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Herbivore-Mediated Effects of Glucosinolates on Different Natural Enemies of a Specialist Aphid

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Abstract The cabbage aphid *Brevicoryne brassicae* is a specialist herbivore that sequesters glucosinolates from its host plant as a defense against its predators. It is unknown to what extent parasitoids are affected by this sequestration. We investigated herbivore-mediated effects of glucosinolates on the parasitoid wasp *Diaeretiella rapae* and the predator *Episyrphus balteatus*. We reared *B. brassicae* on three ecotypes of *Arabidopsis thaliana* that differ in glucosinolate content and on one genetically transformed line with modified concentrations of aliphatic glucosinolates. We tested aphid performance and the performance and behavior of both natural enemies. We correlated this with phloem and aphid glucosinolate concentrations and emission of volatiles. *Brevicoryne brassicae* performance correlated positively with concentrations of both aliphatic and indole glucosinolates in the phloem. Aphids selectively sequestered glucosinolates. Glucosinolate concentration in *B. brassicae* correlated negatively with performance of the predator, but positively with performance of the parasitoid, possibly because the aphids with the highest glucosinolate concentrations had a higher body weight. Both natural enemies showed a positive performance-preference correlation. The predator preferred the ecotype with the lowest emission of volatile glucosinolate breakdown products in each test combination, whereas the parasitoid wasp preferred the *A. thaliana* ecotype with the highest emission of these volatiles. The study shows that there are differential herbivore-mediated effects of glucosinolates on a predator and a parasitoid of a specialist aphid that selectively sequesters glucosinolates from its host plant.

Keywords *Arabidopsis thaliana* · *Brevicoryne brassicae* · *Diaeretiella rapae* · *Episyrphus balteatus* · Parasitoid wasp · Predator · Sequestration

Introduction

Plants have evolved a wide array of traits that confer resistance to herbivores (Karban and Baldwin, 1997; Schoonhoven et al., 2005). Specialists that are adapted to feeding on plants containing specific secondary metabolites, however, often use these compounds for their own benefit, e.g., as oviposition or feeding stimulants (van Loon et al., 1992; Gabrys and Tjallingii, 2002). Some concentrate metabolites actively taken up from host plants in special tissues or organs. This sequestration can make these herbivores unpalatable to natural enemies (Duffey, 1980; Müller, 2009).

Brassicaceous plants contain glucosinolates (GLS) that, upon damage by chewing herbivores, become exposed to the plant enzyme myrosinase that hydrolyzes GLS, resulting in toxic compounds such as (iso)thiocyanates and nitriles that negatively affect a wide variety of generalist herbivores

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(Halkier and Gershenzon, 2006; Hopkins et al., 2009). Phloem-feeding herbivores, however, can ingest GLS without bringing these compounds into contact with plant myrosinases (Andreasson et al., 2001). Thus, aphids prevent the formation of toxic hydrolysis products of most GLS (de Vos et al., 2007; Kim and Jander, 2007). The cabbage aphid *Brevicoryne brassicae* is a specialist that uses GLS as feeding stimulants (Gabrys and Tjallingii, 2002), and sequesters GLS from its food plants (Francis et al., 2001; Kazana et al., 2007; Pratt, 2008; Kos et al., 2011). It contains an endogenous myrosinase, which is stored separately from the GLS (Jones et al., 2001; Bridges et al., 2002; Francis et al., 2002). Upon predator attack, sequestered GLS come in contact with the aphid myrosinase, resulting in the formation of toxic hydrolytic products. Negative effects of this sequestration have been reported for aphid predators, such as ladybird beetles, hoverflies, and lacewings (Francis et al., 2001; Kazana et al., 2007; Pratt, 2008; Chaplin-Kramer et al., 2011; Kos et al., 2011). Most predators kill their prey immediately and feed on multiple individuals during their development. Parasitoids, in contrast, develop inside a single host individual, and koinobiont parasitoids allow the host to continue to grow and feed after parasitization (Godfray, 1994). Parasitoids are probably differentially affected by sequestration of GLS in *B. brassicae* compared to predators, but this rarely has been investigated. Le Guigo et al. (2011) compared the fitness of the solitary endoparasitoid *Diaeretiella rapae* when developing in *B. brassicae* that were feeding on host plant species with different foliar GLS concentrations. Parasitoid performance did not correlate with foliar GLS concentrations. In that study, GLS concentrations in the aphids were not analyzed. It has been shown previously that *B. brassicae* sequesters GLS selectively (Kabouw et al., 2011; Kos et al., 2011). It is still unknown to what extent *D. rapae* performance is affected by GLS sequestration in *B. brassicae*.

GLS not only affect performance of natural enemies of herbivores feeding on brassicaceous plants, but also their behavior. Formation of volatile GLS breakdown products, resulting from herbivore feeding, increase attraction of several specialist parasitoids (Bradburne and Mithen, 2000; Blande et al., 2007; Mumm et al., 2008). Effects of volatile GLS breakdown products on the behavior of generalist predators are largely unknown.

Our objective was to investigate herbivore-mediated effects of GLS on the performance and the behavior of the parasitoid *D. rapae* and the predacious hoverfly *Episyrphus balteatus*. These species represent two different groups within carnivorous insects and are two of the most important natural enemies of *B. brassicae*. To obtain aphids that differ in their sequestered GLS concentrations, we reared them on three ecotypes of *Arabidopsis thaliana* that differ qualitatively and quantitatively in their GLS content (Houshyani et al., in press). Additionally, a genetically transformed line was created to produce higher concentrations of foliar aliphatic (methionine-derived) GLS compared to the wild-type plants. We compared the performance of *B. brassicae* on these different ecotypes/lines, analyzed the GLS in the phloem and in the aphids feeding on it, and determined the performance of *E. balteatus* and *D. rapae* when feeding on these aphids. Additionally, we studied parasitoid and predator preference behavior in response to aphid-induced volatile organic compounds emitted by the different plant ecotypes/lines.

Methods and Materials

Plant Material and Growth Conditions Three *Arabidopsis thaliana* (L.) Heynh. ecotypes were selected, based on their maximal divergence in metabolite profiles (qualitative and quantitative composition of the mix of metabolites) (Houshyani et al., in press). Columbia (Col)-0 was provided by Dr. P. Reymond (Lausanne, Switzerland); Cape Verde Island (Cvi) was obtained from the European *Arabidopsis* Stock Centre (http://nasc.nott.ac.uk/, Cvi = N8580); and Eringsboda (Eri) was 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. unpublished data, see also Supplemental Material Online Resource 1). 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.

Arabidopsis thaliana seeds were surface-sterilized overnight by vapor phase sterilization and inoculated on a growth medium (purified agar 0.8%+2.2 gl^{-1} 0.5 MS+ vitamins; pH 6; containing 30 $\mu g\ ml^{-1}$ kanamycin to select transformed seedlings). After 4 d of stratification at 4°C, plates were transferred to a growth chamber at 21±2°C, 50–70% relative humidity (RH) and a 8:16L:D photo regime, with a light intensity of 200 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density (PPFD).

Two-week-old seedlings with two true leaves were transplanted to pots (5 cm diam) containing autoclaved soil (80°C for 4 h; Lentse potgrond, Lent, The Netherlands). Plants were watered three times a week, and the soil was treated weekly with entomopathogenic nematodes (*Steinernema feltiae*; Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) to control infestation by larvae of sciarid flies. Plants used were 6–7 wk-old, and remained in the vegetative state during experiments.

Insect Rearing *Brevicoryne brassicae* L. (Hemiptera: Aphididae) were reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus). *Episyrphus balteatus* de Geer

(Diptera: Syrphidae) pupae were provided by Koppert Biological Systems and kept in gauze cages (67 × 50 × 67 cm). Adults emerging from the pupae were provided with water, a *B. brassicae*-infested *B. oleracea* plant, organic sugar grains, and bee-collected pollen provided by Koppert Biological Systems. *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae) was reared in gauze cages (30 × 40 × 60 cm) containing *B. brassicae*-infested *B. oleracea* plants. Wasps were provided with water and honey. The *B. brassicae* and *D. rapae* culture originated from individuals obtained from *B. oleracea* in the vicinity of Wageningen (The Netherlands) in 2008. All insect species were reared at 22±2°C, 60–70% RH and a 16:8 h L:D photo regime.

