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## Effects of glucosinolates on a generalist and specialist leaf-chewing herbivore and an associated parasitoid

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1 **Effects of glucosinolates on a generalist and specialist leaf-chewing herbivore and an**  
2 **associated parasitoid**

3

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22

23 **Abstract**

24

25 Glucosinolates (GLS) are secondary plant metabolites that as a result of tissue damage, for  
26 example due to herbivory, are hydrolysed into toxic compounds that negatively affect  
27 generalist herbivores. Specialist herbivores have evolved specific adaptations to detoxify GLS  
28 or inhibit the formation of toxic hydrolytic products. Although rarely studied, GLS and their  
29 breakdown products may also affect parasitoids. The objectives were to test the effects of  
30 GLS in a multitrophic system consisting of the generalist herbivore *Spodoptera exigua*, the  
31 specialist herbivore *Pieris rapae*, and the endoparasitoid *Hyposoter ebeninus*. Three ecotypes  
32 of *Arabidopsis thaliana* that differ in their GLS composition and concentrations and one  
33 transformed line that constitutively produces higher concentrations of aliphatic GLS were  
34 used, the latter allowing a direct assessment of the effects of aliphatic GLS on insect  
35 performance.

36 Feeding by the generalist *S. exigua* and the specialist *P. rapae* induced both higher  
37 aliphatic and indole GLS concentrations in the *A. thaliana* ecotypes, although induction was  
38 stronger for indole than aliphatic GLS. For both herbivores a negative correlation between  
39 performance and aliphatic GLS concentrations was observed. This suggests that the specialist,  
40 despite containing a nitrile-specifier protein (NSP) that diverts GLS degradation from toxic  
41 isothiocyanates to less toxic nitriles, cannot completely inhibit the formation of toxic GLS  
42 hydrolytic products, or that the costs of this mechanism are higher at higher GLS  
43 concentrations. Surprisingly, performance of the parasitoid was positively correlated with  
44 higher concentrations of aliphatic GLS in the plant, possibly caused by negative effects on  
45 host immune responses. Our study indicates that GLS can not only confer resistance against  
46 herbivores directly, but also indirectly by increasing the performance of the parasitoids of  
47 these herbivores.

48

49 **Key words:** *Arabidopsis thaliana*; Brassicaceae; *Hyposoter ebeninus*; Multitrophic  
50 interactions; *Pieris rapae*; *Spodoptera exigua*.

51

## 52 **1 Introduction**

53

54 Plants have evolved a wide array of resistance traits that prevent or reduce insect herbivory.  
55 These traits can affect the performance or behaviour of herbivores directly by chemical  
56 means, such as the production of toxins, repellents and digestibility reducers, or by physical  
57 means, such as the production of trichomes and epicuticular waxes (Karban and Baldwin,  
58 1997; Schoonhoven et al., 2005). Plants can also affect herbivores indirectly, by promoting  
59 the effectiveness of natural enemies that feed on these herbivores. For example, plants emit  
60 herbivore-induced volatiles that attract natural enemies of herbivores to the plant (Dicke and  
61 Baldwin, 2010; Hare, 2011; Kessler and Heil, 2011; Schoonhoven et al., 2005; Vet and  
62 Dicke, 1992). Secondary plant metabolites that confer direct resistance to herbivores can also  
63 negatively influence the performance of the natural enemies of these herbivores, either  
64 directly due to toxicity of the compounds, or indirectly due to reduced growth and  
65 development of their host or prey (Gols and Harvey, 2009; Harvey, 2005; Ode, 2006).

66       Among the best studied secondary plant metabolites are glucosinolates (GLS) that are  
67 characteristic for the plant family Brassicaceae. Upon damage, the GLS that are stored in the  
68 vacuoles become exposed to the enzyme myrosinase, which is stored separately in special  
69 cells. As a result of the myrosinase activity, GLS are hydrolysed into several toxic compounds  
70 such as (iso)thiocyanates and nitriles. These breakdown products negatively affect a wide  
71 variety of generalist herbivores (Bones and Rossiter, 2006; Halkier and Gershenzon, 2006;  
72 Hopkins et al., 2009). Specialist herbivores of Brassicaceae, however, have evolved specific

73 adaptations to detoxify GLS or inhibit the formation of toxic (iso)thiocyanates (Ratzka et al.,  
74 2002; Wittstock et al., 2004), sequester GLS (Francis et al., 2001; Kazana et al., 2007; Kos et  
75 al., 2011b; Müller, 2009), or use GLS and their hydrolysis products as oviposition or feeding  
76 stimulants (Gabrys and Tjallingii, 2002; Miles et al., 2005; van Loon et al., 1992).

77 GLS and their breakdown products may not only affect herbivores, but also natural  
78 enemies, such as predators and parasitoids, that feed on GLS-containing herbivores. Most  
79 studies on the effects of GLS on natural enemies involved predators, and these studies  
80 reported negative effects of GLS and their breakdown products on the performance of  
81 predators (Chaplin-Kramer et al., 2011; Francis et al., 2001; Kazana et al., 2007; Kos et al.,  
82 2011b; Pratt, 2008). Parasitoids, however, have been studied less frequently, and little is  
83 known about the effects of GLS and their breakdown products on the performance of  
84 parasitoids (Gols and Harvey, 2009).

85 The objectives of this study were to test the effects of GLS in a multitrophic system  
86 consisting of the brassicaceous *Arabidopsis thaliana* (L.) Heynh., the generalist herbivore  
87 *Spodoptera exigua* Hübner (Beet Armyworm: Lepidoptera, Noctuidae), the specialist  
88 herbivore *Pieris rapae* L. (Small Cabbage White butterfly; Lepidoptera: Pieridae), and the  
89 solitary koinobiont endoparasitoid *Hyposoter ebeninus* Gravenhorst (Hymenoptera:  
90 Ichneumonidae). *Spodoptera exigua* feeds on many plant species, among which  
91 brassicaceous plants, and is negatively affected by GLS and their breakdown products (Arany  
92 et al., 2008; Gigolashvili et al., 2007; Müller et al., 2010). The brassicaceous specialist *P.*  
93 *rapae* possesses a nitrile-specifier protein (NSP) in the gut that diverts GLS degradation from  
94 toxic isothiocyanates to less toxic nitriles that are excreted with the faeces (Wittstock et al.,  
95 2004). *Pieris rapae* adults use GLS as oviposition stimulants, and their larvae use these  
96 compounds as feeding stimulants (Miles et al., 2005; Müller et al., 2010). The endoparasitoid  
97 *H. ebeninus* parasitizes, among others, the larvae of *P. rapae*. Adult females lay a single egg

98 into a host larva. The hatched parasitoid larva first feeds on haemolymph and fat body of the  
99 host, whereas later in development it consumes all host tissues (except the head capsule and  
100 the outer layer of the cuticle), which leads to the death of the host (Harvey et al., 2010; J.  
101 Harvey, personal communication). The biology of *H. ebeninus* has only recently been studied  
102 in detail (Harvey et al., 2010) and, as far as we know, herbivore-mediated effects of GLS on  
103 *H. ebeninus* have never been tested.

