

IMPACTS OF CLIMATE CHANGE ON HERBIVORE INDUCED PLANT SIGNALING AND
DEFENSES

BY

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DISSERTATION

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ABSTRACT

The accumulation of CO₂ and O₃ in the troposphere alters phytochemistry which in turn influences the interactions between plants and insects. To examine the effect of elevated atmospheric CO₂ and O₃ concentrations on plant-insect interaction, I measured changes in transcription using microarray analysis of field-grown soybean (*Glycine max*). I found that the number of transcripts in the leaves affected by Japanese beetle (JB; *Popillia japonica*) herbivory was greater when plants were grown under elevated CO₂, O₃ and the combination of both compared to ambient atmosphere. The effect of herbivory on transcription diminished strongly with time, and elevated CO₂ interacted more strongly with herbivory than did elevated O₃ in my study. Constitutive levels and the induction by herbivory of key transcripts associated with defense and hormone signaling were down-regulated under elevated CO₂, suggesting susceptibility may be altered.

To examine the impact of elevated CO₂ exposure on susceptibility to herbivory in soybeans in more detail, the magnitude and timing of transcripts related to three major hormone signaling pathways (jasmonic acid [JA], salicylic acid [SA], ethylene [ET]) and related defenses were examined in field environments under elevated CO₂ after JB feeding. In addition, JB preference between elevated and ambient-grown tissue was determined. Elevated CO₂ decreased the induction of JA and ET related transcripts (*lox7*, *aos*, *hpl* and *acc1*), resulting in decreased accumulation of defenses (polyphenol oxidase, protease inhibitors) over time compared to ambient-grown plants. Elevated CO₂-grown tissue was preferred by JB in choice experiments. Elevated CO₂ also increased the accumulation of SA in soybeans. SA and JA are known to have an antagonistic relationship in other plants, and this antagonism may explain the reduction in JA

related transcripts. These results suggest elevated CO₂ exposure could cause increases in insect damage and reduction in diseases caused by pathogen sensitive to the SA defense pathway in the future.

In addition to elevated CO₂, models predict that plants will also experience increased drought in the future, possibly altering plant-insect dynamics in unanticipated ways. To investigate the combined effects of drought and elevated CO₂ on plant-insect interactions, components of susceptibility and palatability in soybeans were examined under field conditions. The effect of elevated CO₂ exposure on phytohormone signaling was consistent with previous studies. Exposure to mild drought stimulated the induction of the JA/ET signaling pathways but had no impact on nutritional components. However, elevated CO₂ exposure in combination with drought amplified the induction of JA and ET signaling transcripts and the accumulation of related defenses in soybeans after beetle herbivory. Overall, in combination with drought exposure, increased susceptibility of soybean to herbivores resulting from elevated CO₂ exposure was removed. This study suggests soybean in areas experiencing elevated drought will have an advantage over well-watered plants grown under elevated CO₂.

It is not known if the impact of elevated CO₂ on phytohormones and induced defenses is a generalized response in soybean or if it varies across plant species. In an attempt to address these questions, phytohormone signaling was examined under ambient and elevated CO₂ concentrations across six soybean cultivars and six different plant species using a common protocol across all experiments to aid in comparison. Elevated CO₂ reduced constitutive levels of JA in some but not all soybean cultivars; there was extensive variation in the response. Unexpectedly, constitutive and induced ET signaling increased in some soybean cultivars. In contrast to the variation seen in

JA and ET, constitutive levels of SA were elevated universally across soybean cultivars grown under elevated CO₂ with little variation in the response. Across species examined, elevated CO₂ had a similar impact as with soybean cultivars, generally reducing constitutive JA signaling transcripts in most species examined. However, in contrast to soybean there was no impact of elevated CO₂ on levels of SA across species. This study suggests some pathways may experience generalized changes for a species with little variation (e.g. SA in soybean), while others may not (JA/ET) and interactions with herbivores can change the response (constitutive versus induced). Thus, the modulation of hormone signaling by elevated CO₂ may cause increases in chewing insect damage and reduction in pathogen infections sensitive to the SA defense pathway in the future. However, the interactions between different aspects of global change with elevated CO₂ may alter the response, playing a more important role in determining plant-insect interactions than previously hypothesized. In conclusion, the impact of elevated CO₂ on phytohormone signaling appears complex, dependent on interactions, individual signaling pathways, cultivars and species examined.

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CHAPTER 1

INTRODUCTION

As a consequence of industrialization and the combustion of fossil fuels, plants will face new environmental conditions in the future, including rising concentrations of CO₂ (Forster *et al.* 2007). Conservative predictions project atmospheric CO₂ to double before the end of this century from current concentrations (Forster *et al.* 2007). This will be a significant change in atmospheric chemistry; current concentrations are already higher than at any previous time in the earth's recent history (Pearson & Palmer 2000). Exposure to elevated CO₂ will increase C₃ photosynthesis and water use efficiency of plants resulting in accelerated plant growth and increased yield (Ainsworth & Long 2005; Long *et al.* 2006). Predicted increases in yield may be lower than expected due to changes in host plant nutritional quality, altering plant-insect dynamics (Hamilton *et al.* 2005; DeLucia *et al.* 2008).

Numerous studies have examined the impact of elevated CO₂ on aspects of plant nutritional quality. In general, concentrations of water and nitrogen decrease, while leaf C/N ratio increases in plants grown under elevated concentrations of CO₂ (Ainsworth *et al.* 2002; Zvereva & Kozlov 2006; Stiling & Cornelissen 2007; Valkama *et al.* 2007). Because nitrogen is generally the limiting component in insect food and is reduced (Mattson 1980; Awmack & Leather 2002), it has been suggested elevated CO₂ reduces host plant quality for insect herbivores. In order to obtain the same amount of nitrogen from plants grown under elevated CO₂, some insects compensate by increasing feeding rates and total leaf area consumed (Bezemer & Jones 1998). In general, results have not been as predictable as this hypotheses would suggest and significant variation in herbivore performance on plants grown under elevated

CO₂ exists in the literature (Peñuelas & Estiarte 1998; Kopper & Lindroth 2003; Zvereva & Kozlov 2006; Stiling & Cornelissen 2007; Bidart-Bouzat & Imeh-Nathaniel 2008), suggesting other aspects of host plant quality are altered.

Another change in the environment plants will face in the future is elevated O₃ concentrations in the troposphere, which have been increasing steadily worldwide in combination with CO₂ (Vingarzan 2004; Forster *et al.* 2007). Typically O₃ interferes with plant physiology, decreasing photosynthesis and yield, while accelerating senescence (Pausch *et al.* 1996; Fuhrer 2003; Morgan *et al.* 2003; Morgan *et al.* 2004; Fiscus *et al.* 2005). Elevated O₃ can also affect phytochemistry by altering production of carbohydrates (Pausch *et al.* 1996; Kopper *et al.* 2001) and phenolic compounds (Runeckles & Krupa 1994). Enriched CO₂ may reduce ozone damage by decreasing stomatal conductance and by increasing carbohydrate pools for the synthesis of antioxidant compounds (Ainsworth & Long 2005; Ainsworth & Rogers 2007). Whereas the physiological effects of elevated CO₂, elevated O₃, and herbivory have been studied individually, the molecular mechanisms that mediate plant responses to these challenges in combination with one another remain unclear.

Soybean (*Glycine max*) is a major crop in the United States, worth an estimated \$31 billion and occupying 32.4 million ha in 2009, (<http://www.soystats.com>). Because of the size and value of the soybean market, it is of undeniable ecological and economic importance, making understanding the response of soybeans to global change a high priority. The Soybean Free-Air Concentration Enrichment (SoyFACE) experiment (<http://soyface.illinois.edu>), exposes large plots of soybean to elevated CO₂ or O₃, but is otherwise unchanged from typical field conditions. This facility provides a unique opportunity to evaluate the effects of these

components of global change on an important agro-ecosystem. In Chapter 2 I explore the molecular mechanisms governing the response of soybean to herbivory under these different atmospheric conditions using the SoyFACE facility.

Previous research at SoyFACE revealed that higher numbers of Japanese beetles (JB; *Popillia japonica* Newman) colonize plants grown in elevated CO₂ compared to ambient CO₂ (Hamilton *et al.* 2005) and that insects that feed on tissue grown under high CO₂ live longer and are more fecund (O'Neill *et al.* 2008). Increased feeding was initially hypothesized to be a consequence of increased sugar content of soybean leaves grown under elevated CO₂, 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 were at work. In addition to nutritional components, plants possess defensive compounds that alter host plant quality and influence plant-insect dynamics. Plant defenses can be constitutively expressed or induced after damage (Howe & Jander 2008). While significant research has demonstrated that elevated CO₂ concentrations can alter plant defense responses to herbivores, there is significant variation in the response (Coviella & Trumble 1999; Zvereva & Kozlov 2006)) and in general inducible defenses have been largely ignored.

The integration of induced defenses in plants is often affected by plant hormones, the best studied being, jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) (Glazebrook 2005; Jones & Dangl 2006; Howe & Jander 2008; McSteen & Zhao 2008; Bari & Jones 2009), which may activate specific defense responses (Kessler & Baldwin 2001; Howe & Jander, 2008; Bari & Jones 2009). These phytohormone signaling pathways are complex, often interacting

antagonistically or synergistically with each other to allow the plant to fine-tune and activate attacker-specific responses (Niki *et al.* 1998; Preston *et al.* 1999; Koornneef *et al.* 2008a, 2008b; Stout *et al.* 2006; McSteen & Zhao 2008; Bari & Jones 2009). Thus, the impact of elevated CO₂ on signaling pathways, as well as the induced defenses they mediate, will be critical to determining plant success against attack in the future. In Chapter 3 I examine the impact of elevated CO₂ on phytohormone signaling and induced defenses and determine their role in changes in insect behavior observed under elevated CO₂.

In combination with increased CO₂ concentrations, air temperature is likely to increase while precipitation is likely to decrease during summer months in the United States (Meehl *et al.* 2007). Thus, soybean will also experience increased drought with increased CO₂ (Meehl *et al.* 2007). In general, increased drought has the opposite effect of elevated CO₂, reducing yield and productivity (Long *et al.* 2006; Goldblum 2009) while increasing the availability of nitrogen (Mattson & Haack 1987; White 1984; Huberty & Denno 2004). Elevated CO₂ exposure may offset the impacts of drought on plants, although few studies take into account the combination of changes. Increased nitrogen in drought-stressed plants has been suggested to improve host plant quality for herbivores (White 1984). However, herbivore performance on plants grown under drought conditions individually and in combination with elevated CO₂ is highly variable in the literature (Huberty & Denno 2004; Joern & Mole 2005). In addition, few studies examine the impact of drought on other aspects of plant palatability, such as defenses. In Chapter 4 I address the impact of drought alone and in combination with elevated CO₂ on nutritional quality and plant palatability, while explicitly determining the importance of interactive effects that more accurately reflect predicted changes.

Induced responses are common across plant species (Karban & Baldwin 1997); although plants show significant inter- and intra-specific variation quantitatively and qualitatively in defensive traits (Gols *et al.* 2008). The relative plasticity of signaling and induction of defenses within and across species under projected CO₂ concentrations will play a major role in determining success of plants in the future. Thus identifying varieties of plants to resistant to herbivory under predicted elevated CO₂ is needed to develop suitable management strategies. Variability in soybean genotypic resistance to herbivory and to elevated CO₂ exposure has been established individually (insect: Dadson *et al.* 2007; McPherson & Buss, 2007; CO₂; Ziska *et al.* 1998; Ziska & Bunce, 2000). To date, however, no information is available concerning variation in the impact of elevated CO₂ in combination with herbivory and defense signaling across varieties of soybean or in other species. There is a need to compare and establish which soybean varieties are compatible with future climates and to determine how species other than soybean will also be affected. While induced defenses vary greatly across species and even within species (Gols *et al.* 2008), in general, phytohormone pathways that mediate the induction of defenses are more universal (Howe & Jander 2008). In Chapter 5 I examine the variation in phytohormone signaling across 6 different cultivars of soybean and six different species of plant grown under elevated CO₂ using the same protocol to aid comparison. This study provides new insight into the extent of the impacts of elevated CO₂ and facilitates selection of resistant varieties and possible crop management strategies in the future.

