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Glucosinolates and other metabolites in the leaves of *Arabidopsis thaliana* from natural populations and their effects on a generalist and a specialist herbivore

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Summary. Because many secondary metabolites in plants act as defense against herbivores it has been postulated that these compounds have evolved under selective pressure by insect herbivores. One explanation for the within-species variation in metabolite patterns in a particular species is that different populations are under selection by different herbivores. We tested this hypothesis, using *Arabidopsis thaliana* plants that originated from dune and inland areas. We analyzed *Arabidopsis thaliana* leaves using NMR spectroscopy and multivariate data analysis. Major differences in chemical composition were found in water-methanol fractions and were due to higher concentrations of sinigrin and fumaric acid in dune plants. Inland plants showed lower levels of glucose. Quantitative analysis of glucosinolates was performed with HPLC. Individual plants and populations demonstrated differences in glucosinolate composition and concentration. In growth chamber experiments, the generalist herbivore, *Spodoptera exigua* grew significantly better on the inland plants, while the specialist herbivore *Plutella xylostella* performed equally well on plants of both origins. Aliphatic glucosinolate as well as total glucosinolate concentrations negatively correlated with larval mass of *Spodoptera exigua*. No significant correlations, however, were found between larval mass of *Plutella xylostella* and glucosinolates in the leaves. A specialist and a generalist herbivore were responding differently to plant secondary chemistry, as was also found in several other studies. This is an important indication that differences in glucosinolate concentrations among populations may result from differential selection by different guilds of herbivores.

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Introduction

Because many secondary metabolites act as defense against herbivores, it has been postulated that insect herbivores have played a dominant role in the evolution of these compounds (Ehrlich & Raven 1964; Rhoades & Cates 1976). Recently it has been shown that the plant metabolome can indeed be under selection by insect herbivores (Shonle & Bergelson 2000; Lankau 2007). One explanation for the variation in concentration as well as in composition of metabolites within a single plant species is selection by different herbivores (Simms 1990, 1992; Mithen *et al.* 1995; van der Meijden 1996). While secondary metabolites may provide effective defense against generalist herbivores, specialist herbivores may use specific plant chemicals as cues to find and identify their food plants. We therefore expect these herbivores to exert contrasting selection pressures and to affect the metabolite composition.

Glucosinolates are secondary metabolites of the Capparales and a few other taxa. At least 120 different glucosinolates have been identified in these plants (Fahey *et al.* 2001). Glucosinolates, especially their breakdown products that result from contact with myrosinase after tissue disruption, have long been known for reducing the palatability of leaf tissue to generalist herbivores (Chew 1988; Giamoustaris & Mithen 1995; Kliebenstein *et al.* 2001). Specialist insect herbivores however, do not respond unequivocally to glucosinolate levels (Nielsen *et al.* 2001).

It is known that the effect of a single chemical factor on the performance of herbivores often depends on its interaction with other chemical factors. Variation in ni-

trogen and sugars (Blau *et al.* 1978; van der Meijden *et al.* 1989) may affect oviposition and larval growth of insect herbivores. It is therefore important to study the effect of different glucosinolates in the context of other chemical compounds. We use two analytical techniques in this paper. NMR spectroscopy produces a wide spectrum chemical analysis, which is rapid, reproducible, and stable in time and gives information on a range of chemical compounds. Though the sensitivity of NMR spectroscopy is not as good as that of some other metabolomic technologies, it is especially suitable for analysing compounds that are present in relatively high concentrations, such as sugars and organic acids. Minor and trace compounds, however, require a targeted approach (Summer *et al.* 2003). HPLC analysis is such a targeted analysis and offers good selectivity and sensitivity and can provide detailed data on single classes of compounds. Therefore we used NMR spectroscopy to find the differences between plants with respect to a large number of chemical substances and we used HPLC to look in more detail at the glucosinolates.

In our study of natural herbivores in *A. thaliana* populations, we compared plants from two different habitats, dune and inland, in the Netherlands. *Arabidopsis thaliana* experienced 40 % fruit damage by the specialist weevils *Ceutorhynchus atomus* and *C. contractus* (Curculinoidae) in the dune habitat (Mosleh Arany *et al.* 2005), but hardly any fruit damage by these weevils was observed on plants growing in the inland habitat. We only observed aphid infection and a small amount of leaf herbivory by unknown herbivores in the inland populations. If generalist and specialist insect herbivores do indeed exert a contrasting selection pressure on *A. thaliana*, they should perform differently on plants based on their chemical compounds. To test this hypothesis, we analysed whether the crucifer specialist herbivore *Plutella xylostella* and the generalist herbivore *Spodoptera exigua* were differently affected by chemical compounds in the leaves.

