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Transcriptional profiling reveals elevated CO_2 and elevated O_3 alter resistance of soybean (*Glycine max*) to Japanese beetles (*Popillia japonica*)

CLARE L. CASTEEL[1,2], BRIDGET F. O'NEILL[3], JORGE A. ZAVALA[2], DAMLA D. BILGIN[2], MAY R. BERENBAUM[2,3] & EVAN H. DeLUCIA[1,2]

[1]*Department of Plant Biology,* [2]*Institute for Genomic Biology and* [3]*Department of Entomology, University of Illinois, Urbana, IL 61801, USA*

ABSTRACT

The accumulation of CO_2 and O_3 in the troposphere alters phytochemistry which in turn influences the interactions between plants and insects. Using microarray analysis of field-grown soybean (*Glycine max*), we found that the number of transcripts in the leaves affected by herbivory by Japanese beetles (*Popillia japonica*) was greater when plants were grown under elevated CO_2, elevated O_3 and the combination of elevated CO_2 plus elevated O_3 than when grown in ambient atmosphere. The effect of herbivory on transcription diminished strongly with time (<1% of genes were affected by herbivory after 3 weeks), and elevated CO_2 interacted more strongly with herbivory than elevated O_3. The majority of transcripts affected by elevated O_3 were related to antioxidant metabolism. Constitutive levels and the induction by herbivory of key transcripts associated with defence and hormone signalling were down-regulated under elevated CO_2; 1-aminocyclopropane-1-carboxylate (*ACC*) synthase, lipoxygenase (*LOX*), allene oxide synthase (*AOS*), allene oxide cyclase (*AOC*), chalcone synthase (*CHS*), polyphenol oxidase (*PPO*) and cysteine protease inhibitor (*CystPI*) were lower in abundance compared with levels under ambient conditions. By suppressing the ability to mount an effective defence, elevated CO_2 may decrease resistance of soybean to herbivory.

Key-words: defence; ethylene; gene expression; global change; herbivory; jasmonic acid; microarray; octadecanoid pathway; plant–insect interactions.

INTRODUCTION

Human activities are confronting plants with new challenges by increasing the concentration of carbon dioxide (CO_2) and ozone (O_3) in the atmosphere. Exposure to elevated CO_2 increases photosynthesis and growth (Drake, Gonzalez-Meler & Long 1997; Ainsworth *et al.* 2002), as well as altering plant chemical composition. In general, nitrogen content is lower and carbohydrate content is greater in plants grown under elevated CO_2 (Lindroth, Kinney & Platz 1993; Bezemer & Jones 1998; Penuelas & Estiarte 1998; Körner 2003), resulting in elevated C : N ratios (Bezemer & Jones 1998). Elevated CO_2 may occur in some locations in concert with elevated O_3. Ozone typically decreases photosynthesis and biomass while accelerating senescence (Pausch *et al.* 1996; Fuhrer 2003; Morgan, Ainsworth & Long 2003; Morgan *et al.* 2004; Fiscus, Booker & Burkey 2005), but elevated O_3 can also affect phytochemistry by altering production of carbohydrates (Pausch *et al.* 1996; Kopper, Lindroth & Nordheim 2001) and phenolic compounds (Runeckles & Krupa 1994). In addition to directly affecting plant physiology, elevated CO_2 and O_3 indirectly affect ecosystem processes by fundamentally changing the relationship between plants and herbivorous insects. Changes in foliar nutrients and secondary chemicals induced by elevated CO_2 and O_3 alter palatability and nutritional quality of plants, affecting the performance of herbivores (Coviella & Trumble 1999; Kopper & Lindroth 2003; Agrell *et al.* 2005).

Soybean (*Glycine max*) is a major crop in the United States (Fleming 1972; Potter & Held 2002), with 30.4 million ha planted in 2004, worth an estimated $18 billion (http://www.ers.usda.gov/News/soybeancoverage.htm). Because of its economic importance, understanding the response of soybeans to global change is a high priority. Previous research suggests that elevated CO_2 may improve production, but given the sensitivity of soybean to elevated O_3 and the dearth of studies of the effect of CO_2 and O_3 on plants in the field (Rogers *et al.* 2004), the future value of the crops under projected increases in tropospheric gases is uncertain (Long *et al.* 2006). The Soybean Free-air Concentration Enrichment (SoyFACE) experiment (http://www.soyface.uiuc.edu/), where large plots of soybean are fumigated with elevated CO_2 or O_3 but are otherwise unchanged from typical field conditions, provides a unique opportunity to evaluate the effects of these components of global change on an important agro-ecosystem.

Colonization and leaf damage of soybean under field conditions by Japanese beetle (*Popillia japonica* Newman) increase under high CO_2 compared with either elevated O_3 levels or ambient atmosphere (Hamilton *et al.* 2005).

Correspondence: E. H. DeLucia. Fax: 217-333-6177; e-mail: delucia@life.uiuc.edu

Japanese beetle, a leaf skeletonizer, is among the most polyphagous insects in the eastern United States, feeding on ~300 species of wild and cultivated plants, including soybean (Potter & Held 1999, 2002). Japanese beetles consuming foliage of soybeans grown under elevated CO_2 lived longer and had higher fecundity than beetles consuming foliage developed under ambient atmosphere or elevated O_3 (O'Neill *et al.*, 2008). Increased feeding was initially postulated to be a consequence of increased sugar content of soybean leaves grown under elevated CO_2, as sugars stimulate feeding by Japanese beetles (Potter & Held 1999, 2002). However, experimentally elevated sugar content in foliage did not contribute to greater beetle longevity or fecundity (O'Neill *et al.*, 2008), suggesting that other mechanisms are at work. While elevated O_3 initiated transcriptional and chemical changes in soybean (Morgan *et al.* 2003), the feeding behavior of *P. japonica* appears to be unchanged when fed foliage grown under elevated O_3 (O'Neill *et al.*, 2008).

Whereas the physiological effects of elevated CO_2, elevated O_3 and herbivory have been studied individually, the molecular mechanisms that mediate plant responses to these challenges in combination remain unclear. To dissect the mechanisms governing the response of soybean plants to herbivory under different atmospheric conditions, we utilized microarray technology to examine transcription of over 35 000 genes under field conditions at the SoyFACE experiment. Examining transcriptional changes in soybeans attacked by Japanese beetle while plants were exposed to elevated CO_2 or elevated O_3 individually and in combination, allowed us to address the following questions: (1) How are transcriptional profiles of soybean altered in response to herbivory by Japanese beetles in field conditions?; and (2) Will elevated CO_2 or O_3, individually or in combination, modulate the global transcriptional response of soybean to herbivory by Japanese beetle?

MATERIALS AND METHODS

Site description

This study was conducted at the SoyFACE facility at the University of Illinois, Urbana-Champaign (40°02′ N, 88°14′ W, 228 m above sea level), where large plots of soybean are exposed to elevated CO_2 or elevated O_3, singly and in combination, but otherwise experience natural conditions. SoyFACE consists of 16 20-m-diameter octagonal plots (total area 282.8 m^2) of soybean distributed in four randomized blocks (e.g. Ainsworth *et al.* 2004). Each experimental plot was circumscribed by a segmented octagon of pipes that injected CO_2, O_3 or a combination of both gases above the soybean canopy (Miglietta *et al.* 2001). The rate and position of gas release were automatically and continuously altered with wind speed and direction to maintain the desired enrichment within the plot. The experimental plots were separated by at least 100 m to prevent cross-contamination of CO_2 and O_3 (Nagy *et al.* 1992).

Within each block, one control plot experienced current ambient CO_2 (~370 μmol mol^{-1}) and O_3, a second plot was fumigated to a target CO_2 concentration of 550 μmol mol^{-1}, a third plot was fumigated to a target O_3 concentration of 26% above ambient levels (average daily exposure from 1000–1800 h: 52 nmol mol^{-1}) and a fourth plot was fumigated simultaneously with both CO_2 and O_3. Plants were fumigated during daylight hours for the entire growing season. One-minute average CO_2 and O_3 were within 20% of the target for >95% of the time. At current rates of anthropogenic emissions, the targets for CO_2 and O_3 represent the atmospheric concentrations predicted for 2050 (Prather *et al.* 2001).

