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PLANT ANIMAL INTERACTIONS

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Elevated CO_2 influences herbivory-induced defense responses of *Arabidopsis thaliana*

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Abstract We experimentally demonstrate that elevated CO_2 can modify herbivory-induced plant chemical responses in terms of both total and individual glucosinolate concentrations. Overall, herbivory by larvae of diamondback moths (*Plutella xylostella*) resulted in no change in glucosinolate levels of the annual plant *Arabidopsis thaliana* under ambient CO_2 conditions. However, herbivory induced a significant 28–62% increase in glucosinolate contents at elevated CO_2. These inducible chemical responses were both genotype-specific and dependent on the individual glucosinolate considered. Elevated CO_2 can also affect structural defenses such as trichomes and insect-glucosinolate interactions. Insect performance was significantly influenced by specific glucosinolates, although only under CO_2 enrichment. This study can have implications for the evolution of inducible defenses and coevolutionary adaptations between plants and their associated herbivores in future changing environments.

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Introduction

Plant defensive responses to herbivory depend on the plant's evolutionary history (e.g., past exposure to herbivory) as well as the physical environment affecting plant–insect associations (Tollrian and Harvell 1999). Although previous research has focused on the evolution of inducible strategies in light of defense costs and the plant likelihood of attack by herbivores (reviewed in Strauss et al. 2002), much less emphasis has been placed on how the environment may affect these inducible responses. There is evidence that induced defense responses of plants to herbivory are influenced by both biotic and abiotic environmental factors including plant density, nutrients, and UV light (reviewed in Agrawal and Karban 1999). Few studies, however, have evaluated increased CO_2 effects on herbivory-induced chemical responses (Lindroth and Kinney 1998; Roth et al. 1998; Bazin et al. 2002), and to our knowledge, no significant $CO_2 \times$ herbivory interactions have been previously reported. Since induced defenses can decrease herbivore damage, and consequently, enhance plant fitness (Agrawal 1999; Kessler and Baldwin 2004), variation in induction levels resulting from environmental changes can have important ecological and evolutionary implications for plants and their interactions with insect herbivores.

In addition, limited information exists regarding potential genotype-specific responses of plant defense-related traits to elevated CO_2. For example, significant $CO_2 \times$ plant genotype interactions have been found for cyanide levels in *Lotus corniculatus* (Bazin et al. 2002), and for concentrations of both phenolic glycosides and condensed tannins in *Populus tremuloides* (Lindroth et

al. 2001). The importance of genotypic diversity has recently gained considerable attention from the emerging field of community genetics. This fairly new discipline as presented first by Antonovics (1992), and later by Whitham et al. (2003) and Neuhauser et al. (2003), emphasizes the significance of the action of genes beyond the population level (i.e., gene effects extended to communities and ecosystems). Whitham et al. (2003) highlighted the increased significance of genotype × environment interactions for global change research, which in turn can affect interactions among species and the structure of populations and communities. Holton et al. (2003) has provided an example of how global atmospheric changes (e.g., elevated ozone and CO_2) differentially affecting genotypes of an aspen species can have effects on its associated insect community, for example, by affecting the performance of a lepidopteran herbivore and its dipteran parasitoid.

Previous studies have concentrated on elevated CO_2 effects on plant nutritional (e.g., nitrogen and carbohydrates) and chemical (mainly phenolics and terpenoids) components, and whether these changes in plant quality affect insect performance (reviewed in Bezemer and Jones 1998; Penuelas and Estiarte 1998). On the other hand, responses of other phytochemical classes such as glucosinolates or structural defenses such as trichomes have been virtually unexplored. Trichomes are cell types protruding from the plant epidermis as root hairs, thorns or papillae, which are known to act as a leaf mechanical and defensive barrier against herbivores (Bird and Gray 2003). Evidence that trichomes can be influenced by environmental factors is scarce, although changes in their numbers have been reported as a result of herbivory and water stress (Quarrie and Jones 1977; Mauricio and Rausher 1997; Agrawal 1999).

Glucosinolates are plant secondary chemicals that can act as herbivore deterrents in the field (Mauricio and Rausher 1997). Glucosinolate production is affected by environmental factors such as temperature, soil moisture, and content of nitrogen, sulfur and boron in the soil (Gershenzon 1984; Ciska et al. 2000; Vallejo et al. 2003). We only know of one study that has assessed the response of three crucifer species to elevated CO_2, in terms of total glucosinolate concentrations (which was species-specific); however, individual glucosinolates were not measured (Karowe et al. 1997). Quantification of individual glucosinolates is fundamental because insects are not likely to respond to total concentrations of a chemical class but rather respond to specific compounds, which may elicit differential responses in insects by acting either in an individualistic or synergistic fashion (Berenbaum and Zangerl 1992).