This paper addresses the following questions:

1. What are the differences in chemical compounds in leaves of plants originating from dune and inland populations of *A. thaliana* when grown for one generation in the lab?

2. Does the glucosinolate profile of *A. thaliana* differentially affect generalist and specialist insect herbivores?

3. Do differences in chemical composition of the leaves correlate with the performance of either herbivore?

Materials and methods

Plants and insects

Arabidopsis thaliana grows naturally in the coastal regions of the Netherlands, in two habitat types. It is locally common along roads in the urban areas (called inland hereafter). It also occurs locally on the calcareous new dunes that were formed partly on top of an old soil profile c. 800 years ago (called dune hereafter).

Seeds were collected in July 2002 from ten plants in two populations in the dunes and two populations in the inland area. The sandy surface of the dune sites at Meijendel, north of The Hague, is covered with mosses, grasses and small herbs with about 10 percent bare soil. Accompanying species included, amongst others, *Erophila verna*, *Cardamine hirsuta, Rubus caesius, Calamagrostis epigejos* with small *Hippophae rhamnoides* shrubs nearby. All populations in the dunes were found within 20 m from woody vegetation with trees like *Populus nigra, P. alba, Betula pubescens* and *Crataegus monogyna.* Seeds from two of the dune populations studied by Mosleh Arany *et al.* (2005) were used for this study (called dune 2 and dune 3 hereafter. The numbering corresponds to Mosleh Arany et al. 2005).

Population 1 in the inland is located in Leiden, 3 m from a paved road and the second one, population 2, is growing near a canal in Noordwijk. Both sites were covered with *Lolium* sp. with about one percent bare soil. Accompanying species included, amongst others, *Erophila verna*, *Cardamine hirsuta* and *Plantago lanceolata.* The distance between the two inland populations is about 8 km and the minimal distance between the dune and the inland habitat is about 6 km.

Caterpillars of *Spodoptera exigua* were obtained from a lab culture, reared on an artificial diet in a growth chamber at 25 °C, 16 h/8 h L/D photoperiod, 70 % RH. Caterpillars of *Plutella xylostella* were obtained from a lab culture reared on *Brassica oleracea* in a growth chamber at 25 °C, 16 h/8 h L/D photoperiod, 40 – 50 % RH.

Metabolomic Analysis

Seeds were collected in the field and after germination plants were grown under controlled conditions in a growth chamber (20 °C, 18-h light, 70 % humidity). Seeds produced by these plants were germinated and the resulting two- month old rosettes were used to make extracts for HPLC and NMR spectroscopy. One hundred mg dry mass of leaves of 5 rosettes of each population was used for HPLC and five hundred mg of each type of plant (6 plant samples for dune 2 and inland 1 and 3 samples for dune 3 and inland 2) for NMR spectroscopy. Extraction, purification and glucosinolate analysis followed the procedure used by van Dam et al. (2003) with sinigrin as the external standard. Glucosinolates were extracted with 70 % methanol solution, desulphatased with arylsulphatase (Sigma, St. Louis, IL, USA) on a DEAE-Sephadex A 25 column and separated on a reversed phase C-18 column on HPLC with an acetonitrile-water gradient. The elution program was a linear gradient starting at 0 % acetonitrile (ACN) and increasing to 35 % ACN in water over 30 minutes. Glucosinolate detection was performed with a PDA detector (200 – 350 nm) with 229 nm as the integration wavelength. Sinigrin (sinigrin monohydrate, ACROS, New Jersey, USA) was used as an external standard. We used the correction factors at 229 nm from Buchner (1987) and the EC (EC, 1990) to calculate the concentrations of the glucosinolates. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with standards kindly provided by M. Reichelt, MPI Chemical Ecology, and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R).