GLS and Primary Metabolites in Phloem of Aphid-Infested Plants After the aphid performance experiment ended, phloem of aphid-infested plants was collected for chemical analysis. We used 8 mM EDTA, following the procedure described in Kos et al. (2011). Four fully-grown leaves of each plant were placed with their petiole for 5 min in the EDTA solution to remove any plant chemicals from the incision. Then, leaves were placed for 4 h in a new vial with 200 μl EDTA solution, under dark conditions. Using this method, a small amount of mesophyll fluids was inherently collected as well. Following incubation, the EDTA solution was collected from the vials, and each vial was rinsed with 50 μl EDTA, resulting in a sample of 250 μl per leaf. The EDTA solution of four plants (16 leaves) was pooled to form one sample of 4 ml, resulting in five replicates per ecotype/line. Leaves were dried at 80°C for 3 d and weighed on an analytical balance (Mettler-Toledo PM200, Tiel, The Netherlands). Phloem samples were frozen at −80°C immediately after collection, freeze-dried, and re-suspended in 2 ml 8 mM EDTA. Half of the collected phloem sample was used for GLS extraction, and half for soluble carbohydrate and amino acid extraction. To extract GLS from the phloem, we used the protocol described by Kos et al. (2011). GLS were separated using high-performance liquid chromatography (HPLC) as described previously by van Dam et al. (2004) and Kabouw et al. (2010). GLS detection was performed with a photodiode array detector set at 229 nm as the integration wavelength. Different concentrations of sinigrin (2-propenylGLS; Acros, NJ, USA) were used as external standard. The retention times of the GLS compounds can be found in Supplemental Material Online Resource 2. 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).

Soluble carbohydrates (50 μl from the one ml sample) and amino acids (50 μl) were extracted and analyzed as described previously by van Dam and Oomen (2008). For the carbohydrates, we used a "10 ppm" reference solution containing 54.9 μM sorbitol and mannitol, 29.21 μM trehalose, sucrose, and melibiose, and 55.51 μM glucose and fructose. This reference solution was diluted to obtain 7.5, 5, and 2.5 ppm calibration standards to obtain a reference curve. To obtain a reference sample containing the 20 most common amino acids, the Sigma AA S 18 amino acid standard (Sigma, St Louis, MO, USA) containing 17 amino acids was supplemented with asparagine, glutamine, and tryptophane (2.5 μmoles ml^{-1} each). This reference solution was diluted to obtain calibration standards ranging from 1 to 8 μM for each amino acid, except for cysteine, which had a range of 0.5–4 μM. For both the carbohydrates and the amino acids, an additional standard was injected after every 10 samples to check for deviations of retention times and the calibration curve.

Dynamic Headspace Collection of Volatiles from Aphid-Infested Plants Six-to-seven week-old *A. thaliana* plants were infested with 100 *B. brassicae* nymphs of mixed instars 3 d prior to headspace collection. Dynamic headspace collection was carried out in a climate chamber at 20± 2°C. Plants were removed from pots, and the soil was wrapped with aluminum foil. Three plants were placed together in a 2.5 l glass jar. Volatiles were collected by sucking air out of the jar at a rate of 90 ml min^{-1} for 3 h through a stainless steel cartridge (Markes, Llantrisant, UK) containing 200 mg Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, MI, USA). Foliar fresh weight of the plants in each pot was measured after volatile collection. For each ecotype/line, 8–11 replicate samples were collected (8 Cvi, 10 Eri, 9 Col-0, 11 Col-0-MYB28).

Headspace samples were analyzed by using a Thermo Trace Gas Chromatography Ultra (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, MA, USA) quadrupole mass spectrometer (MS) (Supplemental Material Online Resource 3). The peak area of each compound was expressed per unit plant fresh weight.

Identification of Compounds Identification of compounds was based on comparison of mass spectra with those in the NIST 2005, Wiley and Wageningen Mass Spectral Database of Natural Products MS libraries. Experimentally calculated linear retention indices (LRI) also were used as additional criterion for confirming the identity of the compounds. Relative quantification (peak areas of individual compounds) was performed using a single (target) ion, in selected ion monitoring (SIM) mode (see Supplemental Material Online Resource 4 for detailed information on the identification methods for each compound).

Plant Morphology and Foliar GLS Concentrations of Uninfested Plants We quantified two plant morphological characteristics, trichome density and plant biomass, because these might influence aphid and natural enemy performance and behavior. For 10 uninfested plants per ecotype/line, we measured foliar biomass and counted the number of trichomes in a 25 mm^2 area in the central part of the abaxial side of the 6th or 7th youngest leaf by using a microscope (Leitz Dialux 20 EB, Wetzlar, Germany; magnification 40×).

For foliar GLS analysis, we harvested all leaf material of 10 uninfested plants per ecotype/line. Samples were frozen at −80°C immediately after collection, freeze-dried, weighed (approximately 100 mg) into micro-centrifuge tubes, and ground to a fine powder.

GLS were extracted and purified from the leaves by using a methanol extraction (van Dam et al., 2004; Kabouw et al., 2010). GLS were separated and detected as described above for the phloem samples.

Insect Performance Individual plants with insects were confined to cylindrical plastic containers (height 13 cm; diam 11 cm) with a gauze lid. Experiments were performed in a climate chamber at 21±2°C, 50–70% RH and a 8:16 L:D photo regime for *B. brassicae* and 16:8 L:D for *E. balteatus* and *D. rapae*. The light intensity at plant level was 200 μmol m^{-2} s^{-1} PPFD. Plants were watered once a week.

Aphid Performance Several 6-wk-old plants of each ecotype/line were inoculated with 10 adult aphids per plant. After 24 h, adult aphids were removed, and the produced offspring were allowed to develop for 3 d until they reached the second instar (L2). Three L2 nymphs were transferred to each of 20 *A. thaliana* plants per ecotype/line, the same ecotype/line as the one on which these nymphs had been feeding before. Until the adult stage, survival of nymphs was recorded daily. The fastest developing adult was kept on the plant, while the other adults were removed. Alate (winged) adults (ca. 5% of all adults) were excluded from the experiment as these contain lower concentrations of GLS than apterous (wingless) aphids (Kazana et al., 2007). The development time until first reproduction ($=T_d$) of the remaining adult was recorded, and the adult fresh weight was measured on a microbalance (Sartorius CP2P, Göttingen, Germany). Adults were allowed to feed on plants and produce offspring, and after a certain number of days (equivalent to T_d), the number of offspring ($=N$) produced by the adult was counted. The estimated intrinsic rate of population increase (r_m) was calculated for each aphid using the formula: $r_m = 0.738 \times (\ln N)/T_d$ (Karley et al., 2002).

Aphid GLS Concentrations After the aphid performance experiment ended, aphids on the 4 plants that were used to obtain one phloem sample (described above) were removed and pooled into one sample. GLS were extracted similarly to the method used for the leaves.

Predator Performance Female *E. balteatus* from the stock rearing were allowed to lay eggs on Brussels sprouts plants infested with *B. brassicae.* After hatching, neonate larvae were transferred to *A. thaliana* plants that had been infested by 10 adult *B. brassicae* from the stock rearing 1 wk earlier. Larvae were allowed to develop on the plants until pupation. Pupae were checked once a day for eclosion of adults. Survival, larva-to-adult development time, sex, and adult dry weight were determined. Newly eclosed adults were frozen, dried to constant weight at 80°C for 3 d, and then weighed on a microbalance. We determined the performance of 35 larvae per *A. thaliana* ecotype/line, one larva per plant.

Parasitoid Performance Aphid mummies containing a *D. rapae* pupa were collected from the stock rearing, and reared until adult parasitoid eclosion. Adult parasitoids were provided with water and honey, allowed to mate, and used for parasitisation when they were 2–4 d-old. Second instar (3-d-old) *B. brassicae* nymphs that had been feeding on one of the *A. thaliana* ecotypes/lines were exposed individually to mated female parasitoids on an aphid-infested leaf until parasitisation was observed (i.e., when the female inserted her ovipositor into the nymph). Four parasitized nymphs were transferred to one *A. thaliana* plant of the same ecotype/line as the one on which these aphids had been feeding before. In total we tested 22 plants per *A. thaliana* ecotype/line. Mummies were collected from plants, and after eclosion, parasitoid sex was determined, and egg-to-adult development time and adult dry weight were measured, as described for the predator. The percentage of successful parasitism of *B. brassicae* by *D. rapae* was calculated per plant by dividing the number of *D. rapae* adults by the total number of *B. brassicae* nymphs that survived (either until the adult stage or until *D. rapae* eclosion) on each plant.

Predator and Parasitoid Preference The preference of predators and parasitoids for volatiles from an ecotype/line was investigated in two-choice bioassays. We tested the ecotypes against each other, and Col-0-MYB28 against Col-0. Plants were treated similarly as described above under *Dynamic Headspace Collection of Volatiles from Aphid-infested Plants.*

Predator Oviposition Preference Mated female hoverflies from the stock rearing were used in the behavioral assays when they were 2–3-wk-old. Females were transferred to a plastic cage (30 × 30 × 30 cm) containing one aphid-infested plant of two different ecotypes/lines, and 10% sugar solution. Females were allowed to oviposit on the plants for

24 h. The number of eggs deposited on each plant was counted. Replicates with females that did not lay any eggs were eliminated from the analysis. For each plant combination, at least 22 replicates with ovipositing females were obtained.

