

1 **Sugar transporters enable a leaf beetle to accumulate plant defense compounds**

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12 **Abstract**

13 Many herbivorous insects selectively accumulate plant toxins for defense against predators;
14 however, little is known about the transport processes that enable insects to absorb and store
15 defense compounds in the body. Here, we investigate how a specialist herbivore, the horseradish
16 flea beetle, accumulates high amounts of glucosinolate defense compounds in the hemolymph.
17 Using phylogenetic analyses of coleopteran membrane transporters of the major facilitator
18 superfamily, we identified a clade of glucosinolate-specific transporters (*PaGTRs*) belonging to
19 the sugar porter family. *PaGTR* expression was predominantly detected in the excretory system,
20 the Malpighian tubules. Silencing of *PaGTRs* led to elevated glucosinolate excretion, significantly
21 reducing the levels of sequestered glucosinolates in beetles. This suggests that *PaGTRs* reabsorb
22 glucosinolates from the Malpighian tubule lumen to prevent their loss by excretion. Ramsay assays
23 performed with dissected Malpighian tubules confirmed a selective retention of glucosinolates.
24 Thus, the selective accumulation of plant defense compounds in herbivorous insects can depend
25 on the ability to prevent excretion.

26 **Introduction**

27 Physiological and chemosensory adaptations of herbivorous insects to plant defense
28 compounds have played an important role in the evolution of insect-plant interactions and insect
29 host range¹⁻⁴. Among the most remarkable insect adaptations is the ability to selectively
30 accumulate (sequester) plant defense compounds for protection from generalist predators⁵⁻⁷.
31 Indeed, it is increasingly recognized that predator pressure has promoted insect adaptations to plant
32 defenses and driven the evolution of specialized host plant associations⁷⁻¹⁰. Sequestration evolved
33 in all major clades of herbivorous insects and is particularly wide-spread in the megadiverse
34 phytophagous Coleoptera and Lepidoptera¹¹; however, the mechanisms by which sequestering
35 insects can accumulate ingested plant defense compounds in their body remain poorly understood.

36 Glucosinolates are hydrophilic defense compounds produced by plants of the order Brassicales
37 that, together with the plant β -thioglucosidase enzyme myrosinase, constitute an activated two
38 component defense system¹²⁻¹⁴. When tissue damage brings both components together,
39 glucosinolates are rapidly hydrolyzed to unstable aglucones that give rise to different products¹⁵
40 of which isothiocyanates are the most detrimental to small herbivores¹⁶⁻¹⁹. Some insects can
41 prevent glucosinolate hydrolysis and accumulate ingested glucosinolates in the body²⁰⁻²⁵. A
42 defensive function of glucosinolate sequestration has been demonstrated in specialist aphids and
43 flea beetles, which convergently evolved insect myrosinases enabling glucosinolate activation in
44 response to predator attack^{21,23,26}. The turnip sawfly, *Athalia rosae*, sequesters glucosinolates but
45 does not possess own myrosinase activity, making a role in defense less likely^{27,28}. Instead, a rapid
46 absorption of ingested glucosinolates across the gut epithelium could represent a detoxification
47 mechanism as it spatially separates glucosinolates from the co-ingested plant myrosinase in the
48 gut lumen^{28,29}. Due to their physicochemical properties, transport of glucosinolates and other plant

49 glucosides such as cyanogenic and phenolic glucosides is proposed to be mediated by membrane
50 carriers^{6,23,29}. For example, Strauss and coworkers demonstrated the role of an ATP-binding
51 cassette (ABC) transporter in the sequestration of the phenolic glucoside salicin in the defense
52 glands of the poplar leaf beetle, *Chrysomela populi*³⁰. More recently, Kowalski and coworkers
53 identified an ABC transporter in the dogbane leaf beetle, *Chrysochus auratus*, with high activity
54 towards a plant cardenolide, which adult beetles sequester in defense glands³¹. Apart from these
55 examples, no other membrane transporters involved in sequestration have been identified to date.

56 We previously investigated the sequestration of glucosinolates in the flea beetle *Phyllotreta*
57 *armoraciae*^{25,26}. This species is monophagous on horseradish in nature, but feeds on various
58 brassicaceous plants in the laboratory³²⁻³⁴ and sequesters glucosinolates mainly in the
59 hemolymph²⁵. *P. armoraciae* prefer to sequester aliphatic glucosinolates over indolic
60 glucosinolates; however, the sequestration pattern depends both on the levels and composition of
61 glucosinolates in the food plant. Interestingly, the beetles also selectively excrete sequestered
62 glucosinolates to balance the accumulation of new glucosinolates and maintain stable
63 glucosinolate levels around 35 nmol per mg beetle fresh weight²⁵. Based on these findings, we
64 hypothesize that glucosinolate transporters are localized in gut and Malpighian tubule epithelial
65 membranes in *P. armoraciae*.

66 Here we use a comparative phylogenetic approach to identify candidate glucosinolate
67 transporter genes within the major facilitator superfamily. We focus on a *P. armoraciae*-specific
68 clade comprising 21 putative sugar porters, which are predominantly expressed in the Malpighian
69 tubules, and demonstrate glucosinolate-specific import activity for 13 transporters. We investigate
70 the role of the identified glucosinolate transporters *in vivo* using RNA interference and establish
71 the transport mechanism of one transporter selected as a model *in vitro*. Our results suggest that

72 transporter-mediated reabsorption of glucosinolates in the Malpighian tubules enables *P.*
73 *armoraciae* to sequester high amounts of glucosinolates in the hemolymph.

74 **Results**

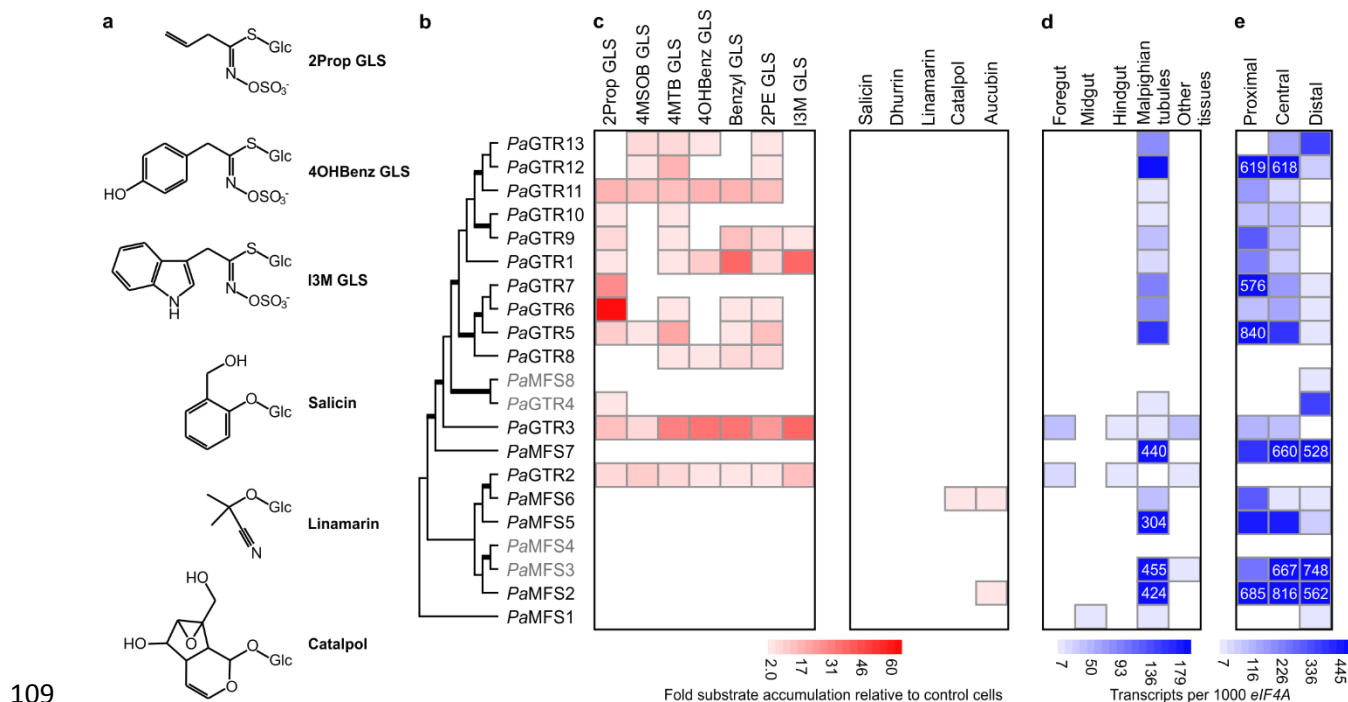
75 **Selection of candidate transporters.** To elucidate the molecular basis of glucosinolate transport
76 in *P. armoraciae*, we generated a gut- and Malpighian tubule-specific transcriptome. In this
77 transcriptome, we predicted a total of 1401 putative membrane transporters using the transporter
78 automatic annotation pipeline (TransAAP)³⁵. Of these, 353 are putative members of the major
79 facilitator superfamily (MFS; Transporter Classification (TC)# 2.A.1; Supplementary Table 1),
80 which are known to transport a broad spectrum of substrates including sugars and amino acids
81 across membranes^{36,37}.

82 We selected MFS transporters as our candidates and additionally assumed that gene
83 duplications have played a role in the evolution of glucosinolate transport activity in *P. armoraciae*.
84 To detect species-specific expansions of MFS transporters, we annotated MFS transporters in the
85 publicly available genomes of three non-sequestering beetle species (Supplementary Data 1). By
86 phylogenetic analysis, we identified the largest *P. armoraciae*-specific clade comprising 21
87 putative sugar porters (TC# 2.A.1.1) for further study (Supplementary Data 2). Members of this
88 clade share between 31 and 93% amino acid sequence identity and are most similar to insect
89 trehalose transporters.