In this study, we experimentally assessed the effects of elevated CO_2 on induced chemical (i.e., individual and total glucosinolate contents) and structural (i.e., trichome density) responses of three *Arabidopsis thaliana* genotypes to herbivory by larvae of the diamondback moth, *Plutella xylostella*. We also evaluated increased CO_2 influences on diamondback moth performance and its association with plant defense-related traits. The pronounced variation in glucosinolate content and composition among *A. thaliana* genotypes selected for this study (Bano 1993), and the well-known ability of the crucifer specialist diamondback moth to rapidly adapt to environmental challenges (e.g., adaptation to pesticides; Talekar and Shelton 1993) provide an ideal system to assess elevated CO_2 effects on induced defense responses and plant–insect interactions. We specifically addressed the following questions:

1. Does CO_2 enrichment affect *A. thaliana* chemical and structural responses to diamondback moth herbivory?
2. Are there plant genotypic differences associated with these induced responses to elevated CO_2?
3. Are insect performance and its association with chemical and structural plant responses influenced by CO_2 enrichment?

We demonstrate that elevated CO_2 can modify herbivory-induced chemical responses in *A. thaliana*, and that these responses can be genotype-specific. Furthermore, we show that structural defenses (i.e., trichomes) and insect–glucosinolate interactions can also be affected by elevated CO_2. The observed CO_2 × genotype effects on induced defenses may have consequences not only for plant–herbivore interactions but also for higher trophic levels (Holton et al. 2003; Whitham et al. 2003). These results highlight the importance of assessing genotypic diversity for predicting the evolution of inducible defenses in future changing environments.

Materials and methods

Selected plant and insect species

The annual mouse-ear cress, *A. thaliana* (Capparales: Brassicaceae), and the diamondback moth, *P. xylostella* (Lepidoptera: Plutellidae), were the plant and insect species selected for this study. *A. thaliana* is an annual plant native to eastern Europe, which is widely distributed throughout temperate regions (Ratcliffe 1961). This plant species has glucosinolates, which are secondary compounds found in the Brassicaceae family. The glucosinolate molecule comprises a common glycone moiety and a variable aglycone side chain that may contain aliphatic, indolyl, or aromatic groups (Mithen and Campos 1996). Glucosinolates in *A. thaliana* are ecotype-specific and their biological activity depends largely on their side chain structure (Bano 1993; Mithen and Campos 1996). Glucosinolate variation in *A. thaliana* is apparently controlled by a flexible modular genetic system, which may allow this chemical class to evolve rapidly in future changing environments (Kliebenstein 2001). For this study, three *A. thaliana* genotypes (highly inbred lines), Can-0, Edi-0 (both late flowering), and Cvi-0 (early flowering), were obtained from the Arabidopsis Biological Resource Center at

Ohio State University (Columbus, OH, USA). These genotypes were selected on the basis of their differences in glucosinolate profiles (Bano 1993), which have been previously shown to influence herbivory in the field (Mauricio and Rausher 1997).

The diamondback moth is a common insect specialist native to southern Europe (Talekar and Shelton 1993) that feeds exclusively on members of the Brassicaceae family. Diamondback moth adults used to start a colony for this study were obtained from larvae collected in cabbage plots at the University of Illinois agricultural fields (Urbana-Champaign, IL, USA). The colony was maintained in rearing cages and emerging adults were fed with 10% (v/v) honey solution. Crucifer seedlings (canola, *Brassica napus*) were used to feed the larvae and for adult oviposition. Second-instar larvae collected from this colony were used for the herbivory treatment.

Experimental design, growth conditions and traits measured

A growth chamber experiment (split-split plot design) was conducted to evaluate the defensive response of *A. thaliana* genotypes to controlled diamondback moth herbivory under both ambient and increased CO_2 conditions. The experiment was performed using four CO_2 chambers located in the Plant Biology Laboratory at the University of Illinois at Urbana-Champaign. The split-split plot design consisted of two CO_2 levels, that is, ambient (360 ppm ± 10) and elevated (720 ppm ± 50) CO_2 acting as main plots; four groups of plants growing in four separate chambers (two distinct groups per CO_2 level, which provided a replication of the CO_2 treatments); two herbivory treatments (i.e., herbivory and control) as subplots (within CO_2 treatments and group), and three genotypes as split-split plots (within herbivory treatments). Each genotype was replicated four times, accounting for a total of 96 plants.

Individual plants were grown from seeds and subjected to a 10-day cold treatment to eliminate dormancy and ensure uniform germination. Five seeds per pot (750 cm^3) were allowed to grow for a week and then, randomly thinned to one individual placed in the center of each pot. To minimize position effects, individual plants were rotated within chambers every 2–3 days. To reduce chamber effects, the four groups of plants were weekly rotated among the four chambers, which were adjusted accordingly to the CO_2 levels originally assigned to each plant group. Light/dark periods were set at 14 h/10 h, respectively. Light intensity and temperature were 150 μM m^{-2} s^{-1} and 22°C, respectively, as recommended by the Arabidopsis Biological Resource Center. Herbivory treatments consisted of placing 2 second-instar moth larvae (approximately 2 mm of long) on each of the plants randomly assigned to this treatment at bolting time, and removed at pupation. This herbivory load resulted in an average of 20% of each plant's total leaf area removed, as measured through a qualitative index of plant damage on rosette leaves (adapted from McCloud and Berenbaum 1999). This qualitative index was calculated by assigning leaves to four size categories (1 = 6–8 cm; 2 = 4–6 cm; 3 = 2–4 cm; 4 = 0–2 cm) and three damage classes depending upon the amount of tissue removed (25% or less, 25–75%, or more than 75%). Each plant from both control and herbivory treatments was protected with a Plexiglas cage with holes for aeration covered with a 135-μ^2-mesh fabric to prevent larval dispersal and infestation by other insects from the greenhouse.