For preparing NMR spectroscopy extracts we followed the procedure of Choi et al. (2004). All spectra were recorded on a Bruker AV-400 NMR spectrometer operating at a proton NMR frequency of 400.13 MHz. After measurements, the ^{1}H-NMR spectra were automatically reduced to ASCII files using AMIX (Analysis of MIXtures software v. 3.8, Bruker Biospin). Spectral intensities were scaled to HMDSO (hexamethyl disilane) and trimethyl silane propionic acid sodium salt (TSP-d_4) for chloroform and water-methanol fractions, respectively, and reduced to integrated regions, called "buckets", of equal width (0.02 ppm) corresponding to the region of δ 10.0 to -0.1. The generated ASCII file was imported into Microsoft Excel for the addition of labels and then imported into SIMCA-P 10.0 (Umetrics, Umeå, Sweden) for PCA analysis.

Table 1 Larval weight (mg ± SE) of *Spodoptera exigua* and *Plutella xylostella*, fed on plants of dune or inland origin.

Herbivore	Dune 2	Dune 3	Inland 1	Inland 2
Larval weight of S. exigua	15.7 ± 3.56 a	19.9 ± 4.02 a	128.5 ± 21.96 b	122.2 ± 5.16 b
Larval weight of P. xylostella	7.20 ± 0.20 a	6.66 ± 0.18 a	7.16 ± 0.19 a	7.11 ± 0.24 a

The values in each row, followed by a different character are significantly different (ANOVA, Tukey test, $P<0.05$). $n=25$.

Experiment with generalist and specialist herbivores

Plants in the rosette stage were used for this experiment. Twenty five plants (five per parent plant) from the same generation of individuals as used for HPLC analysis were used for this experiment. Two second instar caterpillars of *Spodoptera exigua* and two second instar caterpillars of *Plutella xylostella* were placed on each of the rosettes. Larval weight of both herbivores was measured after 5 days.

Statistical analysis

Data were analyzed with SPSS 10 (SPSS Inc., Chicago, USA). Normality of the data was checked by post-hoc analysis of the residuals using the Kolmogorov-Smirnov test for normality. Differences in larval weight of herbivores and differences in glucosinolate concentration between dune and inland populations were tested with ANOVA (General Linear Model, Univariate, type III Sums of Squares). To simplify the analysis we first checked whether mother plants from the same population were significantly different. No such differences were found, so we pooled all data on seedlings of different mother plants within the same population. The correlations between larval weight of herbivores and plant glucosinolates, fumaric acid and glucose were analyzed with a Pearson test.

NMR-data were analyzed with a principal component analysis (PCA) which is a clustering method requiring no knowledge of the data set. PCA acts to reduce the dimensionality of multivariate data while preserving most of the variance within the data (Goodacre *et al.* 2000). The principal components can be displayed in a graphical fashion as a "scores" plot. This plot is useful for observing any grouping in the data set. PCA models were constructed using all the samples in the study. Coefficients by which the original variables must be multiplied to obtain the PC are called loadings. The numerical value of a loading of a given variable on a PC shows how much the variable has in common with that component (Eriksson *et al.* 2001). Thus for NMR spectroscopy data, loading plots can be used to detect the spectral areas responsible for the separation in the data.

Results

Herbivory Assessment

The larval weight of *P. xylostella* fed on plants from dune and inland origin populations was not significantly different (Table 1). The larval weight of *S. exigua* fed on dune plants was significantly lower than when fed on inland plants ($P=0.001$). No differences were found among the two dune populations and among the two inland populations ($P=0.65$, $P=0.88$ respectively) (Table 1).

Table 2 ANOVA's (Type III) on glucosinolate concentration of plants from four populations of dune and inland origin after one generation in a growth room.

Glucosinolates	Source	*df*	*F* value	*p*
Indole	Population	3	0.824	0.500
Aliphatic	Population	3	24.156	<0.001
Total GLS	Population	3	21.827	<0.001

Metabolomic Analysis

NMR spectroscopy analysis

The chloroform fractions of leaves were not clearly separated in the PC1–PC2 score plot of the dune and inland populations (Fig. 1). Data for the water-methanol fractions of the two plant types were clearly separated from each other by PC3 (Fig. 2). The loading plot of PC3 shows that this separation was mainly due to the signals of sinigrin (δ 6.02, 5.30, 5.04, 3.90, 3.52), the signal of fumaric acid (δ 6.52) and the signal of glucose at δ 4.64. In addition, small positive PC3 values are detected in the range of δ 6.5 – δ 10.0. It means that phenolic compounds are more abundant in the dune populations than in the inland populations. The signal from 3.20 is choline and 2.68 is malic acid (Fig. 2 B).