104 To provide the herbivores with plants that differ in GLS content, three ecotypes of *A.*  
105 *thaliana* that differ in their total GLS concentrations, as well as in their GLS profiles (the  
106 qualitative and quantitative composition of the mix of GLS) were used (Houshyani et al.,  
107 2011). Furthermore, a genetically transformed line was developed that over-expresses the  
108 transcription factor HAG1/MYB28 and consequentially produces higher concentrations of  
109 aliphatic GLS compared to the wild-type plant. This allowed for making a direct assessment  
110 of the effects of aliphatic GLS concentrations on herbivore and parasitoid performance. It  
111 was expected that the performance of both herbivores would be negatively affected by higher  
112 levels of GLS in the host plant, but more so for the generalist *S. exigua* than for the specialist  
113 *P. rapae*. Furthermore, it was expected that GLS, through direct negative effects as well as  
114 through negative effects on the performance of *P. rapae*, would also negatively affect  
115 performance of *H. ebeninus*. The cascading effects of GLS in this multitrophic system are  
116 discussed.

117

## 118 **2 Results**

### 119 *2.1 Glucosinolate analysis*

120 The GLS concentrations of each of the three *A. thaliana* ecotypes (Cvi, Eri and Col-0) and the  
121 high-GLS transgenic line (Col-0-MYB28) were analysed. The total concentrations of aliphatic  
122 and indole GLS, as well as the GLS profile (the qualitative and quantitative composition of

123 the mix of GLS), of both uninfested and herbivore-infested plants were measured. We  
124 statistically compared the GLS composition a) among each of the three *A. thaliana* ecotypes,  
125 and b) between Col-0 and the Col-0-MYB28 line. The results of both comparisons are  
126 reported separately.

127

### 128 2.1.1 Aliphatic and indole GLS

129 Ecotype effect: The three *A. thaliana* ecotypes differed in the concentrations of aliphatic GLS,  
130 but not in indole GLS, as averaged for uninfested and herbivore-infested plants (ANOVA,  
131 aliphatic:  $F_{2,81} = 123.81$ ,  $P < 0.001$ ; indole:  $F_{2,81} = 2.33$ ,  $P = 0.104$ ). Cvi plants contained  
132 higher concentrations of aliphatic GLS than Eri and Col-0 plants (Fig. 1). Herbivore feeding  
133 increased concentrations of aliphatic and indole GLS, as averaged over the three ecotypes  
134 (ANOVA, aliphatic:  $F_{2,81} = 14.07$ ,  $P < 0.001$ ; indole:  $F_{2,81} = 30.48$ ,  $P < 0.001$ ). There were no  
135 differences in induction of aliphatic or indole GLS between the two herbivore species (post-  
136 hoc Tukey tests on the effect of herbivore treatment,  $P > 0.05$  for both comparisons). There  
137 was no interaction between ecotype and herbivore treatment for aliphatic and indole GLS  
138 (ANOVA,  $P > 0.05$  for both analyses). The pairwise differences in GLS concentrations  
139 among the nine ecotype x herbivore treatment combinations can be seen in Fig. 1. Based on  
140 the number of statistically significant pairwise differences in GLS concentrations between  
141 uninfested and infested plants, the induction effects of herbivory were most apparent for  
142 indole GLS (Fig. 1).

143 Over-expression effect: As averaged over the three herbivore treatments, Col-0-  
144 MYB28 plants contained higher aliphatic GLS concentrations than Col-0 plants (ANOVA,  
145  $F_{2,54} = 19.18$ ,  $P < 0.001$ ). However, there was an interaction between the effect of the *A.*  
146 *thaliana* line (Col-0 or Col-0-MYB28) and the herbivore treatment (ANOVA,  $F_{2,54} = 5.38$ ,  $P$   
147  $= 0.007$ ), because herbivore feeding did not induce aliphatic GLS in Col-0-MYB28 plants,

148 whereas it did in Col-0 plants. As a result, aliphatic GLS concentrations did not differ  
149 between herbivore-infested Col-0 and herbivore-infested Col-0-MYB28 plants (Fig. 1).  
150 Concentrations of indole GLS did not differ between Col-0 and Col-0-MYB28 plants, but  
151 were induced significantly by herbivore feeding in both Col-0 and Col-0-MYB28 plants  
152 (ANOVA, herbivore treatment:  $F_{2,54} = 22.86$ ,  $P < 0.001$ ; *A. thaliana* line and interaction:  $P >$   
153  $0.05$ ; Fig. 1).

154

### 155 2.1.2 GLS profiles

156 Ecotype effect: GLS profiles differed among the nine plant ecotype x herbivore treatment  
157 combinations (3 PLS-DA principal components,  $R_2X_{cum} = 0.920$ ,  $R_2Y_{cum} = 0.316$ ,  $Q_{2cum} =$   
158  $0.292$ ). The PLS-DA model showed that the largest difference in GLS profiles was due to the  
159 ecotype effect, and that the largest difference among the ecotypes was between Cvi and the  
160 other two ecotypes (Fig. 2). Differences among the ecotypes were both qualitative (different  
161 compounds) and quantitative (different concentrations of the compounds). Feeding by *S.*  
162 *exigua* or *P. rapae* only slightly, and only quantitatively, changed the GLS profile of the plant  
163 compared to the profile of the uninfested control plants. There were no clear differences in  
164 GLS profiles between plants infested by *S. exigua* or *P. rapae* (Fig. 2).

165 Over-expression effect: Also when comparing Col-0 and Col-0-MYB28, GLS profiles  
166 differed among the different plant line x herbivore treatment combinations (3 PLS-DA  
167 principal components,  $R_2X_{cum} = 0.772$ ,  $R_2Y_{cum} = 0.375$ ,  $Q_{2cum} = 0.332$ ). There were only  
168 quantitative differences in GLS profiles between the two lines, as both lines produced the  
169 same GLS compounds, but in different concentrations. Again, herbivore feeding only  
170 quantitatively changed the GLS profile of the plants, and there were no clear differences in  
171 GLS profiles between plants infested by *S. exigua* or *P. rapae* (Fig. 2).

172

## 173 2.2 *Spodoptera exigua* performance

174 Insect performance on each of the four *A. thaliana* ecotypes/lines was determined in no-  
175 choice assays. Similarly as reported for the GLS analyses, we compared herbivore  
176 performance a) among each of the three *A. thaliana* ecotypes, and b) between Col-0 and Col-  
177 0-MYB28.

178 Ecotype effect: Survival of *S. exigua* until the adult stage was on average 33% (25  
179 adults out of 75 larvae), and did not differ among the ecotypes (logistic regression,  $P > 0.05$ ,  
180 Table 1). *Spodoptera exigua* developed fastest into the adult stage on Eri plants, at  
181 intermediate rate on Col-0 plants, and slowest on Cvi plants (ANOVA,  $F_{2,19} = 53.80$ ,  $P <$   
182  $0.001$ ; Fig. 3). Adult body wt of *S. exigua* was highest on Eri plants, intermediate on Col-0  
183 plants, and lowest on Cvi plants (ANOVA,  $F_{2,19} = 27.27$ ,  $P < 0.001$ ; Fig. 3). There was no  
184 effect of sex or the interaction between ecotype and sex on development time and adult dry wt  
185 (ANOVA,  $P > 0.05$  for every analysis).

186 Over-expression effect: survival of *S. exigua* was twice lower on Col-0-MYB28 plants  
187 than on Col-0 plants, but the difference was not statistically different (logistic regression,  $P >$   
188  $0.05$ ; Table 1). *Spodoptera exigua* neonate-to-adult development time and adult dry wt did not  
189 differ between Col-0 and Col-0-MYB28 plants (ANOVA,  $P > 0.05$  for both comparisons; Fig.  
190 3).

