in a TissueLyzer II. The corresponding frozen

¹<https://www.ncbi.nlm.nih.gov/ipg> on February 28, 2020

leaf samples were homogenized with metal beads at 25 Hz for 2 min in a pre-cooled sample holder to prevent thawing. For each replicate, we calculated the ingested fresh weight and extracted the corresponding amount of homogenized plant tissue in the same buffer volume used for feces extraction. After centrifugation at 4°C for 10 min at $16,000 \times g$, the supernatant was directly used for myrosinase activity assays. Assays consisted of 25 μ L extraction buffer, 5 μ L of an aqueous 11 mM 4MSOB glucosinolate solution, and (a) 25 μ L feces homogenate or (b) 25 μ L leaf extract. Assays containing only extraction buffer and substrate served as background control ($n = 3$). To test whether feces extracts have an inhibitory effect on plant myrosinase activity, we additionally performed assays in which we mixed 25 μ L of feces homogenate with 25 μ L of corresponding leaf extract and 5 μ L glucosinolate substrate. Except for three assays with combined feces and leaf extracts, all assays were performed with two technical replicates. Assays were incubated for 30 min at 30°C, stopped by boiling for 5 min at 95°C, and 50 μ L of 60% (v/v) methanol were added. After the activity assay, samples containing feces homogenates were centrifuged at 4°C for 10 min at $16,000 \times g$, the supernatant collected and final samples were stored at -20°C until LC-MS/MS analysis. 4MSOB glucosinolate was quantified as described in *Experiment 2* and myrosinase activity was expressed as nmol 4MSOB glucosinolate hydrolyzed per minute and mg (ingested) plant fresh weight.

Statistical Analyses

Statistical analyses were performed in R 3.3.1 (R Core Team, 2018) or in SigmaPlot 11.0 (Systat Software). Details of statistical analyses performed for each dataset are summarized in **Supplementary Table 1**.

RESULTS

Plant Myrosinase Activity Influences Glucosinolate Sequestration

We examined the influence of plant myrosinase activity on glucosinolate sequestration in *P. armoraciae* by comparing the relative accumulation of 4MSOB and 4MTB glucosinolates in adults that fed on *Arabidopsis* leaves with (wild type) or without (*tgg*) myrosinase activity (*Experiment 1*). *Tgg*-fed beetles accumulated twofold higher levels of glucosinolates than wild type-fed beetles (Mann-Whitney rank sum test, $U = 482.000$, $p < 0.001$; **Figure 2A**), whereas beetle feeding rates and glucosinolate levels did not differ between both plant genotypes (results of statistical analyses are shown in **Supplementary Table 1**).

To determine whether the lower glucosinolate accumulation in wild type-fed beetles is due to glucosinolate hydrolysis, we analyzed the levels of 4MSOB glucosinolate and known hydrolysis products in bodies and feces of wild type- and *tgg*-fed beetles (*Experiment 2*). Because the detected metabolite levels differed greatly between replicates (**Supplementary Table 2**), we compared the amounts of hydrolysis products relative to the total amounts of glucosinolate and hydrolysis products in each sample. We detected a significantly higher proportion of

hydrolysis products in body and feces samples of wild type-fed beetles than in the corresponding samples of *tgg*-fed beetles (**Figure 2B**; beetles: Student's *t*-test, $t = 2.754$, $p = 0.025$, feces: Student's *t*-test, $t = 7.229$, $p < 0.001$). 4MSOB cyanide represented the dominant hydrolysis product in body and feces samples, whereas only traces of free and metabolized isothiocyanates were found (**Supplementary Table 2** and **Figure 2B**). Overall, glucosinolate hydrolysis products accounted for 28% and 2.2% of the total detected metabolites in wild type- and *tgg*-fed beetles, respectively (bodies and feces). Together, our results show that a fraction of ingested 4MSOB glucosinolate was hydrolyzed by the plant myrosinase, but that glucosinolate hydrolysis also occurred independently of plant myrosinase activity.