Parasitoid Preference for Aphid-Induced Plant Volatiles Parasitoid behavior was assessed in a Y-tube olfactometer in a climatized room at 22±2°C as described by Bukovinszky et al. (2005). Compressed air was filtered over charcoal and split into two air streams each at a flow of 2 $l min^{-1}$. Each air stream was led through a 5 l glass jar that contained 4 aphid-infested plants of one of the two ecotypes/lines of a test combination. Each air stream was then led into one of the two arms of the Y-tube. The olfactometer was illuminated from above with artificial light at an intensity of 60 $\mu mol\ m^{-2}\ s^{-1}$ PPFD.

Naïve, mated 2-d-old *D. rapae* females were allowed to oviposit for 1 h in aphids feeding on one of the two ecotypes/lines of a combination (equally divided among the tested wasps) to increase their host-searching behavior. Experienced wasps were released individually at the base of the Y-tube, and their preference for one of both odor sources was recorded. A choice was recorded when a wasp crossed a finish line drawn one cm before the end of each arm, and did not return to the junction within 15 s. Wasps that did not make a choice within 15 min were considered as non-responsive and were omitted from the statistical analysis. Four or five new sets of plants were used for each test combination. For every new set of plants, 20 wasps were tested. After every 10 wasps, the position of the odor sources was exchanged to compensate for any asymmetry in the set-up.

Statistical Analyses Analyses were performed in SPSS for Windows (18th edition, Chicago, IL, USA), unless indicated otherwise. If variables were log-transformed to obtain normality and equal variance, this is indicated in the relevant table of the Results section. To test the effect on the continuous variables, such as development time and body weight, we used ANOVA followed by *post-hoc* Tukey-tests for pair-wise ecotype comparisons and *t*-tests for comparisons between Col-0 and Col-0-MYB28. If assumptions on normality and equal variance were violated, Kruskal-Wallis tests with *post-hoc* Mann–Whitney *U* tests with a Holm's sequential Bonferroni correction were used for pair-wise ecotype comparisons, and Mann–Whitney *U* tests for comparisons between Col-0 and Col-0-MYB28. Survival and the percentage of successful parasitism were calculated per plant (plant was used as the experimental unit), and differences in these variables among ecotypes and between Col-0 and Col-0-MYB28 were analyzed by logistic regression in GenStat (13th edition, VSN International, UK). If over-dispersion was observed, the data were corrected for this by using estimated dispersion instead of fixed dispersion. T-probabilities were calculated to test pair-wise differences between means.

To test whether an equal number of predator eggs was laid on each ecotype/line in a test combination, Wilcoxon matched-pairs signed-rank tests were used. To test whether an equal number of parasitoid wasps chose either ecotype/line in a test combination in the Y-tube olfactometer, *Chi-square* tests were used. Effects of parasitoid experience on the preference of the wasps was tested by logistic regression in GenStat.

To determine whether there were differences in volatile profiles and aphid GLS profiles among the ecotypes and between Col-0 and Col-0-MYB28, we used multivariate discriminant analysis Projection to Latent Structures-Discriminant Analysis (PLS-DA) in SIMCA-P (12th edition, Umetrics, Umeå, Sweden) (Eriksson et al., 2006). For the volatiles, the variable importance in the projection (VIP) was calculated. Variables with a VIP value higher than 1 are most influential for the discrimination among the ecotypes/lines (Eriksson et al., 2006). For volatile compounds with a VIP higher than 1, the difference among the ecotypes and between Col-0 and Col-0-MYB28 was analyzed as described above for the continuous variables. Partial Least Squares Projections to Latent Structures (PLS) in SIMCA-P, a multivariate method for regression analysis, was used to test the relationship between a) metabolite profiles in the phloem and performance of aphids feeding on those plants, and b) the GLS profile in the phloem and in the aphids feeding on those plants. For the latter PLS-analysis, only the GLS compounds that were found in both the phloem and the aphids, as well as the total GLS, total aliphatic GLS and total indole GLS, were included. To pre-process data, metabolite concentrations were log-transformed, mean-centered, and scaled to unit variance. To test whether concentrations of individual GLS compounds or classes in the phloem and aphids were correlated, we used Spearman's correlation test. PLS analyses of the relationships between aphid GLS concentrations and predator/parasitoid performance could not be performed, as we measured these variables in separate experiments. Note that in the Results section 'aliphatic GLS' refers to the total of all aliphatic GLS compounds that were detected, 'indole GLS' refers to the total of all indole GLS compounds, and 'total GLS' refers to the total of all GLS compounds (aliphatic and indole GLS combined).

Results

GLS and Primary Metabolites in Phloem of Aphid-Infested Plants Ecotype effect: Total, aliphatic and indole GLS concentrations in the phloem of aphid-infested plants differed among ecotypes (Kruskal-Wallis H, df=2, total: χ^2=6.48, P=0.039; aliphatic: χ^2=8.07, P=0.018; indole: χ^2=10.82, P=0.004), due to both qualitative and quantitative differences (Table 1). Phloem of Cvi had the highest total and aliphatic GLS concentrations, whereas phloem of Eri plants had the highest indole GLS concentrations. Phloem of Col-0

Table 1 Mean (± SE) concentrations of metabolites in the phloem of aphid-infested plants of three *Arabidopsis thaliana* ecotypes and the transformed COL-0-MYB28 line, and in *Brevicoryne brassicae* aphids reared on these plants

Metabolite			*Arabidopsis thaliana* ecotype			Transformed
			Cvi	Eri	Col-0	Col-0-MYB28
Phloem	Carbohydrates[a]	sorbitol	0.002±0.001	0.005±0.001	0.002±0.002	0.002±0.002
		mannitol	0.008±0.001	0.008±0.002	0.010±0.002	0.014±0.003
		trehalose	0.010±0.009	0.001±0.001	0.002±0.001	0.013±0.008
		glucose	0.392±0.069	0.149±0.020	0.156±0.010	0.216±0.014
		fructose	0.321±0.060	0.097±0.015	0.112±0.008	0.166±0.012
		sucrose	0.988±0.191	2.328±0.305	1.892±0.142	1.949±0.147
		raffinose[b]	0	0.015±0.006	0	0
		Total carbohydrates	1.721±0.312a	2.604±0.340a	2.175±0.139a	2.318±0.148 ns
	Amino acids[a]	arginine	0.095±0.017	0.080±0.011	0.136±0.012	0.157±0.014
		lysine	0.016±0.006	0.024±0.005	0.040±0.006	0.053±0.008
		glutamine	2.474±0.370	2.348±0.292	3.671±0.375	3.552±0.363
		asparagine	0.139±0.022	0.065±0.010	0.032±0.016	0.027±0.014
		alanine	0.026±0.013	0.036±0.005	0.070±0.008	0.099±0.011
		threonine	0.833±0.148	0.313±0.042	0.472±0.037	0.610±0.048
		valine	0.018±0.008	0.036±0.006	0.065±0.011	0.090±0.015
		serine	0.081±0.017	0.065±0.011	0.121±0.013	0.077±0.008
		leucine	0.010±0.006	0	0.008±0.005	0.009±0.006
		methionine	0.001±0.001	0	0.002±0.002	0.001±0.001
		histidine	0.004±0.004	0.012±0.001	0.021±0.003	0.095±0.014
		phenylalanine	0.017±0.004	0.014±0.002	0.033±0.004	0.034±0.004
		glutamate	0.066±0.001	0.040±0.004	0.098±0.009	0.114±0.010
		aspartate	0.058±0.012	0.047±0.009	0.113±0.016	0.091±0.013
		tyrosine	0.007±0.002	0.009±0.001	0.015±0.004	0.020±0.005
		Total amino acids[c]	3.844±0.592a	3.088±0.390a	4.897±0.470a	5.028±0.480 ns
	GLS[d,e]	2-(*S*)-2-hydroxy-butenylGLS	1.958±1.199	0	0	0
		2-propenylGLS	1.208±0.803	1.028±1.028	0	0
		3-butenylGLS	1.030±0.699	0	0	0
		3-methylthiopropylGLS	1.694±0.237	0.384±0.250	0.735±0.051	0.147±0.010
		Total aliphatic GLS	5.889±1.860b	2.426±1.258a	0.915±0.186a	0.147±0.010*
		3-indolylmethylGLS	0.348±0.100	0.705±0.069	0.302±0.068	0.248±0.055
		4-methoxy-3-indolylmethylGLS	0.507±0.073	0.805±0.064	0.309±0.054	0.297±0.052
		Total indole GLS	0.855±0.055a	1.511±0.117b	0.611±0.102a	0.544±0.090 ns
		Total GLS	6.744±1.851b	3.936±1.287ab	1.526±0.253a	0.691±0.086*
Aphid	GLS[e,f]	3-methylsulfinylpropylGLS	0	30.04±17.38	5.61±3.54	2.36±1.49
		4-methylsulfinylbutylGLS	2.13±0.52	5.53±2.77	70.08±35.61	46.96±23.86
		2-propenylGLS	76.59±10.86	7.49±1.35	7.27±2.25	3.05±0.95
		2-hydroxy-4-pentenylGLS	0.57±0.19	0.09±0.09	2.76±1.20	1.16±0.50
		3-butenylGLS	272.87±49.87	1.08±0.86	2.47±1.18	1.04±0.50
		3-methylthiopropylGLS	0.16±0.07	2.19±1.22	0	0
		4-methylthiobutylGLS	0.25±0.12	1.66±0.88	3.16±1.31	1.32±0.55
		3-hydroxypropylGLS	0.42±0.08	99.01±43.23	5.87±3.78	2.47±1.59
		7-methylsulfinylheptylGLS	16.37±2.71	3.72±1.45	6.68±2.67	1.94±0.78
		8-methylsulfinyloctylGLS	210.25±37.20	118.69±45.04	34.49±13.11	2.07±0.79
		Total aliphatic GLS	579.61±98.59b	269.50±111.37ab	138.40±58.95a	62.36±28.58 ns
		3-indolylmethylGLS	29.63±8.45	30.14±18.29	7.20±1.87	9.36±2.43
		4-hydroxy-3-indolylmethylGLS	0.44±0.18	1.11±0.64	0.19±0.19	0.05±0.05