Each pupa was individually placed in a gelatin capsule until adult emergence and subsequent death. Then, insect gender was determined and weight was recorded. Adult weight was used as an index of insect performance. After the herbivory trial, 1 g of fresh rosette leaves per plant was sampled and freeze-dried from plants exposed to herbivory and controls to quantify individual and total glucosinolate concentrations. Glucosinolates were extracted from freeze-dried rosette leaves, converted to desulphoglucosinolates and analyzed using high-pressure liquid chromatography (HPLC) methods as described by Magrath et al. (1993). The following aliphatic and indolyl glucosinolates were detected and quantified: 2-propenyl, 3-butenyl, methylsulphinyloctyl, methylsulphinylheptyl, indolylmethyl, 4-methoxyindolylmethyl, and 1-methoxyindolylmethyl glucosinolates. Total glucosinolates were estimated by adding individual glucosinolate concentrations, and relative amounts of individual glucosinolates were calculated as the proportion of total glucosinolate content. Trichome density was estimated as the total number of trichomes within a 2.4-mm^2 area of the upper central area of the adaxial side of one rosette leaf per plant (Mauricio and Rausher 1997). Leaves sampled were matched for size and age across plants.

Statistical analyses

A split-split linear mixed model (using PROC MIXED, SAS Version 8) was used for the statistical analysis of the measured response variables using the appropriate error terms for this design as shown by Anderson and McLean (1974). In this model, CO_2, herbivory, genotype, and their interactions were considered fixed effects, and group (replication of the CO_2 treatments) and its interactions were assumed to be random. The correct denominator degrees of freedom for all tests in this model were calculated by using the Satterthwaite approximation (PROC MIXED). Response variables were logarithmically or squared-root transformed when necessary to satisfy parametric model assumptions. Variables that despite previous transformations still showed deviations to normality or heteroscedasticity were rank-transformed (Potvin and Roff 1993; Brunner et al. 2002). Significance probabilities of all tests were adjusted using a sequential Bonferroni correction to compensate for the simultaneous comparison of several

traits. Preplanned contrasts were performed to assess mean phenotypic differences in defense-related traits between herbivory treatments (herbivory vs control) for each genotype and CO_2 level, and to evaluate mean differences in insect performance between CO_2 levels (ambient vs elevated CO_2) for each genotype and gender. In addition, analyses of covariance were performed to test for differences in regression relationships between insect performance and defense-related traits at the two CO_2 levels.

Results

Analyses of variance revealed variable responses of *A. thaliana* genotypes to elevated CO_2 and herbivory, in terms of defense-related traits such as glucosinolate concentrations and trichome density (Table 1). Group effects and their interactions are not shown in Table 1 because they were not statistically significant. Overall, the main effect of herbivory was more pronounced than that of CO_2, particularly for glucosinolate concentrations. The $CO_2 \times$ herbivory interaction is especially important in this study as it provides information on how future changes in CO_2 levels might modify plant defensive responses to insect herbivory and thus, potential interactions of plants with their associated herbivore community. In this study, we found a significant $CO_2 \times$ herbivory interaction for several individual glucosinolates (i.e., 2-propenyl, 3-butenyl, indolylmethyl and 4-methoxyindolylmethyl glucosinolates) and for the total concentration of these compounds (Table 1). Table 1 shows that this interaction was not significant for 3-butenyl, when the three genotypes were included in the analysis. However, since 3-butenyl was present in significant amounts only in genotype Cvi-0, we performed a separate analysis for this particular genotype, which revealed a significant $CO_2 \times$ herbivory interaction ($P = 0.016$). Overall, these results showed that CO_2 levels strongly affected chemical induction by herbivory in *A. thaliana* and that these responses were dependent on the individual glucosinolate considered. While glucosinolate levels were, in general, not significantly affected by herbivory at ambient CO_2 conditions, herbivory induced a significant 28–62% increase in glucosinolate concentrations under CO_2 enrichment (see Cvi-0 and Edi-0 genotypes in Fig. 1a–c, f, g). The only significant herbivory-induced decrease in glucosinolate concentrations (by 52%) was observed for indolylmethyl glucosinolate levels at ambient CO_2 (Fig. 1f). In addition, while longer chain aliphatic glucosinolates such as methysulphinyloctyl and methysulphinylheptyl glucosinolates (Fig. 1d, e) were not significantly affected by herbivory at either ambient or elevated CO_2 conditions, 1-methoxyindolyl glucosinolate was significantly induced by herbivory at both CO_2 levels in the Can-0 and Edi-0 genotypes (Fig. 1h). Contrary to the observed changes in induced chemical responses, structural defenses (i.e., trichome densities) were never affected by insect herbivory. However, we detected a marginally significant CO_2 effect on this trait (see Table 1, Fig. 1i). Specifically, a significant elevated CO_2-induced decrease in trichome density was found in the Cvi-0 genotype.