This dissertation examines the impacts of global change on plant-insect interactions. The goals of this research were to 1) investigate transcriptional profiles of soybean in response to beetle herbivory in field conditions and determine if they are altered by elevated CO₂ or O₃,

individually or in combination, 2) determine the role of phytohormone signaling and related induced defenses in soybean-beetle interactions previously observed under elevated CO₂ exposure, 3) examine whether drought conditions will modulate soybean and beetle responses to elevated CO₂, and 4) explore variation in the response of phytohormone signaling within soybean and across species under elevated CO₂ exposure.

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CHAPTER 2

TRANSCRIPTIONAL PROFILING REVEALS ELEVATED CO₂ AND ELEVATED O₃ ALTER RESISTANCE OF SOYBEAN (*Glycine max*) TO JAPANESE BEETLES (*Popillia japonica*)¹

ABSTRACT

The accumulation of CO₂ and O₃ 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 CO₂, elevated O₃ and the combination of elevated CO₂ plus elevated O₃ 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 CO₂ interacted more strongly with herbivory than elevated O₃. The majority of transcripts affected by elevated O₃ were related to antioxidant metabolism. Constitutive levels and the induction by herbivory of key

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transcripts associated with defence and hormone signaling were down-regulated under elevated CO₂; 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 CO₂ may decrease resistance of soybean to herbivory.

INTRODUCTION

Human activities are confronting plants with new challenges by increasing the concentration of carbon dioxide (CO₂) and ozone (O₃) in the atmosphere. Exposure to elevated CO₂ increases photosynthesis and growth (Drake *et al.* 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 CO₂ (Lindroth, *et al.* 1993; Bezemer & Jones 1998; Penuelas & Estiarte 1998; Körner 2003), resulting in elevated C : N ratios (Bezemer & Jones 1998). Elevated CO₂ may occur in some locations in concert with elevated O₃. Ozone typically decreases photosynthesis and biomass while accelerating senescence (Pausch *et al.* 1996; Fuhrer 2003; Morgan *et al.* 2003; Morgan *et al.* 2004; Fiscus *et al.* 2005), but elevated O₃ can also affect phytochemistry by altering production of carbohydrates (Pausch *et al.* 1996; Kopper *et al.* 2001) and phenolic compounds (Runeckles & Krupa 1994). In addition to directly affecting plant physiology, elevated CO₂ and O₃ indirectly affect ecosystem processes by fundamentally changing the relationship between plants and herbivorous insects. Changes in foliar nutrients and secondary chemicals induced by elevated CO₂ and O₃ 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.proxy2.library.uiuc.edu/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 CO₂ may improve production, but given the sensitivity of soybean to elevated O₃ and the dearth of studies of the effect of CO₂ and O₃ 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 CO₂ or O₃ 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 CO₂ compared with either elevated O₃ levels or ambient atmosphere (Hamilton *et al.* 2005). 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 CO₂ lived longer and had higher fecundity than beetles consuming foliage developed under ambient atmosphere or elevated O₃ (O'Neill *et al.* 2008). Increased feeding was initially postulated to be a consequence of increased sugar content of

soybean leaves grown under elevated CO₂, 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 O₃ 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 O₃ (O'Neill *et al.* 2008).

Whereas the physiological effects of elevated CO₂, elevated O₃ 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 CO₂ or elevated O₃ 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 CO₂ or O₃, individually or in combination, modulate the global transcriptional response of soybean to herbivory by Japanese beetle?

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 CO₂ or elevated O₃, singly and in combination, but otherwise experience natural conditions. SoyFACE consists of 16 20-m-diameter octagonal plots (total area 282.8 m²) 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 CO₂, O₃ 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 CO₂ and O₃ (Nagy *et al.* 1992).

Within each block, one control plot experienced current ambient CO₂ (~370 μmol mol⁻¹) and O₃, a second plot was fumigated to a target CO₂ concentration of 550 μmol mol⁻¹, a third plot was fumigated to a target O₃ 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 CO₂ and O₃. Plants were fumigated during daylight hours for the entire growing season. One-minute average CO₂ and O₃ were within 20% of the target for >95% of the time. At current rates of anthropogenic emissions, the targets for CO₂ and O₃ 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 m² plots of soybean within each octagonal treatment plot were encased individually in mesh cages (1 m³) 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. A; Appendix A).

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 CO₂. 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.agilent.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 A (available on request; $P < 0.01$).

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 B (Appendix A). 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 CO₂ or elevated O₃. Three days after leaves under ambient atmospheric conditions were infested with Japanese beetles, 1126 transcripts were significantly affected (Fig. 2.1a; P < 0.01). Exposure to elevated CO₂ or elevated O₃ increased the number of transcripts expressed in beetle-damaged plants. Three days post-infestation, beetle damage in elevated CO₂ alone or in combination with elevated O₃ affected the greatest numbers of transcripts (2847 and 3062, respectively; Fig. 2.1a,c; P < 0.01). Smaller numbers of genes (1731) were regulated in beetle-damaged leaves exposed to elevated O₃ compared to damaged leaves exposed to elevated CO₂ or the combination of elevated CO₂ plus elevated O₃ (Fig. 2.1a–c; P < 0.01). The number of genes affected in common by beetle damage and changes in atmospheric composition was relatively small (B plus CO₂, 379; B plus O₃, 466; B plus CO₂ plus O₃, 272; Fig. 2.1a–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 CO₂ and O₃, only 90 genes remained affected by herbivory after 2 weeks compared to 1126

genes that were affected 3 d after herbivory (Supplementary Table A; $P < 0.01$). Of these 90 genes, the 45 in Table 2.1 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 2.1; $P < 0.01$). These transcripts, including those encoding caffeoyl-CoA O-methyltransferase, isoflavone reductase homolog 1, putative cinnamyl alcohol dehydrogenase, chalcone O-methyltransferase, S-adenosyl-l-methionine : daidzein 7-O-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 2.1; $P < 0.01$).

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 2.1; $P < 0.01$). 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 2.1a and 2.2a; Supplementary Table A; $P < 0.01$). 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 CO₂ (four ferredoxin-dependent glutamate synthases and one NADH-dependent glutamate synthase), although β -2 cytosolic glutamine synthetase and nitrite reductase were up-regulated (Figs 2.1a & 2.2a; Supplementary Table A; P < 0.01).

Exposure of undamaged plants to elevated O₃ elicited no significant changes in transcription of genes associated with starch, sucrose or nitrogen metabolism (Fig. 2.2a; Supplementary Table A; P < 0.01). Our results suggest that, absent any changes in plant genetic composition, elevated CO₂ levels will substantially alter the nutritional value of soybeans for insects prior to damage, while O₃ 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 O₃ 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 CO₂ (Fig. 2.2b; Supplementary Table A; P < 0.01). Twenty-eight transcripts related to flavonoid/phenylpropanoid production were down-regulated in undamaged plants exposed to elevated CO₂ (Fig. 2.2b; Supplementary Table A; 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 (*CUTI*) and *CERI*-like protein] metabolism were significantly down-regulated (Fig. 2.2b; Supplementary Table A; $P < 0.01$).

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 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. 2.2b; Supplementary Table A; $P < 0.01$). 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 A; $P < 0.01$), such as thioredoxin, *LOX* and *PRI0*, 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 (*CERI*) protein, putative very-long-chain fatty-

acid-condensing enzyme (*CUT1*) and cuticle protein (*WAX2*; Fig. 2.2b; Supplementary Table A; $P < 0.01$). 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. 2.3a; Supplementary Table S1; $P < 0.01$).

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. 2.3a; Supplementary Table A; $P < 0.01$).

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 O₃. Only one transcript in nitrogen metabolism was up-regulated in damaged leaves exposed to elevated O₃ (β -2 cytosolic glutamine synthetase; Fig. 2.3a; Supplementary Table A; 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 CO₂ or elevated O₃. In ambient atmosphere, beetle damage up-regulated transcripts involved in flavonoid biosynthesis (Fig. 2.3b; Supplementary Table A; 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. 2.3b; Supplementary Table A; 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. 2.3b; Supplementary Table A; 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. 2.3b; Supplementary Table A; P < 0.01).

Exposure to elevated CO₂ 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 CO₂. Transcription of genes associated with isoprenoid and terpenoid metabolism was intensified by elevated CO₂. Transcripts involved in anthocyanin, isoflavonoid and dihydroflavonol biosynthesis were dampened under elevated CO₂, while those involved in flavonol metabolism were generally intensified (Fig. 2.3b; Supplementary Table A; 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-amyryn synthase) were all down-regulated after beetle attack under elevated CO₂ (Fig. 3b; Supplementary Table A; P < 0.01). Elevated CO₂ resulted in the down-regulation of transcripts related to terpenoid biosynthesis in beetle-damaged leaves, including β-amyryn synthase, limonene cyclase-like protein and transcripts similar to oxidosqualene cyclase (Fig. 2.3b; Supplementary Table A; P < 0.01).

In contrast to elevated CO₂, exposure to elevated O₃ intensified the up-regulation of flavonoids and phenylpropanoids in response to beetle damage (Fig. 2.3b; Supplementary Table A; P < 0.01). Key genes involved in anthocyanin and chalcone biosynthesis were strongly up-regulated by the combination of beetle damage and elevated O₃ (anthocyanin acyltransferase, leucoanthocyanidin dioxygenase-like protein, chalcone isomerase and chalcone synthase; Fig. 2.3b; Supplementary Table A; 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 O₃ (Fig. 2.3b; Supplementary Table A; P < 0.01). Like the flavonoids, the expression of many phenylpropanoid-related transcripts was intensified by damage to leaves in elevated O₃ (caffeic acid O-methyltransferase, N-hydroxycinnamoyl/benzoyltransferase-like protein, S-adenosyl-l-methionine, daidzein 7-O-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. 2.3b; Supplementary Table A; P < 0.01).

Damage to leaves grown under elevated CO₂ plus elevated O₃ ameliorated the responses in plant secondary metabolism, producing transcriptional signatures similar to those resulting from beetle damage in ambient air (Fig. 2.3b; Supplementary Table A; 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. 2.4a–c; P < 0.01; Table 2.2; P < 0.001). Defence-related transcripts such as protease inhibitors (*PIs*) 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. 2.4d,e; P < 0.01; Table 2.2; 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 2.2; $P < 0.001$). Only a single salicylic-acid-binding protein 2 transcript was induced after Japanese beetle feeding (Table 2.2; $P < 0.001$).

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. 2.4a–c; $P < 0.01$; Table 2.2; $P < 0.001$). 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. 2.4a–e; $P < 0.01$; Table 2.2; $P < 0.001$). Exposure of beetle-damaged plants to elevated CO_2 intensified the up-regulation of transcripts involved in lipid degradation (Table 2.2; $P < 0.001$), 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. 2.4; $P < 0.01$; Table 2.2; $P < 0.001$).

Several transcription factors previously shown to be induced after caterpillar feeding were also induced after beetle damage in soybean (Table 2.2; $P < 0.001$). 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 2.2; $P < 0.001$). Exposure to elevated O_3 intensified the induction of these transcription factors, while the effect of elevated CO_2 was less consistent (Table 2.2; $P < 0.001$).

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 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 CystPIs reduced the content of this important soybean defence compound

(Zavala *et al.* 2008, 2009), this change in gene transcription may explain the greater consumption of foliage by beetles under elevated CO₂ (Hamilton *et al.* 2005), because host plants would be more digestible.

In contrast to elevated CO₂, exposure to elevated O₃ 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 CO₂, elevated O₃ 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 CO₂ (Fig. 2.4; P < 0.01; Table 2.2; 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 *et al.* 1992). Transcripts of several key regulatory enzymes involved in the octadecanoic pathway were down-regulated in plants grown under elevated CO₂, indicating a dampening of induced defences. By reducing the up-regulation of genes in the octadecanoid pathway following herbivore induction, exposure to elevated CO₂ 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 CO₂ in growth chambers revealed that jasmonic acid metabolite levels were reduced compared to plants grown in ambient atmosphere (Chapter 3).