Table 1 (continued)

Metabolite	*Arabidopsis thaliana* ecotype			Transformed
	Cvi	Eri	Col-0	Col-0-MYB28
4-methoxy-3-indolylmethylGLS	0.85±0.11	2.30±0.36	3.16±0.40	2.75±0.35
1-methoxy-3-indolylmethylGLS	0.05±0.04	0.08±0.05	0	0
Total indole GLS	30.98±8.71a	33.63±19.20a	10.54±2.14a	12.16±2.60 ns
Total GLS	610.59±104.11b	303.13±130.08ab	148.94±60.76a	74.52±30.89 ns

N=5 for each sample. For every sample, phloem or aphids collected from four plants were pooled

[a] μmol g^{-1} dry weight leaf

[b] Tentatively identified

[c] Parameter was log-transformed in statistical analysis to obtain normality

[d] nmol g^{-1} dry weight leaf

[e] Glucosinolates (GLS) are grouped according to their biosynthetic origin into indole and aliphatic GLS, and analyses were performed separately for total GLS, aliphatic GLS and indole GLS

[f] μmol g^{-1} dry weight aphids

Statistical tests were performed only for the total carbohydrate, amino acid, aliphatic GLS, indole GLS and total GLS concentrations, not for individual compounds. Different letters denote differences in means among the three ecotypes as analyzed by Mann–Whitney *U*-tests with sequential Bonferroni correction (for GLS) or ANOVA and *post-hoc* Tukey tests (for carbohydrates and amino acids)

*denotes significant difference and ns denotes non-significant difference between Col-0 and Col-0-MYB28 as analyzed by Mann–Whitney *U*-tests (for GLS) or t-tests (for carbohydrates and amino acids)

Carbohydrates and amino acids have been identified and quantified based on calibration lines for the corresponding authentic standards. The retention times used for identification of each GLS compound can be found in Supplemental Material Online Resource 2. For quantification of GLS sinigrin (2-propenylGLS) was used as the external standard

plants had the lowest concentration of all GLS classes (Table 1). Ecotypes did not differ in total concentrations of carbohydrates and amino acids in the phloem (ANOVA, $P>0.05$ for both analyses), although there were small qualitative and quantitative differences in the concentrations of the individual compounds (Table 1).

Over-expression effect: The phloem of aphid-infested Col-0-MYB28 plants had lower concentrations of total and aliphatic GLS than Col-0 plants, and similar concentrations of indole GLS (Mann–Whitney *U*-test: total: $U<0.001$, $P=0.008$; aliphatic: $U<0.001$, $P=0.008$; indole: $U=7.00$, $P=0.310$; Table 1). This was unexpected, as foliar tissue of Col-0-MYB28 plants had higher concentrations of aliphatic GLS than Col-0 plants (Supplemental Material Online Resource 5). Total concentrations of carbohydrates and amino acids in the phloem did not differ between Col-0 and Col-0-MYB28 plants (Table 1).

Dynamic Headspace Collection of Aphid-Infested Plants Ecotype effect: The three *A. thaliana* ecotypes differed in volatile profiles of aphid-infested plants (4 PLS-DA principal components, $R_2X_{cum}=0.906$, $R_2Y_{cum}=0.836$, $Q_{2cum}=0.682$). The volatile profile of Cvi was high in breakdown products of GLS such as 3-butenyl isothiocyanate, 3-butene nitrile, and 3-methyl-3-butene nitrile. Col-0 plants emitted larger amounts of the sesquiterpenes δ-selinene and daucene, whereas the headspace of Eri plants was high in the ester methyl salicylate (Fig. 1a, b; Table 2).

Over-expression effect: Volatile profiles of Col-0 and Col-0-MYB28 plants could be separated by PLS-DA (4 PLS-DA principal components, $R_2X_{cum}=0.861$, $R_2Y_{cum}=0.932$, $Q_{2cum}=0.612$; Fig. 1c,d). Of the compounds that had a VIP-value higher than 1 in the PLS-DA model, only one compound was emitted in significantly different amounts by Col-0 and Col-0-MYB28 plants: the GLS breakdown product 3-butene nitrile, which was emitted in larger amounts by Col-0-MYB28 plants (Table 2).

Plant Morphology Ecotype effect: Eri plants had a higher biomass than plants of the other ecotypes. Cvi plants had the highest, and Eri plants the lowest trichome density (Supplemental Material Online Resource 5). Over-expression effect: There was no difference in biomass or trichome density between Col-0 and Col-0-MYB28 plants (Supplemental Material Online Resource 5).

Aphid Performance Ecotype effect: Aphid survival did not significantly differ among ecotypes (logistic regression, $P=0.051$, Table 3). Aphid development time, adult weight, number of offspring, and estimated intrinsic rate of population increase (r_m) differed among ecotypes (ANOVA, respectively $F_{2,57}=15.10$, $P<0.001$; $F_{2,57}=30.24$, $P<0.001$; $F_{2,57}=18.62$,