Because allyl glucosinolate represents the major glucosinolate in the natural host plant of *P. armoraciae*, we quantified the impact of plant myrosinase activity on the metabolic fate of ingested allyl glucosinolate (*Experiment 3*). This experiment was performed with beetles that were reared on *B. rapa* and thus do not contain allyl glucosinolate (**Supplementary Table 3**). *Tgg*-fed beetles accumulated 9% more of the total ingested allyl glucosinolate than wild type-fed beetles (**Table 1**), confirming a negative influence of plant myrosinase activity on sequestration. In a separate feeding experiment, we quantified the emission of the volatile allyl glucosinolate hydrolysis product allyl isothiocyanate (*Experiment 4*), and detected significantly higher amounts of allyl isothiocyanate in the headspace of wild type-fed beetles than in the headspace of *tgg*-fed beetles (**Figure 3A**). However, in this experiment, we found no difference regarding the amounts of sequestered allyl glucosinolate in wild type and *tgg*-fed beetles (**Figure 3B**; results of statistical analyses are summarized in **Supplementary Table 1**).

Feeding on *Arabidopsis* Genotypes With or Without Myrosinase Activity Does Not Affect Beetle Weight and Energy Reserves

To determine whether glucosinolate hydrolysis influences beetle performance, we compared the beetle fresh weight and different energy reserves in males and females after feeding on *Arabidopsis* wild type and *tgg* leaves for 10 days after eclosion. As a control, we compared the nutritional value of wild type and *tgg* leaves and found no differences regarding the total levels of soluble protein, free amino acids, soluble sugars and glucosinolates between wild type and *tgg* plants (**Supplementary Table 4**). The food plant had no influence on the beetle weight or the levels of soluble protein, lipids, glycogen and soluble carbohydrates in both males and females (**Supplementary Table 5**).

P. armoraciae Rapidly Absorbs Glucosinolates Across the Gut

To establish whether *P. armoraciae* can rapidly absorb ingested glucosinolates, we analyzed the distribution of 4MSOB glucosinolate in *P. armoraciae* beetles shortly after feeding for 1 min on wild type and *tgg* leaves. We recovered more than 80% of the total detected 4MSOB glucosinolate from the body (without gut) and this glucosinolate distribution did not differ