Soybean (cv. 93B15; Pioneer Hi-Bred, Johnston, IA, USA) was planted at 0.38 m row spacing in May 2005. This variety is typical of those grown in commercial production in Illinois. According to standard agronomic practice in this region, soybean was rotated annually with corn and was not fertilized.

Insect infestation

Pre-reproductive plants in each experimental plot were infested with Japanese beetles. Two weeks before infestation, two 1 m^2 plots of soybean within each octagonal treatment plot were encased individually in mesh cages (1 m^3) supported by PVC pipes. Cages were used to limit movement of herbivores and prevent damage to the plants prior to the experiment. The plastic mesh covering (mesh size: 1×4 mm) prevented the movement of most insects and had relatively little effect on microenvironment inside the cages. Radiation passing through the mesh was reduced by 14%, and the difference in air temperature and relative humidity inside and outside of the cages was <1% (Supplementary Fig. S1).

Japanese beetles were collected from soybean plants adjacent to the experimental plots, and deprived of food for 24 h prior to infestation. For each plot, 30 female and 30 male adult beetles were released within one of the two cages enclosing plants (damaged). The remaining cage contained plants that received no beetles (undamaged). Most beetle damage occurred on the top three trifoliates. Three days (30 June 2005) and 2 weeks (1 July 2005) after infestation, locally damaged leaves were collected to compare short- and long-term effects of herbivory. Fully expanded leaves were scored in six damage classes (5, 10, 20, 30, 40 and 50%), and one locally damaged leaf in the 5–10% damage class was collected on the first sampling date and one locally damaged leaf in the 20–30% damage class was collected on the second sampling date. Greater damage necessitated collecting leaves in a higher damage class on the second date. For a given sampling date, individual leaves collected experienced the same amount of damage in ambient and elevated CO_2. An undamaged leaf of the same developmental stage was collected from all control and treatment plots. One leaf was harvested from three separate plants in each cage, and the leaves were pooled to create a single sample per plot. Tissue was flash-frozen in liquid nitrogen and stored in a –80 °C freezer. This complete experiment was

replicated four times in the field (four plots for each treatment), resulting in 64 tissue samples ($N = 4$).

RNA preparation and microarray hybridization

Total RNA was extracted from each of the 64 frozen tissue samples using a guanidine thiocyanate acid phenol-based method (Chomczynski & Sacchi 1987). RNA integrity was verified on a 1.2% formaldehyde agarose gel (Sambrook *et al.* 1989) and with a microfluidic visualization tool (Bioanalyzer; Agilent Technologies, Palo Alto, CA, USA, http://www.algilent.com). Methods for the preparation of cRNA from mRNA, as well as the subsequent steps leading to hybridization and scanning of the soybean GeneChip arrays, were performed as in Ko & Han (2004) and Ko *et al.* (2004). The microarrays were hybridized and scanned by Keck Center for Comparative and Functional Genomics at the University of Illinois (http://www.biotech.uiuc.edu/centers/Keck/).

Probe signal intensities were processed with the Affymetrix MicroArray Suite software package (MAS 5.0), and the resulting data files containing raw intensities were imported into an open-source software package (Bioconductor, R; http://www.bioconductor.org/) where data were checked for normality and outliers; subsequent normalization of raw data and estimation of signal intensities were made with the robust multichip average method (Bolstad *et al.* 2003). Average expression values were calculated using the *limma* package in R (Irizarry *et al.* 2003). Expression data were analysed with a three-way analysis of variance (ANOVA) with FDR adjusted *P* values PROC Mixed in SAS (SAS Institute, Inc., Cary, NC, USA). To avoid type I errors, a *P* value of <0.01 was used for data presented in the figures. To reduce false positives while presenting a more easily interpreted number of genes, a more stringent *P* value of <0.001 was used for transcripts presented in the tables. Genes were classified into functional categories with a visualization tool [Mapman; http://gabi.rzpd.de/projects/MapMan; Thimm *et al.* (2004)] that was annotated for soybean by Gillespie (unpublished results). A complete list of significant transcript fold changes can be found in the Supplementary Table S1 ($P < 0.01$).

Real-time RT-PCR

The expression levels of genes coding for lipoxygenase (*LOX*) and 1-aminocyclopropane-1-carboxylate (*ACC*) synthase were confirmed with quantitative real-time RT-PCR (qRT-PCR), with *actin* as an internal standard. This procedure was performed on individual RNA samples from each plot previously used to make the RNA pools hybridized on the microarray. Total RNA (3 μg) was used as the starting material for the qRT-PCR experiments. The first-strand cDNA synthesis was carried out with the 'SuperScript' kit (Invitrogen Technologies, Carlsbad, CA, USA) using oligo-dT as primer. Primer sequences for the selected genes can be found in Supplementary Table S2. Reactions were carried out using 10 μL of the 'Syber green PCR master mix' (Applied Biosystems, Foster City, CA, USA), with 800 nM of primer, in the '7500' instrument (Applied Biosystems). The PCR was initiated with incubation to 95 °C for 10 min to activate the enzyme. Then, the following cycle was repeated 40 times: 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. The CT values were quantified and analysed according to Livak & Schmittgen (2001) with the Applied Biosystems software by averaging the four independently calculated normalized expression values that were duplicated on the plate for each treatment (RT-7500; Applied Biosystems). The responses of transcripts to the treatments were similar in direction, but were of lower magnitude when measured by microarray than by real-time PCR. For example, the response of *LOX* to the treatments was ~39% lower when measured by microarray compared to real-time PCR.

RESULTS

Transcriptome

Gene expression in soybean leaves was strongly affected by Japanese beetle herbivory, and this change in gene expression was amplified when herbivory occurred on leaves grown under elevated CO_2 or elevated O_3. Three days after leaves under ambient atmospheric conditions were infested with Japanese beetles, 1126 transcripts were significantly affected (Fig. 1a; $P < 0.01$). Exposure to elevated CO_2 or elevated O_3 increased the number of transcripts expressed in beetle-damaged plants. Three days post-infestation, beetle damage in elevated CO_2 alone or in combination with elevated O_3 affected the greatest numbers of transcripts (2847 and 3062, respectively; Fig. 1a,c; $P < 0.01$). Smaller numbers of genes (1731) were regulated in beetle-damaged leaves exposed to elevated O_3 compared to damaged leaves exposed to elevated CO_2 or the combination of elevated CO_2 plus elevated O_3 (Fig. 1a–c; $P < 0.01$). The number of genes affected in common by beetle damage and changes in atmospheric composition was relatively small (B plus CO_2, 379; B plus O_3, 466; B plus CO_2 plus O_3, 272; Fig. 1a–c; $P < 0.01$).

Chronically regulated transcripts

The effect of herbivory by Japanese beetles on gene transcription in soybean leaves diminished with time; for leaves grown in ambient atmosphere or any combination of elevated CO_2 and O_3, only 90 genes remained affected by herbivory after 2 weeks compared to 1126 genes that were affected 3 d after herbivory (Supplementary Table S1; $P < 0.01$). Of these 90 genes, the 45 in Table 1 represent those genes that were chronically affected by herbivory when leaves were grown in one or more background atmospheres.

