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

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Abstract

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

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

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

1 Introduction

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

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

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

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

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

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

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

2 Results

2.1 Glucosinolate analysis

The GLS concentrations of each of the three *A. thaliana* ecotypes (Cvi, Eri and Col-0) and the high-GLS transgenic line (Col-0-MYB28) were analysed. The total concentrations of aliphatic 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,

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

2.1.2 GLS profiles

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

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

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 ± 0.1 days and 17.2 ± 0.1 days respectively) (ANOVA, $F_{1,93} = 6.51$, P
221 = 0.012). Females had a higher body wt than males (2.67 ± 0.09 mg and 2.10 ± 0.03 mg
222 respectively) (ANOVA, $F_{1,93} = 65.17$, $P > 0.001$). Although the development time and dry wet

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

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

3 Discussion

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

3.1 Induction of GLS by herbivory

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

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

3.2 Effects of GLS on the two herbivores

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

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

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

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

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

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

3.3 Effects of GLS on the third trophic level

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

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

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

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

3.4 Conclusion

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

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

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

4. Experimental

4.1 Plant material and growth conditions

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

To produce plants with higher foliar levels of aliphatic GLS we over-expressed the transcription factor HAG1/MYB28 in *A. thaliana* ecotype Col-0 (Houshyani et al., in prep.; see also Method S1 in the Supplementary material). This transcription factor represents a key component in the regulation of aliphatic GLS biosynthesis in *A. thaliana* (Gigolashvili et al., 2007). T2 generation seeds of one successfully transformed line (hereafter named Col-0-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^2/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

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

4.3 Glucosinolate analysis

To correlate herbivore performance with GLS profiles of the plants, GLS were extracted from 10 uninfested, 10 *S. exigua*-infested and 10 *P. rapae*-infested plants of each ecotype/line. Infested plants had been inoculated by one neonate larva. After five days, the larvae were removed from the plants and the remaining leaf material was harvested for GLS extraction. GLS could not be extracted from the same plants on which herbivore performance was tested, as there was not enough leaf material left after feeding by the herbivores during their entire larval development. Leaf samples were frozen at -80 °C immediately after collection, freeze-dried, weighed into a micro-centrifuge tube and ground to a fine powder. GLS were extracted and purified by using the methods of Van Dam et al. (2004) and Kabouw et al. (2010) and foliar GLS content was assessed using high-performance liquid chromatography (HPLC). GLS detection was performed with a photodiode array detector set at 229 nm as the integration wavelength. Different concentrations of sinigrin (Acros, New Jersey, USA) were used as an external standard. The correction factors at 229 nm from Buchner (1987) and the European Community (1990) were used to calculate the concentrations of the GLS. DesulfoGLS peaks were identified by comparison of HPLC retention times and ultraviolet spectra with standards provided by M. Reichelt (Max Planck Institute for Chemical Ecology, Jena, Germany) and a certified rapeseed standard (Community Bureau of Reference, Brussels, 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 ± 2 °C, 50-70% relative humidity (RH) and a 16:8 L:D photo regime with a light intensity of
429 200 µmol/m^2/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

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

4.5 Statistical analysis

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

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

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

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

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

Supplementary material

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

Table S1. Artificial diet for *Spodoptera exigua*

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

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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

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

[a] 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); [b] * 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 µmol/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 = glucobrassicanapin, 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 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