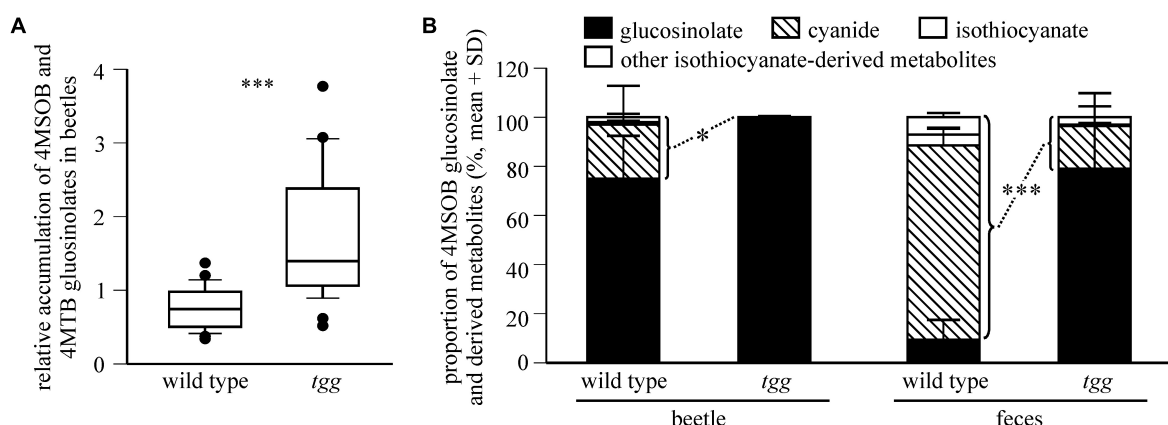


FIGURE 2 | Plant myrosinase activity influences glucosinolate sequestration in *P. armoraciae*. **(A)** Accumulation of 4-methylsulfanylbutyl (4MSOB) and 4-methylthiobutyl (4MTB) glucosinolates in *P. armoraciae* adults after feeding on *Arabidopsis* wild type and myrosinase-deficient *tgg* leaves ($n = 28$ per plant genotype). Glucosinolates in beetles and fed leaves were extracted, converted to desulfo-glucosinolates and analyzed by high performance liquid chromatography coupled to diode array detection (HPLC-DAD). The glucosinolate concentration per mg beetle fresh weight is expressed relative to the concentration per mg plant fresh weight in the corresponding fed leaf, which was set to 1. Boxplots show the median and the 25th and 75th percentile. Dots represent data points that lie outside the 10th and 90th percentiles. Data were compared by Mann-Whitney rank sum test ($***p < 0.001$). **(B)** Relative composition of 4MSOB glucosinolate and hydrolysis products detected in bodies and feces of wild type- or *tgg*-fed adults ($n = 5$ per plant genotype). Beetles that had fed on the *Arabidopsis myb* mutant served as a control ($n = 5$). Glucosinolates and hydrolysis products were extracted with 50% methanol and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Metabolites were quantified using external standard curves. The average amounts of each metabolite detected in control beetles and their feces was subtracted from the corresponding samples of wild type and *tgg*-fed beetles. The proportions of hydrolysis products relative to the total amount of detected glucosinolate and hydrolysis products in bodies and feces of wild type and *tgg*-fed beetles (set to 100%, respectively) were compared by Student's *t*-tests (beetles: $*p = 0.025$, feces $***p < 0.001$). Results of statistical analyses are shown in **Supplementary Table 1**. 4MSOB cyanide corresponds to the nitrile hydrolysis product of 4MSOB glucosinolate. Other isothiocyanate-derived metabolites comprise 4MSOB isothiocyanate-glutathione conjugate, 4MSOB isothiocyanate-cysteinylglycine conjugate, 4MSOB isothiocyanate-cysteine conjugate, 2-(4-(methylsulfanyl)butylamino)-4,5-dihydrothiazole-carboxylic acid, 4MSOB amine, and 4MSOB acetamide.

TABLE 1 | Recovery of ingested allyl glucosinolate in *P. armoraciae*.

	Mean percentage \pm SD ($n = 10$)		t^b	p
	Wild type-fed	<i>tgg</i> -fed		
Beetle	43.8 \pm 8.2	53.0 \pm 4.8	2.924	0.009
Feces	0.2 \pm 0.3	0.3 \pm 0.1	0.655	0.521
Unknown ^a	56.0 \pm 8.4	46.7 \pm 4.8	2.883	0.010

^aProportion of ingested allyl glucosinolate that could not be recovered.

^bData were arcsine square root transformed before statistical analysis by Student's *t*-test.

between wild type- and *tgg*-fed beetles (Student's *t*-test, $t = 0.075$, $p = 0.944$, **Figure 4A**).

Gut Content Extracts of *P. armoraciae* Reduce Myrosinase Activity *in vitro*

To determine whether glucosinolate sequestration may be facilitated by suppression of plant myrosinase activity in the gut lumen of *P. armoraciae*, we analyzed the influence of beetle gut content extracts on plant myrosinase activity in *in vitro* assays. Compared to control assays, gut content extracts significantly reduced myrosinase activity by up to 50% (ANOVA, $F = 85.639$, $p < 0.001$). Boiled gut content extracts reduced myrosinase activity significantly less than untreated extracts (**Figure 4B**).