90 **Functional characterization of candidate transporters.** We expressed the candidate transporters
91 in High Five insect cells and screened them for glucoside import activity. In addition to
92 glucosinolates, we tested different types of non-host glucosides as substrates, namely iridoid,
93 cyanogenic, and phenolic glucosides (Fig. 1a). Insect cells expressing 15 different transporters

94 accumulated significantly higher levels of at least one tested glucoside compared to control cells.
95 Of these, 13 transporters were specific for glucosinolates (Fig. 1b, c), hereafter referred to as
96 *Phyllotreta armoraciae* Glucosinolate Transporter (*PaGTR*) 1 to 13. Most *PaGTRs* showed broad
97 and overlapping substrate spectra; for example, 10 different *PaGTRs* used 2-propenyl
98 glucosinolate, the major glucosinolate in the host plant of *P. armoraciae*, as a substrate. The other
99 two transporters mediated iridoid glucoside uptake into insect cells, whereas phenolic and
100 cyanogenic glucosides were not used as substrates by any of the recombinant transporters (Fig. 1c
101 and Supplementary Fig. 1).

102 **Tissue-specific expression of *PaGTRs*.** To determine the localization of *PaGTRs* in *P.*
103 *armoraciae*, we compared the transcript levels in foregut, midgut, hindgut, Malpighian tubules,
104 and other tissues by quantitative PCR. All except two *PaGTRs* were specifically expressed in the
105 Malpighian tubules (Fig. 1d and Supplementary Fig. 2). Along the tubule, *PaGTRs* were
106 predominantly expressed in the proximal region close to the midgut-hindgut junction (Fig. 1e and
107 Supplementary Fig. 2d). This Malpighian tubule region was previously shown to reabsorb
108 metabolites to prevent their loss by excretion³⁸⁻⁴⁰.



109

110 **Fig. 1 Import activity and expression pattern of candidate MFS transporters.** **a** Chemical structures of
 111 selected glucosinolates (GLS) and non-host glucosides used in transport activity assays. **b** Phylogenetic
 112 relationships of candidate transporters selected based on the diversification pattern of coleopteran MFS
 113 transporters shown in Supplementary Data 2. Branches shown in bold have a bootstrap support higher than
 114 95%. Recombinant proteins expressed in High Five insect cells were detected by Western blotting. For four
 115 candidates, we did not detect recombinant protein (Supplementary Fig. 1); the names of these candidates
 116 are written in grey **c** Recombinant transporters were screened for glucoside uptake activity in assays using
 117 equimolar mixtures of glucosinolates or non-host glucosides. Glucoside accumulation in transfected insect
 118 cells is expressed relative to that in mock-transfected insect cells used as background control. Values
 119 represent the mean of three assays. **d** Expression pattern of candidate MFS transporter genes in different
 120 tissues of *P. armoraciae*. **e** Expression pattern of candidate MFS transporter genes in different regions of
 121 the Malpighian tubule. Copy number estimates are given per 1000 copies of mRNA of the reference gene
 122 *eIF4A*. Low gene expression levels are visualized by limiting the scale to a value of 200 and 500 in **d** and
 123 **e**, respectively. The exact values are provided for genes with higher expression level. Each value represents
 124 the mean of four and three biological replicates in **d** and **e**, respectively. 2Prop, 2-propenyl; 4MSOB, 4-
 125 methylsulfinylbutyl; 4MTB, 4-methylthiobutyl; 4OHBenz, 4-hydroxybenzyl; 2PE, 2-phenylethyl; I3M,
 126 indol-3-ylmethyl.

127 **Function of *PaGTRs in vivo*.** The Malpighian tubules are bathed in hemolymph, the major storage
128 site for sequestered glucosinolates in *P. armoraciae*. We analyzed the glucosinolate levels in the
129 hemolymph and detected total concentrations of up to 106 mM (Supplementary Table 2). Which
130 role does *PaGTR*-mediated glucosinolate transport in Malpighian tubules play in glucosinolate
131 storage? We addressed this question by silencing *PaGTR* expression in adult *P. armoraciae* beetles.
132 We selected *PaGTR1* as a model because this transporter prefers indol-3-ylmethyl (I3M)
133 glucosinolate, a substrate that is transported by only one other Malpighian tubule-specific
134 transporter (Fig. 1c). After confirming the specific silencing of *PaGTR1* expression by quantitative
135 PCR (Fig. 2a; Supplementary Fig. 3a), we fed the beetles with leaves of *Arabidopsis thaliana* Col-
136 0 (*Arabidopsis*) containing I3M glucosinolate.

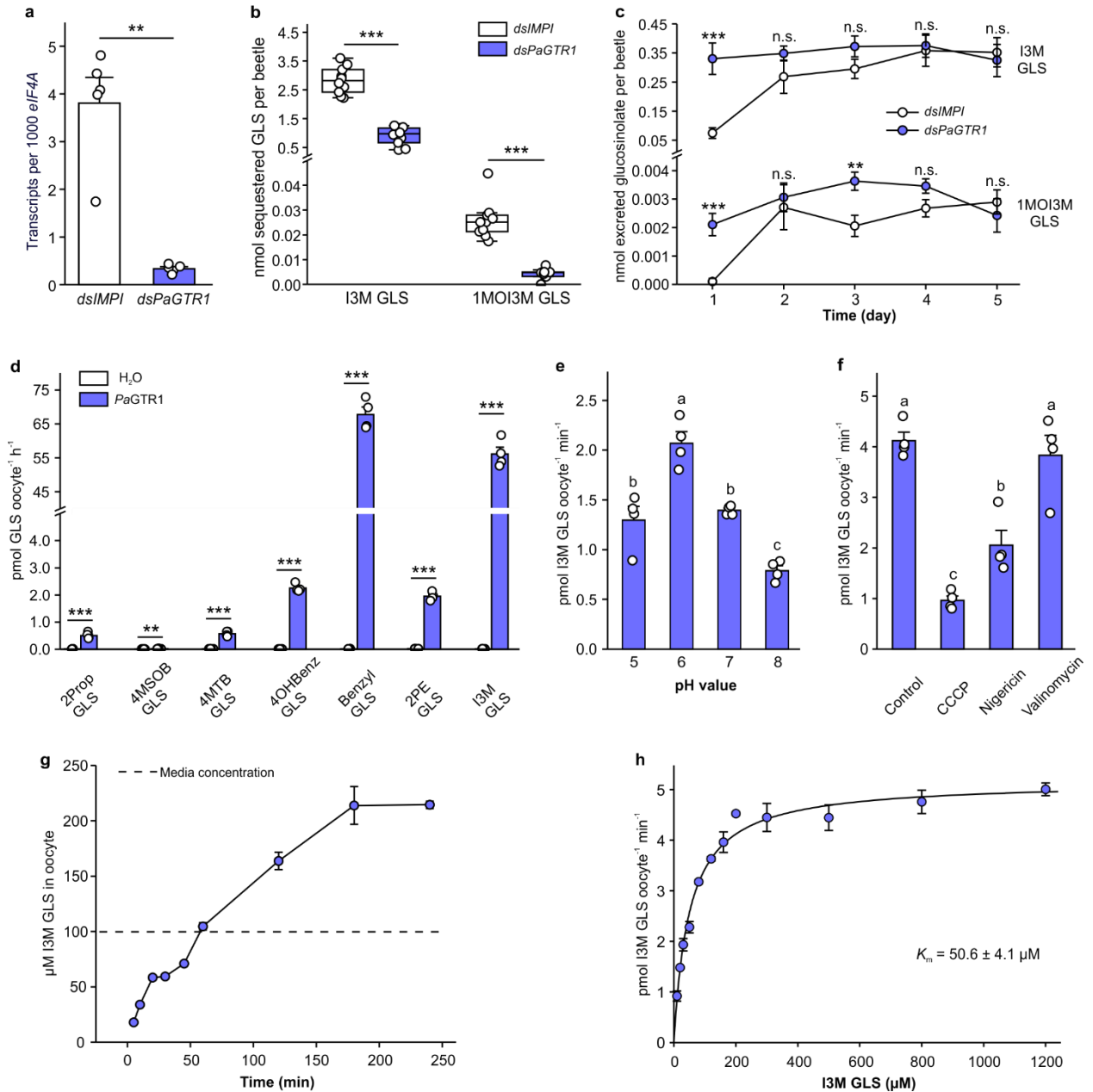
137 Although the treatment did not influence beetle feeding behavior (Supplementary Fig. 3b), we
138 detected significantly less I3M glucosinolate and 1-methoxyindol-3-ylmethyl (1MOI3M)
139 glucosinolate in *PaGTR1*-silenced beetles than in control beetles (Fig. 2b). In addition, *PaGTR1*-
140 silencing led to elevated levels of I3M and 1MOI3M glucosinolates in the feces (Fig. 2c). The
141 levels of other glucosinolates were not reduced in *PaGTR1*-silenced beetles compared to the
142 control (Supplementary Table 3 and Supplementary Fig. 4). The effect of *PaGTR1* knock-down
143 on the accumulation and excretion of specific glucosinolates in *P. armoraciae* is consistent with
144 the substrate preference of recombinant *PaGTR1* in insect cell-based uptake assays, and suggests
145 that *PaGTR1* prevents excretion of two indolic glucosinolates by reabsorbing them from the
146 Malpighian tubule lumen.

147 ***PaGTR1* is a proton-dependent high affinity glucosinolate transporter.** To establish the
148 mechanism of *PaGTR1*-mediated glucosinolate reabsorption, we expressed *PaGTR1* in *Xenopus*
149 *laevis* oocytes and analyzed its biochemical properties. First, we analyzed the substrate preference

150 of oocyte-expressed *PaGTR1*, and found it to be similar to that of insect cell-expressed *PaGTR1*
151 (Fig. 2d). Previously characterized members of the sugar porter family act either as uniporters or
152 as proton-dependent active transporters⁴¹⁻⁴³; thus, we analyzed whether the extracellular pH
153 influences I3M glucosinolate accumulation in *PaGTR1*-expressing oocytes. The glucosinolate
154 uptake rate depended on the external pH and was maximal at pH 6, which is consistent with the
155 acidic pH in the Malpighian tubule lumen of *P. armoraciae* (Fig. 2e and Supplementary Fig. 5).
156 Uptake activity decreased significantly when pH was greater than 6, suggesting that *PaGTR1*-
157 mediated glucosinolate transport is driven by a proton gradient across the membrane. To test this,
158 we performed glucosinolate uptake assays at pH 6 and added either the protonophore carbonyl
159 cyanide *m*-chlorophenyl hydrazine (CCCP), the K⁺/H⁺ exchanger nigericin, or the K⁺ ionophore
160 valinomycin to the assay medium. Addition of CCCP and nigericin decreased the glucosinolate
161 uptake rates by 77% and 50% compared to control assays, respectively, whereas valinomycin did
162 not influence transport by *PaGTR1*-expressing oocytes. The effects of different ionophores on
163 glucosinolate transport show that *PaGTR1*-mediated glucosinolate uptake is proton-driven, but is
164 not influenced by K⁺ (Fig. 2f).