The abundance of transcripts directly related to plant defences was greatly reduced under elevated CO₂ in a manner consistent with decreased resistance to herbivory. Chalcone synthase, *PPO* and *CystPIs* transcripts were induced after beetle damage, and the magnitude of this induction was dampened when beetles attacked leaves exposed to elevated CO₂ (Fig. 2.4e,f; $P < 0.01$; Table 2.2; $P < 0.001$). Chalcone synthase is a key enzyme in phenylpropanoid biosynthesis, catalyzing the production of flavonoids and isoflavonoids that have been implicated in defence against herbivores (Carraoanizzi & 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 CO₂ on expression of *CystPIs*. The accumulation of *PIs* 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 *et al.* 1997; Korth & Dixon 1997; Ryan 2000). *PIs* 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 antidiigestive proteins, leaves grown in elevated CO₂ potentially are more

digestible to beetles than those grown in ambient air. Soybean cysteine PIs are active against beetle proteases, such as those in the gut of adult *Diabrotica virgifera* (Western corn rootworm beetles; Zhao *et al.* 1996). CO₂-associated reductions in PI and PPO content of foliage may explain the higher fecundity and longevity of Japanese beetles on soybean grown in elevated CO₂ (O'Neill *et al.*, 2008). While decreased soybean defence is consistent with higher numbers of Japanese beetles in elevated CO₂ plots, actual quantification of jasmonic acid and plant defences, such as PIs and PPO, will be necessary to confirm the scenario construed from transcription analysis.

Elevated O₃ intensified transcriptional responses to beetle damage, including greater mRNA abundance of defence and antioxidant-related transcripts (Fig. 2.3). Increased antioxidants and stress-response transcripts in the phenylpropanoid pathway under elevated O₃ have been observed previously (Wustman *et al.* 2001; Gupta *et al.* 2005) and made up the majority of induced transcript exposure to elevated O₃ 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 (Carraoanizzi & 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 O₃ (Fig. 2.4) 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 CO₂. In addition to initiating the production of defence

compounds, jasmonic acid promotes senescence, and *LOXI*, a gateway gene in the synthesis of jasmonic acid, is greatly increased during senescence (He *et al.* 2002). Exposure to elevated CO₂ suppressed the expression of *LOXI* and also down-regulated genes (e.g. *ACC* synthase) involved in biosynthesis of ethylene, another phytohormone that promotes senescence (Fig. 2.4d). The rate of senescence of the soybean canopy is retarded under elevated CO₂ (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; Nability *et al.* 2006; Lamp *et al.* 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 2.2). 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 CO₂ (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 CO₂ exposure in the field (Supplementary Table A), but other photosynthesis-related transcripts were significantly down-regulated (Table 2.2). 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 CO₂.

Recently, Long *et al.* (2006) reported that the growth stimulation caused by elevated CO₂ 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 CO₂ 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

TABLE 2.1

Soybean transcripts chronically altered after attack (Log2 fold change)

Gene Description	3 days 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	-	-	2.23	-	-	-	2.49	-
amino acid permease 6	0.75	0.86	-	-	0.71	-	-	-
aspartic-type endopeptidase/ pepsin A	-	-	0.9	-	-	-	1.1	-
vesicle-associated membrane protein 72	0.39	0.68	-	0.83	0.47	-	-	-
ATP binding / kinase/ protein kinase/ protein serine/threoninekinase/ protein-tyrosine kinase	-	-	0.72	-	-	-	0.84	-
caffeoyl-CoA O-methyltransferase	-	-	3.02	-	-	-	3.66	-
calcium ion binding	-	-	0.81	-	-	-	0.92	-
catalytic	1.27	-	-	-	1.4	-	-	-
catalytic/ hydrolase	-	-	1.46	-	-	-	1.93	-
Conserved hypothetical protein	-	-	0.8	-	-	-	1.03	-
cytochrome P450	-	-	1.96	-	-	-	2.2	-
disease resistance-responsive family protein	1.85	-	3.92	-	-	-	3.45	-
DNA binding protein WRKY2	0.64	-	1.15	-	0.7	-	1.07	-
DNA binding protein WRKY2	0.63	-	1.24	1.14	-	-	0.91	-
gibberellin 2-oxidase	1.1	-	1.27	-	-	-	1.5	-
heat shock transcription factor 34	-	-	1.89	-	-	-	1.79	-
hypothetical protein	1.1	-	1.62	-	-	-	1.34	-
hypothetical protein LOC_Os11g36070	-	-	0.74	-	-	-	0.86	-
Isoflavone reductase homolog 1	-	-	3.32	-	-	-	3.82	-
Isoflavone reductase homolog 1	-	-	2.6	-	-	-	3.44	-
OSJNBa0038O10.14	2.23	-	3.14	-	1.93	-	-	-
OSJNBa0038O10.14	2.2	-	3.02	-	1.93	-	-	-
putative cinnamyl alcohol dehydrogenase	-	-	1.41	-	-	-	1.74	-

TABLE 2.1 (continued)

Soybean transcripts chronically altered after attack (Log2 fold change)

Gene Description	3 days 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 heat-shock protein	-	-	2.27	-	-	-	2.73	-
putative leucine-rich repeat protein	-	-	0.68	-	-	-	0.85	-
putative RNA-binding protein	-	-	1.1	-	-	-	1.1	-
receptor-like protein kinase	-	-	2.45	-	-	-	1.67	-
Rhodopsin-like receptor	-	-	-	-	-	-	-	-0.68
S-adenosyl-L-methionine: daidzein 7-O-methyltransferase	-	-	1.73	1.89	-	-	1.71	-
Similar to serine/threonine kinases	-	-	1.57	-	1.31	-	1.97	-
UDP-glucosyltransferase HRA25	1.32	1.86	-	-	1.47	-	-	-
unknown	2.38	-	3.92	-	2.21	-	3.13	-
unknown	-	-	2.42	-	-	-	2.16	-
unknown	-	-	2.06	-	-	-	1.96	-
unknown protein	-	-	1.56	-	1.16	-	1.72	-
unknown protein	-	-	3.04	2.71	-	-	2.47	-
unknown protein	-	-	1.37	-	-	-	1.72	-
unknown protein	-	-	1.41	-	-	-	1.95	-
unknown protein	0.81	-	1.21	-	1.16	-	-	-
unknown protein	-	-	3.7	-	2.53	-	3.84	-
unknown	-	-	2.49	-	-	-	3.02	-
unknown	0.71	0.97	0.79	-	0.71	-	-	-
unknown	0.99	1.29	1.19	1.59	1.01	-	-	-
unknown	-	-	1.84	-	-	-	1.66	-
CHOMT_MEDSA Isoliquiritigenin 2'-O-methyltransferase (ChalconeO-methyltransferase) (ChOMT)	-	-	2.21	-	-	-	2.27	-

Values and colors represent the fold change (log2) for genes affected by herbivory (FDR P-value <0.001)

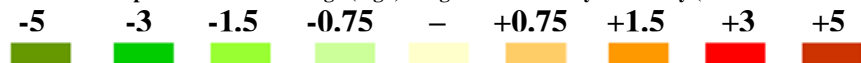


TABLE 2.2

Transcript fold change (Log2) 3 days after attack, FDR p-value <0.001

Gene Description	Affymetrix ID	Beetle	Beetle+CO ₂	Beetle+O ₃	Beetle+CO ₂ +O ₃
<i>Oxidative stress related genes</i>					
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
<i>Photosynthesis related genes</i>					
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 center subunit XI,	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
<i>Lipid Degredation/Beta oxidation</i>					
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	-

TABLE 2.2 (continued)

Transcript fold change (Log2) 3 days after attack, FDR p-value <0.001

Gene Description	Affymetrix ID	Beetle	Beetle+CO₂	Beetle+O₃	
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 signaling 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	GmaAffx.70690.1.S1_at	1.40	1.91	2.14	2.98
lipoxygenase	Gma.1.1.A1_at	0.74	-	-	-
lipoxygenase	Gma.1.1.S1_at	4.64	5.79	-	-
lipoxygenase	GmaAffx.27496.2.S1_at	1.69	2.33	-	-
lipoxygenase	GmaAffx.73072.1.S1_at	2.12	2.95	-	-
lipoxygenase	Gma.11166.1.S1_s_at	0.49	0.43	-	-
lipoxygenase	Gma.16500.1.S1_at	2.34	3.05	2.24	-
lipoxygenase	GmaAffx.60245.1.S1_at	0.51	-	0.77	-
allene oxide synthase	GmaAffx.83504.1.A1_at	1.72	-	1.82	-
allene oxide synthase	GmaAffx.53768.1.S1_at	1.11	-	-	-
allene oxide synthase	GmaAffx.86334.2.S1_at	1.36	-	1.62	-

TABLE 2.2 (continued)

Transcript fold change (Log2) 3 days after attack, FDR p-value <0.001

Gene Description	Affymetrix ID	Beetle	Beetle+CO₂	Beetle+O₃	
allene oxide synthase	GmaAffx.22456.1.S1_at	1.43	-	-	-
putative jasmonic acid regulatory protein	Gma.5331.1.S1_at	2.20	-	2.12	-
<i>Cytochrome P450</i>					
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
<i>Defense/stress related genes</i>					
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	Gma.17733.1.S1_s_at	2.44	-	2.68	-
cysteine protease inhibitor	Gma.3314.1.S1_a_at	1.64	1.66	-	-
cysteine protease inhibitor	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

TABLE 2.2 (continued)

Transcript fold change (Log2) 3 days after attack, FDR p-value <0.001

Gene Description	Affymetrix ID	Beetle	Beetle+CO₂	Beetle+O₃	
polyphenol oxidase	GmaAffx.92650.1.S1_at	3.32	3.02	3.33	-
polyphenol oxidase	GmaAffx.46214.5.S1_at	3.14	2.57	3.61	-
polyphenol oxidase	GmaAffx.46214.1.S1_at	4.02	3.21	4.95	3.93
polyphenol oxidase	GmaAffx.46214.2.S1_s_at	4.95	4.61	5.15	-
polyphenol oxidase	GmaAffx.92267.1.S1_at	-	-	-	1.12
polyphenol oxidase	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 arrayelements is given in supplementary material Table B; Appendix A. Colour scale correlates with fold change expression



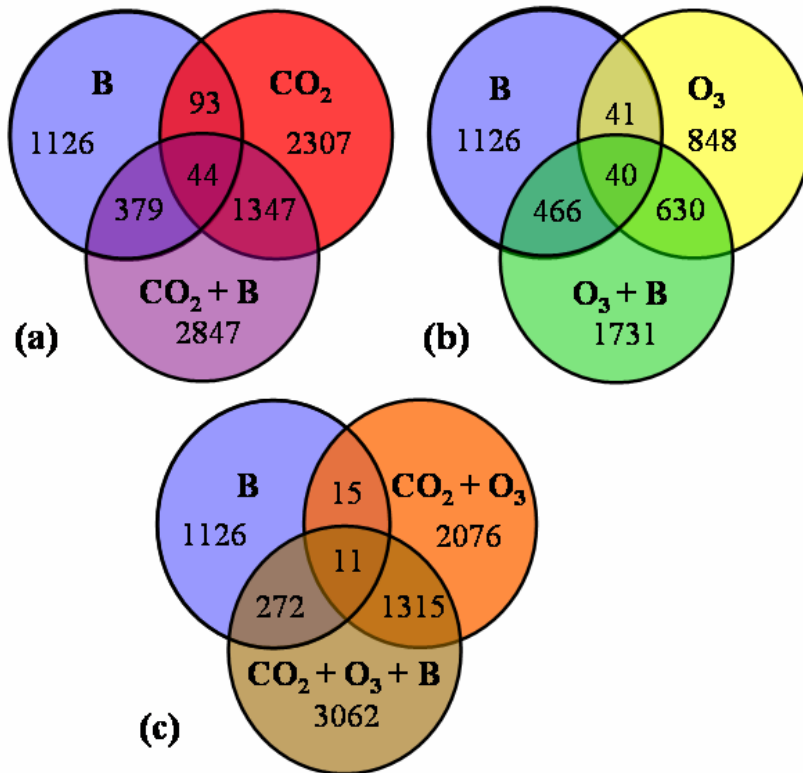


Figure 2.1. The effect of damage by Japanese beetles (B), growth in elevated CO₂ or elevated O₃ and the combination of beetle damage and altered atmospheric composition on transcription of genes in soybean leaves. Values represent the number of transcripts differentially expressed 3 d after infestation by Japanese beetles relative to un-infested leaves growing in ambient atmospheric conditions, or genes differentially expressed in leaves grown in elevated CO₂, elevated O₃ or the combination of elevated CO₂ plus elevated O₃ ($P < 0.01$). (a) Transcripts differentially expressed following damage by beetles under ambient atmospheric conditions, or for leaves grown in elevated CO₂ without beetle damage, or following beetle damage and growth in elevated CO₂; (b) transcripts differentially expressed for leaves grown in elevated O₃ without beetle damage, or following beetle damage and growth in elevated O₃; (c) transcripts differentially expressed for leaves grown in the combination treatment of elevated CO₂ plus elevated O₃ without beetle damage, or following beetle damage and the combination treatment of elevated CO₂ plus elevated O₃.