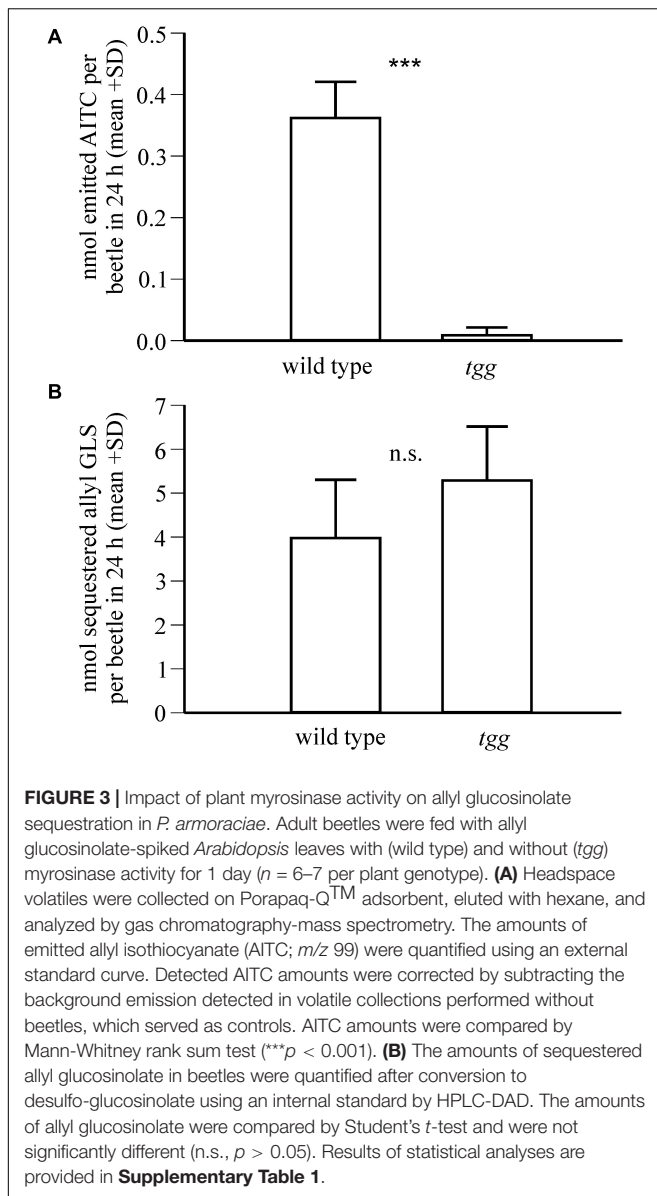
P. armoraciae Excretes Inactive Myrosinase Enzyme

To investigate whether the *Arabidopsis* myrosinases TGG1 and TGG2 are degraded in the gut of *P. armoraciae*, we analyzed the fecal proteome. We detected a total of 14 peptides derived from the *Arabidopsis* myrosinase TGG1 in two protein bands between 55 and 70 kDa covering 34% of the TGG1 amino acid sequence (**Figures 5A,B** and **Supplementary Table 6**). The molecular weight range in which we detected TGG1 peptides corresponds approximately to the predicted (61.1 kDa) and apparent (75 kDa) molecular weight of TGG1 (Zhou et al., 2012). TGG2-specific peptides were not detected in feces with our approach.

Since the proteomic analysis indicates that *P. armoraciae* excretes intact myrosinase, we compared the levels of ingested myrosinase activity with those that were excreted. Myrosinase activity detected in feces corresponded to less than 4% of the ingested activity (**Figure 6**, $t = 10.449$, $p < 0.005$). We additionally spiked plant myrosinase extracts into feces homogenates, but observed similar activity as in control assays (**Figure 6**, $t = 0.158$, $p = 1.000$).