165 A time-course analysis of I3M glucosinolate accumulation in *PaGTR1*-expressing oocytes
166 revealed that glucosinolate levels saturated after three hours at an intracellular concentration that
167 was twofold higher than in the assay buffer. This result confirmed that *PaGTR1* can mediate
168 glucosinolate uptake against a concentration gradient (Fig. 2g). Plotting the transport rate as a
169 function of increasing glucosinolate concentration yielded a saturation curve that was fitted to
170 Michaelis-Menten kinetics with a K_m value of $50.6 \pm 4.1 \mu\text{M}$ (Fig. 2h). Considering the much
171 higher I3M glucosinolate concentration of $2082 \pm 668 \mu\text{M}$ in the hemolymph (Supplementary

172 Table 2), our results characterize *PaGTR1* as a high affinity proton-dependent glucosinolate
 173 transporter in *P. armoraciae*.



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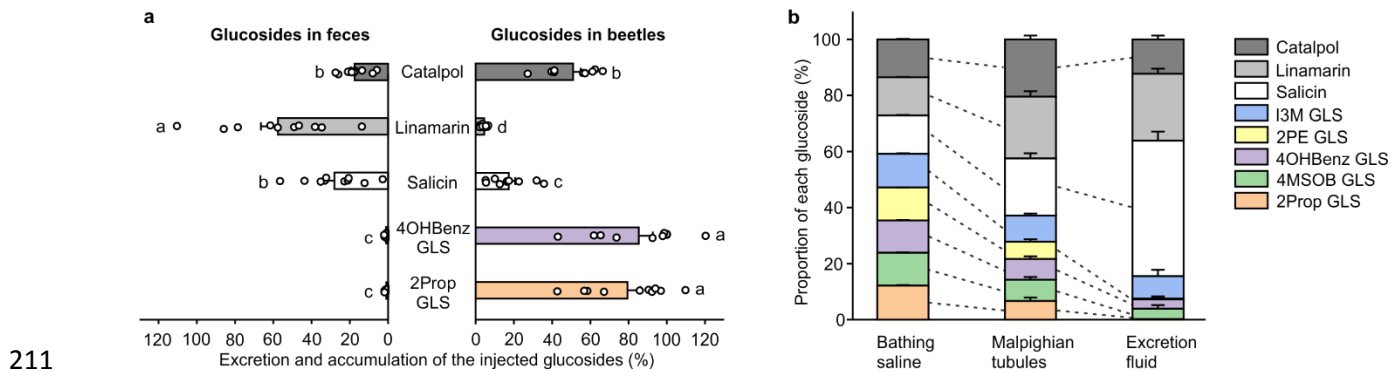
175 **Fig. 2 Functional and biochemical characterization of *PaGTR1*-mediated glucosinolate transport. a**
 176 *PaGTR1* expression in *P. armoraciae* four days after dsRNA injection ($n = 5$). **b** Accumulation of indol-3-
 177 ylmethyl (I3M) GLS and 1-methoxyindol-3-ylmethyl (1MOI3M) GLS in adult *P. armoraciae* beetles after
 178 five days feeding on *Arabidopsis* leaves. Box plots show the median, interquartile range, and outliers of

179 each data set ($n = 10$). **c** Excreted amounts of I3M GLS and 1MOI3M GLS over five days feeding on
180 *Arabidopsis* ($n = 9$ for day 4, $n = 10$ for other days). **d** Substrate preference of *PaGTR1*. Oocytes expressing
181 *PaGTR1* and control oocytes were incubated with equimolar mixtures of seven glucosinolates ($n = 4$). **e**
182 pH dependency of *PaGTR1* ($n = 4$). **f** Effects of different ionophores on *PaGTR1*-mediated glucosinolate
183 transport ($n = 4$). **g** Time course of glucosinolate accumulation in *Xenopus* oocytes expressing *PaGTR1* (n
184 $= 4$). **h** Kinetic analysis of *PaGTR1*-mediated I3M GLS transport ($n = 4$). Data are shown as mean \pm s.e.m.
185 Treatments were compared by two-tailed Student's *t*-test, Mann-Whitney *U* test or the method of
186 generalized least squares (**a**, **b**, **c**, **d**), or one-way analysis of variance (ANOVA) (**e**, **f**). Bars labeled with
187 different letters are significantly different ($P < 0.05$). n.s., not significantly different, ** $P < 0.01$, *** $P <$
188 0.001.

189 ***P. armoraciae* selectively retain glucosinolates.** We further investigated the role of selective
190 transport in Malpighian tubules by analyzing the excretion of two glucosinolates (2-propenyl- and
191 4-hydroxybenzyl glucosinolate) and three non-host glucosides (salicin, linamarin and catalpol)
192 after injection into the hemolymph of adult *P. armoraciae* beetles. Within one day, beetles excreted
193 between 18% and 58% of the injected non-host glucosides and less than 2% of the injected
194 glucosinolates (Fig. 3a). In addition, we compared the recovery of injected glucosides after 30
195 mins with that after one day. While the levels of glucosinolates remained stable, we recovered
196 significantly less non-host glucosides from beetle bodies. However, significantly more catalpol
197 than salicin and linamarin was recovered (Fig. 3a), suggesting that catalpol was excreted or
198 metabolized at a lower rate than the other two non-host glucosides.

199 To confirm that the selective retention of glucosinolates in *P. armoraciae* is due to reabsorption,
200 we analyzed glucoside excretion *in situ* using Ramsay assays (Supplementary Fig. 6). We exposed
201 dissected Malpighian tubules to an equimolar mixture of glucosides and compared the glucoside
202 composition in the bathing saline and Malpighian tubule tissue, and in the Malpighian tubule tissue
203 and the excretion fluid, respectively. The Malpighian tubule tissue contained significantly lower
204 proportions of glucosinolates than the bathing saline and correspondingly higher proportions of

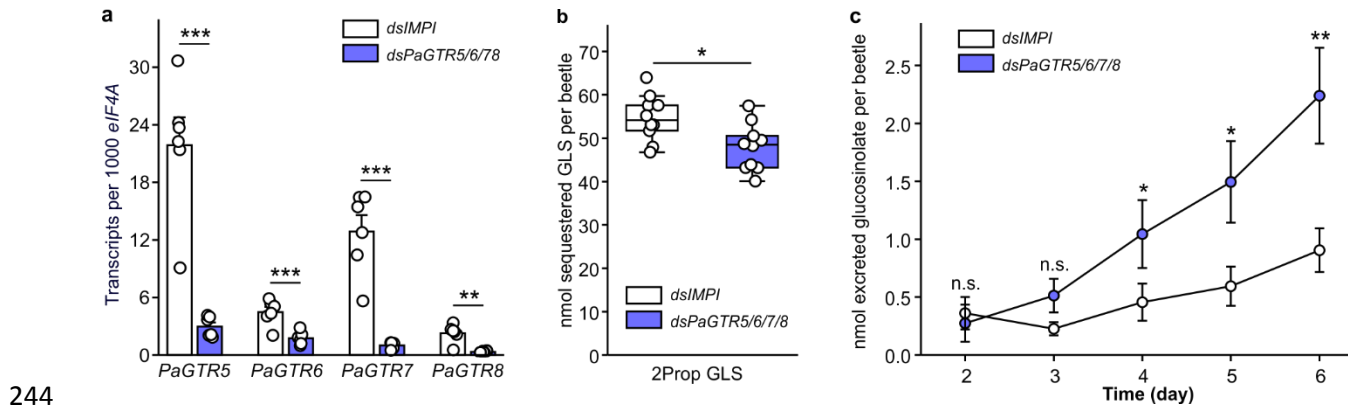
205 non-host glucosides (Fig. 3b). The excretion fluid contained even lower percentages of most
 206 glucosinolates, in particular of 2-propenyl and 2-phenylethyl glucosinolates. In addition, we
 207 detected less catalpol in the excretion fluid than in the Malpighian tubule tissue. Combined, these
 208 findings show that the Malpighian tubules of *P. armoraciae* selectively reabsorb glucosinolates
 209 and catalpol, which is consistent with the substrate spectra of recombinant candidate transporters
 210 (Fig. 1c).



211
 212 **Fig. 3 Selective excretion of plant glucosides *in vivo* and *in situ*.** **a** Excretion and accumulation of plant
 213 glucosides injected in beetles. Each beetle was injected with 100 nL of an equimolar mixture of five
 214 glucosides. Injected beetles were sampled 30 min after the injection and after one day feeding on
 215 *Arabidopsis* ($n = 10$). Glucoside excretion was analyzed by quantifying the amount of excreted glucosides
 216 in the feces ($n = 10$). Glucoside content in beetles and feces is expressed relative to the glucoside amounts
 217 detected in beetles 30 min after injection (set to 100%). The relative accumulation and excretion of
 218 glucosides was compared using the method of generalized least squares, respectively. Bars labeled with
 219 different letters are significantly different ($P < 0.05$). **b** Excretion of plant glucosides by isolated Malpighian
 220 tubules. The dissected Malpighian tubule was placed in a droplet of saline and a mixture of eight different
 221 plant glucosides each at a concentration of 6.7 mM. To visualize excretion, we added 0.1% (w/v) amaranth.
 222 After 2-3 h, bathing saline, Malpighian tubule and excretion fluid were sampled, extracted and analyzed by
 223 LC-MS/MS. Paired *t*-tests were used to compare the relative composition of plant glucosides in bathing
 224 saline and Malpighian tubule, and Malpighian tubule and excretion fluid, respectively. Dashed lines
 225 indicate significant differences between samples ($P < 0.05$, $n = 11$). Data are shown as mean \pm s.e.m.