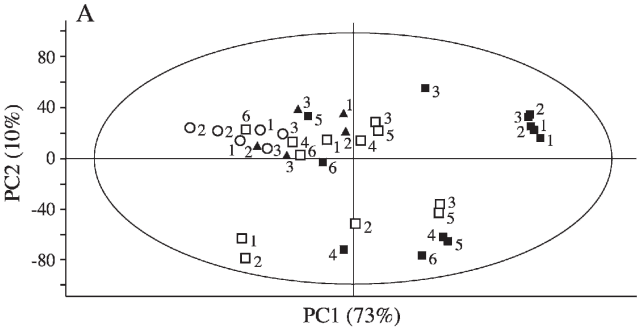

Fig. 1 Score plot (PC1 vs. PC2) of principal component analysis of the chloroform fractions of *Arabidopsis thaliana* leaf extracts. (■) inland 1; (▲) inland 2; (□) dune 2; (o) dune 3 plants. The ellipse represents the Hotelling T^2 with 95 % confidence in score plots. The experiments were based on 2 replicated samples from 9 dune and 9 inland plants. In a few cases there is no replicate. Replicates share the same symbol and number.

The relative area of the signals of fumaric acid at δ 6.52 and of glucose at δ 4.64 of the water-methanol fraction of

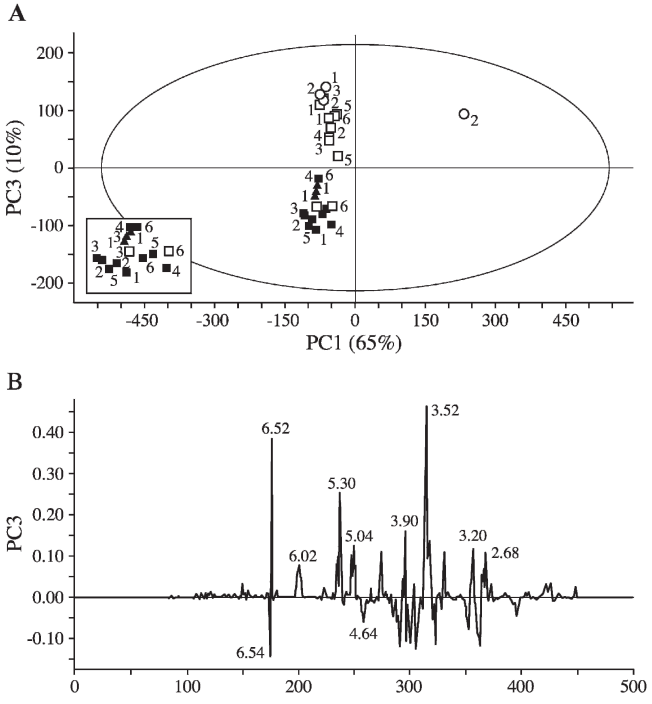

Fig. 2 Score (A) plot and loading (B) plot of principal component analysis of the water-methanol fraction of *Arabidopsis thaliana* leaf extracts. Origin: (■) inland 1; (▲) inland 2; (□) dune 2; (o) dune 3. The ellipse represents the Hotelling T^2 with 95% confidence in the score plot. The experiments were based on the 2 replicated (1–2 plants in 1 replicate) samples from 9 dune and 9 inland plants. The numbers next to each peak (δ) in the loading plot denote chemical shift of NMR spectra.

concentration of aliphatic glucosinolates were highly significant between populations. Also total concentration of glucosinolates was significantly different

The results of posthoc Duncan's multiple range tests (Table 3) on aliphatic and total glucosinolates illustrates the differences between populations and habitats. Dune populations have higher concentrations than inland populations. Differences in concentration of indole glucosinolates were not significant between populations (Table 2).

Overall the aliphatic glucosinolates dominate in the leaves of these four *Arabidopsis* populations. Sinigrin (2-propenyl glucosinolate) is by far the most abundant individual glucosinolate which constitutes almost 90% of the total concentration of glucosinolates of the dune populations. In inland population 2 we found 79% sinigrin. In inland population 1 sinigrin was only detected in one out five plants in an extremely low concentration. In this population 3-hydroxypropyl is with 51% the most abundant glucosinolate.