Analyses of variance also revealed that plant responses to elevated CO_2 and herbivory were genotype-specific. Both $CO_2 \times$ genotype and herbivory $\times$ genotype interactions were significant for several glucosinolates denoting a differential response of genotypes, in terms of chemical defenses, to changes in the physical and biological environment (Table 1). On the other hand, the three-way interactions were not significant, which indicates that, overall, CO_2 levels affected glucosinolate responses to herbivores in a similar fashion across genotypes (i.e., no chemical induction under ambient CO_2, but significant induction under elevated CO_2; see Fig. 1a–c, f). However, separate analyses of variance for each CO_2 level revealed that genotypes differed in their induced chemical responses, although only under CO_2 enrichment (significant genotype $\times$ herbivory interaction for 2-propenyl, $P = 0.001$; 3-butenyl, $P = 0.0007$; and total glucosinolate levels, $P = 0.0015$). These results indicate that CO_2 levels affected the genotypic variation in induced chemical responses to herbivory (i.e., no genotypic variation at ambient CO_2, but significant variation under enriched CO_2).

Overall, relative amounts of individual glucosinolates (as a percentage of the total amount) were less affected by CO_2 and herbivory than absolute concentrations, although some significant effects were detected for

Table 1 ANOVA *P*-values for glucosinolate contents and trichome density in *A. thaliana* under elevated CO_2 and herbivory

Source	Total Gluc	2-Propenyl	3-Butenyl	MSO	MSHEP	IM	4-Meth IM	1-Meth IM	Trichomes
CO_2	0.403	0.385	0.632	0.910	0.405	0.909	0.060	0.186	**0.031**
Herb	**0.002***	**< 0.001***	**0.024**	0.460	0.487	**0.003***	**< 0.001***	**< 0.001***	0.886
$CO_2 \times$ Herb	**0.009***	**0.019**	0.607	0.425	0.169	**< 0.001***	**0.002***	0.275	0.741
Gen	**< 0.001***	**< 0.001***	**< 0.001***	**< 0.001***	**< 0.001***	**0.027**	**< 0.001***	**< 0.001***	**< 0.001***
$CO_2 \times$ Gen	**0.009***	**< 0.001***	**0.023**	0.932	0.387	0.249	0.072	0.140	0.095
Herb $\times$ Gen	**0.014**	**0.004***	0.378	0.502	0.532	0.050	**0.015**	**0.004***	0.418
$CO_2 \times$ Herb $\times$ Gen	0.149	0.343	0.166	0.382	0.725	0.612	0.214	0.259	0.927

Gen genotype, *Herb* herbivory, *Gluc* glucosinolates, *MSO* methylsulphinyloctyl, *MSHEP* methylsulphinylheptyl, *IM* indolylmethyl, *Meth* methoxy

Boldface indicates $P < 0.05$. Asterisk denotes significant *P* values after a sequential Bonferroni correction