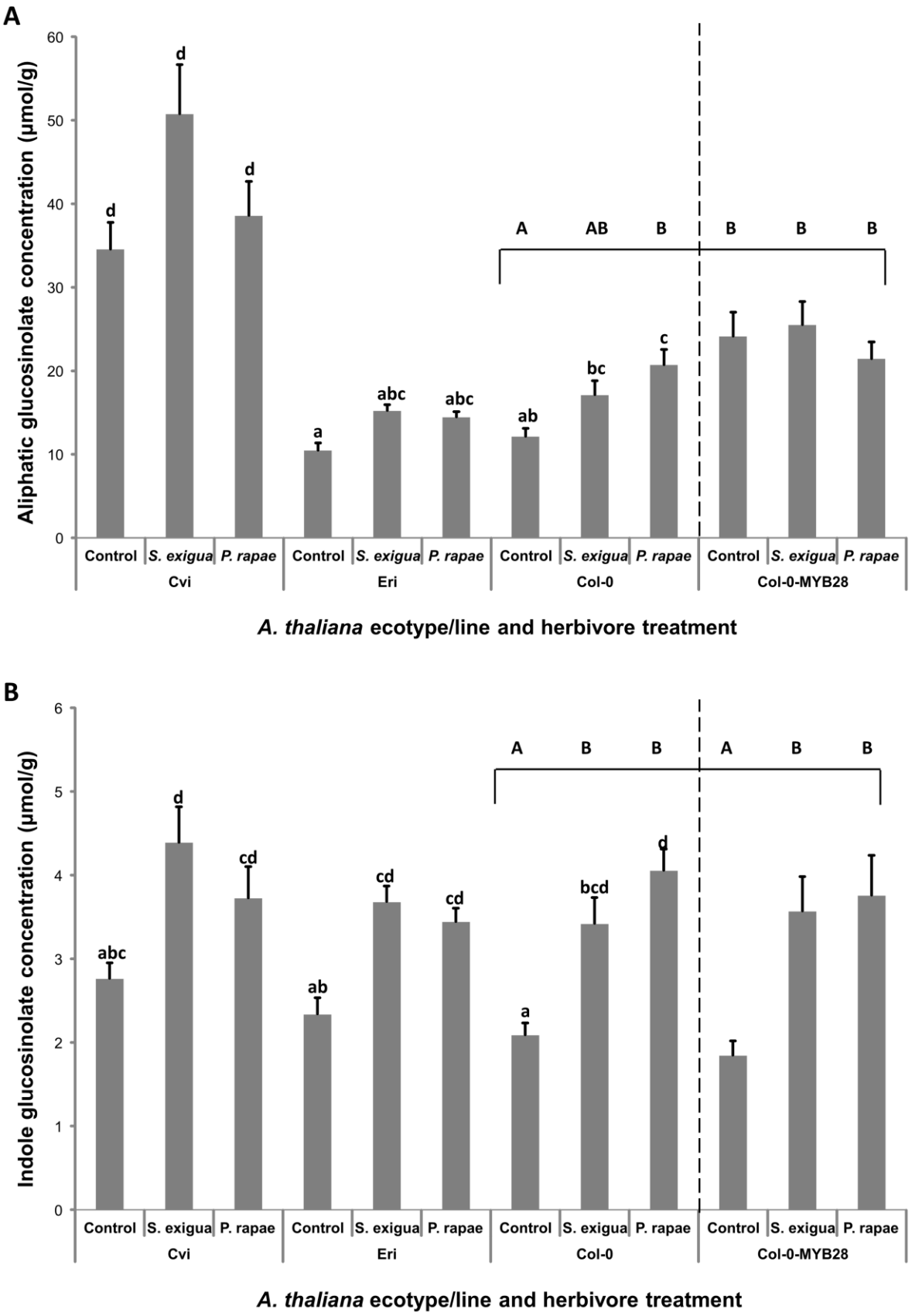

A
Aliphatic glucosinolate concentration (µmol/g)
60
50
40
30
20
10
0
d
d
d
a
abc
abc
ab
bc
c
A
AB
B
B
B
B
Control
S. exigua
P. rapae
Cvi
Eri
Col-0
Col-0-MYB28
A. thaliana ecotype/line and herbivore treatment
B
Indole glucosinolate concentration (µmol/g)
6
5
4
3
2
1
0
abc
d
cd
ab
cd
cd
a
bcd
d
A
B
B
A
B
B
Control
S. exigua
P. rapae
Cvi
Eri
Col-0
Col-0-MYB28
A. thaliana ecotype/line and herbivore treatment