191

## 192 2.3 *Pieris rapae* performance

193 Ecotype effect: Survival of *P. rapae* until the adult stage was on average 77% and did not  
194 differ among the ecotypes (logistic regression,  $P > 0.05$ ; Table 1). *Pieris rapae* development  
195 time was affected by plant ecotype and the interaction between ecotype and sex, but not by  
196 sex itself (ANOVA, ecotype:  $F_{2,52} = 30.79$ ,  $P < 0.001$ ; sex:  $F_{1,52} = 1.83$ ,  $P = 0.182$ ; interaction:  
197  $F_{2,52} = 3.70$ ,  $P = 0.032$ ). Overall, *P. rapae* developed faster into the adult stage on Col-0 and

198 Eri plants than on Cvi plants (Fig. 3). The interaction between ecotype and sex was due to a  
199 slightly faster development of females on Eri plants compared to males, whereas females on  
200 Col-0 and Cvi developed slightly slower than males, although none of the differences between  
201 males and females were significant. Adult dry wt of *P. rapae* was affected only by plant  
202 ecotype, and not by sex or the interaction between both (ANOVA, ecotype:  $F_{2,52} = 11.41$ ,  $P <$   
203  $0.001$ ; sex and interaction  $P > 0.05$ ). Adult dry wt of *P. rapae* was higher on Eri and Col-0  
204 plants than on Cvi plants (Fig. 3).

205 Over-expression effect: survival of *P. rapae* was lower on Col-0-MYB28 plants than  
206 on Col-0 plants (logistic regression,  $df = 1$ , deviance ratio = 4.05,  $P = 0.044$ ; Table 1).  
207 Development time and adult dry wt differed between Col-0 and Col-0-MYB28 plants  
208 (ANOVA, development time:  $F_{1,31} = 9.82$ ,  $P = 0.004$ ; wt:  $F_{1,31} = 7.30$ ,  $P = 0.011$ ). *Pieris*  
209 *rapae* developed slower and into adults with a lower body wt on Col-0-MYB28 plants (Fig.  
210 3).

211

#### 212 2.4 *Hyposoter ebeninus* performance

213 Ecotype effect: Survival of the parasitized *P. rapae* larvae, either until host pupation or until  
214 *H. ebeninus* eclosion, was on average 68% and did not differ among the ecotypes (logistic  
215 regression,  $P > 0.05$ , Table 1). The percentage of successful parasitism of *P. rapae* larvae by  
216 *H. ebeninus* differed among the ecotypes (logistic regression,  $df = 2$ , deviance ratio = 3.69,  $P$   
217 = 0.025) and was higher on Cvi plants than on Eri plants (Table 1). Furthermore, the  
218 percentage of successful parasitism was higher on Col-0 plants than on Eri plants (Table 1),  
219 although this difference was only marginally significant ( $P = 0.051$ ). Females developed  
220 slower than males ( $17.5 \pm 0.1$  days and  $17.2 \pm 0.1$  days respectively) (ANOVA,  $F_{1,93} = 6.51$ ,  $P$   
221 = 0.012). Females had a higher body wt than males ( $2.67 \pm 0.09$  mg and  $2.10 \pm 0.03$  mg  
222 respectively) (ANOVA,  $F_{1,93} = 65.17$ ,  $P > 0.001$ ). Although the development time and dry wet

223 of the host differed among the plant ecotypes, there was no difference in the egg-to-adult  
224 development time or adult dry wt of *H. ebeninus* among the plant ecotypes (Table 1), nor a  
225 significant interaction between parasitoid sex and plant ecotype (ANOVA,  $P > 0.05$  for every  
226 analysis).

227 Over-expression effect: Survival of the *P. rapae* larvae until host pupation or until *H.*  
228 *ebeninus* eclosion and the percentage of successful parasitism did not differ between Col-0  
229 and Col-0-MYB28 plants (logistic regression,  $P > 0.05$  for both comparisons, Table 1).  
230 Although the development time and dry wt of the host differed between Col-0 and Col-0-  
231 MYB28 plants, there were no differences in egg-to-adult development time and adult dry wt  
232 between parasitoids reared on both lines (ANOVA,  $P > 0.05$  for both comparisons; Table 1).

233

### 234 **3 Discussion**

235

236 This study showed that effects of GLS cascaded in a multitrophic system consisting of *A.*  
237 *thaliana* plants, a generalist and a specialist leaf-chewing herbivore and an associated  
238 parasitoid of the latter. Our study indicates that GLS can not only confer resistance against  
239 herbivores by negatively affecting their performance, but also by increasing the performance  
240 of the parasitoids of these herbivores.

241

#### 242 *3.1 Induction of GLS by herbivory*

243 Feeding by the generalist *S. exigua* and the specialist *P. rapae* induced both aliphatic and  
244 indole GLS concentrations in the three *A. thaliana* ecotypes. Effects of herbivore feeding on  
245 the induction of GLS were more apparent for indole GLS than for aliphatic GLS, which is a  
246 general trend in GLS-containing plants that are attacked by herbivores (Gols et al., 2008b;  
247 Mewis et al., 2006; Textor and Gershenzon, 2009). Feeding by *S. exigua* and *P. rapae*  
248 resulted in similar induction strength of GLS and in similar GLS profiles after induction.

249 Herbivory also induced indole GLS concentrations in plants of the genetically  
250 transformed Col-0-MYB28 line that produced higher concentrations of aliphatic GLS.  
251 Unexpectedly, herbivore feeding did not induce aliphatic GLS in these transformed plants.  
252 Most likely, the physiological maximum production of aliphatic GLS by Col-0 had been  
253 reached by inserting the HAG1/MYB28 transcription factor behind a constitutive promoter.

254

### 255 3.2 Effects of GLS on the two herbivores

256 Both the generalist *S. exigua* and the specialist *P. rapae* developed slower and into smaller  
257 adults on the *A. thaliana* ecotype with the highest aliphatic GLS concentrations, and *vice*  
258 *versa* on the *A. thaliana* ecotype with the lowest aliphatic GLS concentrations. Thus, aliphatic  
259 GLS concentrations seemed to be important in determining performance of both the generalist  
260 and the specialist herbivore. As expected, survival was much lower and the difference in  
261 performance among the ecotypes was larger for the generalist *S. exigua* than for the specialist  
262 *P. rapae*. *Pieris rapae* possesses a nitrile-specifier protein (NSP) that diverts GLS degradation  
263 from toxic isothiocyanates to less toxic nitriles (Wittstock et al., 2004). However, despite the  
264 NSP, a negative correlation between GLS concentrations and the performance of *P. rapae*  
265 was observed. There are several potential explanations for this negative effect. *Pieris rapae*  
266 excretes isothiocyanates in the faeces (Agelopoulos et al., 1995), suggesting that it cannot  
267 completely prevent formation of isothiocyanates by NSP-activity. NSP is probably not  
268 equally efficient in inhibiting the breakdown all GLS compounds into toxic products (Gols et  
269 al., 2008b; H. Vogel personal communication). The nitriles formed as a result of NSP-activity  
270 might still be moderately toxic to *P. rapae* (Burow and Wittstock, 2009; Hopkins et al.,  
271 2009). Alternatively, higher GLS concentrations might induce enhanced NSP biosynthesis  
272 that incurs higher energetic costs for the insect. Our finding of a negative correlation between  
273 GLS and herbivore performance are in agreement with other studies using *S. exigua* (Arany et

274 al., 2008; Gigolashvili et al., 2007; Müller et al., 2010) and *P. rapae* (Agrawal and Kurashige,  
275 2003). However, opposite to our findings, two other studies did not find major changes in  
276 larval development of *P. rapae* due to increased aliphatic GLS concentrations (Gols et al.,  
277 2008b; Müller et al., 2010). Perhaps this was due to a difference in the strain of *P. rapae* or  
278 the plant species that was used.