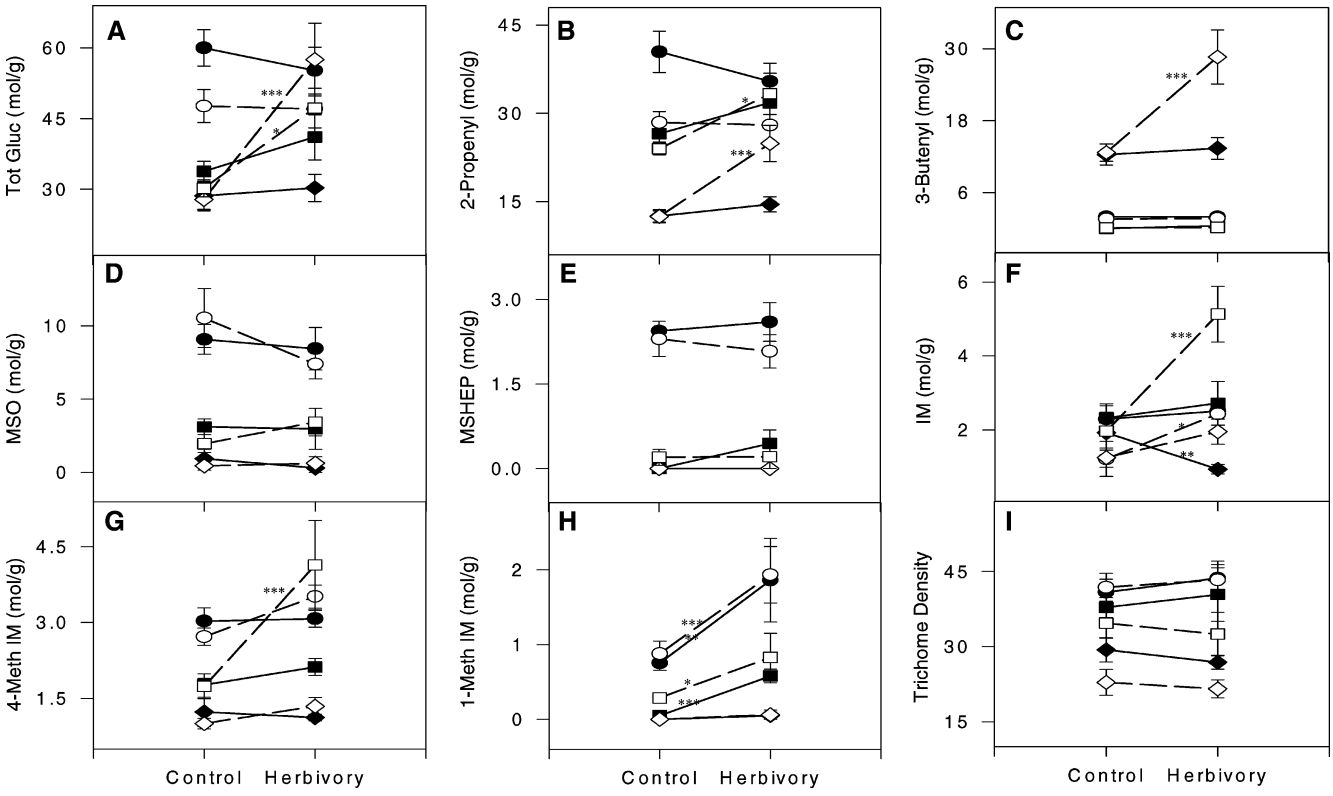

Fig. 1 Induced chemical (**a**–**h**) and structural (**i**) responses of *A. thaliana* genotypes to diamondback moth herbivory in ambient (*solid lines*) and elevated (*dotted lines*) CO_2 environments. Plant genotypes are denoted by *circles* (Can-0), *diamonds* (Cvi-0), and *squares* (Edi-0). *Asterisks on lines* symbolize significant contrasts (herbivory vs control) at each CO_2 and genotype level. Refer to Table 1 for glucosinolate abbreviations. Trichome density was estimated as the total number of trichomes within a 2.4 mm^2 . Significance levels: $*P<0.05$, $**P<0.01$, $***P<0.001$. *Error bars* indicate standard errors

2-propenyl and indolyl glucosinolates (see Table 2, Fig. 2). Results of preplanned contrasts revealed a significant reduction in the proportion of 2-propenyl glucosinolate in the Can-0 genotype resulting from CO_2 enrichment (compared to control conditions) and in the Edi-0 genotype as a result of both herbivory and elevated CO_2 treatments. On the other hand, relative contents of indolyl glucosinolates significantly increased as a result of herbivory and elevated CO_2 combined in the Edi-0 genotype, and due to herbivory in the Can-0 genotype (under elevated CO_2 only).

Elevated CO_2 significantly affected insect performance and insect-glucosinolate interactions (Fig. 3, Table 3). An overall decrease in insect performance resulting from enriched CO_2 was detected. This outcome appeared to be more pronounced for males than for females, although the $CO_2 \times$ gender interaction was not significant. In addition, interactions of genotype with CO_2 and/or gender were not significant, in spite of some differences in insect performance among genders and genotypes. Analyses of covariance performed to infer differences in regression relationships between adult weight (response variable) and glucosinolate concentrations (covariate) across CO_2 treatments (CO_2 effects and $CO_2 \times$ covariate interactions) indicated that some of these associations were environmentally dependent

Table 2 ANOVA *P*-values for relative glucosinolate contents in *A. thaliana* exposed to elevated CO_2 and herbivory

Source	2-Propenyl	3-Butenyl	MSO	MSHEP	IM	4-Meth IM	1-Meth IM
CO_2	**0.004***	0.817	0.879	0.402	0.978	0.215	0.064
Herb	0.227	0.287	0.159	0.118	0.212	0.248	**<0.001***
$CO_2 \times$ Herb	0.093	0.888	0.661	**0.038**	**0.012**	0.398	**0.074**
Gen	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**0.036**	**<0.001***	**<0.001***
$CO_2 \times$ Gen	0.457	0.339	0.515	0.613	0.546	**0.025***	**0.024**
Herb × Gen	0.097	0.279	0.949	0.419	**0.005***	0.083	**0.001***
$CO_2 \times$ Herb × Gen	0.214	0.146	0.449	0.605	0.678	0.247	0.262

Gen genotype, *Herb* Herbivory, *Gluc* glucosinolates, *MSO* methylsulphinyloctyl, *MSHEP* methylsulphinylheptyl, *IM* indolylmethyl, *Meth* methoxy
Boldface indicates $P<0.05$. Asterisk denotes significant *P*-values after a sequential Bonferroni correction