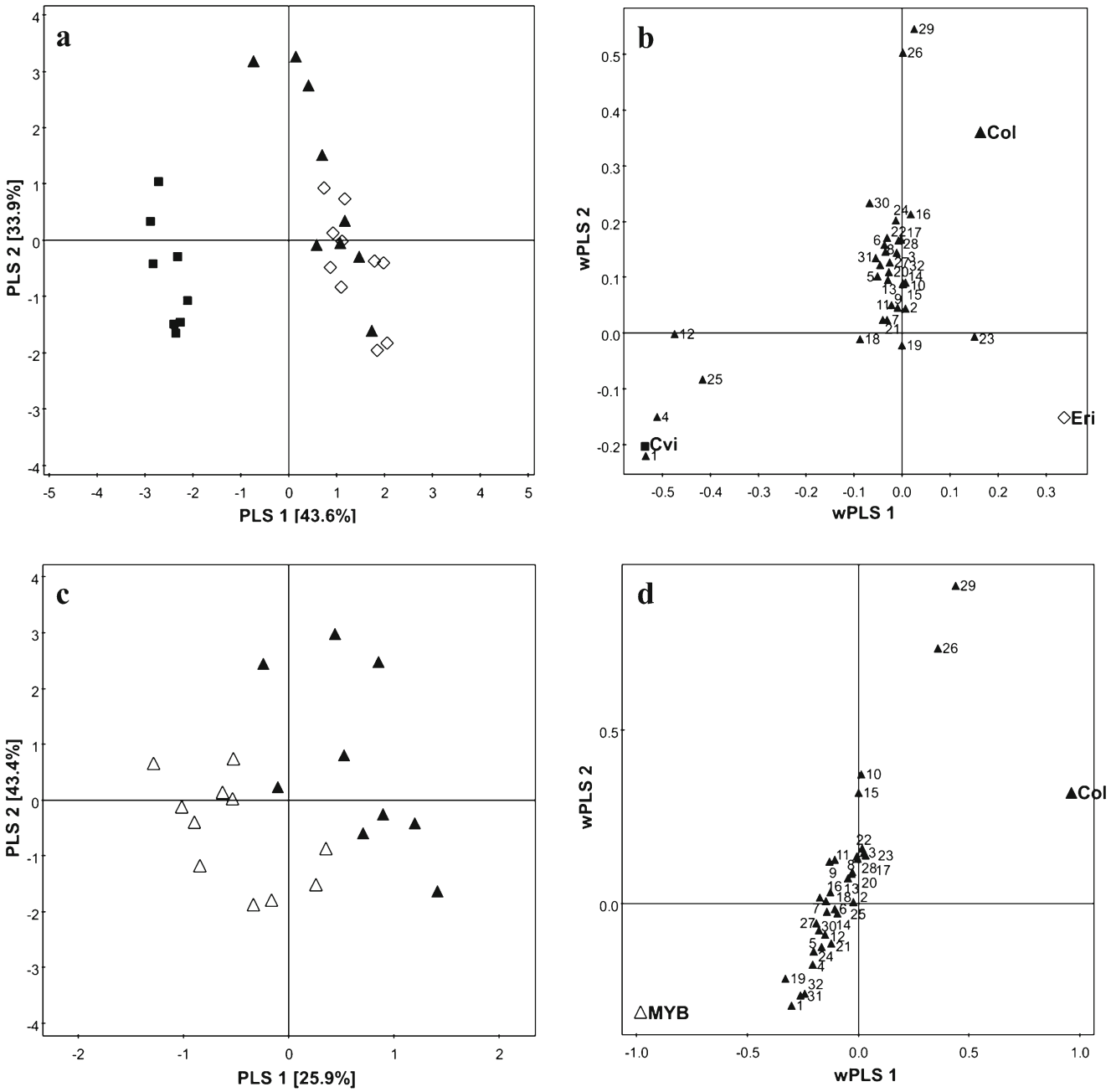

Fig. 1 Projection to Latent Structures-Discriminant Analysis (PLS-DA) score and loading plots of the first two components based on the volatile emission of aphid-infested plants of three *Arabidopsis thaliana* ecotypes (**a** and **b)** and of ecotype Col-0 and the transformed Col-0-MYB28 (**c** and **d)**. Plant ecotypes investigated are Cvi (filled boxes), Eri (open diamonds) and Col-0 (filled triangles); the transformed line is Col-0-MYB28 (open triangles). The score plots (**a** and **c**) show the distinction in volatile profiles of the ecotypes/lines. In brackets the percentage of variation explained is indicated. The loading plots (**b** and **d**) show the contribution of the volatile compounds to the discrimination among the ecotypes/lines. Numbers refer to the volatile compounds listed in Table 2

$P<0.001$; $F_{2,57}=45.66$, $P<0.001$). All aphid performance parameters were higher (development time shorter) on Cvi plants than on plants of the other ecotypes (Table 3).

Over-expression effect: There was no difference in any of the measured performance parameters of *B. brassicae* between Col-0 and Col-0-MYB28 plants ($P>0.05$ for any comparison, Table 3).

All measured aphid performance parameters were significantly positively correlated (development time inversely correlated) with total and aliphatic GLS and several carbohydrates (4 PLS principal components, $R_2X_{cum}=0.757$, $R_2Y_{cum}=0.735$, $Q_{2cum}=0.343$; Fig. 2). Aphid performance parameters were, to a lesser extent, also seemingly positively correlated with indole GLS, but this was significant only for

Table 2 Mean (± SE) amount of volatiles emitted by aphid-infested plants of three *Arabidopsis thaliana* ecotypes and the transformed COL-0-MYB28 line

No.	Compound	*Arabidopsis thaliana* ecotype			Transformed
		Cvi	Eri	Col-0	Col-0-MYB28
1	3-butene nitrile[a]	7216±1024 b	80±15 a	131±37 a	246±44 *
2	2-pentanone	818±98	1519±746	1118±221	1263±264
3	4-methyl-2-pentanone	263±40	178±27	584±277	375±69
4	3-methyl-3-butene nitrile[a]	5731 ±1059 b	58±11 a	147±57 a	204±40 ns
5	1-pentanol	804±117	399±61	797±148	1130±149
6	2-hexanone	474±73	311±56	626±130	735±118
7	butyl acetate	211±37	174±58	255±103	346±131
8	2-pentyl acetate	35±10	32±10	43±14	47±14
9	styrene[a]	2986±708	3892±1755	3860±1457	6055±2879 ns
10	cumene[a]	424±117	602±300	602±185	622±207 ns
11	isocumene	375±83	474±237	446±168	588±210
12	3-butenyl isothiocyanate[a]	253044±35561 b	5109±1809 a	19333±11465 a	17190±4633 ns
13	hemimellitene	932±116	846±295	1093±277	1182±207
14	p-cymene	231±49	305±112	297±80	384±63
15	limonene	1495±504	1519±467	1924±699	1855±577
16	o-cresol	592±188	539±154	1092±297	1495±392
17	m-cymene	436±53	402±88	772±172	669±77
18	γ-terpinene	101±42	112±58	127±48	112±44
19	linalool[a]	97±24	75±18	85±29	564±433 ns
20	cis-limonene-1,2-epoxide	68±10	47±14	75±13	76±9
21	menthol	853±283	695±275	542±42	942±311
22	1-methylene-1H-indene	2662±624	1595±256	3254±626	2912±372
23	methyl salicylate[a]	177±42 a	749±140 b	459±104 ab	422±100
24	diethyl-2-methylene succinate[a]	1485±218	1324±309	3218±985	3867±614 ns
25	cyclosativene[a]	1041±220 b	27±5 a	46±8 a	60±9
26	daucene[a]	216±109 ab	82±14 a	1483±675 b	242±50 ns
27	γ-elemene[a]	706±271 a	453±124 a	974±384 a	1003±224 ns
28	longifolen	669±168	506±101	968±251	955±171
29	δ-selinene[a]	321±206 a	153±54 a	6053±3112 a	261±44 ns
30	6-methyl-alpha-ionone[a]	369±79	153±25	635±210	661±125 ns
31	lilial[a]	1698±146	930±98	1983±450	3391±460 ns
32	farnesylacetaldehyde[a]	16171±3015	9846±1432	18047±3806	29986±4227 ns

N=8–11 for each ecotype/line. Unit is peak area mg^{-1} fresh weight

Numbers correspond to the numbers in Fig. 1

[a] Statistical tests were performed only for the compounds that had a VIP-value higher than 1 in the PLS-DA model shown in Fig. 1 that included either the three ecotypes (Fig. 1a and b) or Col-0 and Col-0-MYB28 (Fig. 1c and d). The compounds with a VIP higher than 1 are most influential for the discrimination among the ecotypes/lines. Different letters denote differences in means among the three ecotypes as analyzed by Mann–Whitney *U*-tests with sequential Bonferroni correction. * denotes significant difference and ns denotes non-significant difference between Col-0 and Col-0-MYB28 as analyzed by Mann–Whitney *U*-tests. Compounds have been identified based on the linear retention index (LRI) and mass spectrum, or mass spectrum only (compounds 4, 5 and 22). See Supplemental Material Online Resource 4 for details on identification methods

development time (inversely correlated). Aphid performance parameters were in general not correlated with amino acids, sucrose, or total carbohydrates.

Aphid GLS Concentrations Ecotype effect: Aphids reared on different ecotypes differed in total and aliphatic GLS concentrations (Kruskal-Wallis H, df=2, total: χ^2=6.98, P=0.031; aliphatic: χ^2=6.98, P=0.031), due to both qualitative and quantitative differences (Table 1). Aphids reared on Cvi plants contained the highest, and aphids reared on Col-0 plants the lowest total and aliphatic GLS concentrations (Table 1). There were no differences in indole GLS

Table 3 Mean (± SE) performance characteristics of *Brevicoryne brassicae*, *Episyrphus balteatus* and *Diaeretiella rapae* reared on three *Arabidopsis thaliana* ecotypes and the transformed COL-0-MYB28 line

Insect species	Performance parameter	*A. thaliana* ecotype[a]			Transformed line[b]
		Cvi	Eri	Col-0	Col-0-MYB28
B. brassicae	Survival until adult stage (%)[c,d]	98 a	92 a	90 a	95 ns
	Development time until first reproduction in days (T_d)[e,f]	8.0±0.1 a	8.4±0.1 a	9.1±0.2 b	9.2±0.2 ns
	Adult fresh weight in mg[f]	0.635±0.025 b	0.471±0.020 a	0.408±0.018 a	0.393±0.015 ns
	Number of offspring (N) in time period equivalent to T_d[e,f]	33.2±1.5 b	22.2±1.2 a	22.2±1.7 a	19.2±1.2 ns
	Estimated intrinsic rate of population increase (r_m)[f]	0.322±0.005 c	0.270±0.006 b	0.250±0.006 a	0.236±0.006 ns
E. balteatus	Survival until adult stage (%)[c]	17 a	40 a	26 a	20 ns
	Larva-to-adult development time in days[e,f,g]	21.5±1.1 c	16.3±0.3 a	18.4±0.6 b	16.7±0.6 ns
	Adult dry weight in mg[e,f,g]	3.30±0.53 a	2.74±0.19 a	2.72±0.16 a	3.25±0.40 ns
D. rapae	Successful parasitism (%)[c,d]	73±5 a	76±5 a	57±7 a	64±7 ns
	Larva-to-adult development time in days[e,f,g]	11.1±0.1 a	11.2±0.1 a	11.1±0.1 a	11.3±0.1 ns
	Adult dry weight in mg[f,g]	0.068±0.002 b	0.058±0.002 a	0.059±0.002 a	0.055±0.002 ns

[a] Different letters denote differences in means among the three ecotypes

[b] ns denotes no significant difference between Col-0 and Col-0-MYB28

[c] Analyzed by logistic regression and *post-hoc* *T*-probability tests

[d] Performance parameter was averaged per plant before statistical analysis

[e] Performance parameter was log-transformed in statistical analysis to obtain normality

[f] Analyzed by ANOVA and *post-hoc* Tukey tests (for the ecotypes), or *t*-test (for the wild-type and transformed Col-0 line)

[g] The data for males and females were combined

among aphids reared on the different ecotypes (Kruskal-Wallis H, $P>0.05$).

