Global Change Biology (2008) **14**, 1–18, doi: 10.1111/j.1365-2486.2008.01574.x

Interactions of elevated carbon dioxide and temperature with aphid feeding on transgenic oilseed rape: Are *Bacillus thuringiensis* (Bt) plants more susceptible to nontarget herbivores in future climate?

SARI J. HIMANEN*, ANNE NISSINEN*†, WEN-XIA DONG‡, ANNE-MARJA NERG*, C. NEAL STEWART JR.§, GUY M. POPPY¶ and JARMO K. HOLOPAINEN*

*Department of Environmental Science, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland, †Agrifood Research Finland, Plant Protection, FIN-31600 Jokioinen, Finland, ‡Tea Research Institute, Chinese Academy of Agricultural Sciences, No. 1 Yungi Road, Hangzhon 31008, China, §Department of Plant Sciences, University of Tennessee, 2431 Joe Johnson Drive, Knoxville, TN 37996, USA, ¶School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

Abstract

Climate change factors such as elevated carbon dioxide (CO_2) and temperature typically affect carbon (C) and nitrogen (N) dynamics of crop plants and the performance of insect herbivores. Insect-resistant transgenic plants invest some nutrients to the production of specific toxic proteins [i.e. endotoxins from *Bacillus thuringiensis* (Bt)], which could alter the C–N balance of these plants, especially under changed abiotic conditions. Aphids are nonsusceptible to Lepidoptera-targeted Bt Cry1Ac toxin and they typically show response to abiotic conditions, and here we sought to discover whether they might perform differently on compositionally changed Bt oilseed rape. Bt oilseed rape had increased N content in the leaves coupled with reduced total C compared with its nontransgenic counterpart, but in general the C : N responses of both plant types to elevated CO_2 and temperature were similar. Elevated CO_2 decreased N content and increased C : N ratio of both plant types. Elevated temperature increased C and N contents, total chlorophyll and carotenoid concentrations under ambient CO_2, but decreased these under elevated CO_2. In addition, soluble sugars were increased and starch decreased by elevated temperature under ambient but not under elevated CO_2, whereas photosynthesis was decreased in plants grown under elevated temperature in both CO_2 levels. *Myzus persicae*, a generalist aphid species, responded directly to elevated temperature with reduced developmental time and decreased adult and progeny weights, whereas the development of the *Brassica* specialist *Brevicoryne brassicae* was less affected. Feeding by *M. persicae* resulted in an increase in the N content of oilseed rape leaves under ambient CO_2, indicating the potential of herbivore feeding itself to cause allocation changes. The aphids performed equally well on both plant types despite the differences between C–N ratios of Bt and non-Bt oilseed rape, revealing the absence of plant composition-related effects on these pests under elevated CO_2, elevated temperature or combined elevated CO_2 and temperature conditions.

Keywords: aphids, *Bacillus thuringiensis*, *Brassica napus*, *Brevicoryne brassicae*, climate change, elevated CO_2, elevated temperature, *Myzus persicae*, transgenic plants

Received 7 August 2007; revised version received 12 November 2007 and accepted 12 December 2007

Introduction

Transgenic insect-resistant crop plants that have been genetically modified to produce *Bacillus thuringiensis* (Bt) crystalline (Cry) endotoxins with specific activity towards certain herbivores are currently grown in over 30 million hectares (James, 2006). Bt plants have been successful in limiting damage by many important insect pests, such as the cotton bollworm (*Helicoverpa zea*), European corn borer (*Ostrinia nubilalis*) and tobacco budworm (*Heliothis virescens*). One of their benefits is

Correspondence: Sari Himanen, tel. + 358 17 163177, fax + 358 17 163191, e-mail: sari.himanen@uku.fi

the reduced need for insecticide applications for pest control (Romeis *et al*., 2006). However, possible unintended pleiotropic effects of genetic modification and the potential for the evolution of resistance in target pests are of regulatory concern (Poppy & Wilkinson, 2005).

In the future, global agriculture will, inevitably, face challenges caused by climate change, which might lead to both global and local alterations in agriculture (IPCC, 2007). Elevated CO_2, temperature and drought can have various effects on different trophic levels in ecosystems (plants, herbivores, predators and parasitoids) (IPCC, 2007). Under these circumstances, transgenic plants may become even more important to sustainable agriculture. However, the performance of transgenic plants, the stability of the transgenic traits and their ecological interactions have been rarely studied under atmospheres with elevated CO_2 and temperature or both factors in combination (Coviella *et al*., 2000, 2002; Chen *et al*., 2005).

Carbon dioxide (CO_2), as the primary determinant of the photosynthetic rate in plants, affects both the physiology and the composition [carbon (C) : nitrogen (N) ratio, allocation of resources] of crop plants (Poorter *et al*., 1997; Long *et al*., 2004). Elevated temperature interacting with elevated CO_2 concentration can influence C–N dynamics of plants by decreasing vegetative developmental periods and enhancing the efficiency of photosynthesis (Morison & Lawlor, 1999). Bt plants, which are modified to produce relatively high levels (up to 1%) of a specific protein constitutively throughout their life cycle and in all parts of the plant, might have altered C and N ratios leading to interactions between the use of N for growth and structural proteins vs. production of Bt toxin (Coviella *et al*., 2000, 2002). Moreover, if C-enriched atmospheres lead to lower N availability and competition for N in the plant (Bryant *et al*., 1983), then reduced allocation could lead to decreased concentrations of active Bt toxin in the plants (Coviella *et al*., 2000, 2002). This would be undesirable with regards to delivering a high dose of Bt toxin for efficacy and minimizing risks for resistance management in target herbivores (Bates *et al*., 2005). Reduced Bt toxin concentration under elevated CO_2 has been reported in Bt cotton (Coviella *et al*., 2000, 2002; Chen *et al*., 2005); however, the biological significance of this change has yet to be determined.

Cruciferous plants (*Brassica* genus) are agriculturally important, as they include numerous crops and vegetables such as cabbage, broccoli and oilseeds. In addition, all these produce C- and N-containing secondary compounds, glucosinolates, as part of their secondary metabolism (reviewed by Halkier & Gershenzon, 2006). Thereby, it can be hypothesized that their N demand is even more pronounced, and they are excellent model species for studying the effects of enhanced CO_2 on C–N metabolism. Bt oilseed rape (*Brassica napus*) was produced by Halfhill *et al*. (2001) principally for the purpose of studying ecological risks of transgene dispersal, as *Brassica* plants have many wild weedy relatives compatible for hybridization (Halfhill *et al*., 2005). In this study, we assessed whether there are differences in the physiology and C–N dynamics between Bt-transgenic and nontransgenic oilseed rape, when both are grown under elevated CO_2, elevated temperature or under both factors in combination. It can be hypothesized that as the synthesis of the Bt toxin protein requires additional N inputs, this could result in competition for the nutrient, because enhanced growth at C excess (in elevated CO_2) also increases N demand (Bryant *et al*., 1983).

*Brassica*s are hosts for many herbivorous aphid species and the generalist aphid *Myzus persicae* is one of the most serious pests of oilseed rape (Desneux *et al*., 2006). For autumn-sown winter oilseed rape, the pest population can cause significant damage (Buntin & Raymer, 1994). The specialist *Brevicoryne brassicae* is the other dominant aphid species attacking *Brassica* plants (Desneux *et al*., 2006). Aphids are among the sap-feeding insects that have responded, either positively or negatively, to elevated CO_2-induced changes in plants (Bezemer *et al*., 1998, 1999; Hughes & Bazzaz, 2001; Holopainen, 2002; Newman *et al*., 2003; Flynn *et al*., 2006). Aphids are not directly affected by Bt Cry1Ac toxin as they lack the specific gut receptors, and Bt toxin is also largely absent in phloem sap (Raps *et al*., 2001). Temperature can affect the development and population biology of aphids directly by increasing their metabolism and leading to increased abundance by decreased developmental times (Bale *et al*., 2002). Interactions between elevated CO_2 and temperature affecting herbivory have been reported (Bezemer *et al*., 1998; Veteli *et al*., 2002; Newman, 2003; Hoover & Newman, 2004; Flynn *et al*., 2006), although there is variability among different plant species in their responses to these factors singly or in combination (Zvereva & Kozlov, 2006). It could be predicted that aphids will have an even greater impact as pest herbivores on agricultural plants in future atmospheres, which is why we chose aphids for screening the performance of potentially important non-Bt-susceptible pests on Bt-transgenic vs. nontransgenic oilseed rape. Our goal was to assess whether future elevated CO_2 and temperature could lead to better adaptation of these pests and to discover the role of Bt toxin production in this. Would these render Bt plants even more susceptible to nontarget pests not directly affected by Bt toxin but that would respond to changes in the plant composition brought by

Bt production? If so, higher pesticide inputs might be necessary for nontarget pest control.

In this study, we addressed whether elevated CO_2, temperature or both factors in combination: (1) change the photosynthetic rate, C and N contents or amounts of C-based (starch and soluble sugars) compounds of Bt-transgenic vs. nontransgenic oilseed rape; (2) change the production of Bt Cry1Ac toxin in Bt oilseed rape; (3) affect the onset of reproduction, number and weight of progeny produced and mean relative growth rate (MRGR) of the non-Bt-susceptible generalist (*M. persicae*) and specialist (*B. brassicae*) aphid species in Bt-transgenic vs. nontransgenic oilseed rape; and finally (4) does aphid feeding itself alter the C–N dynamics of Bt-transgenic and nontransgenic oilseed rape?

Materials and methods

Plant material and growth conditions

B. napus ssp. *oleifera* (oilseed rape) cultivar Westar has been previously transformed to contain a synthetic Bt *cry1Ac* gene and green fluorescent protein (GFP) *mGFP5er* marker gene regulated by independent cauliflower mosaic virus 35S promoters (Halfhill *et al*., 2001). Line GT1 used here showed stable expression of both genes in single insert with no obvious phenotypic effects. Nontransgenic cv. Westar parent line served as a control to the transgenic line.

F_4 seeds of Bt-transgenic and nontransgenic plants were sown in 0.66 L pots in 2 : 1 : 1 fertilized soil (Kekkilä, Finland, NPK: 100, 30, 200 mg L^{-1}) : B2 sphagnum peat (Kekkilä, Finland, NPK: 110, 40, 220 mg L^{-1}) : sand (0.5–1.2 mm) mixture. Plants were grown in four computer-controlled growth chambers (2.6 m^3; Bioklim 2600 T, Kryo-Service Oy, Helsinki, Finland). The limited number of growth chambers available simultaneously (one chamber per treatment) did not allow us to use chambers as replicates to ideally exclude pseudoreplication (Hurlbert, 1984). Therefore, we conducted two consecutive experiments with similar treatments in order to limit the problem of pseudoreplication (measuring responses of the same variables and using replication in time as a random factor in the statistical analysis). Owing to limited space in the chambers and available resources, all variables could not be included in both experiments [i.e. different aphid species were studied in different experiments (*M. persicae* in Experiment 1 and *B. brassicae* in Experiment 2)]. In all analyses, individual plants served as replicates.