Of the 45 genes chronically affected by herbivory, 13 were of unknown function and six represented transcripts related to the phenylpropanoid and flavonoid biosynthesis pathways (Table 1; $P < 0.01$). These transcripts, including those

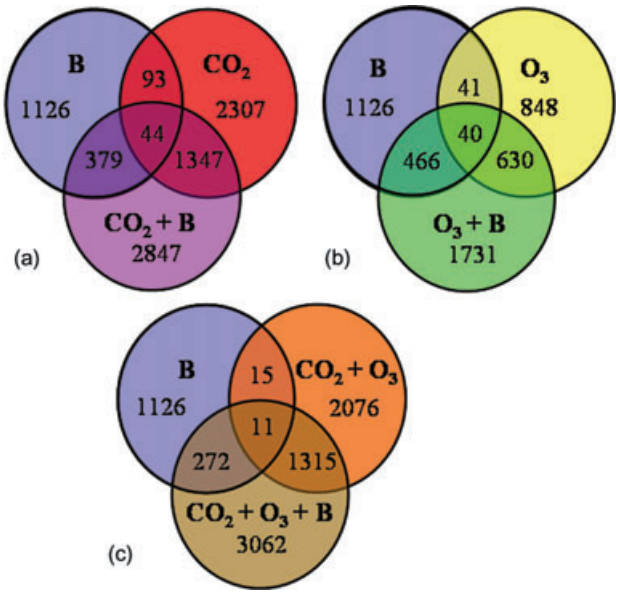

Figure 1. The effect of damage by Japanese beetles (B), growth in elevated CO_2 or elevated O_3 and the combination of beetle damage and altered atmospheric composition on transcription of genes in soybean leaves. Values represent the number of transcripts differentially expressed 3 d after infestation by Japanese beetles relative to un-infested leaves growing in ambient atmospheric conditions, or genes differentially expressed in leaves grown in elevated CO_2, elevated O_3 or the combination of elevated CO_2 plus elevated O_3 ($P < 0.01$). (a) Transcripts differentially expressed following damage by beetles under ambient atmospheric conditions, or for leaves grown in elevated CO_2 without beetle damage, or following beetle damage and growth in elevated CO_2; (b) transcripts differentially expressed for leaves grown in elevated O_3 without beetle damage, or following beetle damage and growth in elevated O_3; (c) transcripts differentially expressed for leaves grown in the combination treatment of elevated CO_2 plus elevated O_3 without beetle damage, or following beetle damage and the combination treatment of elevated CO_2 plus elevated O_3.

encoding caffeoyl-CoA *O*-methyltransferase, isoflavone reductase homolog 1, putative cinnamyl alcohol dehydrogenase, chalcone *O*-methyltransferase, *S*-adenosyl-L-methionine : daidzein 7-0-methyltransferase, were up-regulated after beetle damage, and the accumulation was intensified by exposure to elevated O_3 and dampened by exposure to elevated CO_2 (Table 1; $P < 0.01$).

In ambient atmosphere, only 11 genes that were affected 3 d after herbivory also were affected after 2 weeks, and were considered chronically affected by herbivory (Table 1; $P < 0.01$). Because relatively few genes continued to be affected by herbivory after 2 weeks, only data from the early time point were analysed further.

Effects of tropospheric chemistry in the absence of beetles on herbivory-related plant transcription

Plant nutrient content and constitutive defences can influence host plant suitability, and thus, an insect's decision to feed. Considerably greater numbers of transcripts involved in primary metabolism were altered in undamaged plants under elevated CO_2 (alone and in combination with elevated O_3) compared to elevated O_3 alone (Figs 1a and 2a; Supplementary Table S1; $P < 0.01$). Transcripts involved in starch metabolism consistently were up-regulated by exposure to elevated CO_2, including glucose-1-phosphate adenylyltransferase large subunit, while starch degradation transcripts were down-regulated (glucan water dikinase, β-amylase, α-amylase). In addition, transcripts governing many aspects of nitrogen metabolism were down-regulated under elevated CO_2 (four ferredoxin-dependent glutamate synthases and one NADH-dependent glutamate synthase), although β-2 cytosolic glutamine synthetase and nitrite reductase were up-regulated (Figs 1a & 2a; Supplementary Table S1; $P < 0.01$). Exposure of undamaged plants to elevated O_3 elicited no significant changes in transcription of genes associated with starch, sucrose or nitrogen metabolism (Fig. 2a; Supplementary Table S1; $P < 0.01$). Our results suggest that, absent any changes in plant genetic composition, elevated CO_2 levels will substantially alter the nutritional value of soybeans for insects prior to damage, while O_3 will have less of an effect.

Before induced defences become operational, components of secondary metabolism can act as constitutive defences affecting insect performance and survival. Undamaged plants exposed to elevated O_3 strongly up-regulated genes in phenylpropanoid and flavonoid metabolism that may function in plant defence, while many of these same transcripts were down-regulated in elevated CO_2 (Fig. 2b; Supplementary Table S1; $P < 0.01$). Twenty-eight transcripts related to flavonoid/phenylpropanoid production were down-regulated in undamaged plants exposed to elevated CO_2 (Fig. 2b; Supplementary Table S1; $P < 0.01$) including several flavonol 3-*O*-glucosyltransferase-like proteins, putative flavonol reductases, flavonol synthases, isoflavone reductases, 4-coumarate-CoA ligase-like proteins, ferulate-5-hydroxylase, *O*-methyltransferases, cinnamoyl CoA reductase-like proteins and a putative cinnamyl alcohol dehydrogenase. Isoprenoid transcription in both the non-mevalonate pathway and the mevalonate pathway was significantly up-regulated (putative violaxanthin de-epoxidase precursor, hydroxymethylglutaryl-CoA synthase and geranylgeranyl pyrophosphate synthase) while transcripts involved in terpenoid (β-amyrin synthase, limonene cyclase-like protein, limonene cyclase, putative) and wax [very-long-chain fatty-acid-condensing enzyme (*CUT1*) and *CER1*-like protein] metabolism were significantly down-regulated (Fig. 2b; Supplementary Table S1; $P < 0.01$).

In the absence of herbivory, elevated O_3 increased accumulation of transcripts in flavonoid and phenylpropanoid metabolism, while other components of secondary metabolism were unchanged. The same transcripts that were down-regulated in elevated CO_2 were significantly up-regulated in elevated O_3, as well as new transcripts previously unaffected, including anthocyanin acyltransferase, chalcone isomerase, chalcone synthase, flavanone 3-hydroxylase-like

Table 1. Soybean transcripts chronically altered after attack (log_2-fold change)

Gene description	Affymetrix ID	3 d Post-infestation				2 Weeks post-infestation			
		B+	B + CO_2	B + O_3	B + CO_2 + O_3	B+	B + CO_2	B + O_3	B + CO_2 + O_3
1-Deoxy-D-xylulose 5-phosphate synthase 2	Gma.7728.1.S1_at	–	–	2.23	–	–	–	2.49	–
Amino acid permease 6	Gma.7487.3.S1_at	0.75	0.86	–	–	0.71	–	–	–
Aspartic-type endopeptidase/pepsin A	GmaAffx.49768.1.S1_at	–	–	0.90	–	–	–	1.10	–
Vesicle-associated membrane protein 72	GmaAffx.5717.1.S1_at	0.39	0.68	–	0.83	0.47	–	–	–
ATP binding/kinase/protein kinase/protein serine/threonine kinase/protein–tyrosine kinase	GmaAffx.16476.1.S1_at	–	–	0.72	–	–	–	0.84	–
Caffeoyl-CoA *O*-methyltransferase	GmaAffx.54524.1.S1_at	–	–	3.02	–	–	–	3.66	–
Calcium ion binding	Gma.5733.1.S1_at	–	–	0.81	–	–	–	0.92	–
Catalytic	Gma.1601.1.A1_at	1.27	–	–	–	1.40	–	–	–
Catalytic/Hydrolase	GmaAffx.89629.1.S1_s_at	–	–	1.46	–	–	–	1.93	–
Conserved hypothetical protein	Gma.3658.1.S1_at	–	–	0.80	–	–	–	1.03	–
Cytochrome P450	GmaAffx.30652.1.S1_at	–	–	1.96	–	–		2.20	–
Disease-resistance-responsive family protein	GmaAffx.91568.1.S1_s_at	1.85	–	3.92	–	–		3.45	–
DNA-binding protein WRKY2	Gma.7467.1.A1_at	0.64	–	1.15	–	0.70		1.07	–
DNA-binding protein WRKY2	GmaAffx.48074.1.S1_x_at	0.63	–	1.24	1.14	–		0.91	–
Gibberellin 2-oxidase	Gma.2486.1.S1_at	1.10	–	1.27	–	–		1.50	–
Heat shock transcription factor 34	Gma.2342.1.S1_at	–	–	1.89	–	–		1.79	–
Hypothetical protein	GmaAffx.9040.1.S1_at	1.10	–	1.62	–	–		1.34	–
Hypothetical protein LOC_Os11g36070	GmaAffx.79411.2.S1_at	–	–	0.74	–	–		0.86	–
Isoflavone reductase homolog 1	Gma.16735.2.S1_at	–	–	3.32	–	–		3.82	–
Isoflavone reductase homolog 1	GmaAffx.90806.1.S1_at	–	–	2.60	–	–		3.44	–
OSJNBa0038O10.14	GmaAffx.41564.2.S1_s_at	2.23	–	3.14	–	1.93			–
OSJNBa0038O10.14	GmaAffx.90752.1.S1_at	2.20	–	3.02	–	1.93			–
Putative cinnamyl alcohol dehydrogenase	GmaAffx.86175.1.S1_at	–	–	1.41	–	–		1.74	–
Putative heat shock protein	Gma.3452.1.S1_at	–	–	2.27	–	–		2.73	–
Putative leucine-rich repeat protein	Gma.4507.1.S1_at	–	–	0.68	–	–		0.85	–