Herbivory in relation to glucosinolates and other chemical compounds

In the feeding trial in the growth room, larval weight of the specialist herbivore *P. xylostella* was neither significantly correlated with total glucosinolate concentration (Fig. 3) nor with the aliphatic or indole leaf glucosinolates (Table 4). The larval weight of generalist herbivore *S. exigua* was negatively correlated with total glucosinolate

Table 3 Glucosinolate type and mean concentration (±SE) (μmoles/g dry weight) of leaves of 5 plants of two dune and two inland populations grown in a growth room for one generation.

Glucosinolate type	Dune 2	Dune 3	Inland 1	Inland 2
I	1.01 ± 0.17 a	1.26 ± 0.10a	1.04 ± 0.19 a	0.97 ± 0.07 a
A	14.66 ± 2.51 b	27.12 ± 1.93 a	7.53 ± 1.36 c	9.65 ± 0.95 bc
	(13.89 ± 2.38)	(25.47 ± 1.84)	(0.04 ± 0.04)	(8.35 ± 0.79)
Total glucosinolate concentration	15.66 ± 2.68 b	28.37 ± 2.00 a	8.56 ± 1.53 c	10.63 ± 1.00 bc

I = indole glucosinolates, A = aliphatic glucosinolates (sinigrin concentrations are presented in brackets) an total concentration of glucosinolates per population. The values in each row, followed by a different character are significantly different (Duncan's multiple range test on aliphatic and total glucosinolates, $P < 0.05$).

7 plants from the inland populations and 8 from the dune populations were used as an estimate for the concentrations of these substances.

Glucosinolate patterns by HPLC analysis

Twelve principal glucosinolates were found in the leaves of plants grown in the growth room. They are clustered into two structural groups: indole glucosinolates (I) and aliphatic glucosinolates (A).

ANOVA's (Table 2) were applied to these data to test whether differences were present between populations. Residuals were normally distributed. Differences in

concentration (Fig. 3) and with the aliphatic glucosinolates in the leaves (Table 4). The larval weight of the generalist herbivore *S. exigua* was also negatively correlated with gluconapin, sinigrin and 4-methoxyglucobrassicin ($r = -0.71$, $P < 0.001$; $r = -0.65$, $P = 0.002$; $r = -0.59$, $= 0.006$ respectively). These correlations were still significant (except for 4-methoxyglucobrassicin) after conservative Bonferroni correction (16 correlations, $\alpha = 0.003$).

Within each population, the correlation between larval weight of *S. exigua* and glucosinolates was not significant. Individual population samples only consisted of five plants and covered only a relative small range of glucosinolate concentrations. Because 14 out of 16 (85%) of these latter correlations, between larval weight of *S.*

Table 4 Pearson correlation coefficients (r, n = 20) between larval weight of *P. xylostella* and *S. exigua* and I = indole glucosinolates, A = aliphatic glucosinolates and total glucosinolate concentration, fumaric acid and glucose (** = $P < 0.01$). * Pearson correlation coefficients with fumaric acid and glucose are based on 15 observations.

Herbivores	I	A	Total GLS	Fumaric acid	Glucose
Larval weight of *P. xylostella*	0.14	–0.38	–0.37	–0.27	–0.12
Larval weight of *S. exigua*	–0.32	–0.65**	–0.64**	0.14	–0.47

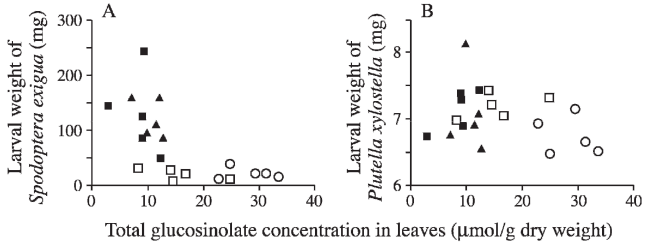

Fig. 3 Larval weight of *Spodoptera exigua* (A) and *Plutella xylostella* (B) versus total glucosinolate concentration of the leaves of dune and inland populations of plants originated from *A. thaliana* that were grown in a growth room. (■) inland 1; (▲) inland 2; (□) dune 2; (o) dune 3.

exigua and glucosinolates, were negative (Binomial test, $P = 0.001$), this supports our conclusion that high glucosinolate levels reduce *S. exigua* growth.