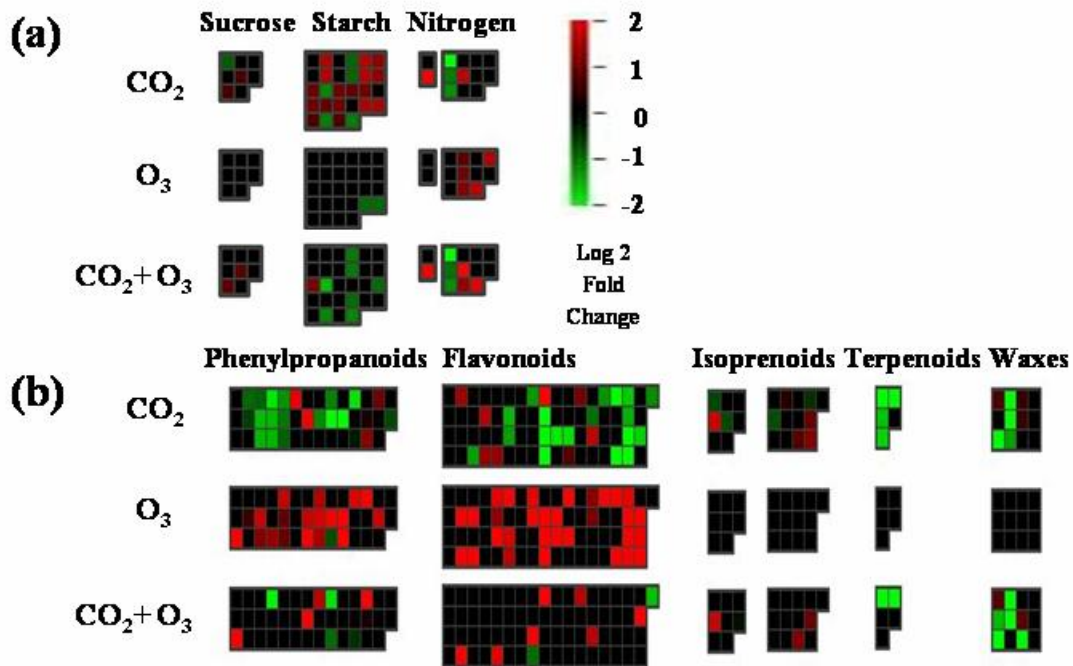


Figure 2.2. The individual and combined effects of elevated CO₂, elevated O₃ and elevated CO₂ plus O₃ without beetle damage on the transcription of genes that may affect leaf palatability to insects. (a) Genes regulating sucrose, starch and nitrogen metabolism may affect nutritional quality, while (b) 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).

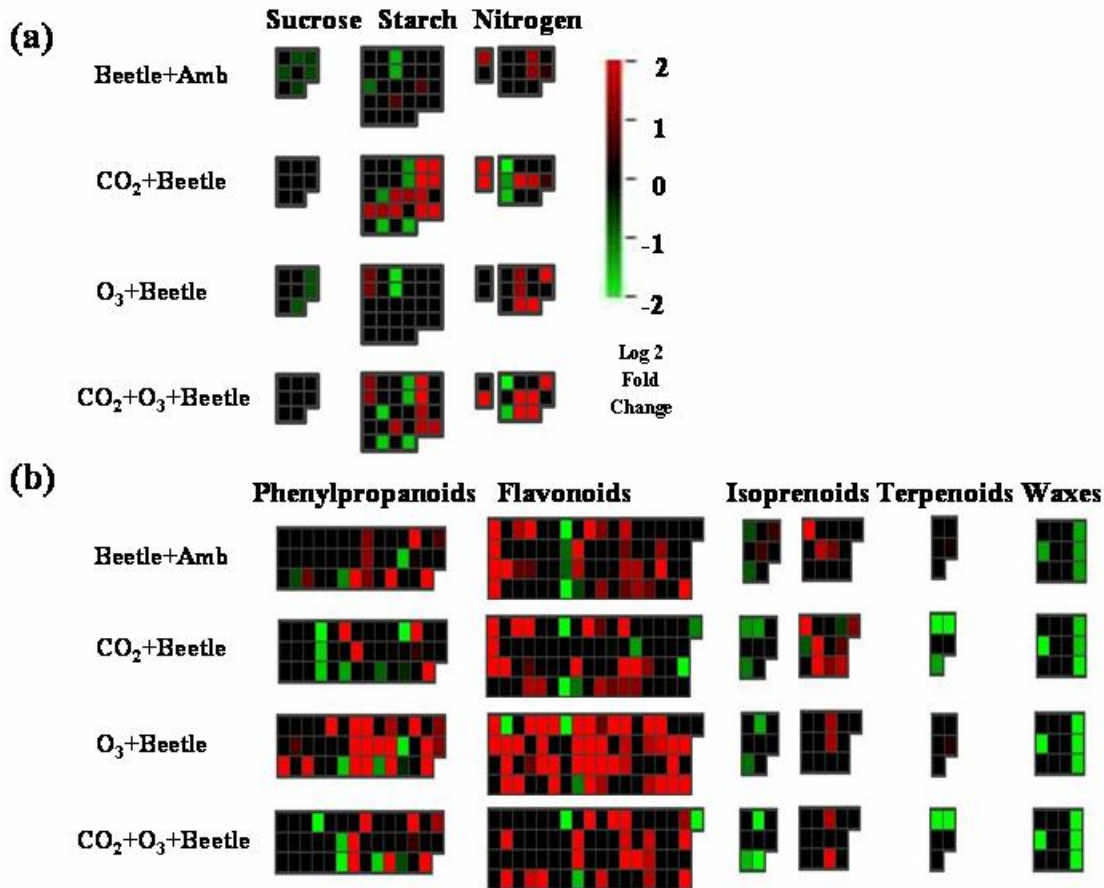


Figure 2.3. Effect of damage by Japanese beetles under the individual and combined effect of elevated CO₂ and elevated O₃ on the transcription of genes that may affect leaf palatability to insects. (a) Genes regulating sucrose, starch and nitrogen metabolism may affect nutritional quality, while (b) 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 (log₂-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).

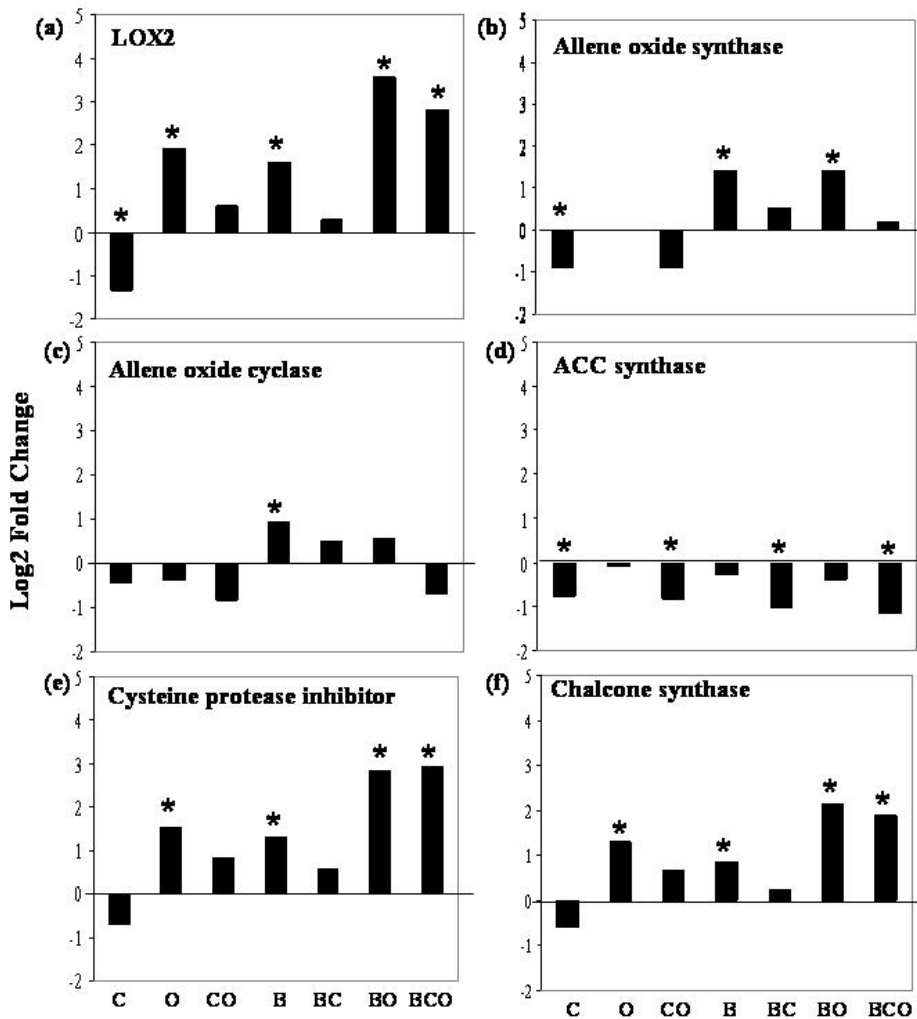


Figure 2.4. The expression level from microarrays of (a) lipoxygenase (LOX), (b) allene oxide synthase (AOS), (c) allene oxide cyclase (AOC), (d) 1-aminocyclopropane-1-carboxylate (ACC) synthase, (e) cysteine protease inhibitor (CystPI) and (f) chalcone synthase transcripts to elevated CO₂ (C), elevated O₃ (O) or damage by Japanese beetles (B), applied singly or in combination. The bars indicate log₂-fold change from the values for undamaged leaves grown in ambient air; asterisks indicate that values are significantly different at P < 0.01.

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CHAPTER 3

THE EFFECTS OF RISING CARBON DIOXIDE ON PHYTOHORMONE SIGNALING AND SUSCEPTIBILITY TO HERBIVORES IN *Glycine max*²

ABSTRACT

Plants encounter myriad challenges in the environment, including insect and pathogen attack. Perception of attack and elicitation of defenses are mediated largely by phytohormones. Elevated CO₂ concentrations in the atmosphere present a new environment for plants to face these challenges. Based on previous studies in soybeans (*Glycine max*), elevated CO₂ is hypothesized to modulate plant resistance through alterations in hormone signaling and defenses against herbivorous pests. To determine the impact of elevated CO₂ exposure on phytohormone signaling in soybeans, the magnitude and timing of transcripts related to three major hormone signaling pathways (jasmonic acid [JA], salicylic acid [SA], ethylene [ET]) and related defenses were examined in field environments under elevated CO₂ after Japanese beetle (JB; *Popillia japonica*) feeding. In addition, JB preference between plants grown under elevated and ambient atmospheres was determined. Elevated CO₂ decreased the induction of JA and ET related transcripts (lipoxygenase 7 (*lox7*), allene oxide synthase (*aos*), hydrogen peroxide lyase (*hpl*), and 1-aminocyclopropane-1-carboxylate synthase (*acc1*) Accumulation of defenses (polyphenol

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oxidase, proteinase inhibitors) initially increased to a higher level after JB attack but over time decreased to levels lower in elevated CO₂ compared to ambient-grown plants. Elevated CO₂ grown tissue was preferred by JB in choice experiments. Elevated CO₂ also increased the accumulation of SA in soybeans. SA and JA have an antagonistic relationship in other plants and this increase in SA may explain the reduction in JA-related transcripts. The modulation of hormone signaling resulted in lower plant defense over time, a response that could increase chewing insect damage and reduce pathogen infections controlled by SA in the future.

INTRODUCTION

As a consequence of the combustion of fossil fuels and land use change, atmospheric CO₂ concentrations are predicted to rise to 550 $\mu\text{mol mol}^{-1}$ by 2050 from the current concentration of 386 $\mu\text{mol mol}^{-1}$ (Forster *et al.* 2007). Elevated CO₂ will increase plant photosynthesis and water use efficiency resulting in increased productivity (Ainsworth & Long 2005; Long *et al.* 2006), although few studies take into account other organisms, such as herbivores, that can directly reduce crop yield (Hamilton *et al.* 2005; DeLucia *et al.* 2008). Elevated CO₂ increases carbon assimilation and alters carbon allocation patterns, reducing host plant food quality. Aspects of quality that are altered include decreased concentrations of water and nitrogen, increased leaf C:N ratio, increased leaf toughness, and increased allocation to phenolic compounds (Ainsworth *et al.* 2002; Zvereva & Kozlov 2006; Stiling & Cornelissen 2007; O'Neill *et al.* 2010). Because CO₂ levels are anticipated to be ~50% greater for soybean grown in the next century (Forster *et al.* 2007), the dynamics of plant-insect herbivore

interactions, such as insect consumption and growth, will be altered as a consequence of the lower quality of plants grown under these conditions.