279 To experimentally test whether aliphatic GLS affect the performance of the two  
280 herbivores, herbivore performance on Col-0 and Col-0-MYB28 plants was compared. As  
281 expected based on the higher concentrations of aliphatic GLS, herbivore survival,  
282 development rate and adult size were lower on Col-0-MYB28 plants than on Col-0 plants.  
283 However, these negative effects were only statistically significant for *P. rapae*, probably due  
284 to the low number of surviving *S. exigua* adults on Col-0-MYB28 plants, possibly because  
285 this generalist species might be at the limit of its host range on *A. thaliana*. The low survival  
286 of *S. exigua* larvae prevented us from studying the effects on a parasitoid of this species. The  
287 higher aliphatic GLS concentration in Col-0-MYB28 plants compared to Col-0 plants was  
288 only significant for uninfested plants, not for herbivore-infested plants. The herbivores in the  
289 performance experiment were transferred to uninfested plants as neonates, and received one  
290 or two additional uninfested plants during their larval development. Therefore, these  
291 herbivores were exposed to the significantly higher GLS concentrations in uninfested Col-0-  
292 MYB28 plants at least several times during their development. We have no indications that  
293 the transformation of Col-0 affected other plant traits besides the production of aliphatic GLS,  
294 because there were no differences in plant traits such as biomass, diameter and trichome  
295 density between Col-0 and Col-0-MYB28 plants (data not shown).

296 Because the *A. thaliana* ecotypes/lines did not differ in indole GLS, effects of indole  
297 GLS on the performance of *S. exigua* and *P. rapae* could not be tested. Negative correlations

298 between indole GLS concentrations and performance of *S. exigua* (Müller et al., 2010) and *P.*  
299 *rapae* (Gols et al., 2008a; Gols et al., 2008b; Müller et al., 2010) have been reported before.

300 Performance of the herbivores might not have been affected only by total aliphatic or  
301 indole GLS concentrations, but also by specific compounds. The multivariate analysis mostly  
302 separated the GLS profile of Cvi plants from the profile of the other two ecotypes, which  
303 corresponded to the largest difference in herbivore performance. It has been proposed that  
304 specific GLS can shape insect performance and abundance more strongly than total  
305 concentrations of these compounds (Kos et al., 2011a; Poelman et al., 2009), and that plants  
306 may maintain variation in their chemical profile to confer resistance to many different  
307 attackers (Jones and Firn, 1991; Kos et al., 2011a; Newton et al., 2009). As expected, Col-0  
308 and Col-0-MYB28 plants did not show qualitative differences in GLS profiles, but only  
309 quantitative differences, i.e. differences in concentrations of each of the produced compounds.

310

### 311 *3.3 Effects of GLS on the third trophic level*

312 The percentage of successful parasitism of *P. rapae* by the parasitoid wasp *H. ebeninus* was  
313 affected by the *A. thaliana* ecotype that its host developed on. Interestingly, the percentage of  
314 successful parasitism was highest on the ecotype on which the performance of the host was  
315 lowest, and *vice versa*, although not all pair-wise differences were statistically significant.  
316 Larvae of *P. rapae* have an immune system that enables them to encapsulate the eggs of their  
317 parasitoids, leading to egg death. Larval wt of *P. rapae* correlates positively with  
318 encapsulation rates and the strength of induced plant defences correlates negatively with  
319 encapsulation rates (Bukovinszky et al., 2009). Perhaps the larger *P. rapae* larvae that  
320 developed on the ecotype Eri had higher parasitoid egg encapsulation rates, leading to a lower  
321 percentage of successful parasitism, than the smaller larvae that developed on Cvi. However,  
322 encapsulation rates of *H. ebeninus* eggs by *P. rapae* were not quantified. Negative

323 correlations between host size and the percentage of successful parasitism were not observed  
324 when comparing Col-0 and Col-0-MYB28 plants, even though there was a difference in *P.*  
325 *rapae* wt between both plant lines.

326 In contrast to the herbivores, development time and adult wt of *H. ebeninus* was not  
327 affected by the host plant ecotype/line. This is in agreement with the hypothesis that adverse  
328 effects of secondary metabolites on insect performance are often less pronounced in the  
329 parasitoid than in the herbivore (Gols and Harvey, 2009). However, it is unclear whether the  
330 *H. ebeninus* larvae were actually exposed to GLS or their hydrolysis products. Direct effects  
331 of GLS and their hydrolysis products on the performance of parasitoids have never been  
332 studied, and it is not known whether parasitoids have the ability to detoxify GLS or their  
333 hydrolysis products (Gols and Harvey, 2009).

334 Volatile breakdown products of GLS have been shown to attract several specialist  
335 parasitoids of herbivores that feed on GLS-containing plants (Blande et al., 2007; Bradburne  
336 and Mithen, 2000; Mumm et al., 2008). Whether this is also true for *H. ebeninus* is presently  
337 not known.

338

### 339 *3.4 Conclusion*

340 This study shows that secondary plant metabolites can affect the performance of insects at the  
341 second as well as the third trophic level. Aliphatic GLS concentrations were negatively  
342 correlated with the performance of not only a generalist herbivore, but also a specialist  
343 herbivore that is adapted to feeding on GLS-containing plants. Perhaps brassicaceous plants  
344 have evolved ways to circumvent the adaptations that specialist herbivores possess to cope  
345 with GLS by increasing the production of specific GLS compounds that these herbivores  
346 cannot detoxify. Host plant ecotype did not affect only the performance of the herbivores, but  
347 also the performance of a parasitoid of one of these herbivores. Surprisingly, the percentage

348 of successful parasitism was highest when the host developed on plants with the highest  
349 aliphatic GLS concentrations, possibly caused by negative effects on host immune responses.

350 Our study indicates that effects of GLS on herbivores can cascade up the food web  
351 and, through changes in host quality or constraints on immune responses, can positively affect  
352 parasitoids feeding on these herbivores. As a result, GLS can not only directly confer  
353 resistance against attacking herbivores by negatively affecting their performance, but also  
354 indirectly by increasing the performance of the parasitoids of these herbivores. It should be  
355 tested whether *H. ebeninus* is attracted to volatile breakdown products of GLS, which would  
356 further enhance the potential of GLS to confer resistance against herbivores in this study  
357 system.

358

## 359 **4. Experimental**

360

### 361 *4.1 Plant material and growth conditions*

362 Three *Arabidopsis thaliana* ecotypes were selected that differ in their GLS concentrations and  
363 profiles (Houshyani et al., 2011): Columbia (Col)-0 (provided by Dr. P. Reymond, Lausanne,  
364 Switzerland), Cape Verde Island (Cvi; obtained from the European Arabidopsis Stock Centre,  
365 <http://nasc.nott.ac.uk/>, Cvi = N8580) and Eringsboda (Eri; collected in Sweden by members  
366 of the Laboratory of Genetics, Wageningen University; Eri-1 = CS22548).