Fig. 2

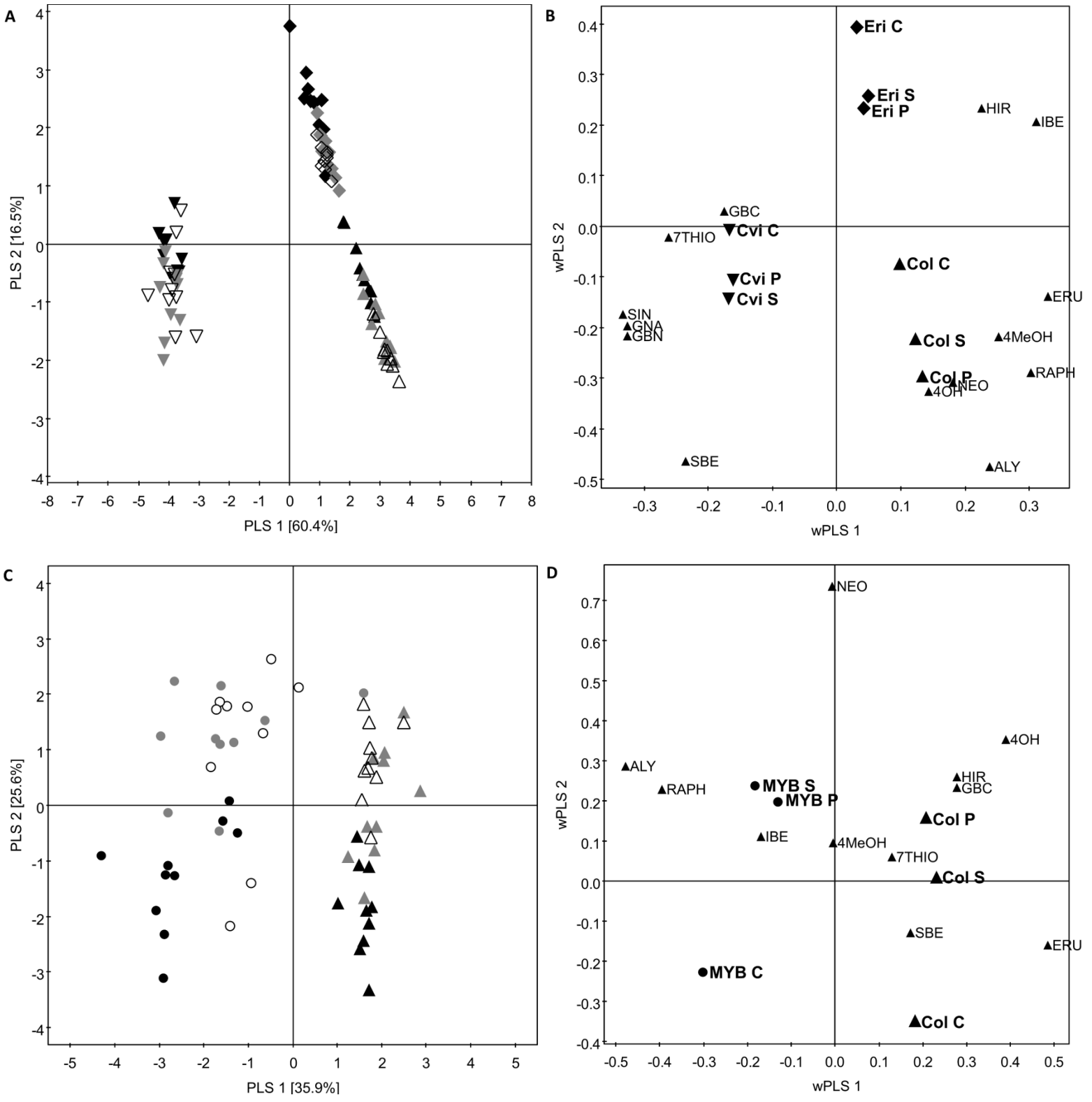
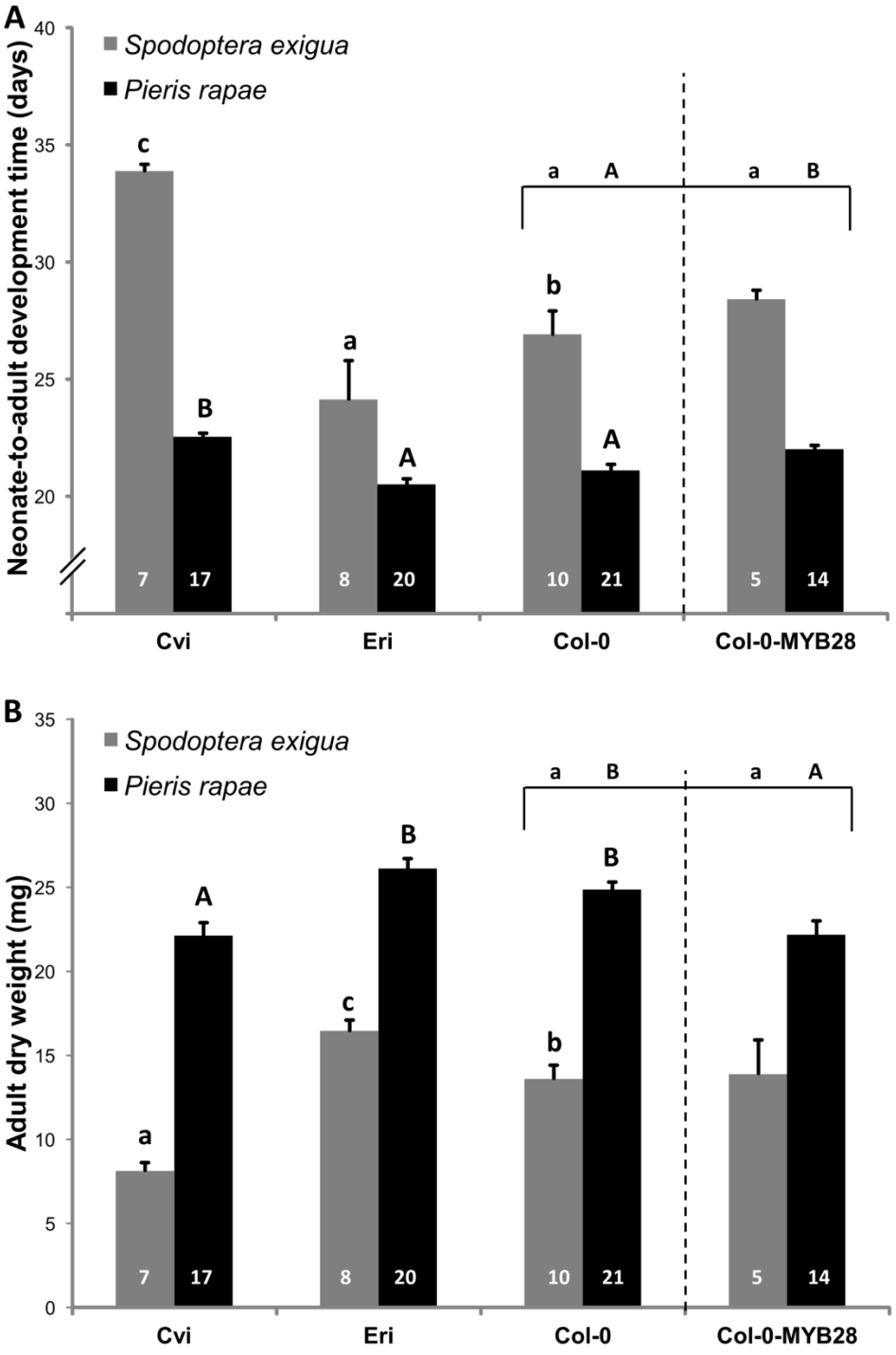

A
PLS 2 [16.5%]
PLS 1 [60.4%]
B
wPLS 2
wPLS 1
Eri C
Eri S
Eri P
HIR
IBE
GBC
Cvi C
7THIO
Col C
Cvi P
Cvi S
ERU
SIN
GNA
GBN
Col S
4MeOH
Col P
RAPH
NEO
4OH
SBE
ALY
C
PLS 2 [25.6%]
PLS 1 [35.9%]
D
wPLS 2
wPLS 1
NEO
4OH
ALY
HIR
GBC
RAPH
MYB S
MYB P
Col P
IBE
4MeOH
7THIO
Col S
SBE
ERU
MYB C
Col C

Fig. 3


A
Spodoptera exigua
Pieris rapae
Neonate-to-adult development time (days)
40
35
30
25
20
c
B
a
A
b
A
a
B
a
A
7
17
8
20
10
21
5
14
Cvi
Eri
Col-0
Col-0-MYB28
B
Spodoptera exigua
Pieris rapae
Adult dry weight (mg)
35
30
25
20
15
10
5
0
a
A
c
B
b
B
a
A
7
17
8
20
10
21
5
14
Cvi
Eri
Col-0
Col-0-MYB28