The SoyFACE (Soybean Free Air Concentration Enrichment) experiment allows examination of the impacts of anthropogenic increases of CO₂ in a common midwestern agricultural ecosystem. Previous research at SoyFACE revealed that higher numbers of Japanese beetles (JB; *Popillia japonica* Newman) colonize plants grown in elevated CO₂ compared to ambient CO₂ (Hamilton *et al.* 2005) and that insects that feed on tissue grown under high CO₂ live longer (O'Neill *et al.* 2008). Japanese beetle was introduced into the United States before 1916 and has since become an important pest (Fleming 1972). The polyphagous adult can feed on >300 host plants (Potter & Held 2002). Subsequent experiments demonstrated that transcripts associated with defense hormone signaling were lower in soybean foliage grown in elevated CO₂ three days after beetle damage compared to ambient-grown tissue (Casteel *et al.* 2008) and that induction of proteinase inhibitors were lower 72 h after continuous beetle damage (Zavala *et al.* 2008, 2009).

The integration of defense mechanisms in plants is mediated largely by three hormone signaling pathways: jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Glazebrook 2005; Jones & Dangl 2006; Howe & Jander 2008; McSteen & Zhao 2008; Bari & Jones 2009). Generally, JA and ET pathways are induced against wounding, chewing insects and necrotrophic pathogens (Maleck & Dietrich 1999; Kessler & Baldwin 2002; Glazebrook 2005; Casteel *et al.* 2008). Jasmonic acid and related compounds act as signals after tissue damage, and the accumulation of JA results in subsequent activation of herbivore-specific defense responses (Howe & Jander 2008). Defense responses include induction of genes with protein products that

contain anti-insect properties that negatively affect insect growth and development (Duffy & Felton 1991; Ryan 2000; Chen *et al.* 2005). In addition to direct defenses, production of green leaf volatiles (GLVs) by plants also is enhanced after biotic stress and JA accumulation. The release of GLVs can reduce herbivore performance and can play an important role in the recruitment of the natural enemies of the herbivores (Matsui *et al.* 2006).

In contrast, the SA signaling pathway is activated against many biotrophic plant pathogens and phloem-feeding insects, such as whiteflies and aphids (Morgan *et al.* 2001; Glazebrook 2005; Zarate *et al.* 2007; Kempema *et al.* 2007). After pathogen attack, SA accumulates in tissue resulting in the synthesis of pathogen specific defenses. Induced responses of the SA signaling pathway are important in mediating local and systemic acquired resistance (SAR), basal resistance, and R-gene mediated defenses to many pathogens (Maleck & Dietrich 1999; Glazebrook 2005, Jones & Dangl 2006). Accumulation of SA often is negatively correlated with the accumulation of JA and herbivore-specific defenses (Stout *et al.* 2006).

To determine the impact of elevated CO₂ exposure on phytohormone signaling in soybeans, the magnitude and timing of transcripts related to three hormone signaling pathways (JA, SA and ET) and related defenses were examined in open field environments under elevated CO₂ after JB feeding. In addition, JB preference between plants grown under elevated or ambient atmospheres was determined in a choice experiment in the laboratory with field-grown tissue.

RESULTS

To determine the effect of elevated CO₂ on the kinetics of soybean signaling transcripts, we analyzed the expression of 1-aminocyclopropane-1-carboxylate synthase (*acc1*), which is

involved in the regulation and biosynthesis of the signaling hormone ethylene (ET), two genes related to the synthesis of the signaling hormone jasmonic acid (JA), lipoxygenase 7 (*lox7*) and allene oxide synthase (*aos*; Saravitz & Siedow 1996; Chen *et al.* 2005), and phenylalanine ammonia-lyase (*pal*) related to the synthesis of phenolics, including salicylic acid (SA). Growth under elevated CO₂ delayed and reduced the accumulation of *acc1* in soybean leaves compared to plants grown in ambient conditions (significant main effect of CO₂, C, $P < 0.05$; significant interaction CO₂ x H, $P = 0.04$; Fig. 3.1A). Two hours after damage by JB, *acc* was 67% higher in leaves grown in ambient CO₂, while there was no increase in accumulation in leaves grown in elevated CO₂. The peak accumulation of *acc1* in leaves grown in elevated CO₂ did not occur until 6 h after damage and was 31% lower compared to plants grown in ambient CO₂ (significant interaction of CO₂ and time after herbivory, CO₂ x H, $P = 0.04$; Fig. 3.1A). No significant main effect of time on the expression level of this transcript was observed.

Elevated CO₂ reduced the accumulation of *lox7* (20%; significant main effect of CO₂, $P = 0.02$; Fig. 3.1B) and there was a trend for *aos* (19%; main effect of CO₂, $P = 0.08$; Fig. 3.1C). As with *acc1*, peak accumulation of *lox7* was delayed by 6 hrs in soybean grown under elevated CO₂ (peak at 24 h) compared to ambient CO₂ (significant main effect of time after herbivory, H, $P < 0.05$; and significant interaction, CO₂ x H, $P < 0.05$; Fig. 3.1B). However, while herbivory induced *aos* abundance over time (main effect time following herbivory, H, $P < 0.05$), there was no effect of CO₂ exposure on the time of peak accumulation for *aos* (interaction: CO₂ x time after herbivory, C x H, $P > 0.05$; Fig. 3.1C). There were no significant effects of any treatments on transcript abundance of *pal* (data not shown).

We analyzed the expression of hydrogen peroxide lyase (*hpl*), a key regulatory gene in the biosynthesis of green leaf volatiles (Matsui *et al.* 2006), after a burst of JB damage in soybean grown in ambient and elevated CO₂. Transcript abundance of *hpl* after JB damage increased significantly over time, and this accumulation was reduced in soybean grown under elevated CO₂ (significant main effect of CO₂, C; significant main effect time following herbivory, H; interaction: CO₂ x time after herbivory, C x H; $P < 0.05$; Fig. 3.1D). Elevated CO₂ reduced the peak accumulation in soybean by 66% compared to ambient-grown plants (Fig. 3.1D).

Prior to the SoyFACE experiment, the effect of elevated CO₂ on constitutive levels of JA and the levels following induction with its methyl ester, methyl jasmonate (MeJA), to mimics leaf damage, were examined in growth chamber experiments when beetles were not available. During the following growing season the interactive effects of CO₂ and JB herbivory on JA levels in field-grown plants under elevated and ambient CO₂ conditions were examined. Growth of soybeans under elevated CO₂ resulted in a 50% and 61% reduction in constitutive JA and MeJA-induced levels of JA, respectively, compared to ambient-grown leaves in growth chambers (main effect of CO₂, $P = 0.03$; Fig. 3.2A). Soybeans under elevated CO₂ in field conditions did not significantly differ in the constitutive levels of JA compared to soybeans grown in ambient CO₂ (0 h, $P > 0.05$; Fig. 3.2B). However, abundance of JA increased 24 h after JB damage in ambient (100%) compared to undamaged soybean in the field, while there was no significant increase under elevated CO₂ (significant main effect of CO₂, $P < 0.05$; interaction: CO₂ x H, $P < 0.05$; Fig. 3.2B). SA levels were not significantly altered after herbivory (main effect of time after herbivory, H, $P > 0.05$; Fig. 3.3); however, elevated CO₂ exposure increased

the abundance of SA in soybean leaves (significant main effect of CO₂, $P < 0.05$; Fig. 3.3). Our results suggest elevated CO₂ reconfigures hormone signaling, possibly altering the dynamics of SA and JA within the plant.

While growth of soybeans under elevated CO₂ did not alter levels of cysteine proteinase inhibitors (Cyst PIs) after a bout of herbivory in the field (main effect of CO₂; interaction; $P > 0.05$; Fig. 3.4A), polyphenol oxidase (PPO) activity was significantly altered (interaction: CO₂ x time after herbivory, H, $P > 0.05$; Fig. 3.4B). Induction of PPO activity peaked after 24 h and began to decline after damage in soybeans grown under elevated CO₂. In contrast, PPO activity continued to increase over the course of the experiment under ambient CO₂ (significant interaction between CO₂ and time after herbivory, CO₂ x H, $P = 0.02$; Fig. 3.4B). Induced PPO activity in soybean grown under elevated CO₂ increased 120% after 24 h compared to undamaged tissue, while ambient CO₂ induction had not yet begun (Fig. 3.4B). However, after 48 h defense activity had returned to constitutive levels in soybeans grown under elevated CO₂, while the PPO activity after herbivory in soybeans grown under ambient CO₂ had just started to increase (12%, significant interaction, CO₂ x H, $P = 0.02$; Fig. 3.4B). While Cyst PIs were not significantly altered, a similar pattern of induction was observed as with PPO activity under elevated CO₂ compared to ambient (Fig. 3.4A).

To determine the consequences of changes in soybean signaling hormones and defense activity after herbivory on behavior, JB were allowed to feed with equal access to leaves grown under ambient or elevated CO₂ conditions in a choice experiment for 24 h. Soybean tissue grown under elevated CO₂ was preferred by beetles compared to tissue grown under ambient CO₂ (Fig.

3.5), and the percentage leaf area removed was greater on soybean grown under elevated CO₂ (12.6%) compared to ambient CO₂ (3.93%, significant main effect of CO₂, $P < 0.001$; Fig. 3.5).

DISCUSSION

By 2050, soybeans will grow in an atmosphere with 50% more CO₂ than today if anthropogenic inputs are not reduced (Forster *et al.* 2007). This study suggests that, under such conditions, phytohormone signaling will be reconfigured in soybean, resulting in a reduction in herbivore-specific direct and indirect defenses. In this study, transcripts and metabolites related to jasmonic acid (JA) and ethylene (ET) signaling were reduced, and the timing of induction of the ET signaling was delayed (Fig. 3.1, A and B). In addition, transcripts related to green leaf volatile (GLV) production were reduced (Fig. 3.1C); GLVs can be utilized as indirect defenses that attract natural enemies of plant herbivores (Matsui *et al.* 2006). Although a statistically significant difference was not resolved in both defenses examined, a reduction of PPO activity was observed along with a trend showing reduced PI activity in plants grown under elevated CO₂ 48 h after herbivory (Fig. 3.4, A and B). Moreover, elevated CO₂ exposure also increased salicylic acid (SA) levels in soybeans when compared to ambient-grown plants (Fig. 3.3), suggesting that interactions with pathogens and phloem feeding insects may also be altered in the future. However, this increase in SA is evidently not transcriptionally regulated, as there was no significant effect on *pal* (data not shown).

Ultimately, these transcriptional and phytochemical changes altered beetle behavior; tissue grown in elevated CO₂ was preferred in choice tests over soybean tissue grown in ambient conditions (Fig. 3.5). These findings are consistent with previous work suggesting that the down-

regulation of herbivore-specific defenses is likely to be the mechanism underlying increased damage observed under conditions of elevated CO₂ in the field (Casteel *et al.* 2008, Zavala *et al.* 2008). To resolve elevated CO₂ impact on the kinetics of induction, our study utilized a short bout instead of continuous feeding utilized in previous studies, which possibly explains the lack of significant difference we observed in PIs. Multiple defense pathways may be altered under an elevated CO₂ atmosphere, indicating that not only susceptibility to beetles will be increased, but the entire trophic structure of agroecosystem interactions between herbivores and pathogens may be altered.

The mechanism by which elevated CO₂ alters the hormonal response to herbivory is not known but may be related to changes in carbohydrate status. In plants, different sugar signals are generated by photosynthesis and carbon metabolism in source and sink tissues to modulate growth, development, and stress responses. Soybean leaves grown at elevated CO₂ have significantly increased photosynthesis rates (Rogers *et al.* 2004) and content of non-structural carbohydrate, including starch and sugars (sucrose, fructose and glucose; Rogers *et al.* 2004, 2006; Ainsworth *et al.* 2007; Sun *et al.* 2009). There are extensive interactions between sugar and plant hormone signaling, and glucose signaling, in particular, has been linked to ET and SA signaling (Rolland *et al.* 2006). In view of the fact that plant-specific sugar signaling mechanisms can interact and modify plant hormone signaling pathways (Rolland *et al.* 2006), elevated CO₂-induced accumulation of sugars may have an influence on the reconfiguration of phytohormone signaling and defenses in soybean.