CO_2 and temperature treatments used were following: (1) ambient CO_2, control temperature (20/16 °C); (2) elevated CO_2 (720 ppm CO_2), control temperature (20/16 °C); (3) ambient CO_2, elevated temperature (24/20 °C, + 4 °C increase to control temperature); and (4) elevated CO_2 (720 ppm), elevated temperature (24/20 °C). Ambient CO_2 was the corresponding background level of air entering the growth chamber facilities and elevated CO_2 was supplied from gas tanks. CO_2 and temperature conditions of the chambers were continuously monitored, and the mean CO_2 and temperature (± SD) values of the treatments, calculated from hourly means, were the following: (1) Experiment 1: 390 ± 23 ppm CO_2, 18.5 °C and Experiment 2: 385 ± 25 ppm CO_2, 18.2 °C; (2) Experiment 1: 716 ± 22 ppm CO_2, 18.2 °C and Experiment 2: 717 ± 28 ppm CO_2, 18.2 °C; (3) Experiment 1: 391 ± 23 ppm CO_2, 22.4 °C and Experiment 2: 385 ± 27 ppm CO_2, 22.4 °C; and (4) Experiment 1: 715 ± 31 ppm CO_2, 22.4 °C and Experiment 2: 718 ± 28 ppm CO_2, 22.2 °C. All treatments had a 16 : 8 h photoperiod and light adjusted to photosynthetically active radiation (PAR) of approximately 250 μmol m^{-2} s^{-2}. The light level used was optimal for the growth of the plants in these type of controlled conditions, but it should be noted that the light intensity is not as high as in field conditions. To avoid any effects of chamber-specific growth conditions, the plants inside the chambers and the treatments among the four chambers used were rotated weekly.

In both experiments, the net photosynthesis, total C and N content and C : N ratio of Bt-transgenic and nontransgenic oilseed rape plants was compared. We also assessed the effects of aphid feeding on the C–N dynamics of these plants. In addition, in Experiment 1, the Bt toxin concentration, chlorophyll pigment concentrations and the reproduction parameters and MRGR of *M. persicae* were measured, whereas in Experiment 2, the concentrations of starch and soluble sugars were determined and similar population growth measurements were conducted with *B. brassicae*. Experiment 1 was started in August and it lasted for a total of 30 days and Experiment 2 (in October) was continued for an additional 3 days (33 days in total) due to the later start of offspring production by *B. brassicae* compared with *M. persicae*. This enabled evaluation of the progeny production of aphids during their exponential population growth close to peak reproduction but not after that, as the experiment had to be limited to the vegetative stage of the plants due to prevailing GM plant biosafety regulations. The details of the two experiments are described below.

Experiment 1: M. persicae

Total leaf biomass, total C and total N. Total leaf biomass from six Bt-transgenic and six nontransgenic plants per CO_2/temperature treatment and six corresponding

plants subjected to *M. persicae* feeding as described below (two aphids and their following progeny on the plant) from 13th day from sowing until the end of the experiment was separately collected at 30 days age and oven dried (60 °C, 72 h). Biomass gain was determined from the intact plants. Total C and N content of the dried samples was measured with a CN analyser (Leco CN-2000, St Joseph, MI, USA) and the C : N ratio was calculated.

Total soluble protein and Bt toxin concentration. The third true leaf from six Bt-transgenic plants (30 days of age) per treatment was separately collected, deep-frozen in liquid N and stored at −80 °C before analysis. Total soluble protein was determined with the Bradford method (Bradford, 1976), and Bt toxin concentration was analysed with an ELISA test (Cry1Ac PathoScreen Kit, Agdia, Elkhart, IN, USA) according to the manufacturer protocol and with Cry1Ac standards for quantitative determination.

Net photosynthetic rate. Net photosynthesis was measured from the third true leaf of six 29–30-day-old Bt-transgenic and nontransgenic plants from each CO_2/temperature treatment with a CI-510 Portable Photosynthesis System (Cid Inc., Vancouver, WA, USA) under saturating light intensity of 1800 PAR. CO_2 level in the cuvette during measurements was adjusted to either 360 ± 3 or 720 ± 2 ppm (supplied from gas tanks).

Chlorophyll pigment concentrations. Chlorophyll (chl) *a*, *b* and total carotenoid concentrations were analysed from the total leaf biomass collected separately from six 30-day-old Bt-transgenic and nontransgenic plants from each CO_2/temperature treatment (same plants as used for measuring net photosynthesis) deep-frozen prior analysis. Chlorophyll was extracted from 0.1 g ground leaf material with 7 mL DMSO by incubating at 65 °C for 1 h and diluting to a 25 mL total volume. The absorbance was read spectrophotometrically (UV-1201 UV–VIS Spectrophotometer, Shimadzu Corporation, Koyoto, Japan) at 665, 648 and 470 nm and results were calculated with the equations proposed by Barnes *et al*. (1992).

M. persicae reproduction and relative growth rate. M. persicae (Sulzer) (Homoptera:Aphididae) originated from the laboratory colony at the University of Kuopio and was reared on *Brassica rapa* cv. Valo at 12 : 12 L : D at approximately 25 °C temperature and 50% RH. Two *M. persicae* adults were transferred to cotyledons or the first or second true leaves of six Bt-transgenic and nontransgenic plants (13 days old) per treatment to produce progeny. After 24 h, the adults were removed and two nymphs per plant were left. If the adult had not produced any progeny at all, a nymph produced by the other adult on the same plant was used, or in a few cases, a nymph produced by an adult on another plant of the same treatment was selected. This resulted in two nymphs per plant. The nymphs were checked at 1-day age and daily from days 5 to 16 to determine the start of reproduction and to calculate the number of progeny produced per aphid. These nymphs produced by the aphids were removed and weighed daily and the mean progeny weight was calculated. If the aphid-infested leaf was mechanically damaged or approached senescence, the herbivore was transferred to the next (younger) intact leaf. On day 16, when the experiment was completed, the adults were weighed and deep-frozen for Bt toxin analysis. Bt toxin was analysed as described earlier after mechanical grinding of the aphids in liquid N.

The MRGR of *M. persicae* in the different treatments was determined by enclosing two individually weighed nymphs (produced by the aphids in the reproduction assay), <24-h old, into clip-cages on the same plants that were used for the reproduction assay described above (26-day old), but on different leaves (leaves 2–5 depending on the previous location of the aphids). The clip-cages were made of 2 mL eppendorf tubes cut at approximately 1 cm height and covered with a fine mesh from the upper side. The tubes were attached to a wire, which formed a loop on the underside of the leaf, and the loop was surrounded with plastic foam to prevent damage of the leaf while also allowing photosynthesis. After 4 days, the aphids were individually weighed again and their MRGR was calculated with the equation: $(\ln W_2 - \ln W_1)/\text{time}$, where W_1 is the initial aphid weight, W_2 is the final aphid weight and time is the duration of the experiment (days) (van Emden, 1969).

Experiment 2: B. brassicae

Total leaf biomass, total C and total N. Total leaf biomass from six Bt-transgenic and six nontransgenic plants per CO_2/temperature treatment and from six corresponding *B. brassicae*-infested plants were collected separately at 33 days of age and oven dried (60 °C, 72 h). Total biomass gain, total C, total N content and C : N ratio was determined as in Experiment 1.

Net photosynthetic rate. Net photosynthesis was measured from six 22-day-old Bt-transgenic and nontransgenic plants as in Experiment 1, but under 360 ppm CO_2 only.

Soluble sugars and starch. Total leaf biomass from six 30-day-old Bt-transgenic and nontransgenic plants per treatment was separately collected, deep-frozen in

liquid N and stored at −80 °C before analysing. Starch, glucose, fructose and sucrose concentrations were analysed enzymatically according to kit manufacturer instructions (Glucose/D-Glucose/D-Fructose kit and Starch kit, Boehringer Mannheim/R-Biopharm, Darmstadt, Germany).

B. brassicae reproduction and relative growth rate. *B. brassicae* (L.) (Homoptera:Aphididae) was obtained from Rothamsted Research, Hertfordshire, UK, and was maintained on *Brassica pekiniensis* cv. Yamiko at 12 : 12 L : D, 25 °C temperature and 50% RH in University of Kuopio. The aphid reproduction measurements were performed as in Experiment 1 for *M. persicae*, but *B. brassicae* aphids were enclosed into clip-cages from the start of the experiment to avoid moving of the aphids on the plant to form colonies (to enable separation of individual aphids). The MRGR measurements of *B. brassicae* were performed on 29-day-old plants.

Statistical analysis

Aphid reproduction and MRGR results were aggregated per plant in both experiments. All data were checked for normality and equality of residual error variances and appropriately transformed (log or square-root) if needed to satisfy the assumptions of analysis of variance. Mixed-model ANOVA was used for analysis of biomass gain and C–N results with the plant type, CO_2 level and temperature as fixed effects and experiment as a random effect. Absolute values were transformed to fold-change values in relation to the control treatment for biomass gain and aphid-induced changes in C and N content results. A separate ANOVA model including plant type, aphid feeding and their interaction was created to study the effects of aphid induction on C–N dynamics under each CO_2/temperature treatment separately. Photosynthesis, Bt toxin and chlorophyll pigment concentrations and aphid performance parameters were tested with ANOVA for the main effects of plant type, CO_2 level and temperature and their interactions. The cumulative reproduction of aphids was analysed with repeated measures ANOVA. Owing to the experimental set-up using individual plants as replicates, the statistical power of the analysis can be higher compared with a set-up using chambers as replicates. The data were analysed with SPSS for WINDOWS 14.0 statistical package.

Results

Biomass gain and net photosynthesis

Elevated CO_2 increased leaf biomass in nontransgenic as well as Bt-transgenic oilseed rape plants (main effect of CO_2 level, $F_{1,86} = 46.87$, $P < 0.001$), whereas Bt plants had lower biomass gain during their early vegetative growth compared with the nontransgenic plants (main effect of plant type, $F_{1,86} = 7.65$, $P = 0.007$) (Fig. 1). Elevated temperature had no significant effect on biomass gain in ambient or elevated CO_2.