367 To produce plants with higher foliar levels of aliphatic GLS we over-expressed the  
368 transcription factor HAG1/MYB28 in *A. thaliana* ecotype Col-0 (Houshyani et al., in prep.;  
369 see also Method S1 in the Supplementary material). This transcription factor represents a key  
370 component in the regulation of aliphatic GLS biosynthesis in *A. thaliana* (Gigolashvili et al.,  
371 2007). T2 generation seeds of one successfully transformed line (hereafter named Col-0-  
372 MYB28) were used in the experiments.

373 *Arabidopsis thaliana* seeds were surface-sterilized overnight by vapour phase  
374 sterilization and inoculated on a growth medium (purified agar 0.8% + 2.2 g/L 0.5 MS +  
375 vitamins; pH 6; containing 30 µg/ml kanamycin to select transformed seedlings). After four  
376 days of stratification at 4 °C, plates were transferred to a growth chamber at 21 ± 2 °C, 50-  
377 70% relative humidity (RH) and a 8:16 light:dark (L:D) photo regime with a light intensity of  
378 200 µmol/m<sup>2</sup>/s photosynthetic photon flux density (PPFD). Two-week-old seedlings with two  
379 true leaves were transplanted to pots (5 cm diameter) containing autoclaved soil (80 °C for 4  
380 h; Lentse potgrond, Lent, The Netherlands). Plants were watered three times per week and  
381 entomopathogenic nematodes (*Steinernema feltiae*; Koppert Biological Systems, Berkel en  
382 Rodenrijs, The Netherlands) were added weekly to the soil to control infestation by larvae of  
383 sciarid flies. Plants were used in the experiments when they were seven weeks old and  
384 remained in the vegetative state during the experiments.

385

#### 386 4.2 Insect rearing

387 *Spodoptera exigua* was reared on artificial diet (Table S1 in the Supplementary material) in a  
388 climatized room at 27 ± 2 °C, 50% RH and a 16:8 h L:D photo regime. Adults were provided  
389 with water-saturated cotton wool. *Pieris rapae* was reared on Brussels sprouts (*Brassica*  
390 *oleracea* L. var. *gemmifera* cv. Cyrus) in a climatized room at 22 ± 2 °C, 40-50% RH and a  
391 16:8 h L:D photo regime. Adults were provided with a sugar water solution in small plastic  
392 tubes. Female *S. exigua* and *P. rapae* were allowed to oviposit on a piece of filter paper (*S.*  
393 *exigua*) or a Brussels sprouts plant (*P. rapae*) for 24 h. Neonate larvae were used in the  
394 experiments.

395 *Hyposoter ebeninus* was reared in *P. rapae* larvae. A leaf of a Brussels sprouts plant  
396 infested with second instar *P. rapae* larvae was exposed to female parasitoids for  
397 approximately one hour. Afterwards, the parasitized larvae were transferred to a gauze cage

398 (30 x 40 x 60 cm) containing Brussels sprouts plants in a greenhouse compartment at  $22 \pm 2$   
399 °C, 60-70% RH and a 16:8 h L:D photo regime. Parasitoid cocoons were collected from the  
400 plants and the eclosed adult wasps were provided with water and honey. Because the survival  
401 of *S. exigua* was very low (see *Results*), it was not possible to include a parasitoid of *S.*  
402 *exigua* in our study.

403

#### 404 4.3 Glucosinolate analysis

405 To correlate herbivore performance with GLS profiles of the plants, GLS were extracted from  
406 10 uninfested, 10 *S. exigua*-infested and 10 *P. rapae*-infested plants of each ecotype/line.  
407 Infested plants had been inoculated by one neonate larva. After five days, the larvae were  
408 removed from the plants and the remaining leaf material was harvested for GLS extraction.  
409 GLS could not be extracted from the same plants on which herbivore performance was tested,  
410 as there was not enough leaf material left after feeding by the herbivores during their entire  
411 larval development. Leaf samples were frozen at -80 °C immediately after collection, freeze-  
412 dried, weighed into a micro-centrifuge tube and ground to a fine powder. GLS were extracted  
413 and purified by using the methods of Van Dam et al. (2004) and Kabouw et al. (2010) and  
414 foliar GLS content was assessed using high-performance liquid chromatography (HPLC).  
415 GLS detection was performed with a photodiode array detector set at 229 nm as the  
416 integration wavelength. Different concentrations of sinigrin (Acros, New Jersey, USA) were  
417 used as an external standard. The correction factors at 229 nm from Buchner (1987) and the  
418 European Community (1990) were used to calculate the concentrations of the GLS.  
419 DesulfoGLS peaks were identified by comparison of HPLC retention times and ultraviolet  
420 spectra with standards provided by M. Reichelt (Max Planck Institute for Chemical Ecology,  
421 Jena, Germany) and a certified rapeseed standard (Community Bureau of Reference, Brussels,  
422 Belgium, code BCR-367 R).

423

#### 424 4.4 Insect performance

425 The performance of *S. exigua*, *P. rapae* and *H. ebeninus* was tested in no-choice situations in  
426 cylindrical plastic containers (height 13 cm; diameter 11 cm) with a gauze lid, each  
427 containing one *A. thaliana* plant. The experiments were performed in a climate chamber at 21  
428  $\pm 2$  °C, 50-70% relative humidity (RH) and a 16:8 L:D photo regime with a light intensity of  
429 200  $\mu\text{mol}/\text{m}^2/\text{s}$  PPFD inside the container. Plants were watered once per week.

430

##### 431 4.4.1 *Spodoptera exigua* and *Pieris rapae* performance

432 Forty plants of each ecotype/line were infested with one neonate *S. exigua* larva and 25 plants  
433 with one neonate *P. rapae* larva. A larger number of replicates was used for *S. exigua* because  
434 a higher mortality of this generalist herbivore was expected. Larvae were allowed to feed on  
435 the plant until pupation. Additional plants were added if the larvae had consumed the first  
436 plant. Pupae were left in the container until adult eclosion, which was checked once a day,  
437 and survival, neonate-to-adult development time, sex and adult dry wt were measured. Adult  
438 dry wt was measured on a microbalance (Sartorius CP2P, Göttingen, Germany) by weighing  
439 freshly eclosed adults that had been dried to constant wt at 80 °C for 3 days.

440

##### 441 4.4.2 *Hyposoter ebeninus* performance

442 Twenty mated female *H. ebeninus* adults of five-to-seven days old were collected from the  
443 stock rearing. Second instar (three day old) *P. rapae* larvae that were reared on one of each of  
444 the *A. thaliana* ecotypes/lines were exposed individually to a female parasitoid on a feeding-  
445 damaged leaf until parasitisation was observed (i.e. when the female inserted her ovipositor  
446 in the larva). Two parasitized larvae were transferred to one *A. thaliana* plant of the same  
447 ecotype/line as the one on which these larvae had been reared. Two larvae per plant instead

448 of one were used because it was expected that parasitisation of the larvae would increase their  
449 mortality. In total 40 plants per *A. thaliana* ecotype/line were tested. Parasitoid cocoons were  
450 collected from the plants and upon adult parasitoid eclosion, parasitoid sex was determined,  
451 and egg-to-adult development time and adult dry wt were measured (similar as described  
452 above). Survival of the parasitized *P. rapae* larvae, either until host pupation or until *H.*  
453 *ebeninus* eclosion, was calculated by adding the number of hosts that pupated to the number  
454 of hosts that developed into a *H. ebeninus* adult and dividing this number by the total number  
455 of larvae that were tested. The percentage of successful parasitism of *P. rapae* larvae by *H.*  
456 *ebeninus* was calculated by dividing the number of *H. ebeninus* adults by the total number of  
457 *P. rapae* larvae that survived (either until host pupation or until *H. ebeninus* eclosion).