Plants tailor defense responses to the type of attack they encounter by activating distinct signal-transduction pathways. For example, chewing herbivores generally activate the JA

signaling pathway (Maleck & Dietrich 1999; Glazebrook 2005; Kessler & Baldwin 2002; Casteel *et al.* 2008), whereas biotrophic pathogens and phloem feeding insects often activate the SA signaling pathway (Maleck & Dietrich 1999; Glazebrook 2005, Jones & Dangl 2006; Zarate *et al.* 2007; Kempema *et al.* 2007). Furthermore, a significant body of literature suggests the induction of the SA signaling pathway suppresses JA signaling (Niki *et al.* 1998; Preston *et al.* 1999; Koornneef *et al.* 2008a, 2008b). Salicylic acid may inhibit JA synthesis or interfere with perception (Doares *et al.* 1995). In addition, JA may negatively affect SA-dependent gene expression (Preston *et al.* 1999). In view of the potential for cross-talk between JA and SA signaling pathways (Pieterse *et al.* 2009), it might be expected that the elevated CO₂-induced accumulation of SA had an impact on the JA-related signaling and defenses. Indeed, elevated CO₂ exposure not only suppressed the expression of key genes involved in the JA signaling pathway (*lox*, *aos*; Fig. 3.1A), but it also suppressed JA production in undamaged plants in growth chambers (Fig. 3.2A). Furthermore, JA-regulated defense induction could not be maintained in plants grown in elevated CO₂ after beetle damage (Fig. 3.4B), which correlates well with the reduction in JA signaling transcripts (Fig. 3.1A). We conclude that elevated CO₂ exposure may also increase resistance to some biotrophic pathogens with increased SA abundance. Considering the well-known relationship between SA and pathogen-induced defenses and recent literature demonstrating that some soybean pathogens are reduced in elevated CO₂ treatments at SoyFACE (Eastburn *et al.* 2010), the impact of elevated CO₂ on pathogen resistance should be investigated in the future.

Rising atmospheric CO₂ levels have the potential to increase soybean susceptibility to herbivores and necrotrophic pathogens and reduce susceptibility to biotrophic pathogens and

phloem feeding insects, altering ecosystem dynamics in the future. By reconfiguring phytohormone signaling transcripts, elevated CO₂ can reduce multiple herbivore-specific defenses in soybean; this reduction may lead to greater damage and thus crop losses in the future. In addition, elevated CO₂ may affect soybean-pathogen interactions through decreased JA and ET, leaving plants more susceptible to necrotrophic pathogens, and increases in SA, leaving plants more resistant to biotrophic pathogens in the future. The impact of these changes on predicted increases in soybean yield are not clear, but the balance is likely to be altered. Established management practices for soybean must adjust to these potential changes in insect and pathogen outbreaks to avoid future loss. Determining what pests have the greatest impact on soybean yield loss and taking effects of elevated CO₂ into future management plans will be important. Examining the variation in the response among soybean cultivars could facilitate future breeding to select resistant varieties. In addition, recent studies demonstrated increased numbers of chewing insects and decreased phloem feeding insects colonizing aspen trees grown under elevated CO₂ compared to ambient conditions (Hillstrom & Lindroth 2008), suggesting that plants besides soybean might respond with similar changes in phytohormones. Further work is needed to identify the mechanisms by which elevated CO₂ reduces the biosynthesis of both JA and ET and increases SA in soybean. Cross-talk between different defense signaling pathways and interactions with future climate change has important consequences for the evolution of plant defense, as it can influence the amount of damage suffered by plants and subsequently influence selection pressure on defense responses. Thus, changes in defense-signaling systems of host plants produced by elevated CO₂ can be expected to alter the dynamics and management of agroecosystems in the future.

METHODS

Site Description

This experiment was conducted at SoyFACE, an open Free Air gas Concentration Enrichment system established at the University of Illinois, Urbana-Champaign (40°02' N, 88°14' W, 228-m above sea level; [http:// soyface.illinois.edu](http://soyface.illinois.edu); Long *et al.* 2004). SoyFACE exposes large field plots of soybean (Pioneer 93B15) to elevated CO₂. It consists of 8, 20-m-diameter octagonal plots (total area 282.8-m²) distributed within four randomized blocks of soybean. Within each block, one control plot was maintained at current ambient CO₂ concentrations (386 μmol mol⁻¹), and one plot was fumigated to a target CO₂ concentration of 550 μmol mol⁻¹ (average fumigated CO₂ concentration was 553 μmol mol⁻¹ for the 2008 season). The target concentrations of elevated CO₂ represented the atmospheric levels predicted by 2050 (Forster *et al.* 2007). Apart from not being Roundup Ready, this variety of soybean is typical of those grown in Midwest commercial production. It was grown according to standard agronomic practice in this region and hence was rotated annually with corn. No nitrogen fertilizer was added.

At the start of the experiment in July 2008, soybeans had been growing for 45 d. In each treatment plot 15 undamaged plants at the vegetative stage were selected, and the first fully expanded trifoliolate on each plant was enclosed in a 1 X 4 mm mesh bag to prevent movement of insects. Twelve of the trifoliolates enclosed in bags were infested individually with five adult beetles each. Japanese beetles were collected using commercially available traps near the experimental plots and starved for 24 h prior to the experiment. Beetles were allowed to feed

until ~10% of the leaf area was removed (~1 h) and then removed. Three locally damaged trifoliates from separate plants were collected and pooled at 2, 6, 24, and 48 h after removal of beetles (12 total plants). The three remaining undamaged trifoliates were collected as a control. Tissue was frozen in liquid nitrogen, ground to a fine powder, and stored at -80°C. This complete experiment was repeated four times in the field (four replicates, two atmospheric treatments, five samples each), resulting in 40 tissue samples (N = 4).

The winter before the field experiment at SoyFACE, preliminary quantification of JA was conducted in growth chamber experiments. Soybean grown from seeds in 3.78 liter pots containing soil-less mix (Sunshine Mix LC1, Sun Gro Horticulture Inc., Bellevue, WA) in controlled environment chambers (Egc, Chagrin Falls, OH) under a 16/8 h day/night cycle. Temperature regime was set to 22/18 °C for day/night and light intensity was ~200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For CO₂ treatments concentrations were set to 400 $\mu\text{mol mol}^{-1}$ for ambient (4 chambers) and 600 $\mu\text{mol mol}^{-1}$ for elevated (4 chambers). In each chamber, 30 d after emergence, 10 undamaged soybean plants at the vegetative stage were selected, and the uppermost fully expanded trifoliolate leaf on each of five plants was treated with 150 μg of methyl jasmonate (MeJA) (Sigma–Aldrich) in 20 μl of lanolin paste. Because Japanese beetles were not available during November, the addition MeJA to leaves was utilized as a proxy for leaf damage (Bolter & Jongsma 1995). An additional 5 plants served as undamaged controls in each chamber. The MeJA-treated plants and control leaves were harvested for analysis 45 minutes after the beginning of the experiment, and the other half were harvested 3 d after infestation (N = 4).

RNA Preparation

Total RNA was extracted from each of the 40 frozen tissue samples using Trizol (Invitrogen, Carlsbad, CA). RNA integrity was verified using a 1.2% formaldehyde agarose gel (Sambrook et al., 1989) and a microfluidic visualization tool (Bioanalyzer, Agilent Technologies, USA, <http://www.agilent.com>).

Quantitative real time RT- PCR

Transcript abundance of genes coding for *lox7* (lipoxygenase), *aos* (allene oxide synthase), *hpl* (hydrogen peroxide lyase), *acc* (1-aminocyclopropane-1-carboxylate synthase), and *pal* (phenylalanine ammonia-lyase) was analyzed with quantitative real-time RT-PCR (qRT-PCR), with *con6* (contig 6) as an internal standard. Contig 6 was identified from a microarray study as constitutively expressed (Libault *et al.* 2008), and stable expression was verified across samples using qRT-PCR. After treatment with RQ1 DNase (Promega, Madison, WI), 3 µg of total RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo-dT₁₂₋₁₈ as a primer. Gene-specific primers used for qRT-PCR were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=NcbiHomeAd) with the following criteria: TM of 60°C, PCR amplicon lengths of 90 to 150 bp yielding primer sequences with lengths of 18 to 24 nucleotides with an optimum at 21 nucleotides, and guanine-cytosine contents of 40% to 60%. Primer sequences for the selected genes can be found in supplementary Table B (Appendix A). Reactions were carried out using 5 µl of the 'Syber green PCR master mix' (Applied Biosystems, Foster City, CA), with 800 nM of primer, in the '7500' instrument (Applied Biosystems, Foster City, CA). The PCR was initiated by incubation at 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 analyzed according to Schmittgen & Livak (2008) when primer set efficiencies were consistent; the standard curve method was used when they were not (http://www.biotech.uiuc.edu/centers/Keck/Functional_genomics/taqman/Guide%20to%20relative%20quantitation.pdf). Applied Biosystems software was used for averaging the four independently calculated normalized expression values that were triplicated on the plate for each treatment.

Phytohormone Analysis

Approximately 150 mg of each frozen tissue from each replicate sample was extracted for analysis of JA and SA according to Wu *et al.* (2007). Plant material was transferred to FastPrep tubes (Qbiogene, Carlsbad, CA) containing 900 mg of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ, USA). One milliliter of ethyl acetate mixed with 100 ng of D₂-JA and 100 ng D₄-SA, used as internal standards, was added to each sample. Samples were then homogenized in a bead beater for 2 min. After centrifugation at 12000 g for 20 min at 4°C, 500 µL were removed and transferred to a new tube. The remaining tissue was then re-extracted with 1-mL ethyl acetate without internal standard and centrifuged as above. The supernatants were combined (250 µL from the re-extraction) and then dried by evaporation on a vacuum concentrator. The dried residue was dissolved in 500 µL 70% (v/v) methanol, vortexed for 10 min and subsequently centrifuged at 12000 g for 10 min.

Measurements were conducted on a liquid chromatograph-mass spectrometry system (Shimadzu 2010 EV, Shimadzu, Columbia, MD, US). A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) was used in a gradient mode

for the separation. The mass spectrometer was operated in a negative electro-spray ionization mode. The most abundant and characteristic fragment ions were quantified by comparing their peak areas with those from internal standards (Wu *et al.* 2007).

Plant Defense Activity

Cysteine proteinase inhibitor (Cyst PI) activity was analyzed according to Zavala *et al.* (2008). Briefly, 200 mg of frozen leaf powder was extracted in 1-mL of a 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 2.0 mM EDTA. Samples were vortexed for 10 s and centrifuged at 12,000 g for 15 min. Cyst PI activity was measured in samples on a plate reader at 37°C for up to 20 min at 410 nm against a papain standard by following the release of p-nitroaniline after the addition of a synthetic substrate p-Glu-Phe-Leu-pNA.

To determine polyphenol oxidase (PPO) activity, frozen leaf powder was extracted according to Anderson & Morris (2001). Briefly, 50 mg of tissue was placed in standard 2-mL microcentrifuge tubes containing 1.5-mL of 10 mM L-DOPA as a phenolic substrate in 50 mM MOPS buffer (pH 6.5). The tubes were then constantly rotated for 0.5 h at room temperature. Following incubation, solutions were centrifuged for 5 min at 12000 g and the change in absorbance was compared with a substrate-only control. The L-DOPA solution was made fresh daily. Absorbance was recorded at 475 nm. One unit of PPO activity was defined as a change of 0.001 absorbance unit/min/mL. All reactions were conducted at room temperature (about 20°C).

Protein concentration was measured (Bradford 1976) using BSA (bovine serum albumin) as a standard. Total Cyst PI and PPO calculations were adjusted to control for differences in protein loading.