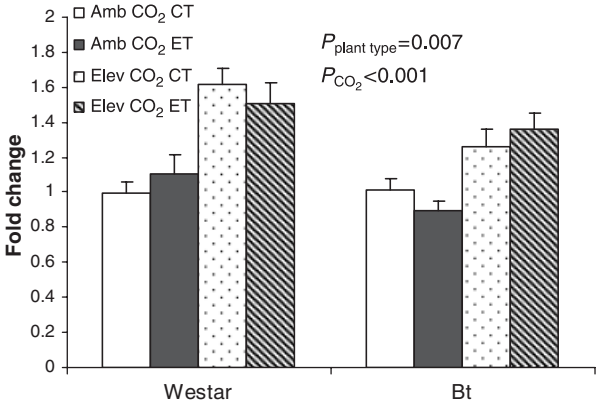

Fig. 1 Biomass gain ± 1 SEM in Westar and Bt-transgenic (Bt) oilseed rape plants grown under ambient or elevated (720 ppm) CO_2 level and under control (CT, 20/16 °C) or elevated (ET, 24/20 °C) temperature indicated as fold change to nontransgenic (Westar) plants grown under ambient CO_2 level and control temperature ($n = 12$). Statistically significant ($P < 0.05$) mixed-model ANOVA results for main effects of plant type, CO_2 level and temperature are shown. Bt, *Bacillus thuringiensis*.

Elevated temperature greatly reduced net photosynthesis in leaves of both plant types (Fig. 2, main effect of temperature: $F_{1,40} = 59.02$, $P < 0.001$; $F_{1,40} = 161.65$, $P < 0.001$; $F_{1,40} = 61.62$, $P < 0.001$ in Experiment 1 under control and elevated CO_2 and Experiment 2 control CO_2 levels, respectively). The net photosynthetic rates measured under elevated CO_2 were higher than the rates under control CO_2 (main effect of measurement CO_2 level, $F_{1,80} = 139.30$, $P < 0.001$). In general, photosynthesis responded to the elevation of CO_2 level and temperature similarly in nontransgenic and Bt-transgenic plants (no significant main effects for plant type). Plants grown under ambient and elevated CO_2 had an equal photosynthetic rate at 22-day age, when measured at ambient CO_2 (Experiment 2) and this was also true for 30-day-old plants, when photosynthesis was measured under 720 ppm CO_2 (Experiment 1). However, when the net photosynthesis was measured under 360 ppm CO_2 at 29-day age, it was reduced for the plants that had been grown under elevated CO_2 compared with plants grown under the control CO_2 level (main effect of CO_2 level, $F_{1,40} = 4.11$, $P = 0.049$). There was also a marginally significant interaction between temperature and CO_2 level ($F_{1,40} = 3.74$, $P = 0.060$), which points to the fact that the reduction in the photosynthetic rate in plants grown under

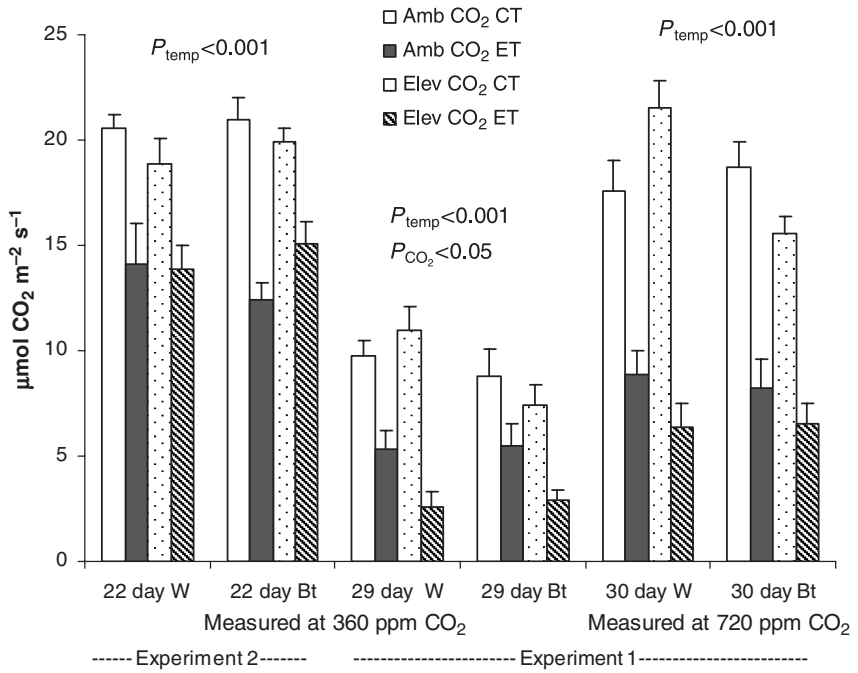

Fig. 2 Net photosynthesis (P_n) in Bt-transgenic (Bt) and nontransgenic (W, Westar) oilseed rape plants grown under ambient or elevated (720 ppm) CO_2 level and under control (CT, 20/16 °C) or elevated (ET, 24/20 °C) temperature. Measurements were made in two separate experiments: in Experiment 1 at time points of 29 days (measured at control CO_2) and 30 days (measured at elevated CO_2), and in Experiment 2 at 22 days ($n = 6$). Statistically significant ($P < 0.05$) ANOVA results for main effects of plant type, CO_2 level and temperature are shown. Bt, *Bacillus thuringiensis*.

enhanced CO_2 was higher under elevated temperature (plants growing faster and being at a later growth stage).

Total C, total N and C : N ratio

Plant N levels were slightly higher in Experiment 2 than in Experiment 1 in overall. The CO_2 and temperature treatments were applied similarly, so this difference was predicted to be caused by some internal regulation of plant growth (Sharma *et al*., 2005) [reproductive growth pursuing over vegetative growth at the end of summer (Experiment 1), with no similar effect later in the autumn (Experiment 2)]. Therefore, for statistical analysis of aphid-induced changes within each experiment, C and N content and C : N ratio levels of control Westar plants were scaled to 1 for both experiments and the treatment effects compared with these set levels.

In the elevated temperature treatment, there was increased C content of intact plants under control CO_2 and decreased C under elevated CO_2 (interaction $CO_2 \times$ temperature, $P < 0.001$) (Table 1). The C content was lower in Bt-transgenic compared with nontransgenic plants (main effect of plant type, $P = 0.006$) and elevated temperature led to a marginally significantly higher C in Bt-transgenic plants under ambient CO_2 (interaction plant type × temperature, $P = 0.056$).

In the elevated CO_2 treatment, there was highly decreased N content of both plant types (main effect of CO_2 level, $P < 0.001$). Elevated temperature increased N content under ambient CO_2 but decreased it under elevated CO_2 (interaction $CO_2 \times$ temperature, $P = 0.006$). In addition, the N content was higher in Bt-transgenic than in the nontransgenic plants (main effect of plant type, $P = 0.045$) and showed a marginally significant trend for a higher decrease by elevated CO_2 in Bt-transgenic than in the nontransgenic plants (interaction plant type × CO_2, $P = 0.054$).

The C : N ratio of both plant types was increased by elevated CO_2 (main effect of CO_2 level, $P < 0.001$). Interestingly, elevated temperature decreased the C : N ratio of plants under control CO_2 level but in contrast increased it under elevated CO_2 ($CO_2 \times$ temperature interaction, $P = 0.025$). Bt-transgenic plants had a reduced C : N ratio compared with nontransgenic plants (main effect of plant type, $P = 0.036$).

In response to *M. persicae* and *B. brassicae* feeding, the C content of both plant types typically decreased, although this was not statistically significant in all treatments (Fig. 3, main effects of aphid feeding). As an exception to this, under elevated CO_2 and temperature, *B. brassicae* feeding resulted in an increase in C content ($F_{1,20} = 4.64$, $P = 0.044$).

Table 1 Total carbon (%), total nitrogen (%) and C : N ratio (mean ± 1 SEM) in intact nontransgenic (Westar) and Bt-transgenic (Bt) oilseed rape plants grown in ambient or elevated (720 ppm) CO_2 level under 20/16 °C (control) or 24/20 °C (elevated) temperature ($n = 12$) and mixed-model ANOVA results for effects of plant type (Bt-transgenic vs. nontransgenic), CO_2 level and temperature and their interactions

	Ambient CO_2				Elevated CO_2			
	20/16 °C		24/20 °C		20/16 °C		24/20 °C	
	Westar	Bt	Westar	Bt	Westar	Bt	Westar	Bt
C (%)	41.31 ± 0.11	40.22 ± 0.13	41.70 ± 0.19	41.65 ± 0.19	41.67 ± 0.14	41.18 ± 0.12	41.22 ± 0.15	40.95 ± 0.30
N (%)	1.30 ± 0.16	1.45 ± 0.10	1.38 ± 0.15	1.77 ± 0.23	1.06 ± 0.06	1.03 ± 0.03	0.88 ± 0.07	0.91 ± 0.05
C : N ratio	36.12 ± 3.30	29.17 ± 1.99	34.13 ± 3.29	28.72 ± 3.64	40.41 ± 1.87	40.22 ± 1.07	49.45 ± 3.32	46.37 ± 2.40

	Plant type		CO_2		Temperature		Plant type × CO_2		Plant type × temperature		CO_2 × temperature		Plant type × CO_2 × temperature	
	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P
C	**8.40**	**0.006**	1.27	0.266	0.51	0.479	0.35	0.555	3.88	0.056	**38.45**	**<0.001**	3.08	0.087
N	**4.27**	**0.045**	**107.54**	**<0.001**	2.49	0.122	3.93	0.054	1.47	0.233	**8.35**	**0.006**	3.56	0.066
C : N ratio	**4.68**	**0.036**	**107.09**	**<0.001**	1.10	0.300	3.29	0.077	0.46	0.500	**5.42**	**0.025**	2.15	0.150

Statistically significant effects ($P < 0.05$) are marked in bold.
Bt, *Bacillus thuringiensis*; CO_2, carbon dioxide.