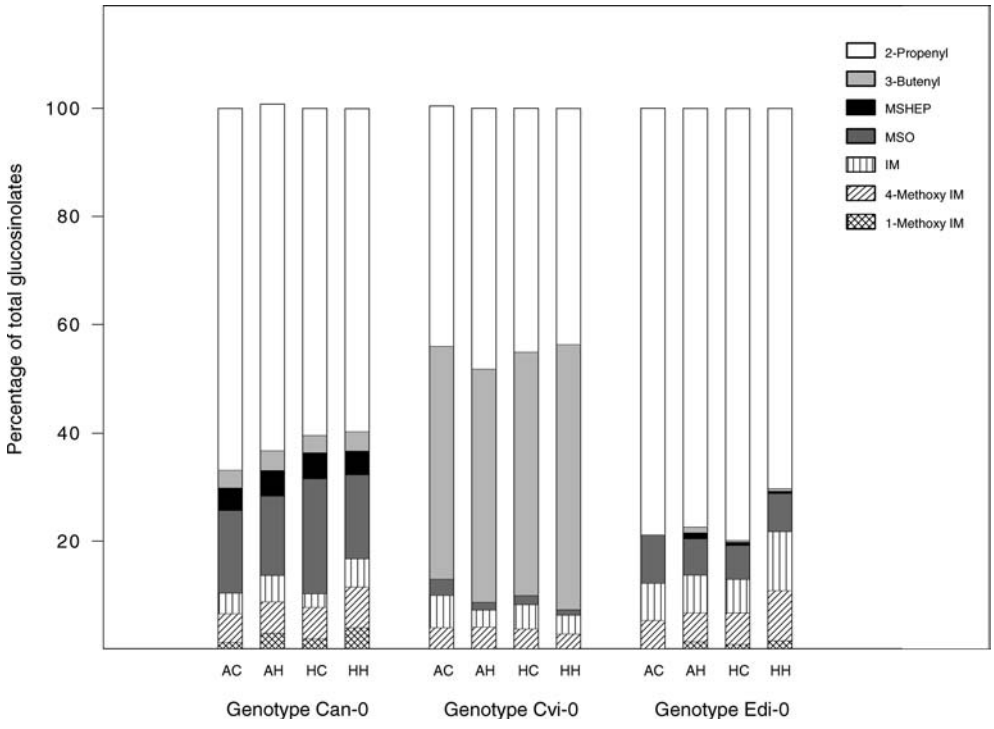

Fig. 2 Effects of CO_2 and herbivory on relative concentrations of glucosinolates in *A. thaliana* genotypes. Refer to Table 1 for glucosinolate abbreviations. Treatments: *A* ambient CO_2; *E* elevated CO_2; *C* control; *H* herbivory

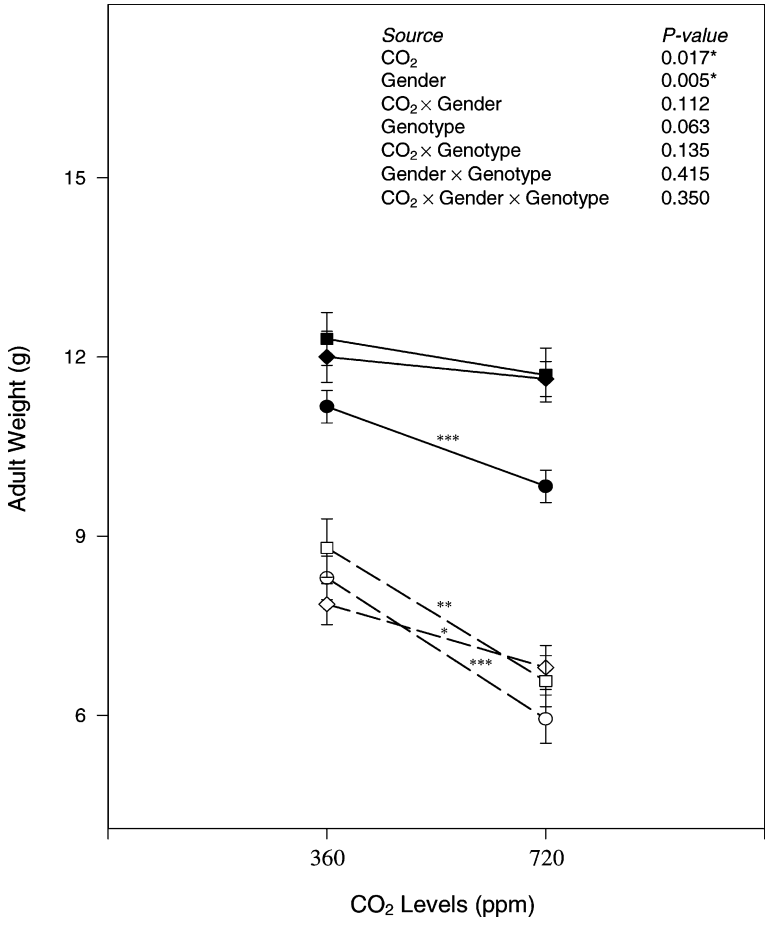

Fig. 3 Responses of female (*solid lines*) and male (*dotted lines*) diamondback moths to feeding on *A. thaliana* genotypes exposed to elevated CO_2. Plant genotypes are denoted by *circles* (Can-0), *diamonds* (Cvi-0) and *squares* (Edi-0). *Asterisks on lines* symbolize significant contrasts (ambient vs increased CO_2) for each gender and genotype. Significance levels: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. *Error bars* indicate standard errors

Table 3 Significance probabilities for analyses of covariance estimating the effect of Covariate (glucosinolate concentrations), CO_2 and their interaction (Covariate $\times CO_2$)

Covariate	Source of variation			Covariate × ambient CO_2		Covariate × elevated CO_2	
	Covariate	CO_2	Covariate $\times CO_2$	β	*P*	β	*P*
2-Propenyl	0.130	**0.003***	**0.003***	−0.097	0.087	0.288	**0.013**
3-Butenyl	0.074	0.077	**0.014**	0.003	0.638	−0.016	**0.001***
Methylsulphinylheptyl	0.165	0.150	0.126	0.001	0.912	−0.010	0.054
Methylsulphinyloctyl	0.523	0.755	0.534	0.000	0.988	−0.010	0.447
Indolylmethyl	0.418	**0.018**	**0.013**	−0.059	0.182	0.114	**0.029**
4-Methoxy indolylmethyl	0.309	0.850	0.843	−0.032	0.430	−0.022	0.521
1-Methoxy indolylmethyl	0.217	0.385	0.440	−0.032	0.102	−0.007	0.767
Total glucosinolates	0.105	**0.007***	**0.008***	−0.103	0.167	0.409	**0.019***