226 ***PaGTRs* prevent excretion of 2-propenyl glucosinolate.** 2-Propenyl glucosinolate represents the
227 major glucosinolate in the host plant of *P. armoraciae* and is sequestered at very high levels in the
228 beetle hemolymph (Supplementary Table 2). To determine whether the identified *PaGTRs* also
229 capable of reabsorbing 2-propenyl glucosinolate in *P. armoraciae*, we simultaneously silenced the
230 expression of a clade comprising four Malpighian tubule-specific transporters (*PaGTR5/6/7/8*),
231 among which three used 2-propenyl glucosinolate as a substrate *in vitro* (Fig. 1b, c). Beetles with
232 silenced *PaGTR5/6/7/8* expression excreted significantly more 2-propenyl glucosinolate than
233 control beetles (Fig. 4a, c and Supplementary Fig. 7, 8). In addition, 2-propenyl glucosinolate
234 levels were significantly lower in *PaGTR5/6/7/8*-silenced beetles than in control beetles (Fig. 4b
235 and Supplementary Table 4). These results support our hypothesis that the major function of
236 Malpighian tubule-expressed *PaGTRs* is reabsorption.

237 **Silencing of *PaGTR2* and *PaGTR3* does not affect glucosinolate accumulation from**
238 ***Arabidopsis*.** Two transporter genes, *PaGTR2* and *PaGTR3*, were found to be expressed in the
239 foregut, hindgut, and other tissues. Because of their expression in the foregut, we investigated
240 whether *PaGTR2* and *PaGTR3*, which showed a broad substrate specificity *in vitro*, play a role in
241 glucosinolate absorption from the gut lumen. However, silencing of *PaGTR2* and *PaGTR3*
242 expression did not influence the accumulation of ingested glucosinolates in beetles in feeding
243 assays with *Arabidopsis* (Supplementary Fig. 9).



244

245 **Fig. 4 In vivo functional characterization of glucosinolate transport mediated by *PaGTR5/6/7/8*.** **a**
246 *PaGTR5/6/7/8* expression in *P. armoraciae* six days after dsRNA injection ($n = 6$). **b** Accumulation of 2-
247 propenyl glucosinolate (2Prop GLS) in adult *P. armoraciae* beetles after six days feeding on *Arabidopsis*
248 leaves followed by one day starvation. Box plots show the median, interquartile range, and outliers of each
249 data set ($n = 10$). **c** Excreted amounts of 2Prop GLS from day 2 until day 6 of feeding on *Arabidopsis* ($n =$
250 10). n.s., not significantly different; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are shown as mean \pm s.e.m.

251 Discussion

252 In this study, we identified glucosinolate-specific transporters in *P. armoraciae* that play an
253 important role in sequestration. We show that *PaGTRs* are mainly expressed in the Malpighian
254 tubules and mediate glucosinolate import *in vitro*. Silencing *PaGTR* expression in Malpighian
255 tubules led to increased glucosinolate excretion, which indicates that *PaGTRs* reabsorb
256 glucosinolates from the Malpighian tubule lumen into the epithelial cells. These findings
257 demonstrate that active reabsorption in the Malpighian tubules is required for glucosinolate
258 accumulation in the hemolymph of *P. armoraciae*.

259 Glucosinolate transporters have previously been identified in the model plant *Arabidopsis* by
260 screening 239 transport proteins for glucosinolate uptake activity in *Xenopus oocytes*⁴⁴. Plant
261 GTRs belong to the nitrate/peptide family (NPF) and presumably evolved from ancestral
262 cyanogenic glucoside transporters⁴⁵. The corresponding transporter family in *P. armoraciae*,

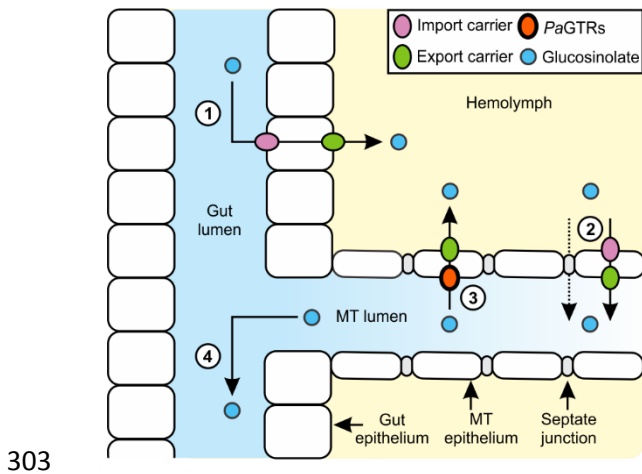
263 annotated as proton-dependent oligopeptide transporter family (POT), comprised only six putative
264 transporters (Supplementary Table 1) and was not investigated in our study. Instead, our
265 phylogenetic analysis of coleopteran MFS transporters provided evidence for species-specific
266 expansions in particular within the sugar porter family, which led us to focus on the largest *P.*
267 *armoraciae*-specific clade of putative sugar porters. All *Pa*GTRs except for *Pa*GTR2 clustered
268 together in one well-supported group (Fig. 1b). Separate of this group we additionally identified
269 two iridoid glycoside-specific transporters. Our study thus highlights the potential of sugar porters
270 to evolve activity towards glycosylated defense compounds.

271 The presence of iridoid glycoside-specific transporters in *P. armoraciae* was surprising, as
272 plants of the order Brassicales do not produce iridoid glycosides^{46,47}. Consistently, we observed
273 reabsorption of the iridoid glycoside catalpol in Malpighian tubules (Fig. 3). Several flea beetles
274 of the genus *Longitarsus* selectively sequester aucubin and catalpol⁴⁸, but this genus is likely not
275 closely related to *Phyllotreta*⁴⁹. Nevertheless, it is possible that iridoid glycoside-specific
276 transporters that had evolved in an ancestor of *Phyllotreta* have retained their function even after
277 flea beetles have adapted to plant families with distinct defense compounds.

278 An evolutionary link between the sequestration of iridoid glucosides and glucosinolates has
279 previously been discovered in the sawfly genus *Athalia* in which a host shift occurred from iridoid
280 glycoside-containing Lamiales to glucosinolate-containing Brassicaceae⁵⁰. Sequestration
281 experiments demonstrated that Brassicaceae-feeders were able to sequester both iridoid glycosides
282 and glucosinolates, whereas Lamiales-feeders only sequestered iridoid glycosides. Based on these
283 findings, Opitz and coworkers hypothesized that glycoside transporters evolved a broader substrate
284 specificity in Brassicaceae feeders. It is also imaginable that the presence of iridoid glycoside-
285 specific transporters in the ancestor of *Phyllotreta* has facilitated the evolution of glucosinolate

286 transport activity when flea beetles specialized on brassicaceous plants. To better understand the
287 evolutionary origin of *PaGTRs*, it will be interesting to investigate the evolution and function of
288 sugar porters across different flea beetle genera.

289 The specific expression of most *PaGTRs* in the Malpighian tubules indicated an important role
290 of the beetle's excretory system in sequestration. In fact, pioneering work by Meredith and
291 coworkers published in 1984 demonstrated that the Malpighian tubules of the large milkweed bug,
292 *Oncopeltus fasciatus*, actively reabsorb the polar cardiac glycoside ouabain, which is sequestered
293 predominantly in the integument, after its passive secretion into the Malpighian tubule lumen³⁸.
294 Although it has generally been recognized that sequestering insects require mechanisms to prevent
295 the excretion of polar metabolites from the hemolymph⁵¹, these mechanisms have not been further
296 investigated. Our physiological assays demonstrate that the Malpighian tubule epithelium of *P.*
297 *armoraciae* discriminates among different types of glucosinolates and non-host glucosides and can
298 reabsorb in particular glucosinolates against a concentration gradient (Fig. 3). Since *PaGTRs*
299 mediated glucosinolate import *in vitro*, we propose that they are located at the apical membrane
300 and thereby enable glucosinolate uptake from the lumen. To complete transepithelial glucosinolate
301 transport, a so far unknown membrane transporter localized at the basolateral membrane is
302 required to export glucosinolates into the hemolymph (Fig. 5).



303

304 **Fig. 5 Proposed model of glucosinolate sequestration in adult *P. armoraciae*.** (1) Absorption of ingested
305 glucosinolates from the gut lumen into the hemolymph; (2) Transcellular or paracellular glucosinolate
306 transport from the hemolymph into the Malpighian tubule (MT) lumen; (3) Selective reabsorption of
307 glucosinolates from the MT lumen into the hemolymph; (4) Excretion of excess glucosinolates. *PaGTRs*
308 localized in the MT are proposed to import glucosinolates from the lumen across the apical membrane into
309 the epithelial cells.

310 The expression profile of putative sugar porter genes in *P. armoraciae* (Supplementary Data 1)
311 and a recent proteomic study performed with the poplar leaf beetle *C. populi*⁵² showed that many
312 sugar porters are specifically expressed in the Malpighian tubules. To our knowledge, only one
313 Malpighian tubule-specific sugar porter has been characterized to date - sugar transporter 8 from
314 the brown plant hopper, *Nilaparvata lugens*, which reabsorbs trehalose⁴². Thus, there is first
315 evidence that sugar porters localized in Malpighian tubules play a role in reabsorption. The
316 localization and function of putative sugar porters we have identified in published beetle genomes
317 has not yet been investigated; however, a phylogenetic analysis suggests that the Malpighian
318 tubule-specific sugar porter CpSP-like17 from *C. populi* and *PaGTRs* evolved from a common
319 ancestor (Supplementary Fig. 10). We thus propose that *PaGTRs* evolved by duplications of a
320 Malpighian tubule-localized sugar porter. Investigating the localization of flea beetle sugar porters

321 that are phylogenetically closely related to *PaGTRs* will help to establish whether *PaGTRs* indeed
322 evolved from Malpighian tubule-specific sugar porters.