 Global Change Biology, doi: 10.1111/j.1365-2486.2008.01574.x

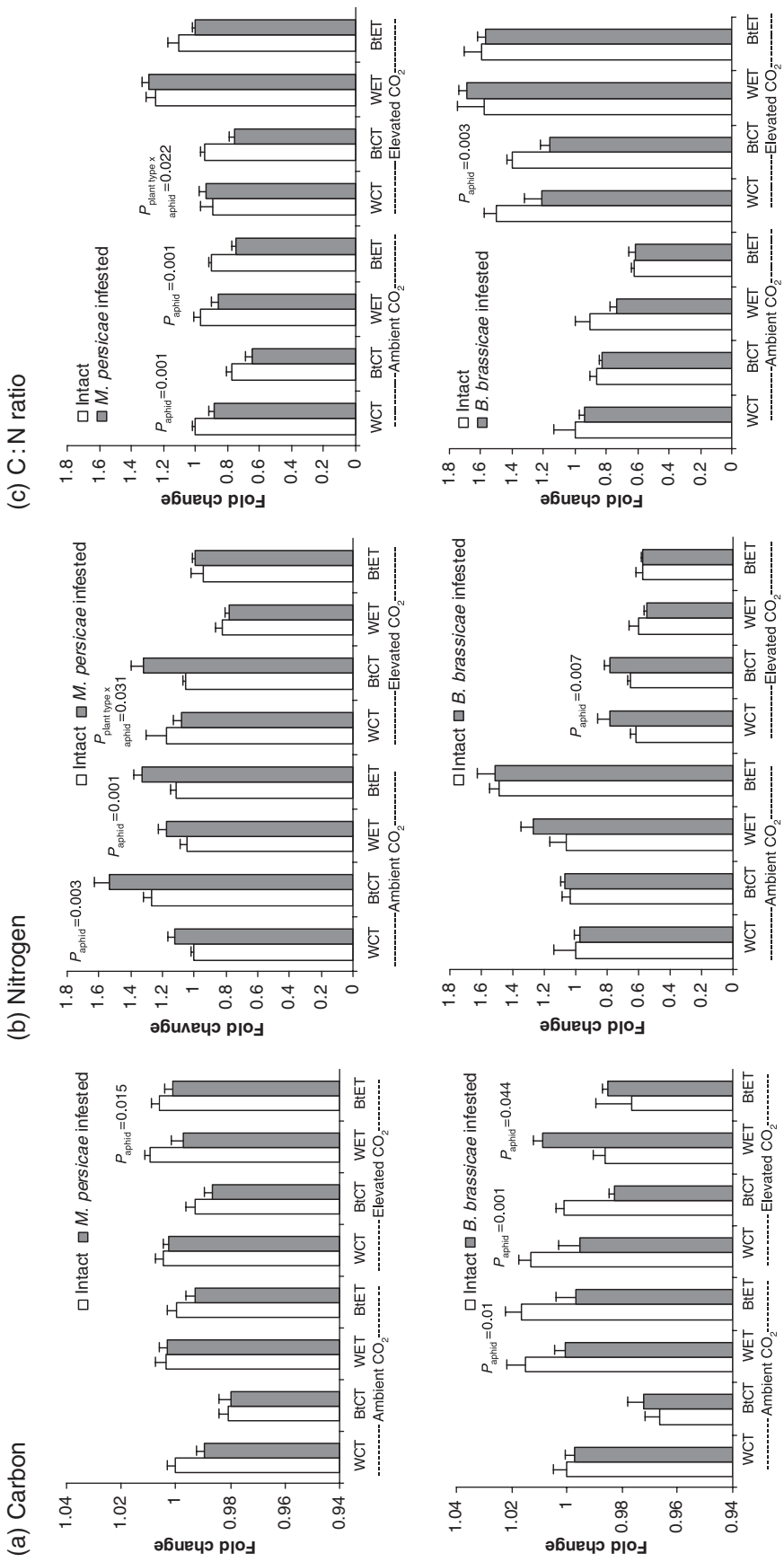

Fig. 3 Carbon (C), nitrogen (N) and C : N ratio in aphid (*Myzus persicae* or *Brevicoryne brassicae*)-infested nontransgenic (Westar) and Bt-transgenic (Bt) oilseed rape plants grown under ambient or elevated (720 ppm) CO_2 level and under control (CT, 20/16 °C) or elevated (ET, 24/20 °C) temperature indicated as fold change to nontransgenic (Westar) plants grown under ambient CO_2 level and control temperature ($n = 6$). Statistically significant ($P < 0.05$) ANOVA effects of aphid feeding and interactions of plant type and aphid feeding within each CO_2 and temperature treatment are shown. Bt, *Bacillus thuringiensis*.

When plants were exposed to *M. persicae* feeding, there was increased N content in nontransgenic and Bt-transgenic plants under control CO_2 level but not under elevated CO_2 (Fig. 3). *M. persicae* feeding induced a differential response in the N content of the two plant types under elevated CO_2 and control temperature: in nontransgenic plants, the N content was slightly decreased, but in Bt plants it was rather increased (interaction plant type × aphid feeding, $F_{1,20} = 5.39$, $P = 0.031$). *B. brassicae* feeding increased the N content of both plant types under elevated CO_2 and control temperature ($F_{1,20} = 9.08$, $P = 0.007$), whereas in other growth conditions, no statistically significant changes were observed in the N dynamics after cabbage aphid feeding.

The C : N ratio was decreased in *M. persicae*-fed plants compared with intact plants grown under ambient CO_2, whereas *B. brassicae* feeding decreased the ratio only under elevated CO_2 and control temperature (Fig. 3). Corresponding to the differential response of the N contents of the two plant types under elevated CO_2 and control temperature, a similar interaction for plant type × aphid feeding ($F_{1,20} = 6.20$, $P = 0.022$) was detected in C : N ratio.

Chlorophyll pigments

Chl *a* concentrations had a trend to increase in elevated CO_2 under control temperature and also to increase at elevated temperature under control CO_2 level (Table 2). In contrast, in the elevated CO_2 and temperature treatment, the concentration was reduced compared with plants from either single elevation treatments (significant CO_2 × temperature interaction, $P = 0.002$). Chl *b*, total chlorophyll and total carotenoid concentrations showed a similar response (CO_2 × temperature interactions: $P = 0.033$, 0.003 and 0.026, respectively). Chl *a*/*b* ratio was reduced by elevated temperature under both CO_2 levels (main effect of temperature, $P = 0.024$). Carotenoid concentrations were lower in Bt-transgenic plants compared with nontransgenic plants (main effect of plant type, $P = 0.025$), but otherwise the pigment concentrations were equal in both plant types and showed similar responses to elevated CO_2 and temperature.

Starch and soluble sugars

Glucose concentrations in Bt-transgenic and nontransgenic oilseed rape leaves at the end of vegetative stage were increased by elevated temperature ($F_{1,40} = 5.90$, $P = 0.021$), particularly under control CO_2 level (Fig. 4). In contrast, elevated CO_2 decreased the glucose concentration ($F_{1,40} = 5.45$, $P = 0.026$). There was a significant interaction between CO_2 level and temperature for fructose ($F_{1,40} = 5.75$, $P = 0.021$) and a similar marginally significant one for glucose ($F_{1,40} = 4.03$, $P = 0.053$). This result indicated that elevated temperature had no such increasing effect on the concentrations of the sugars under elevated CO_2 as appeared under control CO_2 conditions. Sucrose concentrations were not significantly affected by elevated CO_2 or temperature. The main effect of plant type approached statistical significance ($F_{1,40} = 3.96$, $P = 0.055$), since the sucrose levels were slightly decreased in Bt-transgenic plants compared with the nontransgenic plants in all treatments. Glucose and fructose concentrations were similar in Bt-transgenic and nontransgenic plants.

Starch concentration was strongly decreased by elevated temperature treatment under control CO_2 level ($F_{1,40} = 13.21$, $P = 0.001$), whereas it was unaffected by temperature under elevated CO_2 (interaction CO_2 level × temperature, $F_{1,40} = 7.87$, $P = 0.008$) (Fig. 4). Elevated CO_2 increased the starch concentration under both temperature regimes (main effect CO_2, $F_{1,40} = 63.69$, $P < 0.001$). Bt-transgenic and nontransgenic plants had equivalent starch concentrations, and the starch responses of the plant types to elevated CO_2 and temperature were similar.

Bt toxin concentration

Bt-transgenic oilseed rape grown under control CO_2 and temperature in Experiment 1 contained $1.89 \pm 0.18\,\mu g$ Bt Cry1Ac g^{-1} leaf fresh weight, measured from the third true leaf. Elevated CO_2 or temperature did not significantly alter the Cry1Ac concentration ($P > 0.05$); Bt concentrations of leaves were $1.97 \pm 0.12\,\mu g$ (ambient CO_2, elevated temperature), $1.74 \pm 0.13\,\mu g$ (elevated CO_2, control temperature) and $1.63 \pm 0.24\,\mu g$ (elevated CO_2 and elevated temperature) toxin. The proportions of Cry1Ac of total soluble protein ranged from 0.016% to 0.03% (results not shown), and these were not significantly affected by the treatments either. *M. persicae* and *B. brassicae* aphids feeding on Bt oilseed rape did not contain a quantifiable concentration of Bt Cry1Ac in any of the CO_2 and temperature treatments.

M. persicae and B. brassicae reproduction and relative growth rate

Elevated temperature shortened the developmental time of *M. persicae* aphids by approximately 3 days on both plant types (Fig. 5). The developmental time of the specialist *B. brassicae* was 10–11 days in all treatments and not affected by plant type, elevated CO_2 or temperature (Fig. 5, Tables 3 and 4).

Table 2 Chlorophyll pigment concentrations and chl *a*/*b* ratio (mean ± 1 SEM) in leaves of Bt-transgenic and nontransgenic (Westar) oilseed rape plants grown in ambient or elevated (720 ppm) CO_2 level under control (20/16 °C) or elevated (24/20 °C) temperature ($n = 6$) and ANOVA results for effects of plant type (Bt-transgenic vs. nontransgenic), CO_2 level and temperature and their interactions

	Ambient CO_2				Elevated CO_2			
	20/16 °C		24/20 °C		20/16 °C		24/20 °C	
	Westar	Bt	Westar	Bt	Westar	Bt	Westar	Bt
Chl *a* (mg/100 g FW)	49.25 ± 4.77	48.77 ± 2.88	57.84 ± 3.49	56.32 ± 2.05	66.75 ± 5.27	51.01 ± 1.93	51.61 ± 4.46	49.52 ± 1.54
Chl *b* (mg/100 g FW)	11.13 ± 1.26	11.19 ± 0.47	13.00 ± 0.75	13.79 ± 0.82	14.36 ± 1.38	12.11 ± 0.53	13.04 ± 1.06	12.15 ± 0.67
Total chlorophyll (mg/100 g FW)	60.38 ± 6.00	59.96 ± 3.10	70.84 ± 4.22	70.11 ± 2.82	81.11 ± 6.63	63.12 ± 2.03	64.65 ± 5.41	61.67 ± 2.06
Carotenoids (mg/100 g FW)	1.42 ± 0.06	1.36 ± 0.07	1.53 ± 0.05	1.45 ± 0.04	1.57 ± 0.07	1.40 ± 0.05	1.43 ± 0.06	1.33 ± 0.08
Chl *a*/*b* ratio	4.49 ± 0.15	4.39 ± 0.27	4.45 ± 0.08	4.12 ± 0.12	4.69 ± 0.10	4.26 ± 0.24	3.98 ± 0.20	4.11 ± 0.14

	Plant type		CO_2		Temperature		Plant type × CO_2		Plant type × temperature		CO_2 × temperature		Plant type × CO_2 × temperature	
	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P
Chl *a*	3.89	0.056	0.44	0.509	0.01	0.961	2.47	0.124	1.57	0.217	**10.62**	**0.002**	2.13	0.152
Chl *b*	0.78	0.383	0.97	0.331	1.51	0.227	2.34	0.134	0.64	0.427	**4.88**	**0.033**	0.06	0.811
Total chlorophyll	3.20	0.081	0.56	0.459	0.05	0.828	2.57	0.117	1.42	0.241	**9.70**	**0.003**	1.54	0.223
Carotenoids	**5.41**	**0.025**	0.03	0.871	0.01	0.972	0.56	0.458	0.09	0.763	**5.34**	**0.026**	0.24	0.625
Chl *a*/*b*	2.27	0.140	0.65	0.424	**5.53**	**0.024**	0.07	0.792	0.46	0.501	1.23	0.274	2.63	0.113

Statistically significant effects ($P < 0.05$) are marked in bold.
Bt, *Bacillus thuringiensis*; FW, final weight; CO_2, carbon dioxide.