Table 1. *Continued*

Gene description	Affymetrix ID	3 d Post-infestation				2 Weeks post-infestation			
		B+	B + CO_2	B + O_3	B + CO_2 + O_3	B+	B + CO_2	B + O_3	B + CO_2 + O_3
Putative RNA-binding protein	GmaAffx.91863.1.S1_at	–	–	1.10	–	–		1.10	–
Receptor-like protein kinase	Gma.2575.1.S1_at	–	–	2.45	–	–		1.67	–
Rhodopsin-like receptor	GmaAffx.18316.2.S1_at	–	–	–	–	–			–0.68
S-adenosyl-L-methionine:daidzein 7-0-methyltransferase	GmaAffx.92998.1.S1_s_at	–	–	1.73	1.89	–		1.71	–
Similar to serine/threonine kinases	Gma.6650.1.S1_at	–	–	1.57	–	1.31		1.97	–
UDP-glucosyltransferase HRA25	GmaAffx.53338.1.S1_at	1.32	1.86	–	–	1.47			–
Unknown	Gma.15636.2.S1_x_at	2.38	–	3.92	–	2.21		3.13	–
Unknown	GmaAffx.54553.1.S1_at	–	–	2.42	–	–		2.16	–
Unknown	GmaAffx.90383.1.S1_at	–	–	2.06	–	–		1.96	–
Unknown protein	Gma.2842.1.S1_at	–	–	1.56	–	1.16		1.72	–
Unknown protein	GmaAffx.33258.1.S1_at	–	–	3.04	2.71	–		2.47	–
Unknown protein	GmaAffx.70823.2.S1_at	–	–	1.37	–	–		1.72	–
Unknown protein	GmaAffx.76391.1.S1_s_at	–	–	1.41	–	–		1.95	–
Unknown protein	GmaAffx.90685.1.S1_x_at	0.81	–	1.21	–	1.16			–
Unknown protein	GmaAffx.90861.1.S1_at	–	–	3.70	–	2.53		3.84	–
Unknown	Gma.3364.1.S1_at	–	–	2.49	–	–		3.02	–
Unknown	Gma.4492.1.S1_at	0.71	0.97	0.79	–	0.71			–
Unknown	GmaAffx.36338.1.S1_at	0.99	1.29	1.19	1.59	1.01			–
Unknown	GmaAffx.52508.1.S1_at	–	–	1.84	–	–		1.66	–
CHOMT_MEDSA Isoliquiritigenin 2′-*O*-methyltransferase (chalcone *O*-methyltransferase) (ChOMT)	GmaAffx.91504.1.A1_s_at	–	–	2.21	–	–		2.27	–

Values and colours represent the fold change (log_2) for genes affected by herbivory (FDR $P < 0.001$).

−5 −3 −1.5 −0.75 – +0.75 +1.5 +3 +5

Table 2. Specific soybean transcripts altered 3 d after attack by Japanese beetles (log_2 fold change, FDR $P < 0.001$)

Gene description	Affymetrix ID	Beetle	Beetle + CO_2	Beetle + O_3	Beetle + CO_2 + O_3
Oxidative-stress-related genes					
Cationic peroxidase	GmaAffx.36514.1.S1_at	2.29	2.79	2.54	3.13
Cationic peroxidase	GmaAffx.50446.2.S1_at	2.36	2.76	2.55	2.86
Class III peroxidase	GmaAffx.8712.1.S1_s_at	4.46	5.11	4.74	5.28
Class III peroxidase	GmaAffx.90443.1.S1_s_at	4.42	4.95	4.52	5.13
Class III peroxidase	GmaAffx.90703.1.A1_at	3.83	4.16	4.01	4.39
Class III peroxidase	GmaAffx.90703.1.S1_at	–	1.23	1.11	1.83
Peroxidase 1 precursor	Gma.6251.1.S1_at	3.04	4.09	3.12	–
GSTX6_SOYBN	GmaAffx.88762.1.S1_at	2.97	–	3.90	–
GSTX6_SOYBN	GmaAffx.88762.1.S1_x_at	2.85	–	3.75	–
Glutathione *S*-transferase GST 11	Gma.8516.1.S1_at	–	1.13	–	–
Glutathione *S*-transferase GST 14	Gma.1502.1.S1_at	1.76	–	2.07	–
Glutathione *S*-transferase GST 14	Gma.1502.2.S1_a_at	1.52	–	1.90	–
Oxidoreductase	GmaAffx.27632.1.A1_at	1.83	2.32	–	–
Oxidoreductase	GmaAffx.90484.1.S1_at	–	0.55	0.29	0.68
Thioredoxin-like 4	Gma.4359.2.S1_at	2.59	3.09	2.24	–
Heme oxygenase 1	GmaAffx.52114.1.S1_at	4.80	5.44	4.47	4.66
Photosynthesis-related genes					
Rubisco-associated protein	Gma.1303.1.S1_at	1.62	–	–	–
Oxygen-evolving system of photosystem II	Gma.10731.1.S1_x_at	–0.25	–0.34	–0.34	–0.51
Photosystem I reaction centre subunit XI, putative	Gma.1316.5.S1_at	–0.37	–	–0.40	–
Photosystem II family protein	Gma.13377.2.S1_a_at	–0.65	–1.08	–0.89	–1.80
Lipid degradation/beta oxidation					
3-Ketoacyl-CoA thiolase	GmaAffx.72944.1.S1_at	1.57	2.05	1.63	2.22
3-Ketoacyl-CoA thiolase	GmaAffx.72944.1.S1_x_at	1.26	1.49	1.13	–
Acyl-CoA oxidase	Gma.254.1.S1_at	–	–	–	–
Triacylglycerol lipase	Gma.4537.1.S1_at	1.06	1.70	1.04	1.54
Transcription factors					
MYB7	GmaAffx.92964.1.S1_at	2.60	–	3.86	–
NAC domain protein NAC3	GmaAffx.90496.1.S1_s_at	1.89	1.85	2.38	2.57
NAC domain protein NAC3	GmaAffx.57970.1.S1_at	2.72	2.35	2.81	–
WRKY78	Gma.3730.2.S1_a_at	1.53	1.68	1.79	–
WRKY78	GmaAffx.91768.1.S1_s_at	1.60	1.65	1.94	–
WRKY86	Gma.16547.1.S1_at	1.20	–	1.75	–
Putative WRKY transcription factor	Gma.3996.1.S1_at	–	–	1.07	–
Putative WRKY transcription factor	Gma.3996.1.S1_x_at	–	–	1.01	–
Hormone-signalling-related genes					
Ethylene related					
Ethylene-induced epoxide hydrolase	GmaAffx.26545.1.S1_at	–	1.28	–	1.35
Ethylene-induced epoxide hydrolase	GmaAffx.6790.1.S1_at	1.49	1.70	–	–
Salicylic acid related					
Salicylic-acid-binding protein 2	GmaAffx.1242.1.A1_at	1.56	–	–	–
Jasmonic acid related					
Lipoxygenase (*LOX*)	GmaAffx.70690.1.S1_at	1.40	1.91	2.14	2.98
LOX	Gma.1.1.A1_at	0.74	–	–	–
LOX	Gma.1.1.S1_at	4.64	5.79	–	–
LOX	GmaAffx.27496.2.S1_at	1.69	2.33	–	–