323 The distinct expression pattern of *PaGTR2* and *PaGTR3* (Fig. 1d) suggested that *PaGTRs* are
324 not only involved in reabsorption in the Malpighian tubules. Here, the expression in the foregut
325 suggested for example a role in glucosinolate absorption. Although the insect midgut has been
326 proposed to be the major tissue involved in absorption⁴⁰, there is initial evidence that glucosinolate
327 absorption might already occur in the foregut of *A. rosae* larvae²⁹. However, as silencing *PaGTR2*
328 and *PaGTR3* expression had no effect on the accumulation of ingested glucosinolates in beetles,
329 it is likely that additional or other membrane transporters are required. To establish the mechanism
330 of glucosinolate absorption, we currently investigate the localization and specificity of
331 glucosinolate uptake in adult *P. armoraciae*.

332 Transport processes play a central role in the evolution of insect sequestration mechanisms. Our
333 study highlights that sequestering insects require mechanisms that prevent the excretion of target
334 compounds, for example by active reabsorption. We furthermore show that in *P. armoraciae*,
335 reabsorption is selective and thus can control not only the level but also the composition of
336 sequestered metabolites in the beetle. Understanding the molecular basis of sequestration now
337 opens up the possibility to manipulate the accumulation of defense compounds in insects in order
338 to investigate how sequestration influences trophic interactions and shapes the composition of
339 ecological communities.

340 **Methods**

341 **Insect culture.** *P. armoraciae* beetles were reared on potted *Brassica juncea* cultivar “Bau Sin” plants or
342 on potted *Brassica rapa* cultivar “Yo Tsai Sum” plants (Known-You Seed Co. Ltd.) in mesh cages
343 (Bugdorm, MegaView Science Co., Ltd.) in a controlled environment chamber at 24 °C, 60% relative
344 humidity and a 16-h photoperiod. Food plants were cultivated in a growth chamber at 24 °C, 55% relative
345 humidity, and a 14-h photoperiod. Beetles were provided with three- to four-week old plants once per week,
346 and plants with eggs were kept separately for larval development. Larvae were allowed to pupate in the soil
347 and after three weeks, the soil with pupae was transferred into plastic boxes (9 L, Lock&Lock) until the
348 new generation of beetles emerged.

349 **RNA isolation, RNAseq and *de novo* transcriptome assembly.** Total RNA was extracted from dissected
350 foregut, midgut, hindgut, and Malpighian tubule tissue of newly emerged *P. armoraciae* beetles that were
351 reared on *B. juncea* using the innuPREP RNA Mini Kit (Analytik Jena). Tissues from at least 10 beetles
352 were pooled per sample. RNA integrity was verified using an Agilent Technologies 2100 Bioanalyzer with
353 the RNA 6000 Nano Kit (Agilent Technologies). RNA quantity was determined using a Nanodrop ND-
354 1000 spectrophotometer (PEQlab Biotechnologie GmbH). One set of RNA samples was sequenced by
355 GATC Biotech on the HiSeq 2500 System from Illumina in Rapid Run mode, using the paired-end (2 ×
356 125 bp) read technology at a depth of 15-25 million reads for each sample. For a second set of samples
357 consisting of four biological replicates per tissue, we additionally performed an on-column DNA digestion
358 with the innuPREP DNase I Digest Kit (Analytik Jena) according to the manufacturer’s instructions. RNA
359 samples were poly(A)-enriched, fragmented, and sequenced at the Max Planck Genome Centre Cologne on
360 the HiSeq 3000 Sequencing System from Illumina, using the paired-end (2 × 150 bp) read technology at a
361 depth of 22 million reads for each sample. Sequencing reads were filtered to remove bad-quality reads
362 based on fastq file scores and trimmed based on read length using CLC Genomics Workbench software
363 version 10.1. With a randomly sampled set of 420 million reads from the two sets of sequencing data, a
364 transcriptome was assembled *de novo* with the following parameters: nucleotide mismatch cost = 1;

365 insertion = deletion costs = 2; length fraction = 0.6; similarity = 0.9. Conflicts among the individual bases
366 were resolved by voting for the base with the highest frequency. After removing contigs shorter than 250
367 bp, the final assembly contained 36,445 contigs with an N50 contig size of 2,115 bp.

368 **Identification of coleopteran MFS transporters.** We predicted a protein dataset for *P. armoraciae* by
369 translating each contig of the gut and Malpighian tubule-specific transcriptome into all six reading frames.
370 After removing sequences shorter than 50 amino acids, we submitted the protein dataset (267,568
371 sequences) to the Transporter Automatic Annotation Pipeline (TransAAP) hosted at the TransportDB 2.0
372 web portal³⁵. This initial annotation predicted a total of 1,401 putative transporter sequences and revealed
373 the major facilitator superfamily (MFS) and the ATP-binding cassette (ABC) transporters to be the largest
374 transporter families (Supplementary Table 1). We focused on MFS transporters, which are classified into
375 more than 80 families⁵³. We used one protein sequence from each MFS family as query to search candidate
376 MFS transporters in the protein dataset from *P. armoraciae* using Blastp (E-value threshold of 10^{-5}), and
377 assigned each candidate to an MFS family based on sequence similarity to transporter sequences deposited
378 in TCDB. Additional candidates were identified by repeating the search procedure with an extended dataset
379 including the candidate MFS transporters from *P. armoraciae*. The number of TMDs for each candidate
380 was predicted using the TMHMM Server v.2.0⁵⁴. Partial sequences encoding less than six predicted TMDs
381 were removed from the dataset. The same strategy was used to identify putative MFS transporters in protein
382 datasets that were predicted from the genomes of *Leptinotarsa decemlineata*, genome annotations v0.5.3⁵⁵,
383 *Anoplophora glabripennis*, assembly Agla_1.0⁵⁶, and *Tribolium castaneum*, assembly Tcas3.0⁵⁷,
384 respectively. The predicted protein sequences are provided in Supplementary Data 1.

385 **Digital gene expression analysis.** Digital gene expression analysis of putative MFS transporters identified
386 in the *P. armoraciae* transcriptome was carried out using CLC Genomics workbench v 10.1 by mapping
387 the Illumina reads from the second set of samples onto the reference transcriptome, and counting the reads
388 to estimate gene expression levels. For the cloned major facilitator superfamily genes, complete open
389 reading frames were used as reference sequences for mapping. For read alignment, we used the following

390 parameters: nucleotide mismatch cost = 2; insertion = deletion costs = 3; length fraction = 0.6; similarity
391 fraction = 0.9; maximum number of hits for a read = 15. Each pair of reads was counted as two. Biases in
392 the sequence datasets and different transcript sizes were corrected using the TPM (transcripts per kilobase
393 million) normalization method to obtain correct estimates for relative expression levels between samples.

394 **Phylogenetic analyses of coleopteran MFS transporters.** We inferred the lineage-specific diversification
395 patterns of putative MFS transporters from *P. armoraciae*, *L. decemlineata*, *A. glabripennis* and *T.*
396 *castaneum* in phylogenetic analyses with two different datasets, one containing all identified MFS
397 transporters (867 sequences), the other containing a subset of putative sugar porters (120 sequences) from
398 the above four species and 35 sugar porters from *Chrysomela populi*⁵². The corresponding protein
399 sequences were aligned using the MUSCLE algorithm⁵⁸ implemented in MEGA 7 with default parameters.
400 The alignments were trimmed manually and the best substitution models were determined using ProtTest
401 3.4.2⁵⁹. Maximum-likelihood phylogenetic trees were constructed in IQ-TREE version 1.6.0⁶⁰ using the
402 VT+G+F substitution model with 1000 ultrafast bootstrap replicates for the full dataset, and the LG+G+F
403 substitution model with 1000 bootstrap replicates for the subset of putative sugar porters.

404 **Identification and sequencing of candidate transporters.** Based on our phylogenetic analysis, we
405 selected the largest clade of putative MFS transporters that was specifically expanded in *P. armoraciae* for
406 further studies. Transcriptome analysis revealed the presence of a pseudogene (*PaMFS9-ps*) that shares
407 between 43 and 95% nucleotide sequence identity with members of the focal clade. The protein encoded
408 by this pseudogene is predicted to possess only two transmembrane domains due to a premature stop codon
409 caused by frame shift mutations in the coding sequence. To obtain the full-length open reading frames
410 (ORFs) of partial transcripts, we performed rapid amplification of cDNA ends-PCR as described before²³.
411 All full length ORFs were cloned into the pCRTM4-TOPO[®] TA (Thermo Fisher Scientific) for sequence
412 verification.

413 **Tissue-specific expression of candidate transporters.** We used quantitative PCR (qPCR) to analyze the
414 expression of the candidate transporter genes in the foregut, midgut, hindgut, Malpighian tubules, and other
415 tissues of one-day old adult *P. armoraciae* beetles ($n = 4$ biological replicates, each with two technical
416 replicates), respectively. In addition, expression of candidate transporter genes was analyzed in the
417 proximal, central, and distal Malpighian tubule regions (Supplementary Fig. 2d) dissected from four-day
418 old adult *P. armoraciae* beetles ($n = 3$ biological replicates, each with two technical replicates). Primers
419 (Supplementary Table 5) were designed using Primer3web version 4.1.0. Analyses of primer specificity
420 and efficiency, RNA extraction, purification, cDNA synthesis, and qPCR were performed as described
421 before⁶¹. Gene expression was normalized to the expression level of eukaryotic initiation factor 4A (*eIF4A*),
422 which showed the lowest variability across tissues among four tested reference genes (Supplementary Table
423 6).