 Global Change Biology, doi: 10.1111/j.1365-2486.2008.01574.x

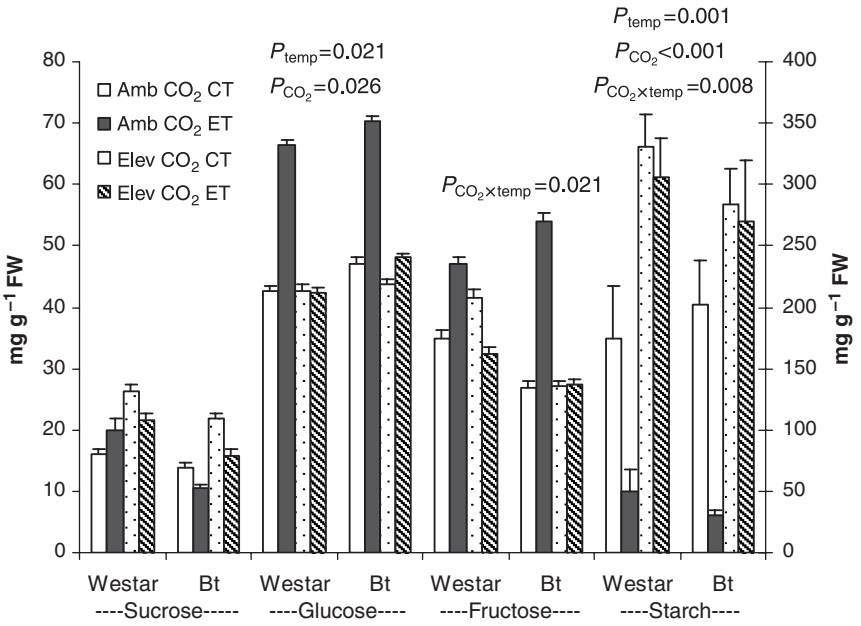

Fig. 4 Concentration of soluble sugars (left axis) and starch (right axis) in 30-day-old Bt-transgenic (Bt) and nontransgenic (W, Westar) oilseed rape plants grown under ambient or elevated (720 ppm) CO_2 level and under control (CT, 20/16 °C) or elevated (ET, 24/20 °C) temperature ($n = 6$). Statistically significant ($P < 0.05$) ANOVA results for main effects of plant type, CO_2 level and temperature and their interactions are shown. Bt, *Bacillus thuringiensis*.

Final weight of adult *M. persicae* was not affected by the plant type, but it was significantly reduced in elevated growth temperature compared with control temperature (main effect of temperature, $P < 0.001$) (Tables 3 and 4). Elevated CO_2 also significantly lowered the *M. persicae* adult weight compared with aphids on plants grown in control CO_2 level ($P = 0.041$, Table 4). Mean weight of <24-h old *M. persicae* nymphs was reduced on plants grown in elevated temperature ($P = 0.007$), but elevated CO_2 or plant type had no significant effect on progeny weight. Performance parameters of *B. brassicae* (developmental time, total number of progeny and final weights of adults) were not affected by plant type, elevated CO_2 or elevated temperature (Table 3, statistical results not shown). Mean progeny weights of *B. brassicae* were marginally significantly increased by elevated CO_2 ($P = 0.088$), and reduced by elevated temperature ($P = 0.055$).

The cumulative fecundity of *M. persicae* was increased by elevated temperature (repeated-measures ANOVA, $F_{1,39} = 142.1$, $P < 0.001$) and reduced by elevated CO_2 (repeated-measures ANOVA, $F_{1,39} = 4.73$, $P = 0.036$) (Fig. 5a,b). The total number of progeny produced by *M. persicae* was highest in plants grown under control CO_2 and elevated temperature, followed by elevated CO_2 combined with elevated temperature, thereafter in the control CO_2 and control temperature and finally lowest in plants grown in elevated CO_2 under control temperature. *M. persicae* showed an equal rate of reproduction in Bt-transgenic and nontransgenic plants. The cumulative fecundity of *B. brassicae* was not affected by plant type, elevated CO_2 or elevated temperature (Fig. 5c,d; statistical results not shown). The MRGR of *M. persicae* and *B. brassicae* nymphs was similar in Bt-transgenic and nontransgenic plants and this was not affected by elevated CO_2 or temperature (Tables 3 and 4).

Finally, Table 5 represents a summary showing the direction (increase or decrease) of the statistically significant effects of the treatments (main effects of plant type, elevated CO_2 and elevated temperature) and the presence of elevated CO_2 × temperature interactions for all the measured parameters.

Discussion

We found significant changes in the C–N dynamics of Bt-transgenic oilseed rape and its nontransgenic parent line at the end of their vegetative growth. Presumably, the extra input requirement for producing Bt toxin could have led to the observed increase in N and the reduction in C content. The Bt toxin proportion from total soluble proteins in this plant line under the growth conditions was relatively low, and still, even this low Bt toxin production resulted in changes in the C : N ratio. Bt Cry1Ac concentrations in Bt oilseed rape show

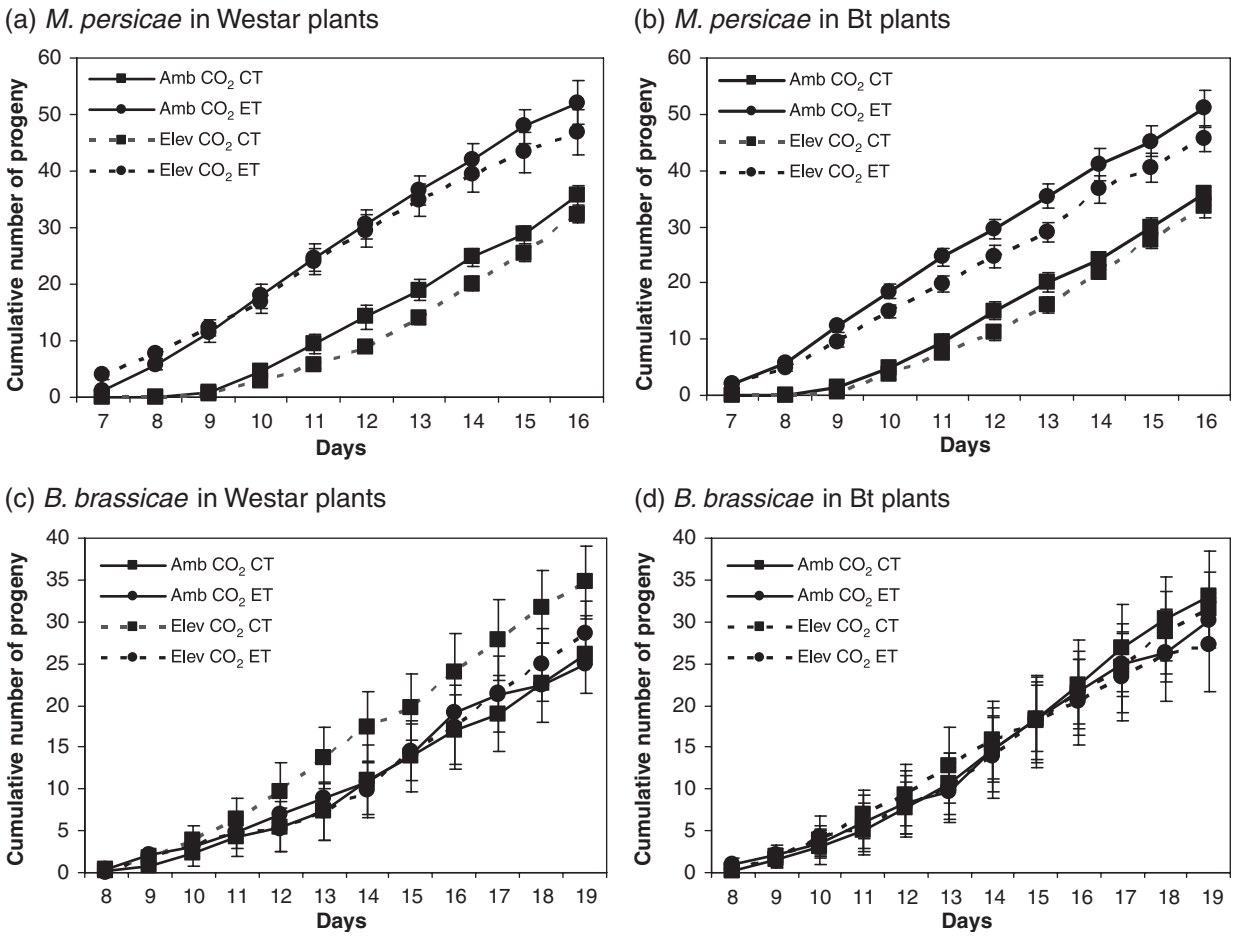

Fig. 5 Cumulative reproduction ± 1 SEM of *Myzus persicae* (a and b) and *Brevicoryne brassicae* (c and d) on nontransgenic (Westar) and Bt-transgenic (Bt) oilseed rape plants grown under ambient or elevated (720 ppm) CO_2 level and under control (CT, 20/16 °C) or elevated (ET, 24/20 °C) temperature. Bt, *Bacillus thuringiensis*.

variation by growth conditions, leaf position and growth stage both in the field (Zhu *et al*., 2004) and also in greenhouse conditions (Wei *et al*., 2005). Therefore, C : N pattern changes in more natural field conditions would be beneficial to assay to reveal whether the change in C : N relations is constitutive or rather specific for growth stage or growth condition. Here, Bt oilseed rape plants in their vegetative stage seemed to have a higher capacity of N uptake or allocation of N to leaves than their nontransgenic parent line in equal chronological age. It could be speculated that the observed difference in N and C could solely be based on the adding to the sum by the incorporated production of the new proteins: Bt toxin, GFP marker protein and *nptII* selectable marker protein in the leaves (Halfhill *et al*., 2001). However, the relatively low concentration of Bt toxin observed in these growth conditions in this line would likely not be totally responsible to cause the observed difference in C–N contents, but would also require additional changes in N allocation in the basic metabolism. The observed differences could also be due to altered growth, because genetic transformation may lead to some additional pleiotropic effects (i.e. reduced growth leading to higher protein content; Rothe *et al*., 2004). In fact, also these 'GT1' Bt-transgenic oilseed rape plants had a slightly lower biomass at the end of their vegetative stage than the nontransgenic plants, which was particularly evident under elevated CO_2 in this work and also showing a similar trend in our earlier study (Himanen *et al*., 2008).