Over-expression effect: Aphids reared on Col-0-MYB28 plants had similar concentrations of total, aliphatic and indole GLS to aphids reared on Col-0 plants (Mann–Whitney *U*, $P>0.05$ for every analysis; Table 1).

Correlations Between GLS Profiles in Phloem and in B. Brassicae Aphids In both the univariate Spearman's correlation tests, as well as the multivariate PLS model, concentrations of most of the GLS compounds or classes in the aphids were not significantly positively correlated with their concentrations in the phloem. Positive correlations were, however, significant for total and aliphatic GLS (PLS model: 1 PLS principal component, $R_2X=0.486$, $R_2Y=0.373$, $Q_2=0.280$; Spearman's correlation: 3-butenylGLS: $r_s=0.53$; $P=0.015$; aliphatic: $r_s=0.72$; $P<0.001$; total: $r_s=0.82$; $P<0.001$). The concentration of the indole 4-methoxy-3-indolylmethylGLS seemed to be negatively correlated, although not significantly, between aphids and the phloem they were feeding on (Fig. 3; Spearman's correlation, $P=0.650$).

The contribution of indole GLS to the total concentration of GLS was lower in aphids than in the phloem of the aphid-infested plants (10% indole GLS in aphids compared to 42% indole GLS in the phloem, as averaged over all four ecotypes/lines; see also Table 1). Additionally, the ratio of the indole compounds was different in the aphids from that in the phloem: in the phloem the concentration of 4-methoxy-3-indolylmethylGLS was higher than the concentration of 3-indolylmethylGLS, whereas this was reverse in the aphids (Table 1).

Predator Performance Ecotype effect: Survival of *E. balteatus* to the adult stage did not differ among ecotypes (logistic regression, $P>0.05$, Table 3). Larva-to-adult development time of the hoverflies was affected by plant ecotype and hoverfly sex (ANOVA, ecotype: $F_{2,23}=27.11$, $P<0.001$; sex: $F_{1,23}=8.10$, $P=0.009$). Hoverflies developed slowest on aphids fed on Cvi plants and fastest on aphids fed on Eri plants (Table 3). Averaged over ecotypes, male hoverflies took longer (18.7±0.8 d) to develop into adults than females (17.3±0.5 d). However, the difference in development time between males and females was only significant on Cvi, and not on Col-0 and Eri, resulting in a significant interaction between ecotype and sex ($F_{2,23}=4.65$, $P=0.020$). Adult dry weight was affected by hoverfly sex (ANOVA, $F_{1,23}=6.63$, $P=0.017$), as male hoverflies (3.18±0.23 mg) were heavier than females (2.49±0.13 mg), but not by plant ecotype (Table 3) or the interaction between ecotype and sex (ANOVA, $P>0.05$ for both analyses).

Over-expression effect: There was no difference in survival, development time, or adult dry weight of *E. balteatus*

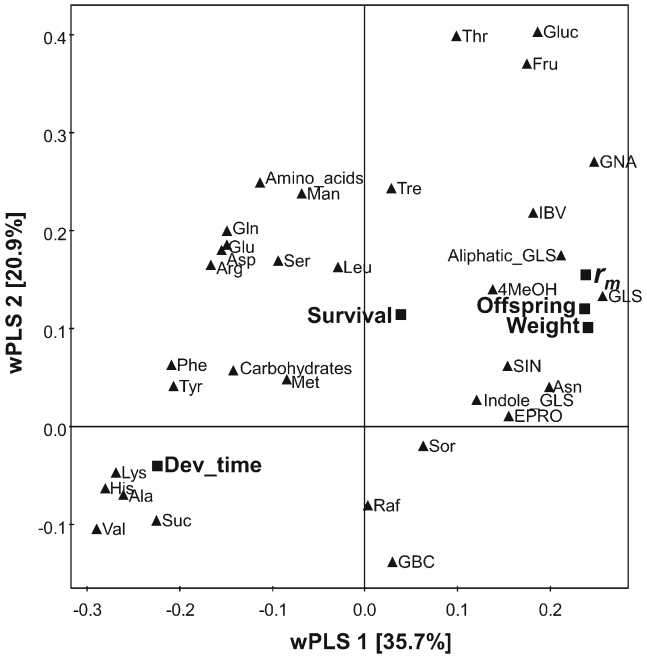

Fig. 2 Loading plot of the first two components of Projection to Latent Structures showing the contribution of each individual compound or compound class measured in the phloem, i.e., glucosinolates (GLS), carbohydrates and amino acids, to the performance of the aphid *Brevicoryne brassicae* in terms of survival, development time, adult weight, number of offspring and estimated intrinsic rate of population increase (r_m). In brackets the percentage of variation explained is indicated. Compound abbreviations: Aliphatic GLS: EPRO = 2-(*S*)-2-hydroxy-butenylGLS (epiprogoitrin), GNA = 3-butenylGLS (gluconapin), IBV = 3-methylthiopropylGLS (glucoiberverin), SIN = 2-propenylGLS (sinigrin). Indole GLS: GBC = 3-indolylmethylGLS (glucobrassicin), 4MeOH = 4-methoxy-3-indolylmethylGLS (4-methoxyglucobrassicin). Carbohydrates: Fru = fructose, Gluc = glucose, Man = mannitol, Raf = raffinose. Sor = sorbitol, Suc = sucrose, Tre = trehalose. Amino acids: Ala = alanine, Arg = arginine, Asn = asparagine, Asp = aspartate, Glu = glutamate, Gln = glutamine, His = histidine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Ser = serine, Thr = threonine, Tyr = tyrosine, Val = valine

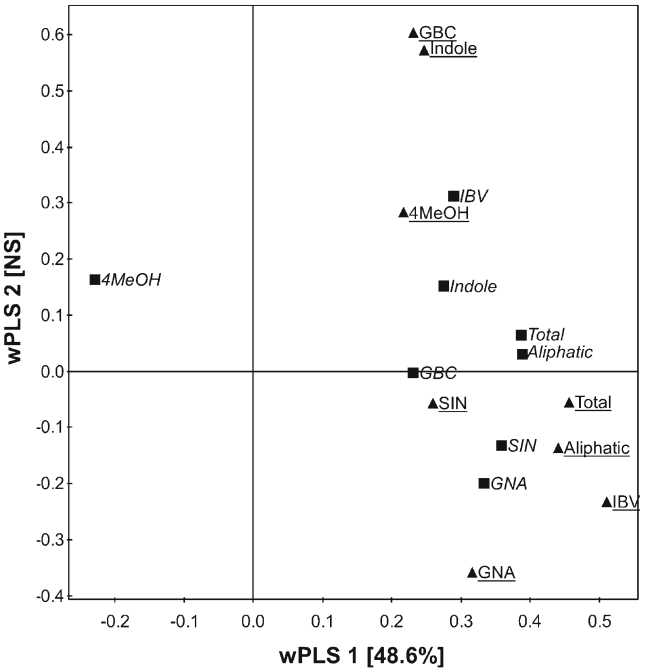

Fig. 3 Loading plot of the first two components of PLS showing the relationship of the concentration of each glucosinolate (GLS) compound or class (aliphatic, indole and total) in the phloem of *Arabidopsis thaliana* (squares, label in *italics*) with the concentration of these compounds or classes in the aphid *Brevicoryne brassicae* (triangles, label underlined) feeding on the phloem. In brackets the percentage of variation explained is indicated. Note that only the first component is significant according to the multivariate model, whereas two components were included to enhance the clarity of the figure. Compound abbreviations: Aliphatic GLS: GNA = 3-butenylGLS (gluconapin), IBV = 3-methylthiopropylGLS (glucoiberverin), SIN = 2-propenylGLS (sinigrin). Indole GLS: GBC = 3-indolylmethylGLS (glucobrassicin), 4MeOH = 4-methoxy-3-indolylmethylGLS (4-methoxyglucobrassicin)

between Col-0 and Col-0-MYB28 ($P>0.05$ for all parameters; Table 3).

Parasitoid Performance Ecotype effect: Plant ecotype did not affect the percentage of successful parasitism of *B. brassicae* by *D. rapae* (logistic regression, $P>0.05$), nor did it affect egg-to-adult development time (ANOVA, $P>0.05$; Table 3). Only adult dry weight was affected by plant ecotype (ANOVA, ecotype: $F_{2,169}=10.16$, $P<0.001$). Adult dry weight was higher on Cvi plants than on plants of the other ecotypes (Table 3). There was no effect of parasitoid sex or the interaction between ecotype and sex for any of the performance parameters ($P>0.05$ for all parameters).

Over-expression effect: There was no difference in the percentage of successful parasitism, development time, or adult dry weight of *D. rapae* between Col-0 and Col-0-MYB28 ($P>0.05$ for all parameters; Table 3).