Table 2. *Continued*

Gene description	Affymetrix ID	Beetle	Beetle + CO_2	Beetle + O_3	Beetle + CO_2 + O_3
LOX	GmaAffx.73072.1.S1_at	2.12	2.95	–	–
LOX	Gma.11166.1.S1_s_at	0.49	0.43	–	–
LOX	Gma.16500.1.S1_at	2.34	3.05	2.24	–
LOX	GmaAffx.60245.1.S1_at	0.51	–	0.77	–
Allene oxide synthase (*AOS*)	GmaAffx.83504.1.A1_at	1.72	–	1.82	–
AOS	GmaAffx.53768.1.S1_at	1.11	–	–	–
AOS	GmaAffx.86334.2.S1_at	1.36	–	1.62	–
AOS	GmaAffx.22456.1.S1_at	1.43	–	–	–
Putative jasmonic acid regulatory protein	Gma.5331.1.S1_at	2.20	–	2.12	–
Cytochrome P450					
Cytochrome P450	GmaAffx.68456.1.S1_at	3.83	–	5.32	4.26
Cytochrome P450	GmaAffx.68456.2.S1_s_at	3.19	–	4.45	3.47
Cytochrome P450	GmaAffx.61184.1.S1_at	2.88	3.40	2.95	–
Cytochrome P450 monooxygenase CYP72B	GmaAffx.78313.1.S1_at	3.04	3.89	4.42	5.11
Cytochrome P450 monooxygenase CYP74C	GmaAffx.86334.1.S1_at	1.53	–	–	–
Cytochrome P450 monooxygenase CYP83A	GmaAffx.60645.1.S1_at	–	1.40	–	1.29
Cytochrome P450 monooxygenase CYP83A	GmaAffx.60645.2.S1_at	–	1.60	–	1.44
Cytochrome P450 monooxygenase CYP83A	GmaAffx.60645.2.S1_s_at	–	1.55	–	1.39
Cytochrome P450 monooxygenase CYP71A10	GmaAffx.93101.1.S1_at	1.24	1.82	–	–
Putative cytochrome P450	Gma.153.1.S1_at	3.50	–	5.24	4.01
Putative cytochrome P450	Gma.153.1.S1_x_at	3.76	–	5.56	4.52
Putative cytochrome P450	GmaAffx.92620.1.S1_s_at	3.58	–	5.36	4.08
Defence-/Stress-related genes					
Trypsin protease inhibitor	Gma.10904.1.S1_s_at	3.90	4.78	–	–
Trypsin protease inhibitor	Gma.10904.1.S1_at	3.03	3.52	–	–
Trypsin protease inhibitor subtype A	Gma.1048.1.S1_at	2.03	2.35	–	–
Serine proteinase inhibitor	Gma.3612.1.S1_s_at	4.98	5.10	4.93	4.35
Serine proteinase inhibitor	GmaAffx.92048.1.S1_at	–	–	1.42	1.30
Cysteine protease inhibitor (*CystPI*)	Gma.17733.1.S1_s_at	2.44	–	2.68	–
CystPI	Gma.3314.1.S1_a_at	1.64	1.66	–	–
CystPI	Gma.3314.1.S1_at	2.57	2.45	–	–
Osmotin precursor (PR 5)	Gma.2821.1.S1_at	2.97	2.10	3.88	2.70
Osmotin precursor (PR 5)	GmaAffx.92699.1.S1_s_at	3.15	–	4.64	3.40
Polyphenol oxidase (*PPO*)	GmaAffx.92650.1.S1_at	3.32	3.02	3.33	–
PPO	GmaAffx.46214.5.S1_at	3.14	2.57	3.61	–
PPO	GmaAffx.46214.1.S1_at	4.02	3.21	4.95	3.93

Table 2. *Continued*

Gene description	Affymetrix ID	Beetle	Beetle + CO_2	Beetle + O_3	Beetle + CO_2 + O_3
PPO	GmaAffx.46214.2.S1_s_at	4.95	4.61	5.15	–
PPO	GmaAffx.92267.1.S1_at	–	–	–	1.12
PPO	GmaAffx.46214.3.S1_at	3.06	2.96	3.07	–
Putative thaumatin-like protein (PR 1)	Gma.2821.2.S1_a_at	3.43	–	4.93	3.75
Thioredoxin-like 4	Gma.4359.2.S1_at	2.59	3.09	2.24	–
Disease resistance protein (CC-NBS-LRR class)	Gma.15155.1.S1_at	1.14	1.22	–	–
Thaumatin family protein (PR 1)	GmaAffx.32256.1.S1_at	–0.36	–0.35	–0.46	–0.61
Germin-like protein precursor	Gma.15727.1.S1_at	–0.96	–	–	

A complete list of significant array elements is given in Supplementary Table S1. Colour scale correlates with fold change expression.

–5 –3 –1.5 –0.75 – +0.75 +1.5 +3 +5

protein, dihydroflavonol 4-reductase, putative flavonol 3-*O*-glucosyltransferase, 2′-hydroxydihydrodaidzein reductase, caffeic acid *O*-methyltransferase II, *S*-adenosyl-L-methionine:2,7,4′-trihydroxyisoflavanone4′-*O*-methyltransferase, *N*-hydroxycinnamoyl/benzoyltransferase-like protein, caffeoyl-CoA *O*-methyltransferase and phenylalanine ammonia-lyase (Fig. 2b; Supplementary Table S1; $P < 0.01$). Other than secondary metabolism, relatively few transcripts in undamaged plants were affected by elevated O_3. The majority of other transcripts responding to elevated O_3 were stress-responsive transcripts (Supplementary Table S1; $P < 0.01$), such as thioredoxin, *LOX* and *PR10*, which were all up-regulated in undamaged plants in elevated O_3. Exposure to the combination of gases produced relatively few significant changes, with a signature similar to plants in ambient air except for transcripts associated with wax metabolism. Five wax-related transcripts were significantly down-regulated under this treatment, including (*CER1*) protein, putative very-long-chain fatty-acid-condensing enzyme (*CUT1*) and cuticle protein (*WAX2*; Fig. 2b; Supplementary Table S1; $P < 0.01$). In summary, elevated CO_2 down-regulated secondary metabolism transcripts prior to beetle damage, while O_3 up-regulated these transcripts.

Effects of beetle damage on transcription

Variation in the content of sugar, starch, nitrogen and amino acids influences the nutritional quality of plant tissues for insects. Damage by Japanese beetles under ambient atmospheric conditions had minimal impact on genes coding for components of primary metabolism. Starch and sucrose synthesis was down-regulated after beetle damage in ambient conditions (Fig. 3a; Supplementary Table S1; $P < 0.01$).

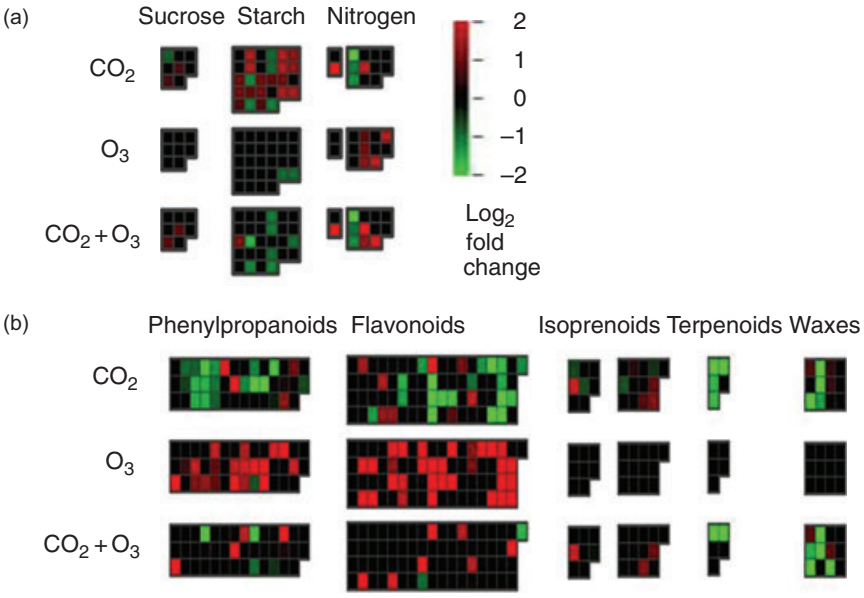

Figure 2. The individual and combined effects of elevated CO_2, elevated O_3 and elevated CO_2 plus O_3 without beetle damage on the transcription of genes that may affect leaf palatability to insects. (a) Genes regulating sucrose, starch and nitrogen metabolism may affect nutritional quality, while (b) genes regulating phenylpropanoid, flavonoid, isoflavonoid, terpenoid and wax metabolism may affect plant defence against herbivores. Up-regulated and down-regulated transcripts relative to plants grown under ambient conditions without beetles are represented by red and green, respectively, using MapMan visualization tool ($P < 0.01$).