An important factor to consider is that the C : N ratio was evaluated during the vegetative growth only and future work should address the overall profile of C : N changes during both vegetative and reproductive stages to reveal, if this difference is more pronounced at either stage. This would enable assessing whether differences in C : N dynamics could affect the reproductive fitness of Bt plants, which is highly important for both their agronomic performance and their persistence in agricultural environments after the growing season being

Table 3 Performance parameters (mean ± 1 SEM) of *Myzus persicae* and *Brevicoryne brassicae* aphids on Bt-transgenic and nontransgenic (Westar) oilseed rape plants grown in ambient or elevated (720 ppm) CO_2 level under control (20/16 °C) or elevated (24/20 °C) temperature ($n = 6$)

	Ambient CO_2				Elevated CO_2			
	20/16 °C		24/20 °C		20/16 °C		24/20 °C	
	Westar	Bt	Westar	Bt	Westar	Bt	Westar	Bt
Experiment 1: *Myzus persicae*								
Development time (days)	10.2 ± 0.3	9.7 ± 0.3	7.5 ± 0.1	7.6 ± 0.3	10.1 ± 0.2	9.8 ± 0.2	6.9 ± 0.2	7.3 ± 0.3
Adult aphid weight (μg)	835.8 ± 24.1	833.7 ± 22.6	597.7 ± 38.6	556.0 ± 26.7	768.6 ± 17.5	797.9 ± 21.3	526.3 ± 37.2	544.2 ± 45.8
Progeny weight (μg)	43.95 ± 1.00	46.40 ± 1.07	42.86 ± 1.22	42.83 ± 1.21	43.76 ± 0.78	45.56 ± 0.86	39.51 ± 1.30	40.19 ± 1.12
Mean relative growth rate	2.15 ± 0.17	2.09 ± 0.09	2.09 ± 0.15	2.11 ± 0.08	2.27 ± 0.14	2.19 ± 0.11	2.22 ± 0.09	2.28 ± 0.14
Experiment 2: *Brevicoryne brassicae*								
Development time (days)	11.6 ± 0.8	11.2 ± 0.8	10.7 ± 0.9	10.9 ± 0.8	10.7 ± 0.9	10.5 ± 1.0	10.8 ± 0.9	11.0 ± 0.8
Adult aphid weight (μg)	532.0 ± 49.6	752.5 ± 70.7	530.8 ± 68.2	506.0 ± 58.6	489.0 ± 19.3	592.0 ± 34.9	629.2 ± 83.7	509.0 ± 116.4
Progeny weight (μg)	32.12 ± 1.43	33.91 ± 2.41	33.93 ± 1.13	33.18 ± 1.43	35.46 ± 0.99	35.09 ± 0.85	34.58 ± 1.64	33.53 ± 2.88
Mean relative growth rate	2.65 ± 0.64	2.55 ± 0.60	1.83 ± 0.23	1.86 ± 0.09	2.20 ± 0.16	1.78 ± 0.31	2.37 ± 0.26	1.85 ± 0.38

Bt, *Bacillus thuringiensis*; CO_2, carbon dioxide.

Table 4 ANOVA results for effects of plant type, CO_2 level and temperature and their interactions on *Myzus persicae* performance

	Plant type		CO_2		Temperature		Plant type × CO_2		Plant type × temperature		CO_2 × temperature		Plant type × CO_2 × temperature	
	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P	$F_{1,40}$	P
Experiment 1: *M. persicae*														
Development time (days)	0.04	0.85	1.39	0.245	**202.9**	**<0.001**	0.37	0.546	2.94	0.094	1.22	0.276	0.02	0.88
Total progeny	0.02	0.891	**4.47**	**0.041**	**56.74**	**<0.001**	0.02	0.888	0.22	0.645	0.39	0.536	0.02	0.877
Adult weight	0.01	0.969	**4.50**	**0.041**	**132.8**	**<0.001**	1.08	0.306	0.34	0.566	0.05	0.824	0.10	0.751
Progeny weight	0.56	0.457	1.53	0.224	**8.24**	**0.007**	0.08	0.783	0.54	0.467	1.11	0.299	0.07	0.796
Mean relative growth rate	0.03	0.857	2.13	0.152	<0.01	0.986	0.01	0.938	0.40	0.529	0.04	0.841	0.02	0.879

Statistically significant effects ($P < 0.05$) are marked in bold.

 Global Change Biology, doi: 10.1111/j.1365-2486.2008.01574.x

Table 5 Summary of the treatment effects on the measured parameters

	Treatment			
Parameter studied	Bt transgene	Elevated CO_2	Elevated temperature	Elevated CO_2 × temperature
Vegetative biomass	↓ **	↑ ***	ns	ns
Photosynthesis	ns	ns	↓ ***	ns
Carbon	↓ **	ns	ns	IA***
Nitrogen	↑ *	↓ ***	ns	IA**
C : N ratio	↓ *	↑ ***	ns	IA*
Bt toxin	na	ns	ns	ns
Soluble carbohydrates				
Glucose	ns	↓ *	↑ *	ns
Fructose	ns	ns	ns	IA*
Sucrose	ns	ns	ns	ns
Starch	ns	↑ ***	↓ **	IA**
Chlorophyll pigments				
Chl *a*	ns	ns	ns	IA**
Chl *b*	ns	ns	ns	IA*
Chl *a*/*b* ratio	ns	ns	↓ *	ns
Total chlorophyll	ns	ns	ns	IA**
Carotenoids	↓ *	ns	ns	IA*
Aphid performance				
Developmental time	ns (M) ns (B)	ns (M) ns (B)	↓ *** (M) ns (B)	ns (M) ns (B)
Adult weight	ns (M) ns (B)	↓ * (M) ns (B)	↓ *** (M) ns (B)	ns (M) ns (B)
Progeny weight	ns (M) ns (B)	ns (M) ns (B)	↓ ** (M) ns (B)	ns (M) ns (B)
Cumulative reproduction	ns (M) ns (B)	↓ * (M) ns (B)	↑ *** (M) ns (B)	ns (M) ns (B)
Mean relative growth rate	ns (M) ns (B)	ns (M) ns (B)	ns (M) ns (B)	ns (M) ns (B)

Increase (↑), decrease (↓) or no effect (ns) is based on statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$) main effects of plant type, CO_2 level, temperature and CO_2 level × temperature interaction.
IA, interaction; na, not applicable; (M), *Myzus persicae*; (B), *Brevicoryne brassicae*.

related to the risks of gene flow into the environment (Halfhill *et al*., 2005). Between the two experiments conducted, differences in the overall status of N levels of plants were observed, which was predicted to be caused by seasonal differences, because the growth conditions were otherwise very similar. The factors leading to seasonal variation are mostly unknown, but it might be caused by internal regulation of plant growth (induction signals for vegetative vs. reproductive growth at different times of year, circannual and circadian rhythms) (Sharma *et al*., 2005). The occurrence of this and other types of seasonal variation even in controlled growth conditions (Ritala *et al*., 2001; Sharma *et al*., 2005), as often found in the greenhouse, is important considering whether there are certain times of year leading to higher incidence of changes in agronomic yield due to uncontrollable intrinsically regulated factors. However, in both experiments, the general patterns (i.e. plant type main effect and interacting CO_2 × temperature effect on C and N contents) were similar.

In general, the responses of biomass gain and photosynthetic rate to elevated CO_2 and temperature were similar in Bt-transgenic and nontransgenic oilseed rape. Elevated CO_2 increased biomass gain and net photosynthesis, total C was increased and total N decreased, which all are highly typical responses to enhanced CO_2 with increased C (Long *et al*., 2004). As oilseed rape has no obvious C storage structures, the positive effect of CO_2 on biomass gain typically decreases later in the development (Reekie *et al*., 1998). Under elevated CO_2 level, carboxylation of ribulose bisphosphate predominates over oxygenation by the Rubisco enzyme, whereas elevated temperature can increase photorespiration (Morison & Lawlor, 1999). Net photosynthesis of oilseed rape leaves was reduced under elevated growth temperature in our study, which was mostly an effect of differences in leaf development (i.e. phenology). The plants grown under elevated temperature were clearly physiologically older due to accelerated vegetative growth under elevated temperature, even though the temporal age of all plants was identical. The reduction in photosynthesis and biomass gain in oilseed rape plants under elevated temperature thereby was affected mostly by reduced leaf duration and

approaching senescence. Also, a decrease in total chlorophyll by elevated CO_2, which is the most typical response of chlorophyll pigments to enhanced CO_2 (Osborne *et al*., 1997; Sallas *et al*., 2003), was apparent in oilseed rape only under elevated temperature, in accordance with a more reduced net photosynthesis under elevated than control temperature with elevated CO_2 level. Elevated temperature alone increased the amount of chlorophyll pigments in oilseed rape leaves.