458

#### 459 4.5 Statistical analysis

460 Analyses were performed in SPSS for Windows (15<sup>th</sup> edition, Chicago, IL, USA), unless  
461 indicated otherwise. Following each ANOVA performed in this study, post-hoc Tukey-tests  
462 were used for pair-wise comparisons.

463 GLS concentrations were log-transformed to obtain normality. Differences in  
464 aliphatic and indole GLS concentrations of leaves a) among the ecotypes and b) between Col-  
465 0 and Col-0-MYB28 were analysed by two-way ANOVA for the factors plant ecotype/line,  
466 herbivore treatment (uninfested, *P. rapae*-infested and *S. exigua*-infested) and the  
467 interactions. Because the only aromatic glucosinolate (gluconasturtiin) detected was present  
468 in only trace amounts, this compound was excluded from statistical analysis.

469 To test if there were differences in GLS profiles of leaves a) among the nine *A.*  
470 *thaliana* ecotype x herbivore treatment combinations and b) among the six *A. thaliana* line  
471 (Col-0 and Col-0-MYB28) x herbivore treatment combinations, the multivariate analysis  
472 Projection to Latent Structures-Discriminant Analysis (PLS-DA) in SIMCA-P (12<sup>th</sup> edition,

473 Umetrics, Umeå, Sweden) (Eriksson et al., 2006) was used. To pre-process data, GLS  
474 concentrations were mean-centred and scaled to unit variance.

475 Differences in development time and adult dry wt of the herbivores and parasitoids a)  
476 among the ecotypes and b) between Col-0 and Col-0-MYB28 were analysed by two-way  
477 ANOVA on the factors plant ecotype/line, sex, and the interactions. The development time of  
478 *P. rapae* was log-transformed to obtain normality. Differences in the survival and the  
479 percentage of successful parasitism a) among the ecotypes and b) between Col-0 and Col-0-  
480 MYB28 were analysed by logistic regression in GenStat (13<sup>h</sup> edition, VSN International,  
481 UK), followed by calculating t-probabilities to test pair-wise differences between means.

482

### 483 **Supplementary material**

484

485 **Method S1.** Generation of HAG1/MYB28 over-expression plants

486 **Table S1.** Artificial diet for *Spodoptera exigua*

487 **Table S2.** Trivial and scientific names of the GLS compounds found in this study

488

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490

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**Table 1. Performance parameters of *S. exigua*, *P. rapae* and *H. ebeninus* reared on three *A. thaliana* ecotypes and one transformed line that produces higher levels of aliphatic glucosinolates**

Insect species	Performance parameter	<i>A. thaliana</i> ecotype <sup>a</sup>			Transformed <i>A. thaliana</i> line <sup>b</sup>
		Cvi	Eri	Col-0	Col-0-MYB28
<i>S. exigua</i>	Survival until adult stage (%)	28 <b>a</b>	32 <b>a</b>	40 <b>a</b>	20 <b>ns</b>
	Number of individuals ( <i>n</i> ) surviving until adult stage	7	8	10	5
<i>P. rapae</i>	Survival until adult stage (%)	68 <b>a</b>	80 <b>a</b>	84 <b>a</b>	56 <b>*</b>
	Number of individuals ( <i>n</i> ) surviving until adult stage	17	20	21	14
<i>H. ebeninus</i>	Survival until host pupation/ <i>H. ebeninus</i> eclosion (%)	70 <b>a</b>	75 <b>a</b>	58 <b>a</b>	70 <b>ns</b>
	Successful parasitism (%)	71 <b>b</b>	48 <b>a</b>	63 <b>ab</b>	46 <b>ns</b>
	Number of individuals ( <i>n</i> ) surviving until adult stage	40	29	31	29 <b>ns</b>
	Egg-to-adult development time (days; mean ± SE)	17.2 ± 0.1 <b>a</b>	17.0 ± 0.1 <b>a</b>	17.4 ± 0.1 <b>a</b>	17.3 ± 0.1 <b>ns</b>
	Adult dry wt (mg; mean ± SE)	2.17 ± 0.04 <b>a</b>	2.27 ± 0.08 <b>a</b>	2.13 ± 0.07 <b>a</b>	2.06 ± 0.04 <b>ns</b>

<sup>a</sup> Different letters denote differences in means among the three ecotypes as analysed by logistic regression and post-hoc T-probability tests (survival) or ANOVA (development time and dry wt); <sup>b</sup>\* denotes significant difference and ns denotes non-significant difference between Col-0 and Col-0-MYB28 as analysed by logistic regression and post-hoc T-probability tests (survival) or ANOVA (development time and dry wt).