Choice Experiments

The first fully expanded soybean trifoliolate was collected from four plants that had been growing for 45 d in each ambient and elevated CO₂ plot. Leaf disks (2.5 cm diameter) were punched from each trifoliolate from both treatments (N = 16), and one disk from each treatment was placed in Petri dishes (15 cm diameter) on filter paper moistened with distilled water. JB were collected as above, starved for 24 h prior to the experiment, and placed in the center of the Petri dish equidistant from the leaf disks. Beetles were allowed to feed for 24 h after which they were removed. Photographs were taken of the leaf disks to analyze leaf area removed, which was quantified using image analysis software (Image J, <http://rsbweb.nih.gov/ij/>).

Statistical Analyses

The qRT-PCR, Hormones, PPO and CystPI activity values were analyzed with a 2 × 5 (CO₂ treatment x time after herbivory) factorial analysis of variance ANOVA followed by Fisher's protected least significant difference (LSD) post hoc comparisons in all experiments. Because the only non-damaged control tissue was measured at the initial time point (time = 0 hrs), the main effect of time represents time after herbivory and is designated as H in the text and figures. Leaf area removed from the choice test was analyzed using a one way analysis of variance ANOVA (Exposure to CO₂). Data were analyzed with SAS, version 9.0 (SAS Institute).

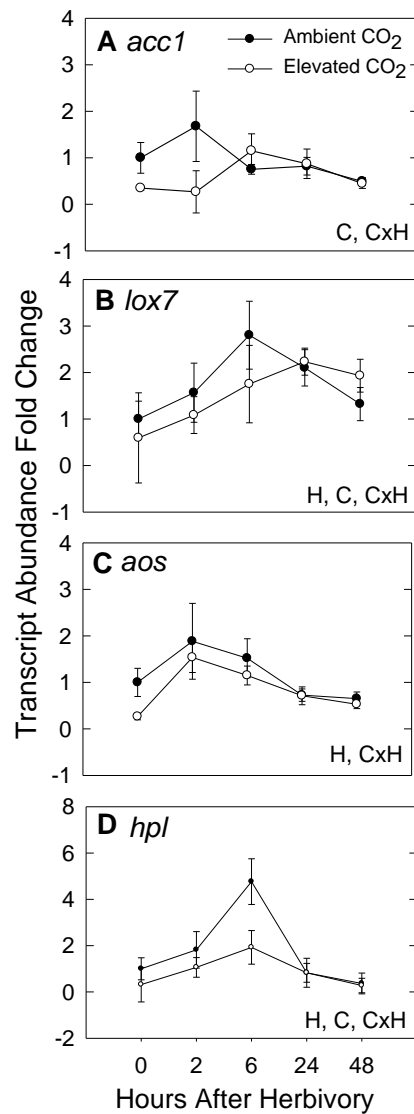


Figure 3.1. Transcript abundance (mean \pm SD) of four genes from fully expanded leaves of soybean grown either under elevated (open symbol) or ambient (filled symbol) CO₂ concentrations after Japanese beetle feeding: (A) 1-aminocyclopropane-1-carboxylate synthase (*acc1*), (B) lipoxygenase 7 (*lox7*), (C) allene oxide synthase (*aos*) and (D) hydrogen peroxide lyase (*hpl*). Significant main effect of CO₂ exposure and time after herbivory are indicated by C and H respectively, while significant interactions are indicated by CxH ($P < 0.05$).

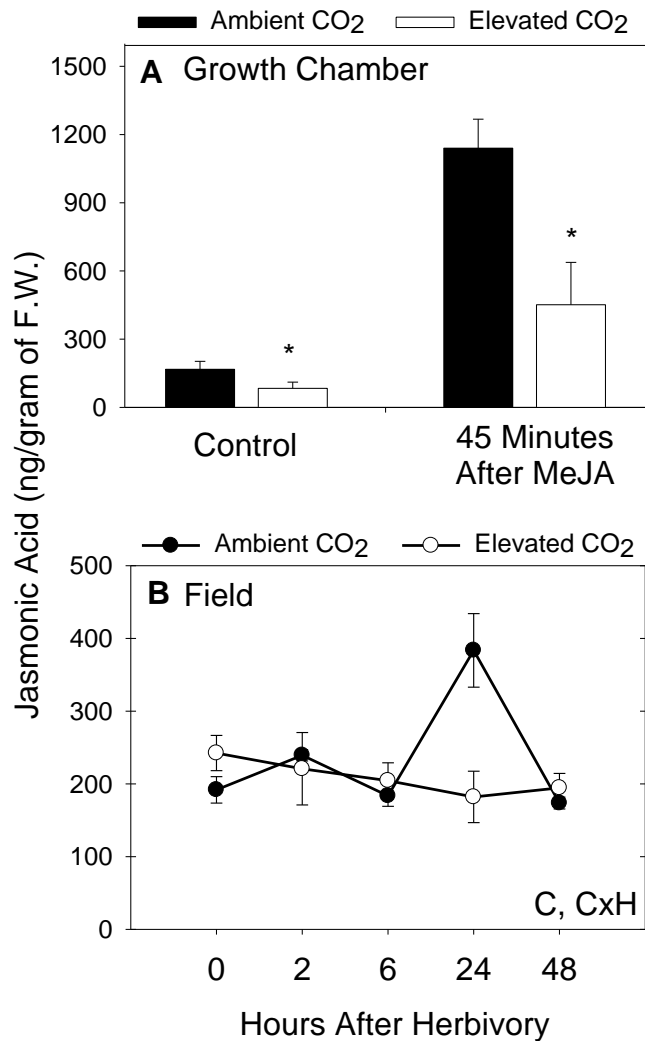


Figure 3.2. Jasmonic acid (JA; mean \pm SEM) from fully expanded soybean leaves grown under ambient CO₂ (filled bars and symbols) and elevated CO₂ (open bars and symbols) in (A) growth chambers or (B) at SoyFACE 45 minutes after induction by either MeJA (A) or after Japanese beetle herbivory (B). Asterisks indicate significant differences between ambient and elevated CO₂ treatments in (A; $P < 0.05$). In (B) significant main effect of CO₂ exposure and time after herbivory are indicated by C and H, respectively, while significant interactions are indicated by CxH ($P < 0.05$).

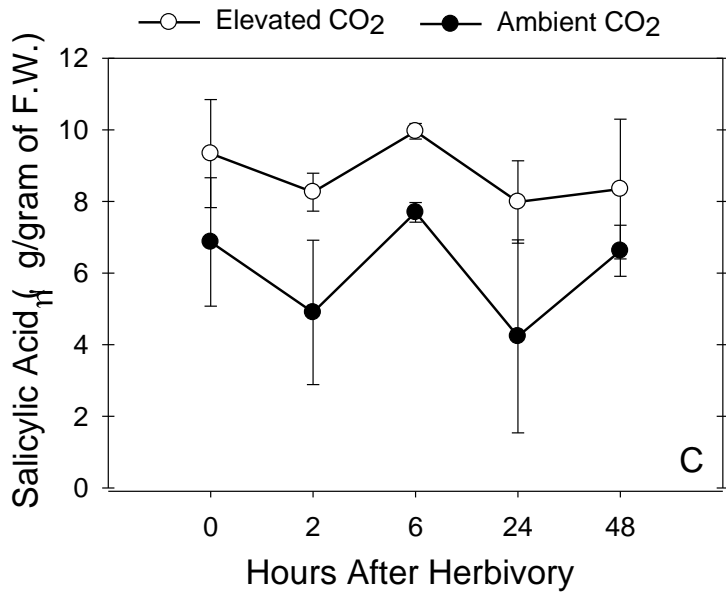


Figure 3.3. Salicylic acid (SA; mean \pm SEM) from fully expanded soybean leaves grown under ambient CO₂ (filled symbols) and elevated CO₂ (open symbols) at SoyFACE after induction by Japanese beetle herbivory. Significant main effect of CO₂ exposure and time after herbivory are indicated by C and H, respectively, while significant interactions are indicated by CxH ($P < 0.05$).

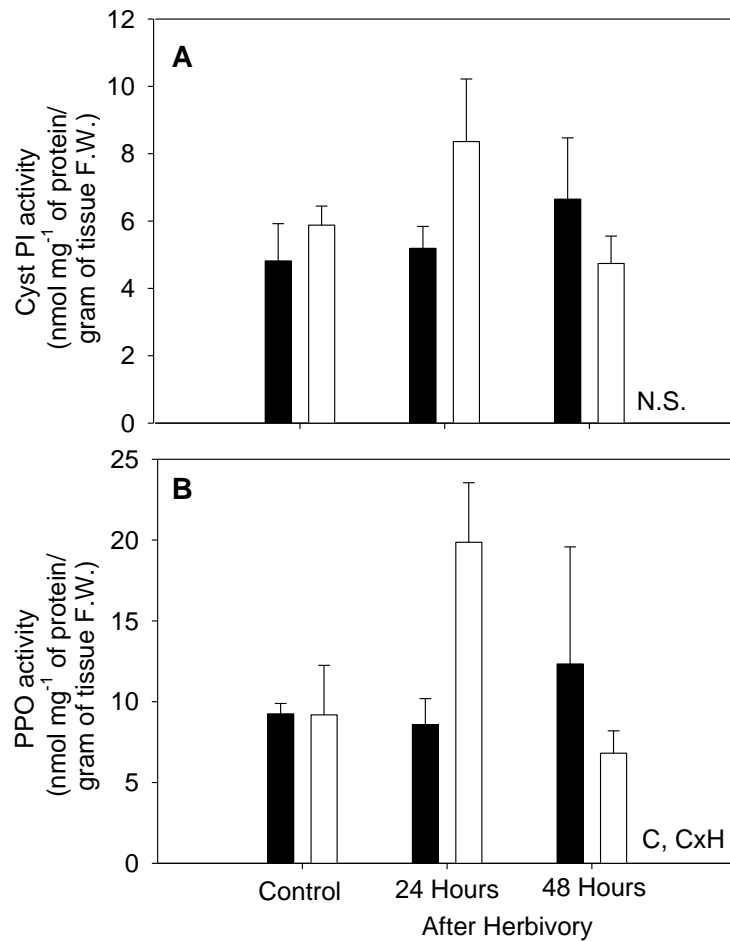


Figure 3.4. Cysteine proteinase (CystPI) activity (mean \pm SEM) (A) and polyphenol oxidase (PPO) activity from fully expanded soybean leaves grown under ambient CO₂ (filled bars) or elevated CO₂ (open bars) at SoyFACE in undamaged plants (control) and after herbivory by Japanese beetles. Significant main effect of CO₂ exposure and time after herbivory are indicated by C and H respectively, while significant interactions are indicated by CxH ($P < 0.05$).

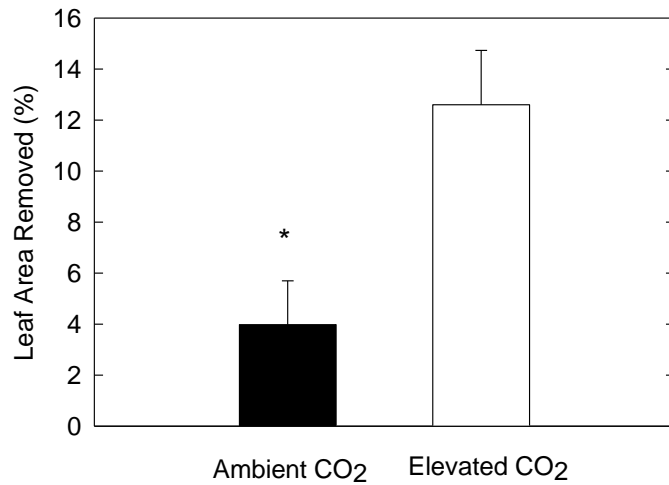


Figure 3.5. Area removed (mean \pm SEM) of fully expanded soybean leaves grown under ambient CO₂ (filled bars) or elevated CO₂ (open bars) after exposure to Japanese beetles for 24 h. Asterisks indicate significant differences between ambient and elevated CO₂ treatments ($P < 0.05$).