Predator Oviposition Preference Female *E. balteatus* preferred to oviposit on aphid-infested Eri plants over aphid-infested Col-0 and Cvi plants, and aphid-infested Col-0 plants over aphid-infested Cvi plants (Wilcoxon: Eri vs. Col-0: $Z=2.63$, $N=27$, $P=0.008$; Eri vs. Cvi: $Z=3.44$, $N=22$, $P=0.001$; Col-0 vs. Cvi: $Z=2.12$, $N=29$, $P=0.034$). Females did not differentiate between aphid-infested plants of Col-0 and Col-0-MYB28 (Wilcoxon, $P>0.05$; Fig. 4).

Parasitoid Preference for Aphid-Induced Plant Volatiles Female *D. rapae* preferred volatiles from aphid-infested Cvi plants over volatiles from aphid-infested Col-0 plants (Chi-square, $\chi^2=5.69$, $P=0.017$). Females neither differentiated between volatiles from any of the other ecotype combinations, nor between Col-0 and Col-0-MYB28 (*Chi-square*, $P>0.05$ for every combination; Fig. 5). There was no effect of previous oviposition experience on the preference of the wasps (logistic regression, $P>0.05$ for any combination).

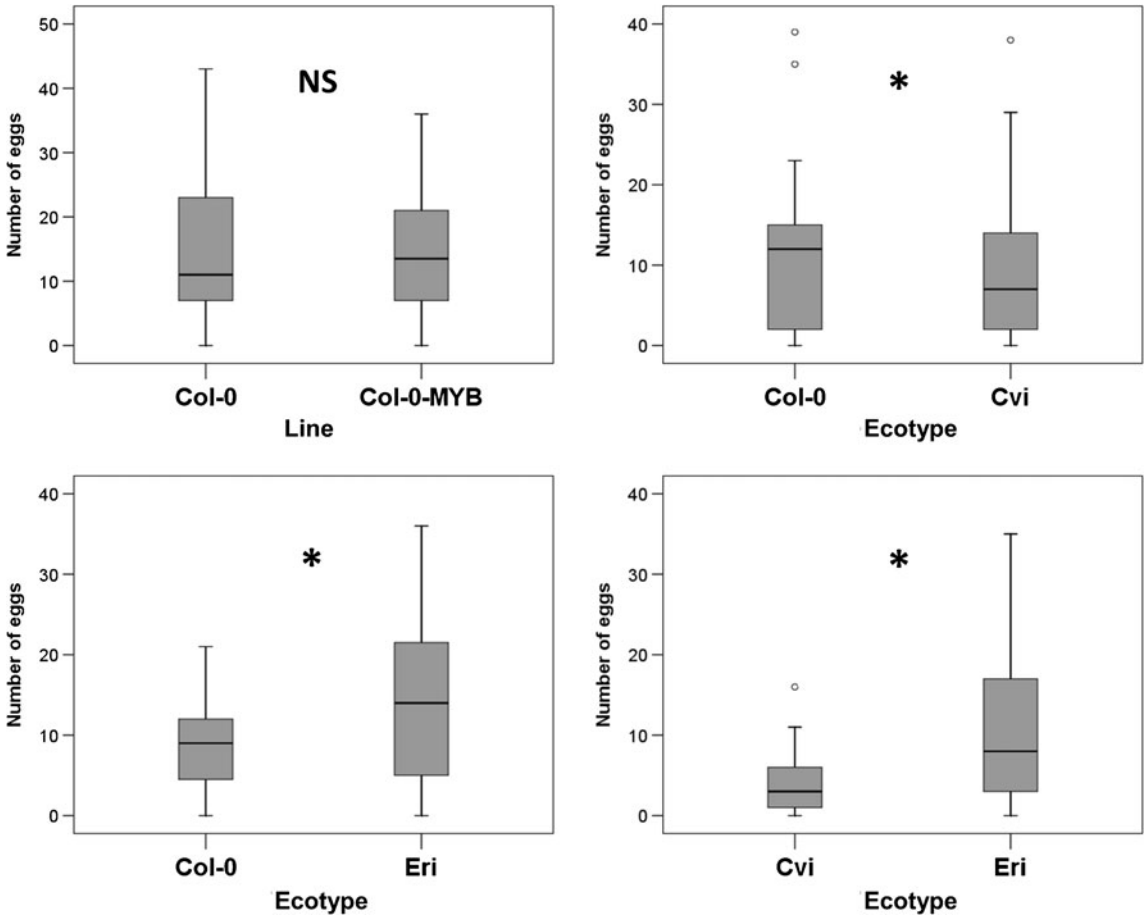

Fig. 4 Oviposition preference of the aphid predator *Episyrphus balteatus* in a two-choice assay with aphid-infested plants of three *Arabidopsis thaliana* ecotypes (Col-0, Cvi and Eri) and one transformed line (Col-0-MYB28). The boxes span the first to third quartile range with the line across the box indicating the median. The whiskers represent the range. Open circles represent outliers. An asterisk indicates a significant difference ($P<0.05$) between the number of eggs deposited on each ecotype/line as analyzed by the Wilcoxon matched-pairs signed-rank test; NS = not significant

Discussion

The performance of *B. brassicae* was best on the *A. thaliana* ecotype with the highest concentrations of aliphatic GLS in the phloem. Furthermore, we found a positive correlation between aliphatic GLS and aphid performance in the multivariate regression analysis. Due to the intercellular path taken by the aphid stylet to the phloem (Tjallingii and Hogen Esch, 1993), aphids can ingest aliphatic GLS from the phloem without bringing these compounds into contact

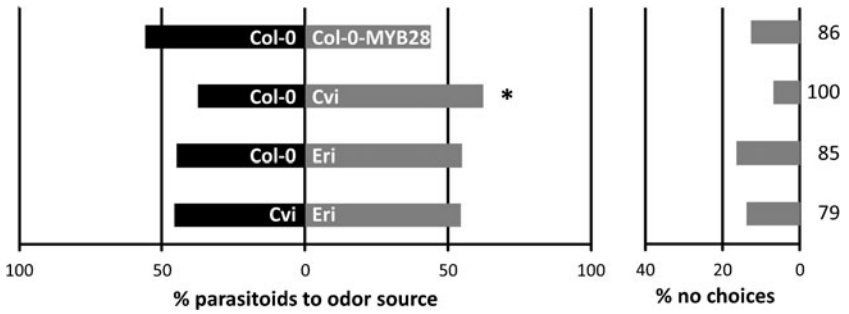

Fig. 5 Responses of *Diaeretiella rapae* females to volatile blends emitted by aphid-infested *Arabidopsis thaliana* ecotypes/lines in a Y-tube olfactometer. Ecotypes investigated are: Col-0, Cvi and Eri; the transformed line is Col-0-MYB28. Each bar represents the percentage of females that made a choice for the indicated odor sources. The percentage of no choice in each experiment and the total number of tested females are indicated on the right. An asterisk indicates a significant preference for one of the two ecotypes/lines in a combination, as analyzed by *Chi-square* tests

with plant myrosinases that are stored in cells adjacent to the phloem (Andreasson et al., 2001). Thus, aphids can prevent the formation of toxic hydrolytic products of aliphatic GLS (de Vos et al., 2007; Kim and Jander, 2007). Together with the observation that *B. brassicae* uses GLS as feeding stimulants (Gabrys and Tjallingii, 2002), our finding of a positive correlation between aliphatic GLS and aphid performance is expected. In contrast to aliphatic GLS, indole GLS are hydrolyzed by aphids into toxic products independently of myrosinase activity (Kim and Jander, 2007; Kim et al., 2008), and negative correlations between the concentrations of indole GLS and performance of *B. brassicae* and other aphid species have been reported (Cole, 1997; Mewis et al., 2005; Kim and Jander, 2007; Kim et al., 2008). Our observation of a slight, but significant, positive correlation between aphid performance and total indole GLS concentrations in the phloem are in disagreement with these latter studies. A possible explanation for this discrepancy is that specific indole GLS may affect aphid performance more strongly than others. The difference in the abundance of specific indole GLS between our study and studies from the literature might be the explanation of differences in effects on aphid performance.

The *A. thaliana* ecotypes did not differ significantly in the concentrations of carbohydrates and amino acids in the phloem, and we did not observe a consistent correlation between aphid performance and concentrations of individual or total carbohydrates and amino acids in the regression analysis. Aphids did not seem to be affected by trichomes, as their performance was best on the *A. thaliana* ecotype with the highest trichome density. We note that we measured trichome density of uninfested plants. It has been demonstrated that feeding by leaf chewers can increase trichome density in *A. thaliana* (Traw and Dawson, 2002), but whether this also is true for aphids is, to our knowledge, unknown.