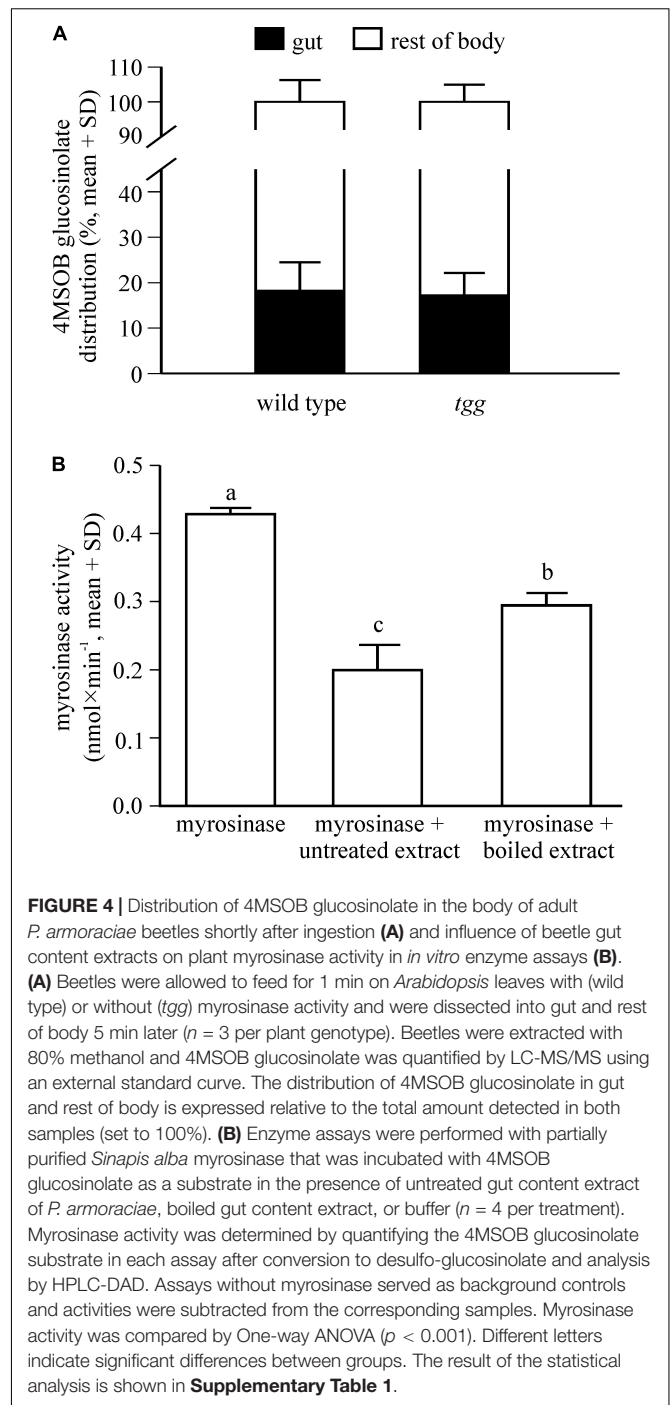
DISCUSSION

The enzymatic activity of defensive plant β -glucosidases is a major barrier to the sequestration of plant glucosides by herbivorous insects (Morant et al., 2008; Pentzold et al., 2014b).



Here, we investigated the impact of plant myrosinase activity on the sequestration of glucosinolates in *P. armoraciae* and explored possible mechanisms that enable adult beetles to suppress glucosinolate hydrolysis during feeding and digestion. We demonstrate a negative influence of plant myrosinase activity on sequestration and confirmed that a fraction of ingested glucosinolates is hydrolyzed by plant myrosinases. We found two mechanisms that can reduce the glucosinolate hydrolysis rate in the gut: the rapid absorption of ingested glucosinolates across the gut epithelium and the suppression of plant myrosinase activity in the gut lumen.

In previous quantitative feeding studies with *P. chrysocephala*, plant myrosinases hydrolyzed approximately 75% of the total ingested glucosinolates (Beran et al., 2018; Ahn et al., 2019), whereas only about 10% of ingested allyl glucosinolate were hydrolyzed in our quantitative study with *P. armoraciae*.



Although *P. chrysocephala* and *P. armoraciae* belong to different genera, they have similar body sizes and feeding modes and therefore cause comparable feeding damage. Our results thus indicate that *P. armoraciae* adults are better adapted to overcome plant myrosinase activity than *P. chrysocephala* adults.

Plant myrosinases hydrolyzed a fraction of ingested glucosinolates and thus negatively influenced glucosinolate sequestration in *P. armoraciae* (Figures 2, 3 and Table 1). Although the sequestration rates of 4MSOB glucosinolate and