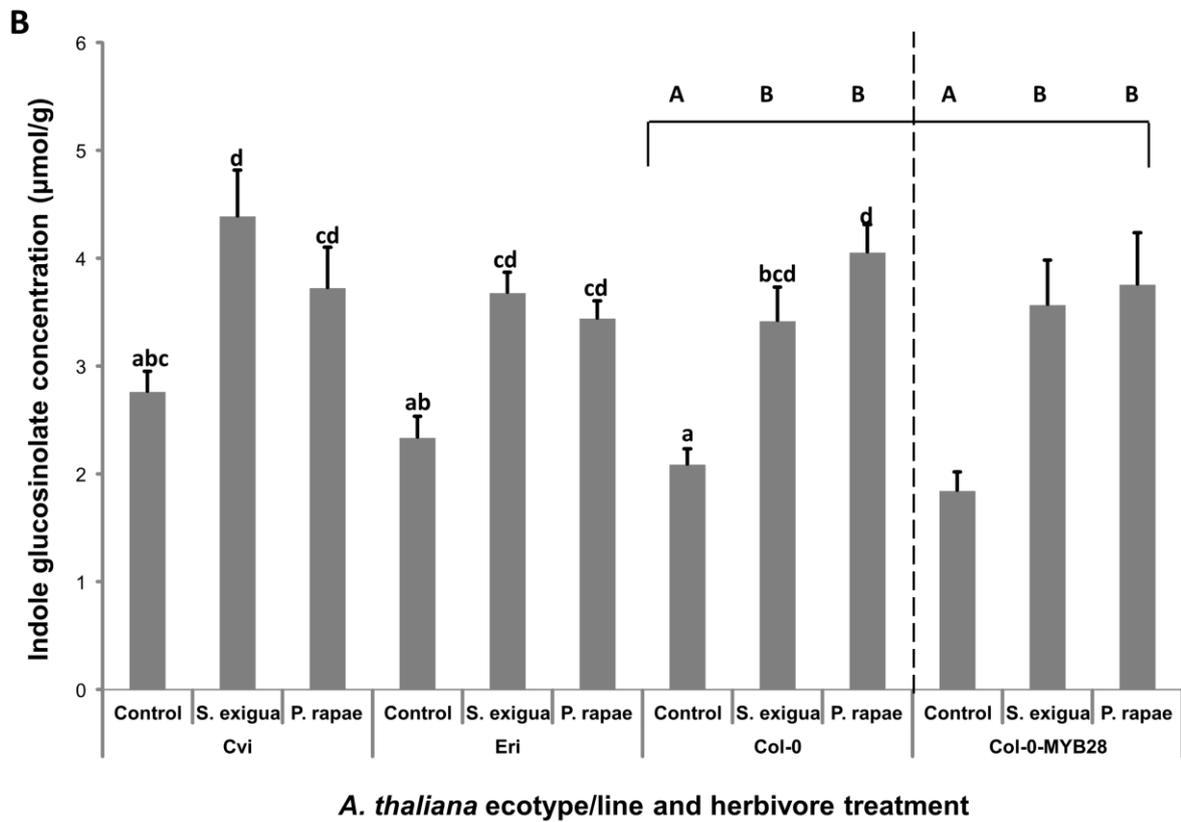
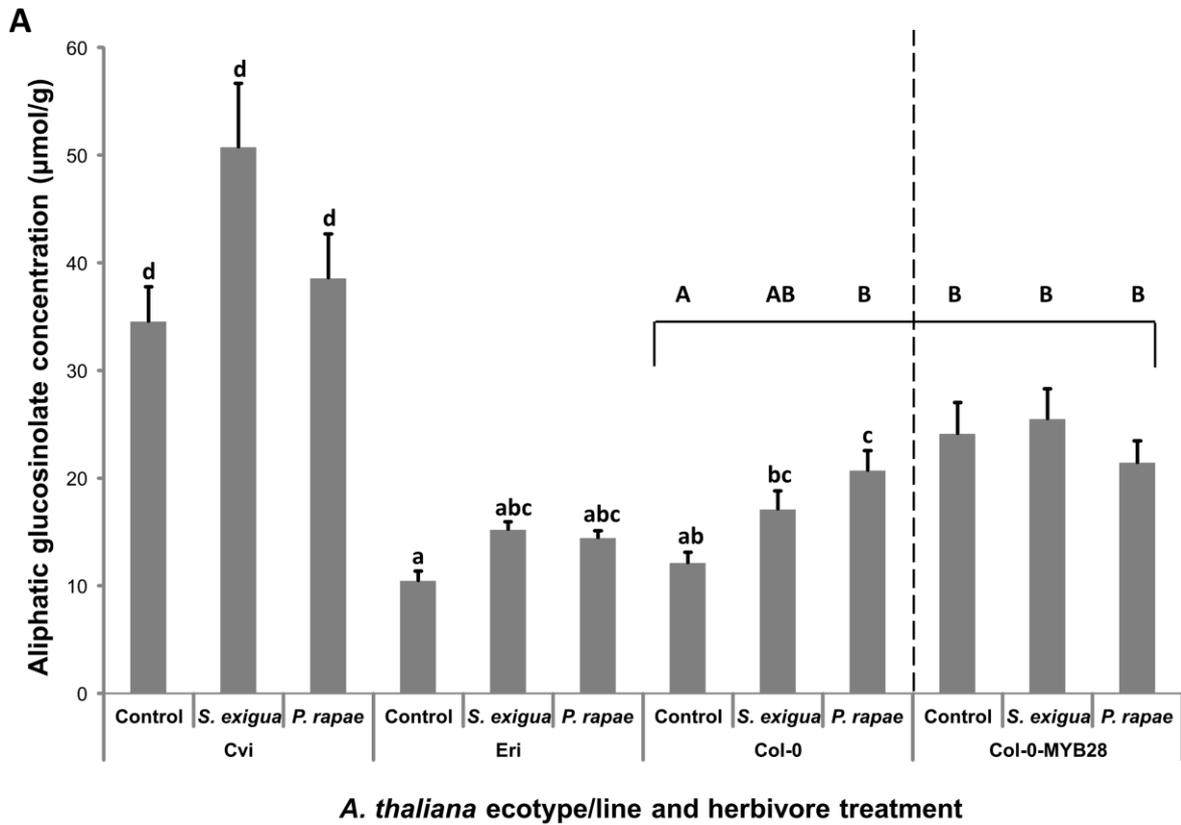
**Fig. 1.** Aliphatic (A) and indole (B) glucosinolate (GLS) concentration (in  $\mu\text{mol/g}$  dry wt; mean + SE) of plants of three *Arabidopsis thaliana* ecotypes (Cvi, Eri and Col-0) and one transformed line that produces higher levels of aliphatic GLS (Col-0-MYB28). Plants were either uninfested (control), infested for five days by one neonate *Spodoptera exigua* larva or infested for five days by one neonate *Pieris rapae* larva. GLS were divided into indole and aliphatic GLS based on their biosynthetic origin.  $n = 10$  for each bar. Different small case letters indicate differences between the nine ecotype x herbivore treatment combinations at the level of  $P < 0.05$  (post-hoc Tukey tests). Different capital letters are used to compare Col-0 and Col-0-MYB28 plants and indicate differences between the six line x herbivore treatment combinations at the level of  $P < 0.05$  (post-hoc Tukey tests).

**Fig. 2.** PLS-DA score and loading plots of the first two components showing the glucosinolate (GLS) profiles of three *Arabidopsis thaliana* ecotypes (**A** and **B**) and one *A. thaliana* ecotype and its transformed line that produces higher levels of aliphatic GLS (**C** and **D**). Plant ecotypes are Cvi (inverted triangles), Eri (diamonds) and Col-0 (triangles); transformed line is Col-0-MYB28 (circles). Herbivore treatments are: uninfested control (black labels in score plot, C in loading plot), infested for five days by one neonate *Spodoptera exigua* larvae (grey labels, S) or infested for five days by one neonate *Pieris rapae* larva (white labels, P). The score plots (**A** and **C**) show the distinction in GLS profiles of the different ecotype/line x herbivore treatment combinations. In brackets the percentage of variation explained is indicated for each axis. The loading plots (**B** and **D**) show the contribution of each of the GLS compounds to the discrimination among the different ecotype/line x herbivore treatment combinations. Aliphatic GLS: ALY = glucoalyssin, GBN = glucobrassicinapin, GNA = gluconapin, HIR = glucohirsutin, IBE = glucoiberin, ERU = glucoerucin, RAPH = glucoraphanin, SBE = glucosiberin, SIN = sinigrin. 7THIO = 7-