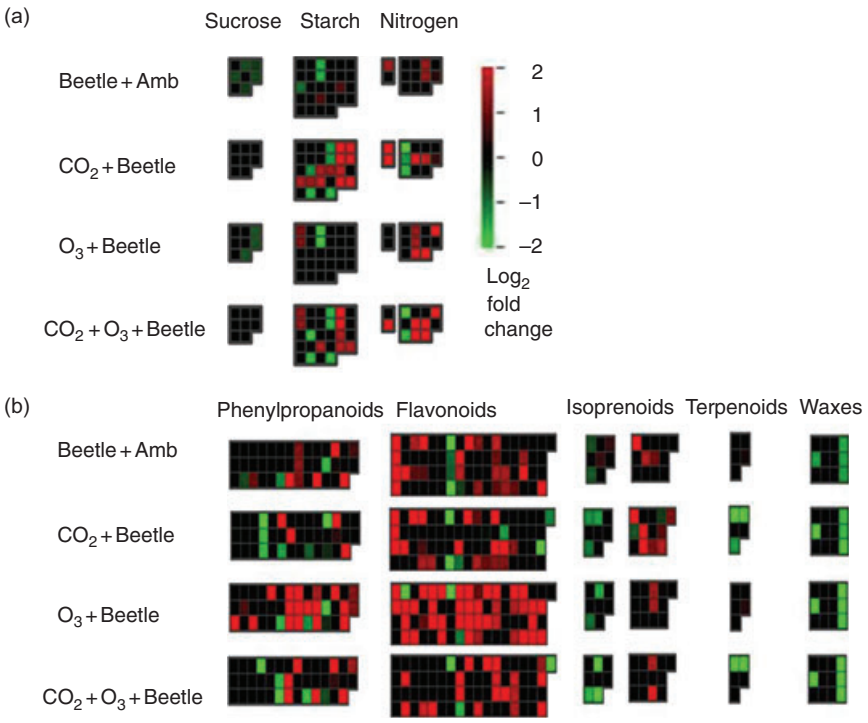

Figure 3. Effect of damage by Japanese beetles under the individual and combined effect of elevated CO_2 and elevated O_3 on the transcription of genes that may affect leaf palatability to insects. (a) Genes regulating sucrose, starch and nitrogen metabolism may affect nutritional quality, while (b) genes regulating phenylpropanoid, flavonoid, isoflavonoid, terpenoid and wax metabolism may affect plant defence against herbivores. The response of gene transcription was measured 3 d after plants were infested with beetles, and values (log_2-fold change) are expressed relative non-infested plants grown in ambient air. Up-regulated and down-regulated transcripts relative to plants grown under ambient conditions without beetles are represented by red and green, respectively, using MapMan visualization tool ($P < 0.01$).

Beetle damage to leaves grown under elevated CO_2 or elevated O_3 elicited a stronger response of gene expression than damage to leaves grown in ambient air. Beetle damage to leaves exposed to elevated CO_2 up-regulated starch biosynthesis transcripts (starch synthase-like protein, isoamylase-like protein, starch branching enzyme, ADP-glucose pyrophosphorylase and granule-bound starch synthase), while simultaneously down-regulating transcripts involved in starch degradation (phosphoglucan water dikinase and glycogen phosphorylase). In nitrogen metabolism, damage by beetles to leaves grown in elevated CO_2 alone or in combination with elevated O_3 down-regulated transcripts related to glutamate synthase (multiple NADH-dependent and ferredoxin-dependent glutamate synthases), but up-regulated nitrite reductase (Fig. 3a; Supplementary Table S1; $P < 0.01$).

Beetle damage did not interact strongly with elevated O_3, suggesting that most of the genes affected in the three-way interaction between damage, elevated CO_2 and elevated O_3 were affected by the combination of beetle damage and elevated CO_2. Transcripts coding for genes in starch metabolism were not altered by beetle damage in leaves grown under elevated O_3. Only one transcript in nitrogen metabolism was up-regulated in damaged leaves exposed to elevated O_3 (β-2 cytosolic glutamine synthetase; Fig. 3a; Supplementary Table S1; $P < 0.01$).

In contrast to genes involved in primary metabolism, beetle damage altered the expression of many genes representing several aspects of secondary metabolism, and the magnitude and direction of the response to herbivory were modified by exposure to elevated CO_2 or elevated O_3. In ambient atmosphere, beetle damage up-regulated transcripts involved in flavonoid biosynthesis (Fig. 3b; Supplementary Table S1; $P < 0.01$). Transcripts related to anthocyanin metabolism (two putative leucoanthocyanidin dioxygenases and four putative anthocyanidin synthases), dihydroflavonol metabolism (dihydroflavonol-4-reductase), flavonol metabolism (putative flavonol synthase, 2′-hydroxydihydrodaidzein reductase) and isoflavonoid metabolism (isoflavone reductase-like protein) were all up-regulated following beetle damage in ambient conditions (Fig. 3b; Supplementary Table S1; $P < 0.01$). However, some key transcripts in phenylpropanoid metabolism were down-regulated (chalcone synthase, *O*-methyltransferase, putative anthranilate *N*-hydroxycinnamoyl/benzoyltransferase and cinnamoyl-CoA reductase-like protein), while transcripts related to lignin biosynthesis were up-regulated (cinnamyl alcohol dehydrogenase, 4 *O*-methyltransferases/*S*-adenosylmethionine-dependent methyltransferase; Fig. 3b; Supplementary Table S1; $P < 0.01$).

Beetle damage in ambient air down-regulated several transcripts related to wax production [cuticle protein (*WAX2*) and two very-long-chain fatty-acid-condensing enzyme (*CUT1*), although one wax-related transcript was significantly up-regulated (*CER1*-like protein) (Fig. 3b; Supplementary Table S1; $P < 0.01$).

Exposure to elevated CO_2 had only a small effect on the response of transcription in soybean to damage by beetles. The up-regulation of transcripts involved in flavonoid and phenylpropanoid metabolism by beetle damage in ambient air was largely unchanged when damage occurred on leaves in elevated CO_2. Transcription of genes associated with isoprenoid and terpenoid metabolism was intensified by elevated CO_2. Transcripts involved in anthocyanin, isoflavonoid and dihydroflavonol biosynthesis were dampened under elevated CO_2, while those involved in flavonol

metabolism were generally intensified (Fig. 3b; Supplementary Table S1; $P < 0.01$). Transcripts related to isoprenoid metabolism from the non-mevalonate pathway [1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*)] including carotenoid-related transcripts (phytoene synthase, violaxanthin de-epoxidase precursor, zeta-carotene desaturase *ZDS2* and β-amyrin synthase) were all down-regulated after beetle attack under elevated CO_2 (Fig. 3b; Supplementary Table S1; $P < 0.01$). Elevated CO_2 resulted in the down-regulation of transcripts related to terpenoid biosynthesis in beetle-damaged leaves, including β-amyrin synthase, limonene cyclase-like protein and transcripts similar to oxidosqualene cyclase (Fig. 3b; Supplementary Table S1; $P < 0.01$).

In contrast to elevated CO_2, exposure to elevated O_3 intensified the up-regulation of flavonoids and phenylpropanoids in response to beetle damage (Fig. 3b; Supplementary Table S1; $P < 0.01$). Key genes involved in anthocyanin and chalcone biosynthesis were strongly up-regulated by the combination of beetle damage and elevated O_3 (anthocyanin acyltransferase, leucoanthocyanidin dioxygenase-like protein, chalcone isomerase and chalcone synthase; Fig. 3b; Supplementary Table S1; $P < 0.01$). Expression of transcripts related to biosynthesis of dihydroflavonols (dihydroflavonol-4-reductase *DFR1*, 2′-hydroxydihydrodaidzein reductase, flavanone 3-hydroxylase-like protein, flavonoid 3′,5′-hydroxylase-like protein and flavonol 3-*O*-glucosyltransferase-like protein), flavonols (flavonol synthase) and isoflavonoid (isoflavone reductase) also were significantly increased in damaged leaves exposed to O_3 (Fig. 3b; Supplementary Table S1; $P < 0.01$). Like the flavonoids, the expression of many phenylpropanoid-related transcripts was intensified by damage to leaves in elevated O_3 (caffeic acid *O*-methyltransferase, *N*-hydroxycinnamoyl/benzoyltransferase-like protein, *S*-adenosyl-L-methionine, daidzein 7-0-methyltransferase, cinnamyl-alcohol dehydrogenase-like protein, 4-coumarate : coenzyme A ligase, cinnamyl alcohol dehydrogenase, cinnamoyl CoA reductase-like protein, *O*-methyltransferase, ferulate-5-hydroxylase and hydroxycinnamoyl transferase; Fig. 3b; Supplementary Table S1; $P < 0.01$).