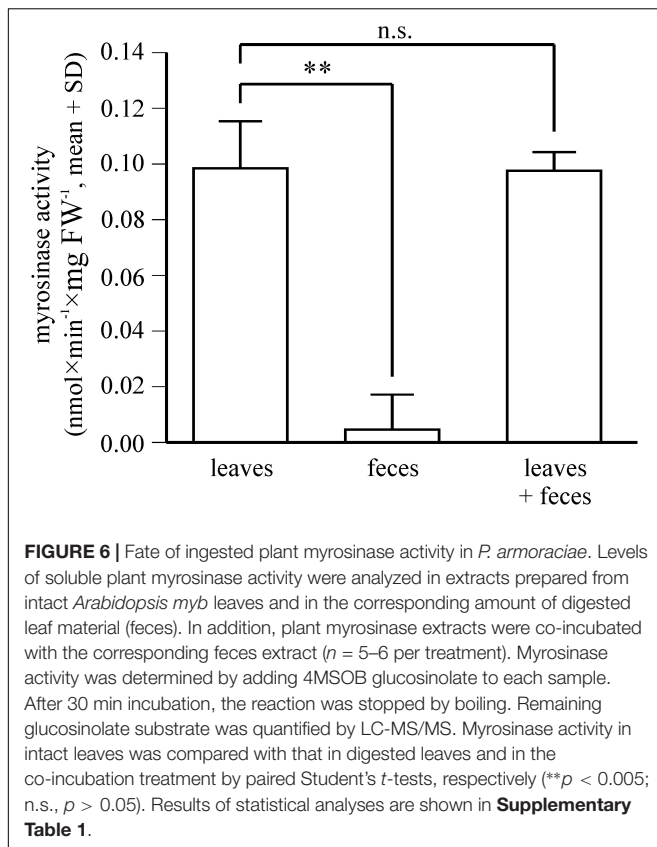
FIGURE 5 | Detection of *Arabidopsis* myrosinases in the feces of *P. armoraciae*. **(A)** One dimensional SDS/PAGE gel of crude feces protein extract. Numbers indicate samples excised for proteomic analysis by nano-UPLC-MS² (only odd numbers are shown). TGG1-derived peptides were detected in bands marked with asterisks (*). **(B)** Amino acid sequence alignment of the *Arabidopsis* myrosinases TGG1 (AT5G26000.1) and TGG2 (AT5G25980.2). Identical amino acids in the TGG2-sequence are represented by a dot. Peptides highlighted with black background were detected by nano-UPLC-MS² (Supplementary Table 6). Only one detected peptide matched both TGG1 and TGG2.

allyl glucosinolate cannot be compared directly because different methods were used for quantification, our results indicate a stronger influence of plant myrosinase activity on 4MSOB glucosinolate than on allyl glucosinolate. Biochemical studies with *Arabidopsis* myrosinases TGG1 and TGG2 revealed similar activities of both enzymes toward these two glucosinolates (Zhou et al., 2012), making it unlikely that the substrate preferences of *Arabidopsis* myrosinases have affected sequestration. A similar glucosinolate-dependent effect of plant myrosinase activity on sequestration was also observed in *P. armoraciae* larvae (Sporer et al., 2020). Larvae sequestered allyl glucosinolate from *B. juncea* leaves but almost no glucosinolates from *Arabidopsis* wild type leaves. Although we detected similar levels of soluble myrosinase activity in crude protein extracts of *Arabidopsis* and *B. juncea* leaves in enzyme assays, we cannot rule out that different levels of myrosinase activity in *Arabidopsis* and *B. juncea* were responsible for this result. Nevertheless, our results suggest that the metabolic fate of ingested allyl glucosinolate in *P. armoraciae* is less affected by plant myrosinase activity than that of *Arabidopsis* glucosinolates. Since allyl glucosinolate represents the dominant glucosinolate in horseradish (Li and Kushad, 2004), *P. armoraciae* might have developed specific mechanisms to avoid the hydrolysis of the characteristic glucosinolate of its natural host plant.

Glucosinolate hydrolysis products, in particular isothiocyanates, are well-known to have a negative impact on insect growth and development by interfering with nutrition (Agrawal and Kurashige, 2003; Jeschke et al., 2016b, 2017; Sun et al., 2019). Under our experimental conditions, feeding beetles

with *Arabidopsis* with or without myrosinase activity did not influence the weight or energy reserves of *P. armoraciae* adults. In addition, the food plant did not influence the developmental time, weight, and energy reserves of *P. armoraciae* larvae (for details refer to **Supplementary Material and Supplementary Table 7**). Although the exposure to glucosinolate hydrolysis products could influence other fitness parameters such as female fecundity or egg hatching rate (Sun et al., 2019), our current results indicate that *P. armoraciae* can tolerate glucosinolate hydrolysis.