Direct effects of elevated CO_2 atmospheres include decreases in the stomatal conductance and stomatal sensitivity in plants as an acclimation to elevated CO_2 (Lodge *et al*., 2001). Rubisco enzyme activity and content can also show adaptation to higher CO_2 concentration, the magnitude of this depending on e.g. leaf age and temperature (Morison & Lawlor, 1999; Pérez *et al*., 2007). Indications of photosynthetic acclimation to elevated CO_2 level (Martínez-Carrasco *et al*., 2005) were found in transgenic and nontransgenic oilseed rape as well (i.e. lowered photosynthesis in elevated CO_2-grown plants compared with control CO_2-grown plants). The concentrations of soluble sugars and starch in Bt-transgenic vs. nontransgenic plant leaves responded similarly to elevated CO_2 and temperature. Nonstructural carbohydrates typically increase under elevated CO_2 and the response can be mitigated (a decreasing effect) by elevated temperature (Poorter *et al*., 1997; Zvereva & Kozlov, 2006). We found increased starch concentration in oilseed rape under elevated CO_2 under both of our temperature regimes, similarly as Sallas *et al*. (2003), who studied the responses of Scots pine seedlings. The other clear effect observed, the reduction in starch and the increase in soluble sugars under ambient CO_2 with elevated temperature, could be related to a higher assimilate targeting to other parts of the plant at the late vegetative stage (i.e. leaves were starting to senescence, a phenological effect). In the elevated CO_2 treatment, the leaves were still active and functioning as sources of C with higher photosynthesis, as seen from the photosynthetic rate measurements, and such allocation from the leaves had not started yet.

Aphid feeding seemed to increase N content of oilseed rape leaves under ambient CO_2, indicating that aphid damage enhances allocation of N to leaves or its uptake by the plant. *M. persicae* has previously been shown to be able to activate multiple genes involved in C assimilation, photosynthesis and N and C mobilization (Divol *et al*., 2005), to alter biomass allocation of plants at elevated CO_2 (Hughes & Bazzaz, 2001) and to increase the concentration of glucosinolates (Mewis *et al*., 2006). In our study, however, under elevated CO_2, aphids did not increase the N content of oilseed rape leaves, which could be a result of reaching the limit for N uptake from maximal plant growth. The feeding of the generalist aphid *M. persicae* had a greater effect in changing the N dynamics than that of the specialist aphid *B. brassicae*, although both species are piercing herbivores (i.e. they cause rather similar feeding damage). *M. persicae* adult weight was higher than that of *B. brassicae* aphids, so the intake of nutrients by *M. persicae* could be assumed to be also higher and could this way lead to more pronounced effects on these plants. Another thing is that *M. persicae* was able to move freely on the plants, whereas *B. brassicae* was enclosed into clip-cages to prevent its colonization behaviour. Therefore, it might also be that for effectively activating N allocation in the plant, the actual place where the feeding is occurring is important.

The response of both aphid species to elevated CO_2 and temperature was highly similar in Bt-transgenic and nontransgenic plants, even though there were differences in C–N ratios between the plant types. Bt Cry1Ac toxin should not affect aphids directly: firstly because aphids feed on phloem sap, which does not contain significant amounts of Bt toxin (Raps *et al*., 2001; Burgio *et al*., 2007), and secondly, because they lack specific receptors for Cry1 toxin binding. We also screened aphids for traces of Bt toxin, but the amounts were undetectable. Schuler *et al*. (2001) also found no indications of pleiotropic effects of Bt *B. napus* on *M. persicae*. Previously, major factors affecting aphid performance have been reported to be, e.g., the nutrient level of the plant, the C : N ratio, the composition of amino acids, the amount of soluble carbohydrates and proteins, the presence of tannins or other carbohydrate-based insoluble compounds and the concentrations of secondary compounds (Cole, 1997). Therefore, the differences in C–N ratios between Bt-transgenic and nontransgenic plants alone may be negligible with regards to aphid effects. There are also aphid species-specific N requirements (Newman *et al*., 2003), which limits making generalizations on their responses.

Of the two aphid species studied, the generalist *M. persicae* was more responsive to both temperature and CO_2 on oilseed rape than the specialist *B. brassicae* and it had nearly 50% higher progeny production than *B. brassicae*. However, on *Brassica oleracea*, Stacey & Fellowes (2002) found *B. brassicae* aphids to be larger with greater fat gain under elevated CO_2, whereas individually reared *M. persicae* had higher fecundity under elevated CO_2. Differences in irradiance (greenhouse vs. growth chamber) and total soil fertility (Newman, 2003; Newman *et al*., 2003) have been associated with variable CO_2 effects on aphids. In addition, variation in amino acid compositions among *Brassica* species (Cole, 1997) could be reasons for differential performance of these aphids on *Brassica* plants. Temperature was the

dominant determinant of *M. persicae* fecundity on oilseed rape, whereas elevated CO_2 counteracted its effect by affecting the reproduction negatively, a phenomenon previously modelled by Newman (2003), which predicted there would be no final change with these interacting factors. The decrease in aphid weights and the increase in total progeny produced under elevated temperature are presumably related and a similar response was reported by Flynn *et al*. (2006) on another aphid species. The role of temperature in accelerating population growth of herbivores by shortening development times has been previously described with numerous species (e.g. Bale *et al*., 2002; Johns & Hughes, 2002; Williams *et al*., 2003), but there are interaction effects between elevated CO_2 and various abiotic factors including temperature (Newman, 2003, 2006). Our results describing the exponential reproduction phase of the aphids do not differentiate between direct effects of CO_2 and temperature on aphid physiology and indirect plant-mediated effects on aphid performance. However, previous studies with aphids indicate that temperature is acting both directly, and through plants, on aphid performance (Bale *et al*., 2002; Newman, 2003), whereas the effects of elevated CO_2 are caused mainly by alterations in plant material as food for herbivores (Agrell *et al*., 2000). In nature, population interactions between different aphid species and also multitrophic interactions with higher trophic levels are involved (Bezemer *et al*., 1998; Awmack *et al*., 2004; Hoover & Newman, 2004), which makes predicting aphid performance in future climate even more challenging (Newman, 2006).

Risk analysis of Bt plants towards nontarget herbivores has focused mainly on screening the potential direct toxic effects of purified and plant-expressed Bt toxin (e.g. Howald *et al*., 2003; Vojtech *et al*., 2005). Because it is well known that herbivores are affected by plant food quality, we might expect certain lines of insect resistance transgenic plants to sometimes indirectly perturb food webs (Schuler *et al*., 1999; Pons *et al*., 2005; Sisterson *et al*., 2007), although this has not been observed in field studies (e.g. Head *et al*., 2005). Even more, the evaluation of nontarget herbivore performance on transgenic plants under altered environmental conditions (elevated CO_2 and temperature) is still not well understood (Coviella *et al*., 2000, 2002; Chen *et al*., 2005). There is no doubt that in future, with the ever-expanding use of GM plants, it will be increasingly important to understand how they respond to climate change in terms of performance and pest susceptibility.

Conclusions

We found changes in C–N ratios in transgenic oilseed rape compared with the nontransgenic parental type, but there was no evidence of effects caused by these changes on Bt Cry1Ac nonsusceptible aphid performance, which could have raised concerns about increased susceptibility of transgenic oilseed rape to nontarget herbivory in future climates. The increased N content observed in Bt plants, however, warrants further research to reveal whether this effect is growth stage-specific, limited to plants in their early vegetative growth, or whether this also occurs or becomes even more profound during the reproductive growth stage, when it could further affect plant fitness, invasion potential or herbivore dynamics. Nevertheless, studies on herbivore pest species responding to the state of N content are encouraged to be continued on Bt plants. Although Bt oilseed rape had an overall lower biomass gain during their early vegetative growth than the nontransgenic plants in this study, the photosynthetic and carbohydrate responses to elevated CO_2 and temperature, singly and in combination, were highly similar in both plant types, revealing equal performance and abilities to resource allocation under these altered abiotic conditions. Most importantly, our study emphasizes the need for further investigations, i.e. assessing the changes occurring in interactions of elevated CO_2 and temperature on Bt plants over the entire growing season with sufficient replication and a natural light environment in field conditions.

Acknowledgements

We thank V. Tiihonen for conducting laboratory analyses for chlorophyll, starch and soluble sugars, T. Oksanen for programming and maintaining the growth chambers, E. Häikiö for advice on photosynthetic measurements, M. A. Ibrahim, N.-L. Nuotio and M. Valkonen for help on aphid weighing and J. Blande for supplying aphids to us. This work was supported by the Academy of Finland (S. J. H., A. N., A.-M. N and J. K. H.) (ESGEMO programme, decision no. 105209) and enabled by USDA Biotechnology Risk Assessment grants to C. N. S.

References

Agrell J, McDonald EP, Lindroth RL (2000) Effects of CO_2 and light on tree phytochemistry and insect performance. *Oikos*, **88**, 259–272.

Awmack CS, Harrington R, Lindroth RL (2004) Aphid individual performance may not predict population responses to elevated CO_2 or O_3. *Global Change Biology*, **20**, 1414–1423.

Bale JS, Masters GJ, Hodkinson ID *et al*. (2002) Herbivory in global climate change research: direct effects of rising temperature on insect herbivores. *Global Change Biology*, **8**, 1–16.

Barnes JD, Balaguer L, Manrique E, Elvira S, Davison AW (1992) A reappraisal of the use of DMSO for the extraction and determination of chlorophylls a and chlorophylls b in lichens and higher plants. *Environmental and Experimental Botany*, **32**, 85–100.

Bates SL, Zhao JZ, Roush RT, Shelton AM (2005) Insect resistance management in GM crops: past, present and future. *Nature Biotechnology*, **23**, 57–62.

Bezemer TM, Jones TH, Knight KJ (1998) Long-term effects of elevated CO_2 and temperature on populations of the peach potato aphid *Myzus persicae* and its parasitoid *Aphidius matricariae*. *Oecologia*, **116**, 128–135.

Bezemer TM, Knight KJ, Newington JE, Jones TH (1999) How general are aphid responses to elevated atmospheric CO_2? *Annals of the Entomological Society of America*, **92**, 724–730.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, **72**, 248–254.

Bryant JP, Chapin FS III, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos*, **40**, 357–368.

Buntin GD, Raymer PL (1994) Pest status of aphids and other insects in winter canola in Georgia. *Journal of Economic Entomology*, **87**, 1097–1104.

Burgio G, Lanzoni A, Accinelli G, Dinelli G, Bonetti A, Marotti I, Ramilli F (2007) Evaluation of Bt-toxin uptake by the non-target herbivore, *Myzus persicae* (Hemiptera: Aphididae), feeding on transgenic oilseed rape. *Bulletin of Entomological Research*, **97**, 211–215.

Chen FJ, Wu G, Ge F, Parajulee MN, Shrestha RB (2005) Effects of elevated CO_2 and transgenic *Bt* cotton on plant chemistry, performance, and feeding of an insect herbivore, the cotton bollworm. *Entomologia Experimentalis et Applicata*, **115**, 341–350.

Cole RA (1997) The relative importance of glucosinolates and amino acids to the development of two aphid pests *Brevicoryne brassicae* and *Myzus persicae* on wild and cultivated brassica species. *Entomologia Experimentalis et Applicata*, **85**, 121–133.