Damage to leaves grown under elevated CO_2 plus elevated O_3 ameliorated the responses in plant secondary metabolism, producing transcriptional signatures similar to those resulting from beetle damage in ambient air (Fig. 3b; Supplementary Table S1; $P < 0.01$).

Signalling

The octadecanoic pathway produces jasmonic acid, a hormone that is central to the induction of anti-herbivore defences. Several key transcripts in the octadecanoic pathway, including *LOX*, allene oxide synthase (*AOS*) and allene oxide cyclase (*AOC*), were up-regulated in soybean after beetle damage (Fig. 4a–c; $P < 0.01$; Table 2; $P < 0.001$). Defence-related transcripts such as protease inhibitors (*PI*s) and polyphenol oxidases (*PPO*), important defence compounds, were also up-regulated after beetle damage in ambient air compared to undamaged plants, as were transcripts coding for pathogenesis-related (PR) proteins, osmotin precursor (*PR 5*) and one putative thaumatin-like protein (*PR 1*; Fig. 4d,e; $P < 0.01$; Table 2; $P < 0.001$). Another large group of transcripts induced by beetle damage were those coding for cytochrome P450s involved in the biosynthesis of secondary metabolites and hormones, including jasmonic acid (Table 2; $P < 0.001$). Only a single salicylic-acid-binding protein 2 transcript was induced after Japanese beetle feeding (Table 2; $P < 0.001$).

Exposure to elevated CO_2 strongly dampened the induction of genes governing defence signalling and the actual production of defence compounds after beetle damage. Constitutive levels of transcription for the signalling genes in the octadecanoid pathway, *LOX* and *AOC*, as well as *ACC* synthase involved in ethylene production, were down-regulated by elevated CO_2 (Fig. 4a–c; $P < 0.01$; Table 2; $P < 0.001$). Except for *ACC* synthase, these genes and the genes coding for cysteine protease inhibitor (*CystPI*), chalcone synthase and polyphenol oxidase were strongly induced by beetle infestation, and this induction was dampened or absent when plants were grown in elevated CO_2 (Fig. 4a–e; $P < 0.01$; Table 2; $P < 0.001$). Exposure of beetle-damaged plants to elevated CO_2 intensified the up-regulation of transcripts involved in lipid degradation (Table 2; $P < 0.001$), suggesting that the genes involved in this lipid-based signalling pathway were altered.

In contrast to the antagonistic effect of elevated CO_2 and beetle damage, elevated O_3 intensified plant defence responses on the transcriptional level; compared to undamaged leaves in ambient air, *LOX*, *CystPI*, *CHS* and *PPO* all were up-regulated in elevated O_3 with or without beetles (Fig. 4; $P < 0.01$; Table 2; $P < 0.001$).

Several transcription factors previously shown to be induced after caterpillar feeding were also induced after beetle damage in soybean (Table 2; $P < 0.001$). These included several WRKYs and MYBs (Hui *et al.* 2003; Izaguirre *et al.* 2003; Ralph *et al.* 2006), as well as a novel transcription factor family, NACs, not previously demonstrated to be induced by herbivory (Table 2; $P < 0.001$). Exposure to elevated O_3 intensified the induction of these transcription factors, while the effect of elevated CO_2 was less consistent (Table 2; $P < 0.001$).

DISCUSSION

Exposure to elevated CO_2 and O_3 altered the transcriptional response to beetle damage and may influence the susceptibility of plants to herbivores. Growth in elevated CO_2 down-regulated aspects of secondary metabolism, plant defences and signalling-related transcripts, possibly leaving plants more vulnerable to herbivores. In contrast, exposure to elevated O_3 elicited a transcriptional response that was similar to insect damage, up-regulating defence-related transcripts in secondary metabolism. Japanese beetle damage induced plant defence transcripts in the field; this induction was ameliorated by exposure to elevated CO_2. Relatively few transcripts were regulated after 2 weeks of continuous herbivory by Japanese beetle

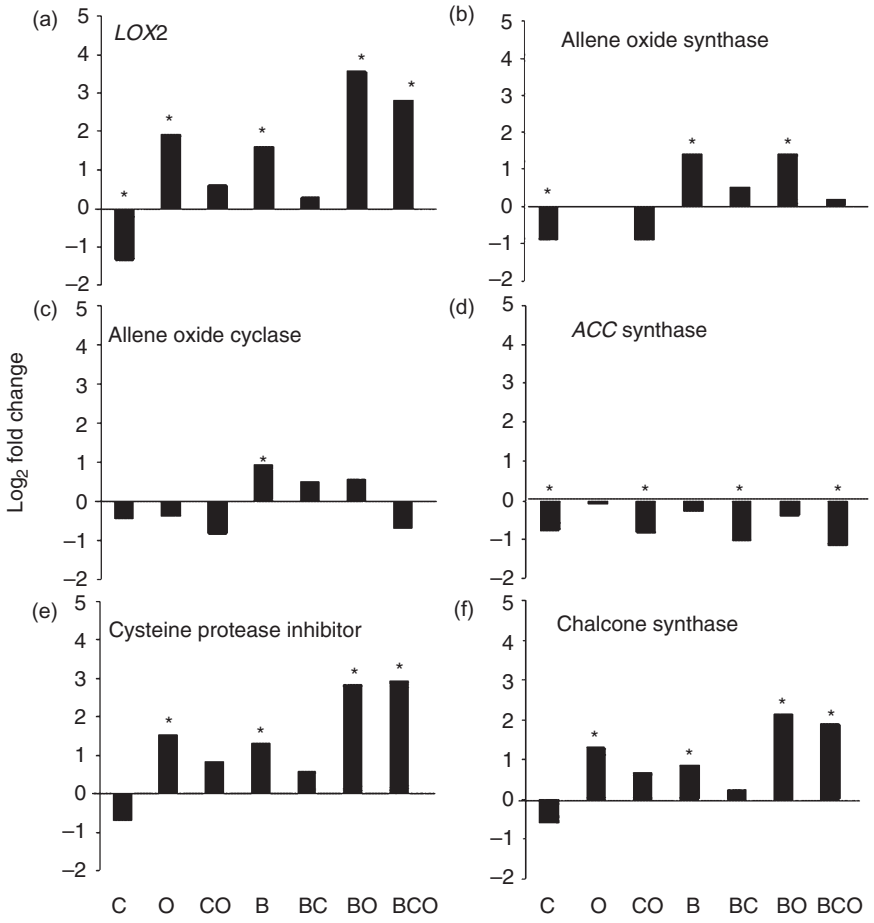

Figure 4. The expression level from microarrays of (a) lipoxygenase (*LOX*), (b) allene oxide synthase (*AOS*), (c) allene oxide cyclase (*AOC*), (d) 1-aminocyclopropane-1-carboxylate (*ACC*) synthase, (e) cysteine protease inhibitor (*CystPI*) and (f) chalcone synthase transcripts to elevated CO_2 (C), elevated O_3 (O) or damage by Japanese beetles (B), applied singly or in combination. The bars indicate log_2-fold change from the values for undamaged leaves grown in ambient air; asterisks indicate that values are significantly different at $P < 0.01$.

under all treatments, suggesting that the majority of transcriptional regulation of plant responses to herbivory happens relatively quickly after damage.