Previous feeding experiments performed with generalist lepidopteran herbivores revealed 4MSOB isothiocyanate to be the major hydrolysis product of 4MSOB glucosinolate in *Arabidopsis* (Schramm et al., 2012; Jeschke et al., 2017). However, instead of the isothiocyanate, we detected mainly the nitrile hydrolysis product of 4MSOB glucosinolate in *P. armoraciae* bodies and feces (**Figure 2B**). There are several possible explanations for this unexpected result: 4MSOB isothiocyanate may not have been recovered completely with our extraction methods because it has reacted with proteins or has been metabolized by the beetle or associated gut microbes (Brown and Hampton, 2011; Jeschke et al., 2015; van den Bosch and Welte, 2017; Friedrichs et al., 2020; Shukla and Beran, 2020). Alternatively, *P. armoraciae* may be able to manipulate the outcome of glucosinolate hydrolysis and promote the formation of the less reactive nitrile instead of the isothiocyanate. Manipulation of glucosinolate hydrolysis occurs for example in larvae of the cabbage white butterfly, which express a so-called nitrile specifier protein in the gut (Wittstock et al., 2004). In fact, a redirection of glucosinolate



hydrolysis toward less toxic nitriles could explain why plant myrosinase activity had no measurable effect on *P. armoraciae* performance. Further research is needed to understand the mechanism underlying the unusual composition of glucosinolate hydrolysis products in *P. armoraciae*.

How chewing insects that cause extensive tissue damage prevent the hydrolysis of ingested plant glucosides is currently not well understood. One proposed mechanism is a rapid absorption of plant glucosides across the gut epithelium separating substrate and enzyme (Abdalsamee et al., 2014; Pentzold et al., 2014b; van Geem et al., 2014). Although we observed a very rapid uptake of ingested glucosinolates in *P. armoraciae*, it remains unclear whether this indeed prevents glucosinolate hydrolysis in the gut lumen. Moreover, we had expected to find less glucosinolates in the guts of wild type-fed beetles due to the presence of myrosinase activity. Surprisingly, we recovered similar proportions of glucosinolates in the guts of *tgg*- and wild type-fed beetles, although we cannot rule out that glucosinolates detected in the gut were spatially separated from plant myrosinases, either in remaining intact plant tissue or in the gut epithelium.

The absorption of polar plant defense compounds such as glucosinolates across the gut is proposed to be mediated by membrane transporters (Discher et al., 2009; Petschenka and Agrawal, 2016). We recently identified glucosinolate-specific transporters belonging to the major facilitator superfamily (MFS) in *P. armoraciae* and found two glucosinolate

transporters to be expressed in the foregut, suggesting a role in glucosinolate uptake. However, silencing the expression of these transporters did not affect the uptake of ingested glucosinolates in adults, which indicates that additional or other transporters mediate glucosinolate absorption in *P. armoraciae* (Yang et al., 2021).

Another explanation for the detection of glucosinolates in the gut of *P. armoraciae* is the inhibition of plant myrosinase activity in the gut. Gut content extracts reduced plant myrosinase activity by 30 to 50% in *in vitro* assays, with boiled extracts inhibiting myrosinase activity significantly less than untreated extracts (**Figure 4B**). These results indicate that the activity of ingested plant myrosinases is possibly reduced in the gut lumen of *P. armoraciae*. Interestingly, gut content extracts of the closely related flea beetle *P. chrysocephala* also reduced plant myrosinase activity *in vitro*, but, in contrast to *P. armoraciae*, there was no difference between the boiled and the untreated extract (refer to **Supplementary Material, Supplementary Figure 1**). However, the observed effects of gut content extracts on myrosinase activity may not be specific for glucosinolate-sequestering flea beetles. To investigate this further, additional controls such as protein standard solutions and gut content extracts of other insect species should be included in future experiments. In general, plant myrosinase activity can be influenced by various factors including the levels of ascorbic acid (cofactor of myrosinases), sulfate, sodium chloride and silver ions (Shikita et al., 1999; Andersson et al., 2009; Bhat and Vyas, 2019; Marcinkowska and Jelen, 2020). In addition, the gut pH can have a strong influence on the activity of ingested plant enzymes (Pentzold et al., 2014b). For example, the highly alkaline pH of the midgut lumen of burnet moth larvae drastically reduced cyanogenic β -glucosidase activity in *Lotus corniculatus* leaf macerates (Pentzold et al., 2014a). In contrast, the neutral pH of gut homogenates of glucosinolate-sequestering turnip sawfly larvae had only minor influence on ingested plant myrosinase activity (Abdalsamee et al., 2014). In Coleoptera, gut pH values ranging from 5 to 8 have been reported (Terra and Ferreira, 1994). For example, a comparison of the gut pH in two closely related flea beetles of the genus *Longitarsus* revealed species-specific pH values of 7.0 and 5.3 (Pankoke and Dobler, 2015). Gut homogenates of *P. armoraciae* showed an acidic pH (details are described in the **Supplementary Material**), which is unlikely to have a strong influence on plant myrosinase activity.