Parameter estimates (β) and their associated *P*-values denote the relationship between female diamondback moth weights and glucosinolate concentrations in *A. thaliana* at each CO_2 level
Asterisk indicates significant *P*-value after sequential Bonferroni correction

(significant $CO_2 \times$ covariate interactions), although only for females (Table 3). It is worth noting that all significant regression relationships were found only under elevated CO_2, and that they were not always consistent in the direction of their response (i.e., signs of estimated regression coefficients were different). For example, while female adult weight was positively associated with levels of 2-propenyl, indolylmethyl and total glucosinolates, they were negatively related to concentrations of 3-butenyl glucosinolates (in the Cvi-genotype). Results for males are not shown because they were not statistically significant (*P*-values for covariates and $CO_2 \times$ covariates ranged from 0.16 to 0.98). Likewise, no significant association was found between adult weights and trichome densities (*P*-values >0.1).

Discussion

Despite the widespread acceptance that abiotic environmental factors affect plant inducible responses to insect herbivores, this interaction has not commonly been investigated (Siemens and Mitchell-Olds 1998). To our knowledge, this is the first study demonstrating that induced chemical defense responses of plants to insect herbivory can be modified by atmospheric CO_2 levels (i.e., significant $CO_2 \times$ herbivory interactions), and that these induced responses can differ among genotypes. Overall, herbivory by the diamondback moth did not significantly induce glucosinolate concentrations at ambient CO_2 levels. However, herbivory-induced increases in glucosinolate contents, ranging from 28% to 62% above basal levels, were found under elevated CO_2 in two out of the three genotypes studied (Cvi-0 and Edi-0). Our results also revealed that elevated CO_2 and herbivory affected relative concentrations of individual glucosinolates, although to a lesser extent than their effects on absolute amounts. Induced defenses can increase plant fitness by reducing subsequent herbivore attacks (Agrawal 1999; Kessler and Baldwin 2004). Thus, natural selection acting in future changing environments may favor those plant genotypes showing enhanced induced defense responses, which in turn can alter plant–insect interactions and community dynamics.

Previous studies have shown herbivory-induced changes in structural defenses such as trichomes (Mauricio and Rausher 1997; Agrawal 1999). For example, Agrawal (1999) found that trichome numbers increased in plants exposed to herbivory by *Pieris rapae* and sprayed with a natural plant response elicitor, jasmonic acid. In this study, in contrast to the pronounced changes in chemical responses, trichome density was not affected by herbivory either at ambient or elevated CO_2. However, elevated CO_2 induced a decrease in trichome density in one of the genotypes studied (Cvi-0), which indicates that structural defenses can also be influenced by future changes in CO_2 levels.

The evaluation of $CO_2 \times$ genotype interactions for defense responses is fundamental for predicting potential evolutionary consequences of global change, not only on plant–herbivore interactions but also on associated higher trophic levels (Whitham et al. 2003). Despite the reduced sample of genotypes assessed in this study, the observed genotypic variation in the response of total and several individual glucosinolates to elevated CO_2 implies a potential of this chemical class to evolve in future enriched CO_2 environments. In addition, we found that CO_2 levels influenced the amount of genotypic variation in the induced chemical response to herbivory (i.e., genotypic variation in inducibility found only under elevated CO_2). These results are particularly relevant for plant species such as *A. thaliana*, which has shown pronounced among-population differences in defense profiles (Bano 1993; Mauricio and Rausher 1997, Kliebenstein 2001). Natural populations of *A. thaliana* consist mostly of single genotypes, likely as a result of this species' high selfing rates (>0.99) and short-distance seed dispersal (approximately within 1 m) (Bergelson et al. 1998). If indeed plants with distinct defense profiles have differential fitness, one could expect that differences in the induced defense response of *A. thaliana* genotypes to elevated CO_2 may cause divergent evolutionary outcomes among natural populations of this annual plant. More information is needed

to assess this prediction and its potential implications for the conservation of genetically distinct plant populations, which as locally adapted units represent fundamental components of biodiversity (Hughes et al. 1997).

As previously mentioned, to demonstrate that enriched CO_2 could have an evolutionary impact on induced chemical responses of plants, it is necessary to evaluate not only the existence of genotypic variation but also the presence of a link between induced responses and fitness (Agrawal 1999). Since tissue sampling for analyzing glucosinolates was destructive (due to the small size of *A. thaliana*), fitness responses were evaluated in the same experiment but on a different set of plants (reported in Bidart-Bouzat 2004). We did not find a significant correlation between mean glucosinolate and fitness levels either in the absence or presence of herbivores, although the lack of association in this study likely resulted from sample size limitations. Nonetheless, it is worth noting that the most plastic genotype (Cvi-0), in terms of total glucosinolate concentrations and predominant aliphatic glucosinolates, was also the most responsive to elevated CO_2 in terms of lifetime fitness (Bidart-Bouzat 2004), suggesting it may be favored by natural selection in future elevated CO_2 environments. However, this predicted outcome would depend on both the presence of other interacting environmental factors affecting plant responses to enriched CO_2 and the specific insect community associated with the plants. For example, while higher glucosinolate levels induced by elevated CO_2 may benefit plants by increasing their resistance to generalist insects, these enhanced levels may render plants more vulnerable to specialist herbivores (Mithen et al. 1995; Stowe 1998).