Larval weight of the two species was not significantly correlated with glucose or fumaric acid levels (Table 4), which were the main additional chemical differences between the two plant types in the NMR spectroscopy. To see if there was any complementary effect of the different groups of compounds, a linear regression was calculated with larval weight of *S. exigua.* No significant effect was found ($P > 0.05$).

Discussion

Metabolites in the leaves of dune and inland origin plants of *A. thaliana.*

Both NMR and HPLC analysis demonstrated differences between glucosinolate patterns in *Arabidopsis* plants growing at dune and inland sites. Sinigrin was the main glucosinolate in the leaves of dune plants, but was either absent or occurred at low concentrations in inland plants (NMR analysis, Fig. 2). When analyzed using HPLC, sinigrin was again found at high concentrations in dune plants. These results confirmed that combining these two analytical methods can provide detailed data on target compounds.

We found differences in types and quantities of glucosinolates between individual plants that were grown together in a controlled environment and analyzed with HPLC. These observations show that there are genetic components linked to the observed glucosinolate variation within and between *A. thaliana* plants found in dune and in inland populations. Glucosinolate levels are highly variable in wild-collected accessions (Mithen *et al.* 1995; Mithen & Campos 1996; Kliebenstein *et al.* 2001). Mithen & Campos (1996) found polymorphisms at gsl-elong and gsl-ohp loci among landraces of *A. thaliana* in Europe. Ecotypes from central and eastern Europe had only propyl glucosinolates, whereas ecotypes from Western Europe mostly had both propyl and butyl glucosinolates. Mauricio (1998) found genetic variation for total glucosinolate concentration in four discrete neighboring natural populations of *A. thaliana* in North Carolina. Glucosinolates accumulation and hydrolysis products are controlled by genetic variation by a number of enzymes that function in a sequential pathway (Kliebenstein *et al.* 2001; Lambrix *et al.* 2001). Thus a mutation in one of the enzymes or a cross-pollination event could lead to a rapid glucosinolate profile shift from one generation to the next (Kliebenstein 2004).

Glucose and fumaric acid were the two other compounds that differed in concentration between the two plant origins. These compounds were the main compounds that separated nine *A. thaliana* ecotypes analyzed by NMR spectroscopy in Ward et al's study (2003) as well. In our study the reason of the highly elevated glucose levels in inland plants is unclear. Variation in glucose ecologically may affect oviposition and larval growth of herbivores (van der Meijden *et al.* 1989).

Does the glucosinolate profile of *A. thaliana* differentially affect the performance of generalist and specialist insect herbivores?

Similar to some previous studies, we found that total glucosinolate levels were negatively correlated with generalist performance. Fig.3 demonstrates a six-fold decrease in larval weight of *Spodoptera* with an increase in total glucosinolate concentration of *Arabidopsis* from 10 to 30 μmol/gdw. That would be a considerable effect if it was only brought about by glucosinolates. A comparison with other studies gives support for this result: Li et al. (2000) found a four to five-fold decrease in leaf area damaged by *Spodoptera eridania* between low and high glucosinolate lines of *Brassica juncea* and a five-fold decrease in larval weight gained by feeding on artificial diets ranging from 3 to 30 μmol allyl glucose/g diet. Glucosinolate composition in *A. thaliana* has a negative impact on the generalist herbivore *Trichoplusia ni* (Kliebenstein *et al.* 2002). Over a relatively small range of variation in total aliphatic glucosinolates (from 2 to 10 μmol/gdw), they found a more than 60% decrease in herbivory. The data presented by Giamouraris & Mithen (1995) on natural herbivory by generalist birds of rapeseed leaves demonstrate a ten-fold reduction between 5 and 30 μmol/gdw.

Mauricio & Rausher (1997) found that insect herbivory and damage by plant pathogens in the field was negatively correlated with glucosinolate concentration. The herbivores in their study were not identified, so that it is not clear whether they were specialist or generalist herbivores. Generalist herbivores are more likely to be

negatively influenced by glucosinolates (Chew 1988). Only total glucosinolate concentration was measured and not the glucosinolate type or hydrolysis products (Chew 1988). Experiments with lines specifically varying in glucosinolate composition could lead to a better understanding of the biological basis for glucosinolate variation maintenance in *A. thaliana* (Kliebenstein 2004).