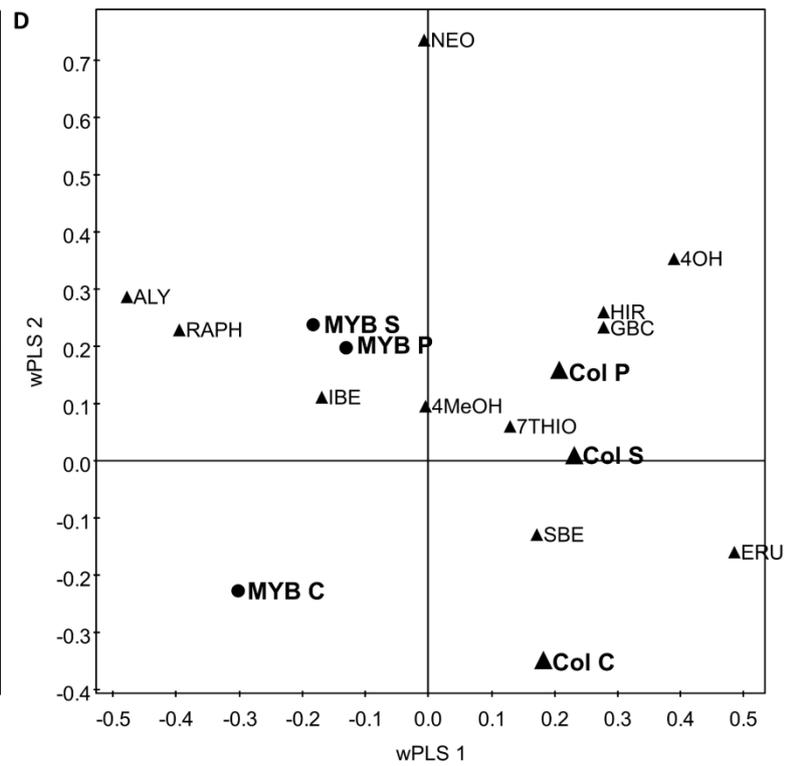
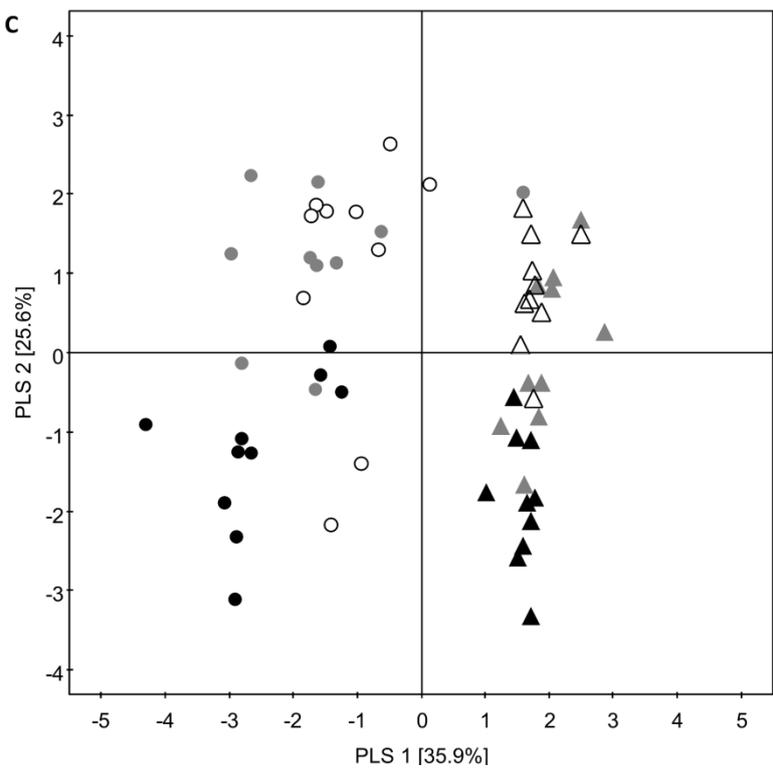
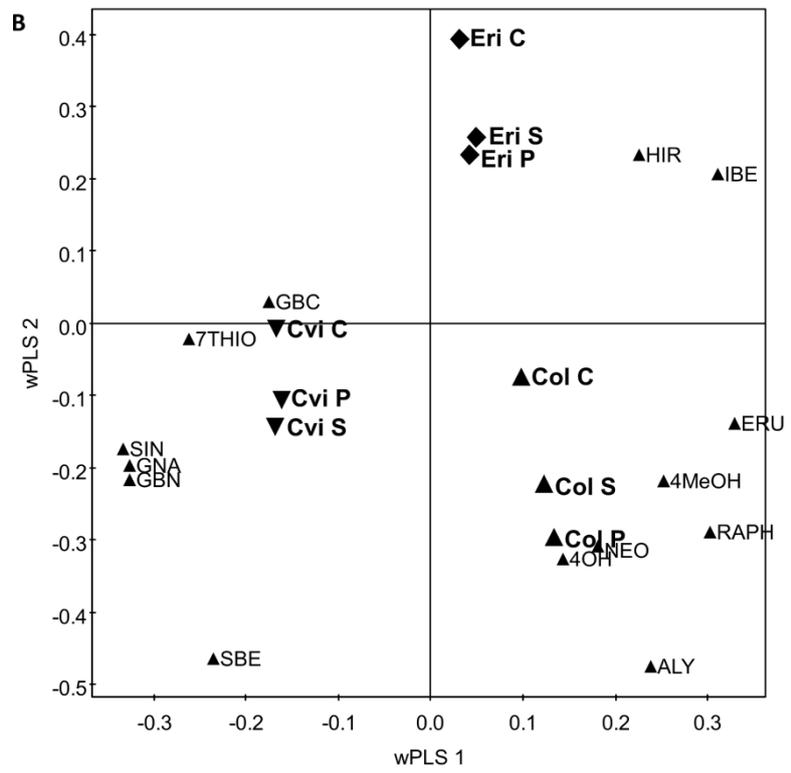
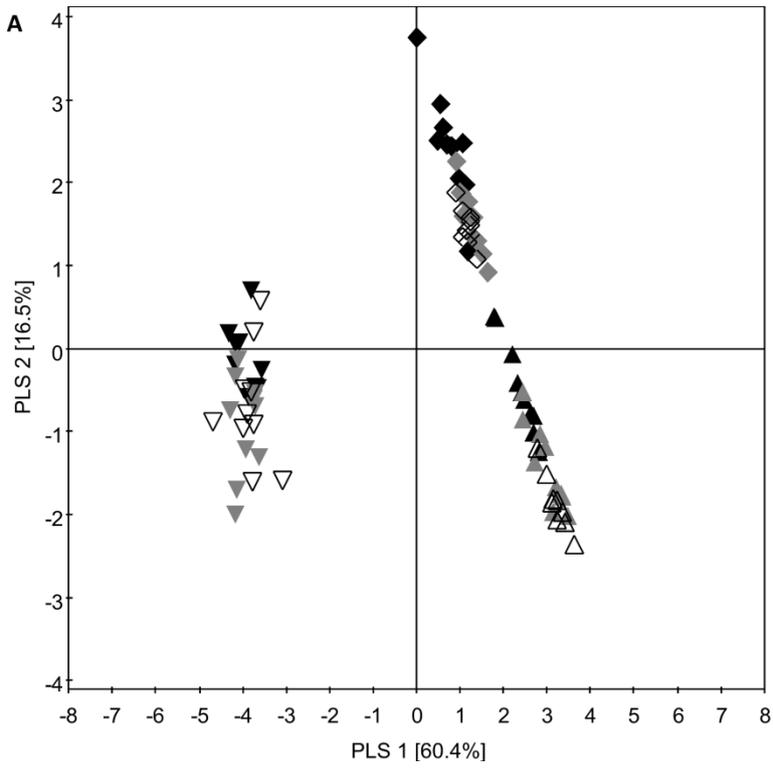
methylthioheptylGLS. Indole GLS: GBC = glucobrassicin, NEO = neo-glucobrassicin, 4MeOH = 4-methoxyglucobrassicin, 4OH = 4-hydroxyglucobrassicin. See Table S2 in the Supplementary material for the scientific names of all GLS compounds.

**Fig. 3.** Neonate-to-adult development time in days (A) and adult dry wt in mg (B) (mean + SE) of *Spodoptera exigua* and *Pieris rapae* when feeding on three *Arabidopsis thaliana* ecotypes (Cvi, Eri and Col-0) and one transformed line that produces higher levels of aliphatic glucosinolates (Col-0-MYB28). Within a herbivore species, different letters (small letters for *S. exigua* and capital letters for *P. rapae*) above the bars indicate significant differences among the *A. thaliana* ecotypes at the level of  $P < 0.05$  (post-hoc Tukey tests). Differences in herbivore performance between Col-0 and Col-0-MYB28 plants are indicated by the letters above the horizontal line (small letters for *S. exigua* and capital letters for *P. rapae*).  $n$  tested is indicated in each bar.

**Fig. 1**



**Fig. 2**



**Fig. 3**