A substantial increase in the susceptibility of soybean exposed to elevated CO_2 to herbivory has been attributed to elevated levels of leaf sugars (Hamilton *et al.* 2005), which stimulates feeding by Japanese beetles (Potter & Held 1999). Consuming foliage grown under elevated CO_2 also increased fecundity of Japanese beetles, but this response could not be explained by greater sugar content because beetles that were fed leaves supplemented with sugar did not exhibit the same increase in fecundity (O'Neill *et al.*, 2008). In so far as the reduction of gene transcripts for *CystPI*s reduced the content of this important soybean defence compound (Zavala, unpublished results), this change in gene transcription may explain the greater consumption of foliage by beetles under elevated CO_2 (Hamilton *et al.* 2005), because host plants would be more digestible.

In contrast to elevated CO_2, exposure to elevated O_3 worked in concert with beetle damage to enhance defence transcripts and presumably aspects of plant defence. Future changes in the chemical composition of the troposphere caused by human activities may modulate well-established patterns of inducible defence. It will be important to explore genetic variation in soybean and other crop plants to identify genotypes that are 'pre-adapted' to future conditions of elevated CO_2, elevated O_3 and other aspects of global change.

The expression of transcripts involved in signalling pathways and the induction of defences to herbivores were dampened by elevated CO_2 (Fig. 4; $P < 0.01$; Table 2; $P < 0.001$). The octadecanoid pathway controls the synthesis and accumulation of jasmonic acid, a hormone that regulates the induction of plant defence responses (Ryan 1990; Farmer, Johnson & Ryan 1992). Transcripts of several key regulatory enzymes involved in the octadecanoic pathway were down-regulated in plants grown under elevated CO_2, indicating a dampening of induced defences. By reducing the up-regulation of genes in the octadecanoid pathway following herbivore induction, exposure to elevated CO_2 compromised the ability of soybean to produce jasmonic acid and mount an effective defence. Consistent with these findings, examination of constitutive levels of jasmonic acid in soybean leaves grown in elevated CO_2 in growth chambers revealed that jasmonic acid metabolite levels were reduced compared to plants grown in ambient atmosphere (Casteel *et al.*, unpublished results).

The abundance of transcripts directly related to plant defences was greatly reduced under elevated CO_2 in a manner consistent with decreased resistance to herbivory. Chalcone synthase, *PPO* and *CystPI*s transcripts were

induced after beetle damage, and the magnitude of this induction was dampened when beetles attacked leaves exposed to elevated CO_2 (Fig. 4e,f; $P < 0.01$; Table 2; $P < 0.001$). Chalcone synthase is a key enzyme in phenylpropanoid biosynthesis, catalyzing the production of flavonoids and isoflavonoids that have been implicated in defence against herbivores (Carraopanizzi & Kitamura 1995). Polyphenol oxidase transcripts and metabolites are induced after herbivory, specifically generating *o*-quinones which are thought to covalently modify and cross-link dietary proteins during herbivore feeding, resulting in decreased amino acid assimilation (Felton *et al.* 1989, 1992). The amino acids most susceptible to attack by *o*-quinones are those most limiting in herbivore diets (lysine, histidine, cysteine and methionine); by reducing the availability of dietary amino acids, the production of *o*-quinones negatively affecting herbivores by increasing mortality and reducing growth rates (Wang & Constabel 2004).

Perhaps even more important in the context of beetle herbivory was the effect of elevated CO_2 on expression of *CystPIs*. The accumulation of *PI*s is elicited by various biotic and abiotic stresses, including mechanical wounding and insect attack as well as signalling molecules such as systemin, methyl jasmonate and larval oral secretions (O'Donnell *et al.* 1996; Koiwa, Bressan & Hasegawa 1997; Korth & Dixon 1997; Ryan 2000). *PI*s inhibit proteases in the insect gut, which prevents the acquisition of proteins, resulting in reduced growth and survivorship (Ryan 1990; Zhao *et al.* 1996; Jongsma & Bolter 1997; Zavala *et al.* 2004). By lowering levels of the transcript that produces these antidigestive proteins, leaves grown in elevated CO_2 potentially are more digestible to beetles than those grown in ambient air. Soybean cysteine *PI*s are active against beetle proteases, such as those in the gut of adult *Diabrotica virgifera* (Western corn rootworm beetles; Zhao *et al.* 1996). CO_2-associated reductions in *PI* and *PPO* content of foliage may explain the higher fecundity and longevity of Japanese beetles on soybean grown in elevated CO_2 (O'Neill *et al.*, in press). While decreased soybean defence is consistent with higher numbers of Japanese beetles in elevated CO_2 plots, actual quantification of jasmonic acid and plant defences, such as *PI*s and *PPO*, will be necessary to confirm the scenario construed from transcription analysis.

Elevated O_3 intensified transcriptional responses to beetle damage, including greater mRNA abundance of defence and antioxidant-related transcripts (Fig. 3). Increased antioxidants and stress-response transcripts in the phenylpropanoid pathway under elevated O_3 have been observed previously (Wustman *et al.* 2001; Gupta *et al.* 2005) and made up the majority of induced transcript exposure to elevated O_3 in this study. Specific responses of Japanese beetles to antioxidants have not been studied, although isoflavonoids and stress-induced transcripts have been implicated in resistance to other arthropods in soybean (Carraopanizzi & Kitamura 1995). Antioxidant-rich food sources may increase the nutritive quality of plants for insects by enhancing insect immune responses (Ojala *et al.* 2005). The observed increase in octadecanoid signalling and defence-related transcripts in plants grown in elevated O_3 (Fig. 4) suggests that possible nutritional benefits would be nullified by increases in direct defence, perhaps even reducing insect fitness.

Suppression of genes in the octadecanoid pathway may also have contributed to delayed canopy senescence under elevated CO_2. In addition to initiating the production of defence compounds, jasmonic acid promotes senescence, and *LOX1*, a gateway gene in the synthesis of jasmonic acid, is greatly increased during senescence (He *et al.* 2002). Exposure to elevated CO_2 suppressed the expression of *LOX1* and also down-regulated genes (e.g. *ACC* synthase) involved in biosynthesis of ethylene, another phytohormone that promotes senescence (Fig. 4d). The rate of senescence of the soybean canopy is retarded under elevated CO_2 (Dermody *et al.* 2006), and suppression of these phytohormones may contribute to this change in phenology.

Many types of insect herbivory reduce photosynthesis in damaged leaves (Zangerl *et al.* 2002; Nabity, Heng-Moss & Higley 2006; Lamp, Alexander & Nguyen 2007). In agreement with this observation, photosynthesis-related transcripts were down-regulated after treatments with beetle damage under a variety of atmospheric conditions in this experiment (Table 2). Down-regulation of small subunit of ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) at the mRNA level has also been observed after exposure to elevated CO_2 (Makino *et al.* 2000). The reduction of a small subunit of Rubisco is thought to be a negative feedback caused by increased hexose and sugar cycling (Long *et al.* 2004). In contrast with previous reports, we found little change specifically in Rubisco mRNA abundance after CO_2 exposure in the field (Supplementary Table S1), but other photosynthesis-related transcripts were significantly down-regulated (Table 2). Gupta *et al.* (2005) and Taylor *et al.* (2005) also failed to detect changes in Rubisco mRNA in young leaves of poplar (*Populus tremuloides*) grown in elevated CO_2.

Recently, Long *et al.* (2006) reported that the growth stimulation caused by elevated CO_2 was less when experiments were conducted with FACE technology than when conducted in chambers. FACE experiments provide free access to herbivores, and increased damage by arthropods may partially explain the observed reduction in productivity, perhaps because of decreased resistance to herbivores. Thus, predicted increase in soybean productivity under projected CO_2 levels may be reduced by alterations at the transcriptional level that leave soybean more susceptible to herbivory in the field. Decreased resistance of this important agro-ecosystem to insect damage may have implications for future agricultural productivity.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Figure S1. The effect of insect cages on environmental conditions: (a) temperature, (b) humidity, (c) wind speed and (d) direction in open field (control), inside cages with openings in field (sham enclosure) and inside enclosed cages (complete enclosure).

Table S1. Total number of soybean transcripts regulated after 3 d beetle damage in ambient conditions compared to under elevated CO_2, elevated O_3 and the combination of gases with and without beetle damage, all relative to un-infested ambient atmospheric conditions ($P < 0.01$).

Table S2. Primers used for RT-PCR for lipoxygenase (*LOX*), 1-aminocyclopropane-1-carboxylate (*ACC*) and *actin*.

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