We found negative correlations between the aliphatic glucosinolates, sinigrin and gluconapin and *S. exigua* performance. Several other studies have shown the same relationship between variation in the presence and /or concentration of aliphatic glucosinolates and behaviour of generalist herbivores. The generalist herbivores *Aphis fabae*, *Aulacorthum solani*, *Mamestra configurata*, *Myzus persicae* and *Trichoplusia ni* were negatively affected by various aliphatic glucosinolates and their pungent degradation products (for reviews see Raybould & Moyes 2001 and Kliebenstein 2004). Although total indole glucosinolate concentration was not significantly correlated with *S. exigua* growth, the 4-methoxyglucobrassicin was negatively affecting the performance of this generalist herbivore. Generally it is assumed that indole glucosinaltes are less effective defense compounds than aliphatic glucosinolates, because upon contact with myrosinase they form unstable isothiocyanates (Wittstock *et al.* 2003). A recent study, however, revealed that 4-methoxyglucobrassicin specifically and more effectively reduced performance of the generalist aphid *Myzus persicae* than sinigrin (Kim & Jander, 2007). Our results thus confirm that specific glucosinolates such as 4-methoxyglucobrassicin present at low concentrations may also contribute to resistance against generalist herbivores.

The negative correlation between glucosinolates and *S. exigua* weight does not provide absolute proof that glucosinolates directly reduce feeding. In Fig. 3 several genotypes are included and theoretically the possibility exists that these genotypes also differ in unknown other factors that covary with glucosinolates. From a chemical perspective, there was no significant correlation with the other main chemical compounds that were different between two types of plants. However morphological differences, such as differences in trichome densities, between dune and inland plants may have also be involved in the suitability of *S. exigua.*

In contrast to some other studies, we did not find that higher levels of glucosinolates increased the performance of the specialist in our study. However, also Kliebenstein *et al.* (2002) found herbivory by the specialist *Plutella xylostella* to be uncorrelated with variation in glucosinolates. Nielsen *et al.* (2001) who used transgenic *A. thaliana* plants with a four- fold increase in total glucosinolate levels neither did find any effect on the suitability of *A. thaliana* for two specialist flea beetle species, *Phyllotreta nemorum* and *P. cruciferae.* The flea beetles did not discriminate between transgenic and wild type plants. Studies on the interaction between specialist herbivores and other members of the Cruciferae were consistent with our results. A survey of the literature (Nielsen *et al.* 2001) shows that the majority of experiments demonstrate no effect of glucosinolates on specialist herbivores.

Despite these results we can not be sure that a positive effect is really absent in these cases. It is very well possible that we are not dealing with a linear relationship, but with an asymptotic relationship between preference or performance of the herbivore and a particular metabolite or metabolite group, that reaches the asymptote already at a very low concentration. Many studies have demonstrated that cue metabolites stimulate specialists to start oviposition or feeding. Chapman (2003) found that the thresholds for such secondary compounds that stimulate phagostimulatory cells of caterpillars are usually much lower than those that stimulate deterrent cells (in generalists). If an herbivore species is sequestering the metabolite, for its own defense or for other reasons, selection of food plants with high concentrations of a particular metabolite seems more likely.

Spodoptera and *Plutella* do not feed naturally on *A. thaliana* populations in our study site. The main naturally occurring herbivores at the dune site are *Ceutorhynchus atomus* and *C. contractus* (Curculionidae). Because the dune type is less affected by the weevils than inland type, it is suggested that these common specialist herbivores exerted a selection pressure on the plants growing in the dunes. However, in a field experiment we did not find any correlation between the glucosinolate concentration in seeds and herbivory damage by these two specialist weevils (Mosleh Arany 2006.).

A range of studies (cited above) demonstrates different effects of glucosinolates on specialist and generalist herbivores. These studies and our data suggest that the evolution of glucosinolate levels and composition may indeed be driven by different selection pressures from generalist and specialist herbivores. A recent paper by Lankau (2007) provides experimental field evidence for *Brassica nigra.* All evidence on generalist herbivory points in the same direction: herbivory is reduced by higher concentrations of glucosinolates. The evidence on specialists is less straightforward: in some cases oviposition or herbivory increased with the concentration of glucosinolates, in many other studies no effect was found. In general, based on all these results, we suggest that differences in glucosinolate patterns or concentrations may indeed be the result of selection by different herbivore guilds. At the same time it should however be realized that selection pressure by the same herbivore guild under different environmental circumstances (e.g. in a harsh versus a benign environment) might lead to different plant defenses (Coley *et al.* 1985).

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