Myrosinase from *S. alba* and other plant defensive β -glucosidases were largely resistant to digestion in the larval gut of the generalist lepidopteran *Spodoptera littoralis* and thus retained most of the activity after digestion (Vassão et al., 2018). Our proteomic analysis of beetle feces also indicates that *Arabidopsis* myrosinase TGG1 resisted digestion in *P. armoraciae*, whereas TGG2 was not detected in feces (**Figure 5** and **Supplementary Table 5**). Because TGG2 expression is restricted to phloem-associated cells (Barth and Jander, 2006), beetles have likely ingested less TGG2 than TGG1 by avoiding the leaf midrib and veins (personal observation). Despite the detection of TGG1 enzyme, we found almost no myrosinase activity in feces of *P. armoraciae*. We tested for the presence of myrosinase inhibitor(s) in feces homogenates but

observed no suppression of spiked myrosinase activity under our assay conditions (**Figure 6**). We thus hypothesize that ingested TGG1 has been inactivated during gut passage in *P. armoraciae*. Previous studies with the turnip sawfly and the diamondback moth also indicated that plant myrosinases are not fully active in the gut (Abdalsamee et al., 2014; Sun et al., 2019). However, the underlying mechanism(s) of myrosinase inhibition in the gut of specialist herbivores including *P. armoraciae* remain to be determined.

P. armoraciae possesses endogenous myrosinase activity, which enables larvae to use sequestered glucosinolates for defense against predators (Sporer et al., 2020). Interestingly, we detected glucosinolate hydrolysis products in the bodies and feces of larvae and adults that had fed on the myrosinase-deficient *tgg* mutant (adults: **Figures 2B, 3A**, larvae: refer to **Supplementary Material, Supplementary Table 8** and **Supplementary Figure 2**). These findings suggest that the beetle myrosinase also plays a role in the endogenous metabolism of sequestered glucosinolates. Similarly, volatile hydrolysis products derived from sequestered glucosinolates have also been detected in the headspace of the striped flea beetle, *Phyllotreta striolata*, which led to the initial discovery of a glucosinolate-myrosinase defense system in *Phyllotreta* flea beetles (Beran, 2011; Beran et al., 2014). To investigate the role of the beetle myrosinase in glucosinolate metabolism and defense in *P. armoraciae*, we currently perform experiments using beetles with suppressed myrosinase activity.

CONCLUSION

Defensive plant β -glucosidases represent a major target of herbivore adaptation to plants equipped with a two-component chemical defense. Our study demonstrates that the specialist herbivore *P. armoraciae* tolerates the hydrolysis of a fraction of ingested glucosinolates and is able to rapidly absorb glucosinolates across the gut. Moreover, we provide evidence that *P. armoraciae* can inactivate plant myrosinases in the gut. The ability to tolerate plant myrosinase activity could be an important prerequisite to develop mechanisms that enable the sequestration of glucosinolates.

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DATA AVAILABILITY STATEMENT

Source data of this study is available at the open access data repository of the Max Planck Society (Edmond) under doi: 10.17617/3.5b.

AUTHOR CONTRIBUTIONS

TS, JK, and FB designed the experiments and wrote the manuscript. TS, JK, YH, and FB performed the experiments. TS, JK, MR, NW, and FB analyzed the data. SG-J performed the bioinformatic analyses. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.645030/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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