Brevicoryne brassicae sequestered GLS from the phloem, and total aliphatic GLS concentrations in the phloem were significantly positively correlated with their concentrations in the aphids. In contrast, whereas in the phloem 4-methoxy-3-indolylmethylGLS was the most abundant indole GLS, aphids sequestered this compound in low concentrations compared to its precursor, 3-indolylmethylGLS. This is in accordance with what we reported previously in aphids feeding on *B. oleracea* plants (Kos et al., 2011). Although the concentrations of most aliphatic GLS in phloem were positively correlated with their concentrations in aphids, selective sequestration also was observed for aliphatic GLS. For example, whereas 3-butenylGLS was the least abundant GLS in the phloem of Cvi plants, it dominated in the aphids feeding on these plants. These findings suggests that *B. brassicae* selectively sequestered GLS from the phloem, a phenomenon we previously reported (Kos et al., 2011). The mechanism underlying the selective sequestration of GLS could be that transporters for GLS in the aphid gut wall may be specific. In other GLS-sequestering species, such as the sawfly *Athalia rosae*, selective sequestration of aliphatic GLS has been reported (Müller and Wittstock, 2005; Müller, 2009). Little is known about the specificity of GLS transporters or the specific mechanisms underlying GLS sequestration (Opitz et al., 2010). In our study, aliphatic GLS dominated the profile of sequestered GLS, in agreement with previous work (Kos et al., 2011). Because hydrolysis into toxic products requires myrosinase activity circumvented by aphids (de Vos et al., 2007; Kim and Jander, 2007), aphid performance itself is most likely little affected by high sequestration of aliphatic GLS. Interestingly, aliphatic GLS are degraded more by purified aphid myrosinase, whereas the lowest activities of the aphid myrosinase are observed with indole GLS (Francis et al., 2002). Thus, higher sequestration of aliphatic GLS by *B. brassicae* may lead to higher toxicity to predators, without affecting aphid performance itself. We note that not all GLS detected in the aphids were detected in the phloem, probably because the concentrations of some GLS in the phloem were below the detection limit of the HPLC. However, we cannot rule out that the aphids converted certain GLS from the phloem into other compounds that were subsequently stored in their body.

As expected, over-expressing Col-0-MYB28 plants produced higher foliar concentrations of aliphatic GLS and similar concentrations of indole GLS compared to the wild-type plants. Unexpectedly, we observed that aliphatic GLS concentrations in the phloem were lower in Col-0-MYB28 plants than in Col-0 plants. Probably, GLS biosynthesis in Col-0-MYB28 plants occurred mainly in the mesophyll, and phloem loading of GLS was limited.

Performance of the generalist aphid predator *E. balteatus* was lowest in terms of development time when fed *B. brassicae* aphids that contained the highest aliphatic GLS concentrations (i.e., aphids reared on Cvi). It is likely that this led to highest concentrations of GLS hydrolysis products after breakdown by the aphid myrosinase, as purified aphid myrosinase quickly degrades aliphatic GLS (Francis et al., 2002), although we did not quantify GLS and their hydrolytic products separately. Our results are in agreement with other studies that have reported negative effects of GLS sequestration by *B. brassicae* on the performance of *E. balteatus* (Vanhaelen et al., 2002; Kos et al., 2011) and other aphid predators (Francis et al., 2001; Kazana et al., 2007; Pratt, 2008; Chaplin-Kramer et al., 2011; Kos et al., 2011). There are several other Brassicaceae-feeding insects that sequester GLS for defense against predators. Similarly to *B. brassicae*, the turnip aphid (*Lipaphis erysimi*) sequesters GLS from phloem into the haemolymph and contains an endogenous myrosinase, a mechanism that is expected to affect negatively predators (Bridges et al., 2002).

Episyrphus balteatus performance did not differ between Col-0 and Col-0-MYB28 plants. This was expected as aphid GLS concentrations did not differ between these plant lines. Hoverfly performance in terms of development time was better when their prey had been feeding on ecotype Eri than on Col-0. However, Eri-fed aphids had higher but not statistically different total GLS concentrations in their phloem from Col-0-fed aphids. This points to the importance of qualitative effects of GLS profiles on hoverfly performance. It was reported previously that differences in *B. brassicae* GLS profiles affect the performance of *E. balteatus* (Kos et al., 2011). Furthermore, haemolymph of *A. rosae* larvae, containing a mix of several GLS compounds, deterred ants and predatory wasps more strongly than the individual major GLS compounds (Müller et al., 2002; Müller and Brakefield, 2003). This suggests that GLS profiles rather than total concentrations influenced these predators, although the stronger deterrence also could have been due to completely different compounds from GLS present in the haemolymph. We cannot rule out effects of other plant or aphid traits on predator performance. Epicuticular plant characteristics, such as leaf waxes and trichomes, may affect predator attachment to the plant (Eigenbrode, 2004). Trichomes have been shown to negatively affect the performance of hoverfly larvae due to entrapment by glandular trichomes, reduced mobility, or falling off the plant (Verheggen et al., 2009). Although we do not know whether this is also true for non-glandular trichomes on *A. thaliana*, the lower trichome density of Eri plants may have contributed to the better performance of hoverfly larvae on this ecotype.

Parasitoid performance in terms of adult weight was best when developing in the largest aphids, containing the highest GLS concentrations. The positive correlation between host size and parasitoid size is in agreement with other studies (Harvey, 2005; Bukovinszky et al., 2008). Our results suggest that the performance of *D. rapae* is not negatively affected by GLS concentrations in the host, supporting the findings of Le Guigo et al. (2011). Although *D. rapae* parasitizes several aphid species, it is the main parasitoid of *B. brassicae* (Bukovinszky et al., 2008), and may be relatively tolerant to GLS. We do not know, however, how *D. rapae* copes with GLS in its host. In fact, there is not much known about detoxification of plant secondary metabolites by parasitoids in general (Ode, 2006; Gols and Harvey, 2009). Negative effects of breakdown products of GLS on *D. rapae* might be prevented by the feeding strategy of the parasitoid larvae. *Brevicoryne brassicae* stores GLS in the haemolymph, but the aphid's myrosinases are stored in the non-flight muscles (Jones et al., 2001; Bridges et al., 2002; Francis et al., 2002). Tissue-feeding endoparasitoids, such as *D. rapae*, consume host haemolymph during most of their larval development and only consume other host tissues shortly before egression (Godfray, 1994; Harvey et al., 2000), thereby possibly preventing the breakdown of GLS into toxic products during the major part of their development. *Diaeretiella rapae* performance did not differ between Col-0 and Col-0-MYB28 plants, which was expected as both aphid size and aphid GLS concentrations did not differ between these lines.

Aphid-infested plants of the three *A. thaliana* ecotypes differed in their volatile profiles. Both the predator (*E. balteatus*) and the parasitoid wasp (*D. rapae)* preferred the ecotype on which their offspring performed best. This demonstrates that preference and performance of these natural enemies are positively correlated, in agreement with other studies (Soler et al., 2007; Gols et al., 2009). The predator always laid fewest eggs on the ecotype within a test combination that had the highest emission of volatile GLS hydrolysis products, suggesting that volatile breakdown products of GLS were repellent for the predators. *Episyrphus balteatus* had access to the aphid-infested plants in the bioassays. We do not know if other plant characteristics or aphid cues also played a role in the selection of an oviposition site by *E. balteatus*. In particular, the preferred ecotype in each test combination had the lowest trichome density. It has been shown previously that adult hoverflies have problems with landing on plants with high trichome densities (Verheggen et al., 2009). In contrast to the predator, the parasitoid preferred volatile cues from the ecotype with the highest emission of volatile GLS hydrolysis products (Cvi), but only when offered in combination with ecotype Col-0. A preference for a high emission of volatile GLS hydrolysis products was expected, as *D. rapae* is known to be attracted to host plants emitting volatile breakdown products of GLS (Read et al., 1970; Bradburne and Mithen, 2000; Blande et al., 2007). Neither the predator nor the parasitoid wasp differentiated between cues from Col-0 and Col-0-MYB28 plants. The relatively small difference in volatile profiles between these lines might not allow olfactory discrimination.

In summary, the four main findings of our study are: 1) The performance of the specialist cabbage aphid *B. brassicae* is positively correlated with concentrations of both aliphatic and indole GLS in the phloem of *A. thaliana* plants; 2) *Brevicoryne brassicae* selectively sequestered GLS from the phloem; 3) The performance of the aphid predator *E. balteatus* is negatively correlated with aphid GLS concentrations. The performance of the aphid parasitoid *D. rapae* is positively correlated with aphid GLS concentrations, probably because the aphids with the highest GLS concentrations have a higher body weight; 4) Both natural enemies prefer the *A. thaliana* ecotype on which their offspring perform best, indicating a positive performance-preference correlation. The predator preferred the *A. thaliana* ecotype with the lowest emission of volatile breakdown products of GLS in each test combination, whereas the parasitoid wasp preferred the *A. thaliana* ecotype with the highest emission of these volatiles, but only in one test combination. Our study shows that there are differential herbivore-

mediated effects of GLS on a predator and a parasitoid of a specialist aphid that selectively sequesters GLS from its host plant.

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