424 **Expression of candidate transporters in insect cells.** For protein expression, we cloned each ORF without
425 stop codon into the pIEx-4 expression vector (Novagen) in frame with the vector-encoded carboxy terminal
426 $6 \times$ His-tag and sequenced the resulting constructs. Primer sequences are listed in Supplementary Table 5.
427 One construct of each candidate gene was used for transfection of High Five™ insect cells (Gibco) cultured
428 in Express Five® SFM medium (Gibco) supplemented with 20 mM glutamine (Gibco) and 50 μ g/mL
429 gentamicin (Gibco). Confluent insect cells were diluted 1:5, dispensed in 500 μ L-aliquots into 24-well
430 plates, and incubated at 27 °C. On the next day, we transfected the cells using FuGENE HD Transfection
431 Reagent (Promega) according to the manufacturer's protocol. Cells treated with transfection reagent only
432 were used as a negative control. After 48 h we harvested the cells for Western blotting and uptake assays.

433 **Western blotting.** To confirm protein expression, transfected insect cells were washed twice with
434 phosphate buffered saline (PBS; pH 7.4), collected by centrifugation, and resuspended in hypotonic buffer
435 (20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM DTT, 0.1% Benzonase nuclease (Merck Millipore) (v/v),
436 and protease inhibitors (cComplete Mini, EDTA-free, Roche Diagnostics GmbH)). After incubation on ice
437 for 10 min, the samples were frozen in liquid nitrogen, thawed, and centrifuged ($16,000 \times g$ for 15 min at

438 4 °C). The resulting cell pellet was resuspended in hypotonic buffer and used for Western blotting using
439 HRP-conjugated anti-His antibody (1: 10,000; Novex, Life technologies).

440 **Glucoside uptake assays with transfected insect cells.** Cells were washed with PBS (pH 5.5) by pipetting
441 and incubated with different glucoside substrates at 200 μ M in PBS (pH 5.5) for 1 h at 27 °C. Assays were
442 performed with substrate mixtures containing seven different glucosinolates (2-propenyl glucosinolate
443 (Roth), 4-methylsulfinylbutyl glucosinolate (Phytoplan), 4-methylthiobutyl glucosinolate (Phytoplan), 2-
444 phenylethyl glucosinolate (Phytoplan), benzyl glucosinolate (Phytoplan), 4-hydroxybenzyl glucosinolate
445 (isolated from *Sinapis alba* seeds as described before⁶²), and indol-3-ylmethyl (I3M) glucosinolate
446 (Phytoplan)), or five other plant glycosides (salicin (Sigma-Aldrich), linamarin (BIOZOL), dhurrin (Roth),
447 catalpol (Sigma-Aldrich), and aucubin (Sigma-Aldrich). After incubation, cells were washed three times
448 with ice-cold PBS (pH 5.5) by pipetting, collected in 300 μ L 80% (v/v) methanol, frozen in liquid nitrogen,
449 thawed, and centrifuged at 3,220 \times g for 10 min at 4 °C. The supernatant was dried by vacuum centrifugation,
450 dissolved in ultrapure water and analyzed by liquid chromatography coupled with tandem mass
451 spectrometry (LC-MS/MS). All glucoside uptake assays were performed in triplicates. The amount of each
452 substrate in transporter-expressing cells was compared with that detected in control cells. Transporters were
453 considered active towards a substrate when the average amounts detected in transporter-expressing cells
454 were at least twofold higher than those detected in control cells.

455 **Cloning of *PaGTRI* into the pNB1u vector and cRNA synthesis.** We amplified the open reading frame
456 of *PaGTRI* without stop codon by PCR using uracil-containing primers (Supplementary Table 5). The 3'
457 primer was designed to encode a Human influenza hemagglutinin (HA)-tag to enable the detection of
458 recombinant protein by Western blotting if necessary. The pNB1u vector was digested overnight at 37 °C
459 with PacI and Nt.BbvCI (New England Biolabs) to generate 8-nt overhangs. One microliter gel-purified
460 PCR product (100 ng/ μ L) was combined with 1 μ L gel-purified vector (50 ng/ μ L), 1 unit USER enzyme,
461 2 μ L 5 x PCR reaction buffer and 5 μ L H₂O, incubated at 37 °C for 25 min followed by 25 min at room
462 temperature. After transformation of chemically competent *E. coli* cells, colonies containing the appropriate

463 insert were identified by Sanger sequencing. The DNA template for cRNA synthesis was amplified by PCR
464 from the *X. laevis* expression construct using pNB1uf/r primers (Supplementary Table 5) and cRNA was
465 synthesized using the mMESSAGE mMACHINE™ T7 Transcription Kit (Invitrogen) according to the
466 manufacturer's manual.

467 **Biochemical characterization of PaGTR1 in *Xenopus* oocytes.** The cRNA concentration was adjusted to
468 800 ng/μL with RNase-free water for oocyte injection. *X. laevis* oocytes (Ecocyte Bioscience) were injected
469 with 50 nL containing 40 ng cRNA or with 50 nL pure water as a control using a Drummond NANOJECT
470 II (Drummond Scientific Company) or a Nanoliter 2010 Injector (World Precision Instruments).

471 Injected oocytes were incubated in Kulori buffer (90 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂
472 and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4) supplemented with 50
473 μg/mL gentamicin at 16 °C for three days until assaying. All transport assays were performed at room
474 temperature. Injected oocytes were pre-incubated for 5 min in Kulori buffer (90 mM NaCl, 1 mM KCl, 1
475 mM MgCl₂, 1 mM CaCl₂ and 5 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0) before they were
476 transferred into the same buffer containing the substrate(s). To determine the substrate preference of
477 PaGTR1, we incubated oocytes in Kulori buffer (pH 6.0) containing an equimolar mixture of 2-propenyl
478 glucosinolate, 4-methylsulfinylbutyl glucosinolate, 4-methylthiobutyl glucosinolate, 2-phenylethyl
479 glucosinolate, benzyl glucosinolate, 4-hydroxybenzyl glucosinolate, and I3M glucosinolate, each at 200
480 μM, for 1 h.

481 The pH dependency of glucosinolate transport was determined by incubating the injected oocytes with
482 100 μM I3M glucosinolate for 10 min in Kulori buffer adjusted to different pH values. The effects of
483 different ionophores on glucosinolate transport was studied by incubating the PaGTR1-expressing oocytes
484 in Kulori buffer at pH 6.0 containing either 20 μM carbonyl cyanide *m*-chlorophenyl hydrazone (H⁺
485 ionophore, Sigma-Aldrich), 20 μM nigericin (K⁺/H⁺ exchanger, Abcam), or 20 μM valinomycin (K⁺
486 ionophore, Abcam), for 15 min. Afterwards, we incubated the oocytes in Kulori buffer containing 100 μM

487 I3M glucosinolate and the corresponding ionophore for 10 min. Assays performed with oocytes incubated
488 in Kulori buffer without any ionophore served as a control.

489 The time course of I3M glucosinolate uptake was analyzed by incubating oocytes with 100 μ M I3M
490 glucosinolate in Kulori buffer (pH 6.0) for 5, 10, 20, 30, 45, 60, 120, 180, and 240 min, respectively. The
491 apparent K_m value of *PaGTR1* for I3M glucosinolate was determined by incubating injected oocytes in
492 Kulori buffer (pH 6.0) for 10 min with different substrate concentrations. The K_m value was calculated by
493 nonlinear regression analysis in SigmaPlot 14.0 (Systat Software Inc.).

494 Each assay consisted of 14-15 oocytes and was stopped by washing oocytes four times with Kulori
495 buffer. Afterwards, 12 of the washed oocytes were distributed into four Eppendorf tubes, with three oocytes
496 per tube and immediately homogenized in 100 μ L of 50% (v/v) methanol. After centrifugation ($21,380 \times g$
497 or $16,000 \times g$ for 15 min), the supernatant was incubated at -20°C for at least 1 h to precipitate proteins,
498 which were pelleted by centrifugation ($21,380 \times g$ or $16,000 \times g$ for 15 min). Finally, 60 μ L sample was
499 diluted with 120 μ L ultrapure water, filtered through a 0.22 μ m PVDF-based filter plate (Merck Millipore),
500 and analyzed by LC-MS/MS. The glucosinolate concentration in oocytes was calculated by assuming an
501 oocyte volume of 1 μ L⁶³.

502 **LC-MS/MS.** Glucosinolates were quantified by LC-MS/MS using an Agilent 1200 HPLC system
503 connected to an API3200 tandem mass spectrometer (AB SCIEX). Separation was achieved on an
504 EC 250/4.6 NUCLEODUR Sphinx RP column (250 mm \times 4.6 mm, 5 μ m; Macherey–Nagel) using a binary
505 solvent system consisting of 0.2% (v/v) formic acid in water (A) and acetonitrile (B), with a flow rate of
506 1 mL/min at 25°C . The elution gradient was: 0-1 min, 1.5% B; 1-6 min, 1.5–5% B; 6-8 min, 5–7% B; 8-
507 18 min, 7–21% B; 18-23 min, 21–29% B; 23-23.1 min, 29–100% B; 23.1-24 min, 100% B; 24-24.1 min,
508 100 to 1.5% B; 24.1-28 min, 1.5% B. Glucosinolates were detected in negative ionization mode. The ion
509 spray voltage was set to $-4,500$ V. Gas temperature was set to 700°C , curtain gas to 20 psi, collision gas to
510 10, nebulizing gas to 70 psi, and drying gas to 60 psi. Non-host glucosides were quantified using an Agilent
511 1200 HPLC system connected to an API5000 tandem mass spectrometer (AB SCIEX). Separation was

512 achieved on an Agilent XDB-C18 column (5 cm × 4.6 mm, 1.8 μm) using a binary solvent system
513 consisting of 0.05 % (v/v) formic acid in water (A) and acetonitrile (B) with a flow rate of 1.1 mL/min at
514 25 °C. The elution gradient was: 0-0.5 min, 5% B; 0.5-2.5 min, 5–31% B; 2.5-2.52 min, 31–100% B; 2.52-
515 3.5 min, 100% B; 3.5-3.51 min, 100–5% B; 3.51-6 min, 5% B. Compounds were detected in negative
516 ionization mode with ion spray voltage set to -4,500 V. The gas temperature was set to 700 °C, curtain gas
517 to 30 psi, collision gas to 6, both nebulizing gas and drying gas to 60 psi. Multiple reaction monitoring
518 (MRM) was used to monitor the transitions from precursor ion to product ion for each compound
519 (Supplementary Table 7). Compounds were quantified using external standard curves. Analyst Software
520 1.6 Build 3773 (AB Sciex) was used for data acquisition and processing.