The selected *A. thaliana* genotypes, which have naturally distinct glucosinolate profiles and contents, revealed varied responses to the same herbivore impact (i.e., significant genotype × herbivory interactions). A significant genotype × herbivory interaction may be indicative of a tradeoff between constitutive (prior to herbivory) and induced levels of secondary compounds in populations with divergent chemical profiles (Siemens and Mitchell-Olds 1998). The existence of this tradeoff has been predicted by theory, in relation to the evolution of inducible defenses (Zangerl and Berenbaum 1990). Theory predicts that defenses are costly, and that while high levels of constitutive defenses are likely to be selected in plant populations subject to high herbivory rates, inducible strategies may be favored when herbivory incidence is either low or unpredictable. Consistent with this prediction, our results revealed that genotypes with lowest levels of constitutive defenses (i.e., Cvi-0 and Edi-0), for example in terms of total glucosinolate content, had highest levels of induction. The opposite trend was true for genotype Can-0, which had highest basal levels and no induced response. These apparent tradeoffs between induced and constitutive levels of total glucosinolates were detected only under elevated CO_2 conditions ($P < 0.01$), which suggests that tradeoffs do not only result from past plant evolutionary history (e.g., life history traits, previous exposure to herbivores) but they are also modified by environmental factors affecting plant defensive strategies against herbivores. The effect of the environment on the type of plant defense response (i.e., inducible or constitutive) certainly deserves further investigation.

Regarding insect responses, elevated CO_2 decreased the overall performance of diamondback moths. This is consistent with previous studies evaluating responses of insects feeding on plants grown under CO_2 enrichment, which showed decreased growth and increased developmental times and larval mortality (Fajer et al. 1989; Traw et al. 1996; Joutei et al. 2000). Changes in insect performance may result from CO_2 effects on plant chemical composition; for example, production of secondary compounds or foliar nitrogen levels (Bezemer and Jones 1998). However, no consistent association has apparently been found between elevated CO_2-induced changes in plant chemistry and insect responses (Kopper et al. 2001).

Analyses of covariance, performed to assess whether the relationship between insect weight and both structural and chemical defenses (i.e., trichome densities and glucosinolate concentrations) was modified by CO_2 levels, showed that insects were unaffected by trichome numbers but influenced by certain glucosinolates. This association was dependent on the insect gender and CO_2 treatment. Only females growing in plants exposed to CO_2 enrichment revealed a significant association with glucosinolate concentrations. Overall, female adult weights were directly related to concentrations of 2-propenyl, indolylmethyl and total glucosinolates, and inversely related to 3-butenyl levels (in the Cvi-0 genotype). It has been shown 3-butenyl glucosinolates can be toxic to diamondback moths, at least in high concentrations, and both 2-propenyl and indolyl glucosinolates to be common feeding and oviposition stimulants for specialist herbivores (Nayar and Thorsteinson 1963). In this study, pronounced elevated CO_2-induced increases in both 2-propenyl and 3-butenyl glucosinolates in genotypes Cvi-0 and Edi-0 (see Fig. 1) resulted in unchanged mean female weight responses to elevated CO_2 (see Fig. 3). This outcome suggests that 2-propenyl and 3-butenyl may be acting antagonistically on female weight, with 2-propenyl apparently compensating for the potential negative effects that increased 3-butenyl concentrations could have had on mean female responses. This finding highlights the importance of assessing the entire glucosinolate profile (rather than total glucosinolates) to understand interactions between insects and this important chemical class. In addition, the significant elevated CO_2-induced decrease in mean adult weight of females raised on the Can-0 genotype cannot either be explained by changes in glucosinolate concentrations since glucosinolate levels in this particular genotype were virtually unaffected by elevated CO_2 levels. Overall, the decrease in insect performance in this study could not be explained by elevated CO_2-induced variation in glucosinolate levels. This lack of association

could be due to an insufficient magnitude of change in glucosinolate content, or it may also represent that observed changes in insect weight resulted from changes in plant chemicals other than glucosinolates (e.g., N content or other secondary compounds).

In conclusion, this study demonstrates that chemical responses to herbivory (in terms of glucosinolates) and structural defenses such as trichomes could be affected by global CO_2 changes. Glucosinolate responses to biotic and abiotic environmental factors were complex and strongly dependent on both the plant genotype and glucosinolate type. The existence of genetic variation for glucosinolate responses to elevated CO_2 suggests a potential for this chemical class to evolve under future environmental challenges and, thus, to affect interactions of plants with herbivores and possibly higher trophic levels. In addition, the pronounced increase in glucosinolate levels under CO_2 enrichment may pose a threat not only for insect generalists that are likely to be more influenced by rapid changes in the concentration of these chemicals, but also for other insect specialists more susceptible than diamondback moths to high glucosinolate levels (Stowe 1998; Kliebenstein et al. 2002). This work set basis for further research on how future CO_2 enrichment may affect plant inducible strategies and the so-called coevolutionary arms race between plants and their associated insect communities.

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