Coviella CE, Morgan DJW, Trumble JT (2000) Interactions of elevated CO_2 and nitrogen fertilization: effects on production of *Bacillus thuringiensis* toxins in transgenic plants. *Environmental Entomology*, **29**, 781–787.

Coviella CE, Stipanovic RD, Trumble JT (2002) Plant allocation to defensive compounds: interactions between elevated CO_2 and nitrogen in transgenic cotton plants. *Journal of Experimental Botany*, **53**, 323–331.

Desneux N, Rabasse J-M, Ballanger Y, Kaiser L (2006) Parasitism of canola aphids in France in autumn. *Journal of Pest Science*, **79**, 95–102.

Divol F, Vilaine F, Thibivilliers S, Amselem J, Palauqui J-C, Kusiak C, Dinant S (2005) Systemic response to aphid infestation by *Myzus persicae* in the phloem of *Apium graveolens*. *Plant Molecular Biology*, **57**, 517–540.

Flynn DFB, Sudderth EA, Bazzaz FA (2006) Effects of aphid herbivory on biomass and leaf-level physiology of *Solanum dulcamara* under elevated temperature and CO_2. *Environmental and Experimental Botany*, **56**, 10–18.

Halfhill MD, Richards HA, Mabon SA, Stewart CN Jr (2001) Expression of *GFP* and *Bt* transgenes in *Brassica napus* and hybridization with *Brassica rapa*. *Theoretical and Applied Genetics*, **103**, 659–667.

Halfhill MD, Sutherland JP, Moon HS *et al*. (2005) Growth, productivity, and competitiveness of introgressed weedy *Brassica rapa* hybrids selected for the presence of *Bt cry1Ac* and *gfp* transgenes. *Molecular Ecology*, **14**, 3177–3189.

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology*, **57**, 303–333.

Head G, Moar M, Eubanks M, Freeman B, Ruberson J, Hagerty A, Turnipseed S (2005) A multiyear, large-scale comparison of arthropod populations on commercially managed Bt and non-Bt cotton fields. *Environmental Entomology*, **34**, 1257–1266.

Himanen SJ, Nissinen A, Auriola S, Poppy GM, Stewart CN Jr, Holopainen JK, Nerg A-M (2008) Constitutive and herbivore-inducible glucosinolate concentrations in oilseed rape (*Brassica napus*) leaves are not affected by *Bt Cry1Ac* insertion but change under elevated atmospheric CO_2 and O_3. *Planta*, **227**, 427–437.

Holopainen JK (2002) Aphid response to elevated ozone and CO_2. *Entomologia Experimentalis et Applicata*, **104**, 137–142.

Hoover JK, Newman JA (2004) Tritrophic interactions in the context of climate change: a model of grasses, cereal aphids and their parasitoids. *Global Change Biology*, **10**, 1197–1208.

Howald R, Zwahlen C, Nentwig W (2003) Evaluation of Bt oilseed rape on the non-target herbivore *Athalia rosae*. *Entomologia Experimentalis et Applicata*, **106**, 87–93.

Hughes L, Bazzaz FA (2001) Effects of elevated CO_2 on five plant–aphid interactions. *Entomologia Experimentalis et Applicata*, **99**, 87–96.

Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. *Ecological Monographs*, **54**, 187–211.

IPCC (Intergovernmental Panel on Climate Change) (2007) *Working group I report: the physical science basis*. Technical Summary, http://www.ipcc.ch/

James C (2006) *Executive summary of global status of commercialized biotech/GM crops: 2006*. ISAAA Briefs No. 35, ISAAA, Ithaca, NY, http://www.isaaa.org

Johns CV, Hughes A (2002) Interactive effects of elevated CO_2 and temperature on the leaf-miner *Dialectica scalariella* Zeller (Lepidoptera: Gracillariidae) in Paterson's Curse, *Echium plantagineum* (Boraginaceae). *Global Change Biology*, **8**, 142–152.

Lodge RJ, Dijkstra P, Drake BG, Morison JIL (2001) Stomatal acclimation to increased CO_2 concentration in a florida scrub oak species *Quercus myrtifolia* Willd. *Plant, Cell and Environment*, **24**, 77–88.

Long SP, Ainsworth EA, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide: plants face the future. *Annual Review of Plant Biology*, **55**, 591–628.

Martínez-Carrasco R, Pérez P, Morcuende R (2005) Interactive effects of elevated CO_2, temperature and nitrogen on photosynthesis of wheat grown under temperature gradient tunnels. *Environmental and Experimental Botany*, **54**, 49–59.

Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C, Gershenzon J (2006) Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry*, **67**, 2450–2462.

Morison JIL, Lawlor DW (1999) Interactions between increasing CO_2 concentration and temperature on plant growth. *Plant, Cell and Environment*, **22**, 659–682.

Newman JA (2003) Climate change and cereal aphids: the relative effects of increasing CO_2 and temperature on aphid population dynamics. *Global Change Biology*, **10**, 5–15.

 Global Change Biology, doi: 10.1111/j.1365-2486.2008.01574.x

Newman JA (2006) Using the output from global circulation models to predict changes in the distribution and abundance of cereal aphids in Canada: a mechanistic modeling approach. *Global Change Biology*, **12**, 1634–1642.

Newman JA, Gibson DJ, Parsons AJ, Thornley JHM (2003) How predictable are aphid population responses to elevated CO_2? *Journal of Animal Ecology*, **72**, 556–566.

Osborne CP, Drake BG, LaRoche J, Long SP (1997) Does long-term elevation of CO_2 concentration increase photosynthesis in forest floor vegetation? *Plant Physiology*, **114**, 337–344.

Pérez P, Zita G, Morcuende R, Martínez-Carrasco R (2007) Elevated CO_2 and temperature differentially affect photosynthesis and resource allocation in flag and penultimate leaves of wheat. *Photosynthetica*, **45**, 9–17.

Pons X, Lumbierres B, Lopez C, Albajes R (2005) Abundance of non-target pests in transgenic Bt-maize: a farm scale study. *European Journal of Entomology*, **102**, 73–79.

Poorter H, van Berkel Y, Baxter R *et al*. (1997) The effect of elevated CO_2 on the chemical composition and construction costs of leaves of 27 C_3 species. *Plant, Cell and Environment*, **20**, 472–482.

Poppy GM, Wilkinson MJ (2005) *Gene Flow from GM Plants*. Blackwell Publishing, Kundli, India.

Raps A, Kehr J, Gugerli P, Moar WJ, Bigler F, Hilbeck A (2001) Immunological analysis of phloem sap of *Bacillus thuringiensis* corn and of the nontarget herbivore *Rhopalosiphum padi* (Homoptera: Aphididae) for the presence of Cry1Ab. *Molecular Ecology*, **10**, 525–533.

Reekie EG, MacDougall G, Wong I, Hicklenton PR (1998) Effect of sink size on growth response to elevated atmospheric CO_2 within the genus *Brassica*. *Canadian Journal of Botany*, **76**, 829–835.

Ritala A, Mannonen R, Oksman-Caldentey K-M (2001) Factors affecting the regeneration capacity of isolated barley microspores (*Hordeum vulgare* L.). *Plant Cell Reports*, **20**, 403–407.

Rothe R, Hartung H, Marks G, Bergmann H, Götz R, Schöne F (2004) Glucosinolate contents in vegetative tissues of winter rape cultivars. *Journal of Applied Botany and Food Quality*, **78**, 41–47.

Romeis J, Meissle M, Bigler F (2006) Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. *Nature Biotechnology*, **24**, 63–71.

Sallas L, Luomala E-M, Utriainen J, Kainulainen P, Holopainen JK (2003) Contrasting effects of elevated carbon dioxide concentration and temperature on Rubisco activity, chlorophyll fluorescence, needle ultrastructure and secondary metabolites in conifer seedlings. *Tree Physiology*, **23**, 97–108.

Schuler TH, Denholm I, Jouanin L, Clark SJ, Clark AJ, Poppy GM (2001) Population-scale laboratory studies of the effect of transgenic plants on nontarget insects. *Molecular Ecology*, **10**, 1845–1853.

Schuler TH, Potting RPJ, Denholm I, Poppy GM (1999) Parasitoid behaviour and *Bt* plants. *Nature*, **400**, 825–826.

Sharma VK, Hänsch R, Mendel RR, Schulze J (2005) Seasonal effect on tissue culture response and plant regeneration frequency from non-bombarded and bombarded immature scutella of barley (*Hordeum vulgare*) harvested from controlled environment. *Plant Cell, Tissue and Organ Culture*, **81**, 19–26.

Sisterson MS, Carriere Y, Dennehy TJ, Tabashnik BE (2007) Nontarget effects of transgenic insecticidal crops: implications of source–sink population dynamics. *Environmental Entomology*, **36**, 121–127.

Stacey DA, Fellowes MDE (2002) Influence of elevated CO_2 on interspecific interactions at higher trophic levels. *Global Change Biology*, **8**, 668–678.

Van Emden HF (1969) Plant resistance to *Myzus persicae* induced by a plant regulator and measured by aphid relative growth rate. *Entomologia Experimentalis et Applicata*, **12**, 125–131.

Veteli TO, Kuokkanen K, Julkunen-Tiitto R, Roininen H, Tahvanainen J (2002) Effects of elevated CO_2 and temperature on plant growth and herbivore defensive chemistry. *Global Change Biology*, **8**, 1240–1252.

Vojtech E, Meissle M, Poppy GM (2005) Effects of Bt maize on the herbivore *Spodoptera littoralis* (Lepidoptera: Noctuidae) and the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). *Transgenic Research*, **14**, 133–144.

Wei W, Schuler TH, Clark SJ, Stewart JCN, Poppy GM (2005) Age-related increase in levels of insecticidal protein in the progenies of transgenic oilseed rape and its efficacy against a susceptible strain of diamondback moth. *Annals of Applied Biology*, **147**, 227–234.

Williams RS, Lincoln DE, Norby RJ (2003) Development of gypsy moth larvae feeding on red maple saplings at elevated CO_2 and temperature. *Oecologia*, **137**, 114–122.

Zhu B, Lawrence JR, Warwick SI, Mason P, Braun L, Halfhill MD, Stewart CN Jr (2004) Stable *Bacillus thuringiensis* (Bt) toxin content in interspecific F_1 and backcross populations of wild *Brassica rapa* after Bt gene transfer. *Molecular Ecology*, **13**, 237–241.

Zvereva EL, Kozlov MV (2006) Consequences of simultaneous elevation of carbon dioxide and temperature for plant–herbivore interactions: a metaanalysis. *Global Change Biology*, **12**, 27–41.