521 Samples from the pH-dependency experiment were analyzed by LC-MS/MS using an Advance UHPLC
522 system (Bruker) connected to an EVOQ Elite TripleQuad mass spectrometer (Bruker) equipped with an
523 electrospray ion source. Separation was achieved on an Kinetex 1.7u XB-C18 column (100 × 2.1 mm, 1.7
524 μm, 100 Å, Phenomenex) using a binary solvent system consisting of 0.05 % (v/v) formic acid in water (A)
525 and acetonitrile with 0.05% (v/v) formic acid (B), with a flow rate of 0.4 mL/min at 40 °C. The elution
526 gradient was: 0-0.2 min, 2% B; 0.2-1.8 min, 2-30% B; 1.8-2.5 min 30-100% B, 2.5-2.8 min 100% B, 2.8-
527 2.9 min, 100-2% B and 2.9-4.0 min 2% B. Glucosinolates were detected in negative ionization mode. The
528 instrument parameters were optimized by infusion experiments with pure standards. The ion spray voltage
529 was set to -4,000 V. Cone temperature was set to 350 °C, cone gas to 20 psi, heated probe temperature to
530 200 °C, and probe gas flow to 50 psi. Nebulizing gas was set to 60 psi, and collision gas to 1.6 mTorr.
531 MRM parameters are provided in Supplementary Table 7. Bruker MS Workstation software (Version 8.2.1,
532 Bruker) was used for data acquisition and processing of glucosinolates. All other samples from experiments
533 using oocyte expression system were analyzed using the LC-MS/MS method described above for insect
534 cell-based assays. The concentrations of all glucosinolates in the substrate preference assay were
535 determined using external standard curves. Assays performed to characterize I3M glucosinolate transport
536 were quantified using 2-propenyl glucosinolate as internal standard.

537 **Double-stranded RNA synthesis.** We synthesized seven different double-stranded RNAs (dsRNAs)
538 between 120 and 298 bp in length, one specific for each *PaGTR1/2/3/5/6/7/8*, respectively, and a 223-bp
539 fragment of the inducible metalloproteinase inhibitor (*IMPI*) from the greater wax moth *Galleria mellonella*
540 (AY330624.1) (*dsIMPI*) using the T7 RiboMAX™ Express RNAi System (Promega). *In silico* off-target
541 prediction was done by searches of all possible 21-mers of both RNA strands against the local *P.*
542 *armoraciae* transcriptome database allowing for two mismatches. Except for *PaGTR5*, the prediction did
543 not find any off-target towards putative transporter genes in the transcriptome (Supplementary Table 8).

544 **Function of *PaGTR1* in vivo.** To analyze the function of *PaGTR1*, we injected newly emerged adult *P.*
545 *armoraciae* beetles (reared on *B. juncea*) with 100 nL ultrapure water containing 80 ng of *dsPaGTR1* or 80
546 ng of *dsIMPI*, respectively, using a Nanoliter 2010 Injector (World Precision Instruments). Injected beetles
547 were provided with detached leaves of three to four-week old *B. juncea* plants and kept in plastic containers
548 with moistened tissue in the laboratory under ambient conditions. Four days after dsRNA injection, we
549 collected *dsIMPI*-injected and *dsPaGTR1*-injected beetles for gene expression analysis ($n = 5$ replicates,
550 three beetles per replicate) and glucosinolate analysis ($n = 10$ replicates, three beetles per replicate),
551 respectively. The remaining beetles were used for a sequestration experiment with *Arabidopsis thaliana*
552 Col-0 (*Arabidopsis*) plants that had been cultivated in a growth chamber at 21 °C, 55% relative humidity,
553 and a 10-h photoperiod. To compare the accumulation and excretion of ingested glucosinolates in *dsIMPI*-
554 injected and *dsPaGTR1*-injected beetles, we fed beetles with detached *Arabidopsis* leaves in Petri dishes
555 (60 mm diameter) that contained 50 μ L of ultrapure water and were sealed with parafilm to prevent leaf
556 wilting ($n = 9$ replicates for day 4, $n = 10$ replicates for other days, 5 beetles per replicate). Feeding assays
557 were performed in the laboratory under ambient conditions, and leaves were exchanged every day for five
558 consecutive days. To estimate how much the beetles fed, we determined the weight of each leaf before and
559 after feeding. Feces were collected every day using 100 μ L of ultrapure water per replicate, combined with
560 300 μ L of pure methanol in a 1.5 mL Eppendorf tube and dried by vacuum centrifugation. Feces samples
561 were then homogenized in 200 μ L 80% (v/v) methanol using metal beads (2.4 mm diameter, Askubal) in a

562 tissue lyzer (Qiagen) for 1 min at 30 Hz. After feeding, adults were starved for one day, weighed, frozen in
563 liquid nitrogen, and stored at -20 °C until glucosinolate extraction. Beetle samples were homogenized using
564 a plastic pestle in 200 μ L 80% (v/v) methanol. All samples were then extracted with 1 mL 80% (v/v)
565 methanol containing 25 μ M 4-hydroxybenzyl glucosinolate as internal standard. After centrifugation
566 (16,000 \times g for 10 min), glucosinolates were extracted from the supernatant, converted to desulfo-
567 glucosinolates, and analyzed by HPLC-UV as described before²³. The glucosinolate content in adults or
568 feces was calculated in nanomole per adult, respectively.

569 To confirm the specificity of *PaGTR1* knock-down we analyzed the effect of *dsPaGTR1* injection on
570 the expression of *PaGTR2*, *PaGTR3*, *PaGTR9*, and *PaGTR10*. *PaGTR9* and *PaGTR10* share the highest
571 nucleotide sequence similarity (69% sequence identity) with *PaGTR1*. *PaGTR2* and *PaGTR3* expression
572 was analyzed because the recombinant transporters also used indol-3-ylmethyl (I3M) glucosinolate as a
573 substrate. RNA extraction, purification, cDNA synthesis, and qPCR were performed as described before⁶¹.

574 **Function of *PaGTR5/6/7/8* in vivo.** To analyze the function of *PaGTR5/6/7/8*, we injected newly emerged
575 adult *P. armoraciae* beetles that had fed for two days on *B. juncea* leaves with 100 nL ultra pure water
576 containing 100 ng of *dsIMPI* or each 100 ng of *dsPaGTR5/6/7/8* using a Nanoliter 2010 Injector (World
577 Precision Instruments). A subset of the dsRNA-injected beetles were fed with detached leaves of
578 *Arabidopsis* plants in plastic containers with moistened tissue for gene expression analysis ($n = 6$ replicates,
579 two beetles per replicate). The remaining dsRNA-injected beetles were used for a sequestration experiment
580 with *Arabidopsis* plants to compare the accumulation and excretion of previously stored and ingested
581 glucosinolates in *dsIMPI*-injected and *dsPaGTR5/6/7/8*-injected beetles. We fed the injected beetles with
582 detached *Arabidopsis* leaves in Petri dishes (60 mm diameter) that contained 30 μ L of ultrapure water and
583 were sealed with parafilm to prevent leaf wilting ($n = 10$ replicates, 6 beetles per replicate). Feeding assays
584 were performed in the laboratory under ambient conditions, and leaves were exchanged every day for six
585 consecutive days. To estimate how much the beetles fed, we determined the weight of each leaf before and
586 after feeding. Starting from the second day, feces were collected as above for five days. After drying by

587 vacuum centrifugation, feces were homogenized in 1 mL 80% (v/v) methanol using metal beads (2.4 mm
588 diameter, Askubal) in a tissue lyzer (Qiagen) for 1 min at 30 Hz. Fed adults were starved for one day,
589 weighed and frozen in liquid nitrogen until glucosinolate extraction. All samples were extracted and
590 analyzed as described above except that an EC 250/4.6 NUCLEODUR 100-5 C18ec column (250 mm ×
591 4.6 mm, 5 µm; Macherey–Nagel) was used for analyzing glucosinolates by HPLC-UV.

592 To confirm the specificity of *PaGTR5/6/7/8* knock-down, we analyzed the effect of *dsPaGTR5/6/7/8*
593 injection on the expression of *PaGTR9* and *PaGTR10*, which share the highest nucleotide sequence
594 similarity (67%-69% sequence identity) with *PaGTR5/6/7/8*. RNA extraction, purification, cDNA
595 synthesis, and qPCR were performed as described before⁶¹.

596 **Function of *PaGTR2/3* *in vivo*.** To analyze the functions of *PaGTR2* and *PaGTR3*, we injected third instar
597 larvae of *P. armoraciae* (reared on *B. rapa*) with 100 nL ultrapure water containing 80 ng of *dsPaGTR2*-
598 and 80 ng of *dsPaGTR3* (*dsPaGTR2/3*) or 80 ng of *dsIMPI*, respectively, using a Nanoliter 2010 Injector
599 (World Precision Instruments). Injected larvae were provided with detached *B. rapa* petioles and kept in
600 plastic tubes with moistened tissue in the laboratory under ambient conditions until pupation. Newly
601 emerged adults were injected with dsRNAs as described above and provided with detached *B. rapa* leaves.
602 Three days after the second dsRNA injection, we collected *dsIMPI*-injected and *dsPaGTR2/3*-injected
603 beetles for gene expression analysis ($n = 6$ replicates, two beetles per replicate) and glucosinolate analysis
604 ($n = 10$ replicates, three beetles per replicate), respectively. The remaining beetles were used for a feeding
605 experiment with *Arabidopsis*. Each replicate consisted of one *Arabidopsis* leaf and three beetles that were
606 placed in a Petri dish (60 mm diameter) with 50 µL of ultrapure water ($n = 13$ replicates for *dsIMPI*-injected
607 beetles, $n = 12$ replicates for *dsPaGTR2/3*-injected beetles). Each leaf was photographed before and after
608 feeding to determine the consumed leaf area. Fed leaves were frozen in liquid nitrogen, freeze-dried, and
609 homogenized using metal beads (2.4 mm diameter, Askubal) in a tissue lyzer (Qiagen) for 2 min at 30 Hz.
610 Fed beetles were starved for one day, weighed, frozen in liquid nitrogen, and stored at -20 °C until
611 glucosinolate extraction. Beetle samples were homogenized using a plastic pestle in 200 µL 80% (v/v)

612 methanol. All samples were extracted with 1 mL 80% (v/v) methanol containing 25 μ M 1-methylethyl
613 glucosinolate (extracted from *Sisymbrium officinale* seeds) as internal standard. Glucosinolates were
614 analysed after conversion to desulfo-glucosinolates by HPLC-UV as described before²³. All the samples
615 were additionally analyzed by another chromatographic run with the same method as above except that an
616 EC 250/4.6 NUCLEODUR 100-5 C18ec column (250 mm \times 4.6 mm, 5 μ m; Macherey–Nagel) was used
617 for analyzing glucosinolates that were not separated in the first chromatographic run. The glucosinolate
618 content in adults and fed leaves was calculated in nanomole per adult and nanomole per cm² leaf,
619 respectively. The ingested glucosinolate amount was calculated based on the ingested leaf area and the
620 corresponding leaf glucosinolate content. To elucidate which proportion of the ingested glucosinolates was
621 sequestered, we expressed the glucosinolate amount detected in the beetles relative to the ingested
622 glucosinolate amount from the leaves, which was set to 100%.

623 **Glucosinolate concentration in the hemolymph of adult *P. armoraciae*.** Hemolymph was collected from
624 seven-day old adult *P. armoraciae* reared on *B. juncea* by cutting off an abdominal leg and collecting the
625 extruding droplet using glass capillaries (0.5 μ L, Hirschmann[®] minicaps[®]) ($n = 6$ replicates, 50 beetles per
626 replicate). The capillaries were marked with 1 mm intervals (corresponding to 15.6 nL) to estimate the
627 volume of collected hemolymph. The hemolymph was diluted in 500 μ L 90% (v/v) methanol, homogenized
628 using metal beads (2.4 mm diameter, Askubal) in a tissue lyzer (Qiagen) for 1 min at 30 Hz, and boiled for
629 5 min at 95 °C. After two centrifugation steps (13,000 $\times g$ for 10 min each), the supernatant was dried by
630 vacuum centrifugation, dissolved in 50 μ L 50% (v/v) methanol, diluted in water, and analyzed by LC-
631 MS/MS.

632 **Morphology of the Malpighian tubule system of *P. armoraciae*.** To investigate the structure of the
633 Malpighian tubule system, we dissected the gut including attached Malpighian tubules of four-day old *P.*
634 *armoraciae* adults in PBS (pH 6.0) under a stereomicroscope. The tracheae that attach Malpighian tubules
635 to the gut were removed using fine forceps to release the tubules. Pictures were taken with a Canon EOS
636 600D camera.

637 **pH of hemolymph and excretion fluid of isolated Malpighian tubules of *P. armoraciae*.** The pH of the
638 hemolymph and Malpighian tubule excretion fluid of five-day old *P. armoraciae* adults was assessed using
639 the pH indicator bromothymol blue (Alfa Aesar). Hemolymph was collected by cutting off an abdominal
640 leg and collecting the extruding droplet using a pipette ($n = 3$ replicates, six to ten beetles per replicate).
641 Excretion fluid was collected from dissected Malpighian tubules that were incubated in saline A as
642 described for the Ramsay assay ($n = 4$ replicates). Hemolymph and excretion fluid were immediately mixed
643 with the same volume of 0.16% (w/v) bromothymol blue dissolved in 10% (v/v) ethanol, respectively,
644 under water-saturated paraffin oil in a Sylgard-coated petri dish. The resulting color of the droplet was
645 compared with those of citric acid– Na_2HPO_4 buffer solutions ranging from pH 5.2 to 6.6 in 0.2 increments
646 mixed with 0.16% (w/v) bromothymol blue.

647 **Fate of plant glucosides injected in *P. armoraciae* beetles.** To analyze the fate of plant glucosides *in vivo*,
648 we injected 100 nL of an equimolar mixture of 2-propenyl glucosinolate, 4-hydroxybenzyl glucosinolate,
649 linamarin, salicin and catalpol, each at 10 mM, and 0.15% (w/v) amaranth into the hemolymph of two-day
650 old adult *P. armoraciae* (reared on *B. rapa*). One group of beetles was sampled 30 minutes after injection
651 by freezing beetles in liquid nitrogen ($n = 10$ replicates, five beetles per replicate). The remaining beetles
652 were fed with detached leaves of *Arabidopsis* in Petri dishes (60 mm diameter) in the laboratory under
653 ambient conditions ($n = 10$ replicates, 5 beetles per replicate). We added 30 μL of ultrapure water to each
654 Petri dish and sealed them with parafilm to prevent leaf wilting. After one day, we sampled the beetles as
655 described above ($n = 10$ replicates, five beetles per replicate). Feces were collected using 100 μL of
656 ultrapure water per replicate and combined with 300 μL of pure methanol in a 1.5 mL Eppendorf tube. All
657 samples were stored at -20°C until extraction. Beetle and feces samples were homogenized as described in
658 the RNAi experiment. After centrifugation ($16,000 \times g$ for 10 min), the supernatant was dried by vacuum
659 centrifugation, dissolved in 100 μL of ultrapure water and analyzed by LC-MS/MS. The glucoside content
660 in adults or feces was calculated as nanomole per adult, respectively.

661 **Ramsay assay.** To analyze the excretion of plant glucosides *in situ*, we performed Ramsay assays⁶⁴ with
662 dissected Malpighian tubules from four to five-day old *P. armoraciae* adults reared on *B. rapa*
663 (Supplementary Fig. 6). Malpighian tubules were dissected in saline A (100 mM NaCl, 8.6 mM KCl, 2 mM
664 CaCl₂, 8.5 mM MgCl₂, 4 mM NaH₂PO₄, 4 mM NaHCO₃, 24 mM glucose, 10 mM proline, 25 mM 3-(N-
665 Morpholino)propanesulfonic acid (MOPS), pH 6.0)⁶⁵. Single tubules were transferred into a 10 µL droplet
666 of saline B (60 mM NaCl, 10.3 mM KCl, 2.4 mM CaCl₂, 10.2 mM MgCl₂, 4.8 mM NaH₂PO₄, 4.8 mM
667 NaHCO₃, 28.8 mM glucose, 12 mM proline, 30 mM MOPS, 1 mM cyclic AMP, pH 6.0) under water-
668 saturated paraffin oil in a Sylgard-coated petri dish. The proximal end of the tubule was pulled out of the
669 droplet, attached to a metal pin and cut using a glass capillary to allow the collection of excretion fluid. To
670 start the assay, we added two microliters of an equimolar glucoside mixture consisting of 2-propenyl
671 glucosinolate, 4-methylsulfinylbutyl glucosinolate, 4-hydroxybenzyl glucosinolate, 2-phenylethyl
672 glucosinolate, I3M glucosinolate, linamarin, salicin, catalpol, each at 40 mM, and 0.6% (w/v) amaranth)
673 to saline B. After two to three hours, we collected the excretion fluid and 2 µL of the bathing droplet in 300
674 µL 80% (v/v) methanol, respectively. The Malpighian tubule was washed three times in about 15 mL of
675 saline A and afterwards transferred into 300 µL of 80% (v/v) methanol. All samples were stored at -20 °C
676 until extraction. Malpighian tubule samples were homogenized using a plastic pestle. After centrifugation
677 (16,000 × g for 10 min), the supernatant was dried by vacuum centrifugation, dissolved in 70 µL ultrapure
678 water and analyzed by LC-MS/MS. Out of 36 assays, 25 assays were excluded because no excretion fluid
679 was visible within 2-3 hours.

680 **Statistical analysis.** No statistical methods were used to predetermine sample size. Statistical analyses were
681 conducted in R 3.5.1⁶⁶ or in SigmaPlot 14.0 (Systat Software, Inc). Two groups were compared by two-
682 tailed Student's *t*-test, two-tailed Student's paired *t*-test, Mann-Whitney *U* test or the method of generalized
683 least squares⁶⁷, depending on the variance homogeneity and the normality of residuals. Three or more
684 groups were compared by one-way analysis of variance (ANOVA) followed by post hoc multiple
685 comparisons test, or the method of generalized least squares⁶⁷. If necessary, data were transformed prior to

686 analysis. For comparisons using the method of generalized least squares, we applied the varIdent variance
687 structure which allows each group to have a different variance. The *P*-value was obtained by comparing
688 models with and without explanatory variable using a likelihood ratio test⁶⁸. Significant differences between
689 groups were revealed with factor level reductions⁶⁹. Information about data transformation, statistical
690 methods, and results of the statistical analyses are summarized in Supplementary Tables 3, 4 and 9.

691 **Reporting summary.** Further information on research design is available in the Nature Research
692 Reporting Summary linked to this article.

693 **Data availability**

694 The data that support the findings of this study are available from the corresponding author upon
695 request. Source data of this study is available at the open access data repository of the Max Planck
696 Society (Edmond) under <https://dx.doi.org/10.17617/3.4z>. The sequences of the genes cloned in
697 this paper have been deposited in the GenBank database (accession nos. MN433061–MN433082).

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926 **Author contributions**

927 Z.-L.Y., and F.B. designed the study. F.B. supervised the study. H.H.N. supervised the
928 experiments with oocytes. Z.-L.Y. performed most of the experiments. S.H. contributed to RACE-
929 PCR and gene cloning. M.R. and C.C. contributed to LC-MS/MS and HPLC-UV analyses. F.B.
930 and H.V. performed transcriptome analysis. F.S. performed analysis of glucosinolate
931 concentration in hemolymph. Z.-L.Y. and F.B. conducted data analysis. Z.-L.Y., and F.B. wrote
932 the manuscript. All authors commented on the manuscript.

933 **Competing interests**

934 The authors declare no competing interests.

935 **Additional information**

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