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CHAPTER 4
ELEVATED CO₂ AMPLIFIES PHYTOHORMONE SIGNALING AND
DEFENSE RESPONSES TO HERBIVORY UNDER DROUGHT
CONDITIONS IN *Glycine max*³

ABSTRACT

Plants will experience increased atmospheric CO₂ and drought in the future, possibly altering plant-insect dynamics. To investigate the combined effects of these elements of global change on plant-insect interactions, transcripts and metabolites in three major hormone signaling pathways (jasmonic acid [JA], salicylic acid [SA], ethylene [ET]) and related defenses in soybean (*Glycine max*) were examined after Japanese beetle (JB; *Popillia japonica*) feeding. Nutritional quality and JB preference for tissue grown in the four treatments also were determined. Elevated CO₂ increased the concentration of leaf sugars and dampened JA/ET signaling, but increased the abundance of SA compared to plants grown in ambient atmospheres. Exposure to drought had no effect on leaf sugars and stimulated the induction of JA/ET. When applied in combination, elevated CO₂ and drought greatly amplified the induction of JA and ET transcripts and the accumulation of related defenses in soybeans after herbivory. Exposure to elevated CO₂ alone decreased defenses and increased susceptibility of soybean to beetle damage. However, exposure to elevated CO₂ and drought together greatly amplified the induction of signaling genes and

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susceptibility to herbivory was reduced, highlighting the importance of examining interactions among the major components of global change under field conditions.

INTRODUCTION

If current trends in global atmospheric change continue unabated, by 2050 plants will be growing in an atmosphere with 50% more CO₂ than today (Forster *et al.* 2007). In combination with increased CO₂ concentrations, it is likely that air temperature will increase while precipitation will decrease during summer months in the United States, leading to increased drought (Meehl *et al.* 2007). Typically, drought reduces yield and agricultural productivity (Goldblum 2009), while elevated CO₂ has the opposite effect (Ainsworth & Long 2005; Long *et al.* 2006). However, the interactive effect of both components of global climate change is not well understood.

Increased CO₂ concentrations and drought may alter plant interactions with herbivores (Lindroth 2010). Exposure to elevated CO₂ decreases the nutritional quality of leaves by decreasing nitrogen concentration and increasing C:N ratios (Ainsworth *et al.* 2002). In contrast, when plants experience drought, nitrogen availability increases in plant tissues (White 1984; Mattson & Haack 1987; Huberty & Denno 2004). Because nitrogen is generally the component in plant foliage limiting the growth of insect herbivore (Mattson 1980; Awmack & Leather 2002), host plant quality is thought to improve for herbivores feeding on certain drought-stressed plants (White 1984) and to decline for herbivores feeding on plants grown under elevated CO₂. However, there is substantial variation in herbivore performance on plants grown under elevated CO₂ and drought conditions individually and in combination (Penuelas & Estiarte 1998;

Huberty & Denno 2004; Joren & Mole 2005; Zvereva & Kozlov 2006; Stiling & Cornelissen 2007), suggesting that other aspects of host plant quality are altered.

Phytohormones are universally employed by plants to coordinate responses to biotic and abiotic stresses. Three phytohormones, jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) have been studied extensively in the mediation of defense responses to biotic stressors, such as pathogens and herbivores (Glazebrook 2005; Jones & Dangl 2006; Howe & Jander 2008). Defense responses can be direct, negatively influencing herbivore performance (Wittstock & Gershenzon 2002) or indirect, attracting the natural enemies of herbivores (Dicke *et al.* 1999; Halitschke *et al.* 2008). Recently, we demonstrated that elevated CO₂ reconfigures these phytohormone signaling networks in soybean (*Glycine max*) plants, resulting in increased susceptibility to herbivores (Casteel *et al.* 2008; Zavala *et al.* 2008, 2009; Chapter 3). Transcripts associated with JA and ET signaling and related defenses (direct and indirect) were lower in soybean grown in elevated CO₂ after Japanese beetle (JB; *Popillia japonica*) damage compared to soybean grown in ambient atmospheres, while SA levels were elevated (Casteel *et al.* 2008; Chapter 3). In addition, the induction of cysteine proteinase inhibitors (CystPIs) and polyphenol oxidase (PPO), important anti-herbivore defenses, were lower after beetle damage (Zavala *et al.* 2008, 2009; Chapter 3), resulting in increased susceptibility to beetle herbivores (Zavala *et al.* 2008, 2009; Chapter 3). Plants may experience greater losses in future environments because of alteration in phytohormone signaling, although the combined impact of drought and elevated CO₂ has not been investigated.

Abiotic stress responses also are regulated by phytohormone signaling pathways. Drought responses are regulated largely by the phytohormone abscisic acid (ABA), which

accumulates in water-stressed plants (Walton 1980; Zeevaart & Creelman 1988). Phytohormone signaling pathways often interact synergistically and antagonistically with each other, allowing the plant to fine-tune and activate stress/attacker-specific responses (Stout *et al.* 2006; McSteen & Zhao 2008; Bari & Jones 2009). In addition, plants often experience multiple stresses at the same time, and the interactions among responses to each stress may influence the severity or the ability of plants to respond to the additional stress (Maleck & Dietrich 1999; Bostock *et al.* 2001; de Bruxelles & Roberts 2001; Bruce & Pickett 2007). Thus, the impact of elevated CO₂ on phytohormone signaling after herbivory in soybean may be altered under drought conditions.

In this study, the interacting effects of water supply and CO₂ exposure on components of susceptibility in soybeans were examined at the Soybean Free Air Concentration Enrichment (SoyFACE) experiment in plants damaged by JB. Plants were grown in a full factorial experiment with elevated CO₂ and drought. To determine the effect of water supply and CO₂ exposure on soybean phytohormone signaling, we analyzed the expression of transcripts in ET and JA signaling pathways. Transcript abundance of 1-aminocyclopropane-1-carboxylate synthase (*acc1*), which is involved in the regulation and biosynthesis of the signaling hormone ethylene, and two genes in the octadecanoid signaling pathway, allene oxide synthase (*aos*) and hydrogen peroxide lyase (*hpl*), were examined. Abundance of *aos* is related to the synthesis of signaling hormone JA and direct defenses, while *hpl* is a key regulatory gene in the biosynthesis of green leaf volatiles, an indirect defense (Halitschke *et al.* 2004; Matsui 2006). Difficulties measuring these dynamic metabolites under field conditions made examining transcript abundance essential. In addition, abundance of the phytohormones JA and SA and related direct defense metabolites, as well as protein and sugar content, important components of insect

nutrition, were measured in tissue under all four treatments. To determine if changes in leaf chemistry altered feeding behavior, JB preferences among tissues grown under the four treatments were quantified in a 4-way choice experiment in the laboratory with tissue samples collected from field-grown plants.

RESULTS

Transcript Abundance

Elevated CO₂ and drought had opposite effects on the expression of genes involved in defense signaling. Elevated CO₂ decreased *hpl* and there was a trend for decreased *aos* (main effect of CO₂ exposure; $P = 0.01$, $P = 0.09$, respectively; Fig. 4.1a, c). In contrast, drought increased the abundance of *aos*, *hpl* and *acc* in soybean leaves (main effect of water supply, $P < 0.01$; 266%, 203%, and 399%; respectively; Fig. 4.1a, b, c). Herbivory by Japanese beetle (JB) increased abundance of both *aos* and *hpl* in soybean (main effect of herbivory: $P = 0.0003$, $P = 0.0045$; respectively; Fig. 4.1a, c). Exposure to drought modified the response of transcripts to elevated CO₂ (Fig. 4.1a, b; significant two-way and three-way interaction, respectively, CO₂ exposure x water supply; CO₂ exposure x water supply x herbivory; $P < 0.05$). The reduction in transcription caused by elevated CO₂ was lost following damage by JB, and exposure to elevated CO₂ and drought in combination greatly intensified the transcription of *aos*, *hpl* and *acc* following induction by JB, respectively (267%, 1143%, 157%; respectively; Fig. 4.1a,b,c).

Hormones and defenses

Levels of SA were higher in plants grown under elevated CO₂ compared to those grown in ambient air (Fig. 4.2a; 25% increase in SA; main effect of CO₂ exposure; $P = 0.005$). Growth

of soybeans under drought had no significant impact on SA levels, and neither water supply nor CO₂ exposure, singly or in combination, affected the concentration of JA in soybeans (main effect and interactive effect $P > 0.05$; Fig. 4.2b). Because of the dynamic nature of ET it was not measured in the field.

While there was no significant main effect of elevated CO₂, water supply or herbivory on the plant defenses examined, there were significant interactions (Fig. 4.3a, b; $P > 0.05$); drought modified the impact of CO₂ on polyphenol oxidase activity (PPO; CO₂ exposure x water supply; $P = 0.047$; Fig. 4.3b). PPO activity was reduced in well-watered plants exposed to elevated CO₂ (reduced 45%; Fig. 4.3b) and activity increased in plants exposed to the combination of drought and elevated CO₂ (increased 75%; Fig. 4.3b). There was no main effect or interactive effect of CO₂ exposure or water supply on cysteine proteinase inhibitor (CystPI) activity in undamaged or damaged soybean tissue (Fig. 4.3a).

Leaf chemistry

Elevated CO₂ increased leaf carbohydrate content, JB herbivory reduced carbohydrate levels, and water supply had no effect (Table 4.1; main effect of CO₂ exposure, $P < 0.01$; herbivory $P < 0.01$; water supply; $P > 0.05$). Total protein content was not affected by CO₂ exposure, water supply or herbivory (Table 4.1; $P > 0.05$). Leaf fructose and glucose content increased significantly in foliage grown under elevated CO₂ (main effect of CO₂ exposure; $P < 0.01$; Table 4.1), while there was a trend for increased sucrose (main effect of CO₂ exposure; $P = 0.065$; Table 4.1). After herbivory, glucose and sucrose content decreased (main effect of herbivory; $P < 0.05$; Table 4.1), while fructose content increased (main effect of herbivory; $P = 0.0046$; Table 4.1), and the reduction of fructose and glucose in leaves was more pronounced

under elevated CO₂ compared to ambient conditions (CO₂ exposure x herbivory; $P < 0.001$; Table 4.1).

Feeding behavior of Japanese beetles

To determine the direct and interacting effects of elevated CO₂ and drought on herbivory, JB were allowed to feed with equal access to leaves grown under ambient and elevated CO₂ atmospheres under normal watering conditions and drought conditions, in a four-way choice experiment for 24 hrs. Soybean tissue grown under elevated CO₂ was preferred by beetles compared to foliage grown under ambient CO₂ (main effect of CO₂ exposure; $P = 0.035$; Fig. 4.4). Beetles removed 13.2% of leaves grown under elevated CO₂, compared to only 3.5% of leaves grown in ambient CO₂ (Fig 4.4). There was no significant effect of drought on leaf area removed by JB ($P > 0.05$; Fig. 4.4).

DISCUSSION

Elevated CO₂ and drought had opposing effects on defense signaling in soybean; reduced water supply attenuated the increased susceptibility to herbivory caused by elevated CO₂ (Fig. 4.3b; Fig. 4.4). Consistent with previous studies, exposure to elevated CO₂ increased susceptibility to herbivores by increasing sugar content, down-regulating constitutive phytohormone signaling (JA and ET) and dampening soybean's ability to induce a defensive response (Ainsworth *et al.* 2002, 2007; Casteel *et al.* 2008, Zavala *et al.* 2008, 2009; Chapter 3; Fig. 4.1a, b, c; Fig. 4.3b; Fig. 4.4). Exposure to drought had the opposite impact on phytohormone signaling transcripts, stimulating the induction of the JA/ET signaling pathways (Fig. 4.1a, b). Drought in combination with elevated CO₂ amplified the induction of JA and ET

signaling transcripts and the accumulation of related defenses after beetle herbivory (Fig. 4.1a, b, c; Fig. 4.3b). When applied in combination, drought removed the increased susceptibility of soybean to JB by elevated CO₂ (e.g. defense abundance, beetle preference; Fig. 4.3; Fig. 4.4).

Responses to drought are regulated mainly by the phytohormone abscisic acid (ABA), which accumulates in water-stressed plants (Walton 1980; Zeevaart & Creelman 1988). Drought and ABA can interact with JA signaling; water stress and exogenous ABA application predominately activates JA-related genes and promotes induced plant defenses (Reinbothe *et al.* 1992; Wasternack & Parthier 1997; Chao *et al.* 1999; Adie *et al.* 2007; Fan *et al.* 2009). In addition, JA levels are elevated in soybean leaves in response to water deficit (Creelman & Mullet 1995), as well as in other species (Deng *et al.* 2008). Considering the established induction of ABA signaling pathways in drought-stressed plants and the synergism between JA and ABA signaling (Walton 1980; Zeevaart & Creelman 1988; Reinbothe *et al.* 1992; Wasternack & Parthier 1997; Chao *et al.* 1999; Adie *et al.* 2007; Fan *et al.* 2009), it might be expected that drought-induced responses affect herbivore-induced signaling and defenses.

Indeed, we demonstrated that drought enhanced the induction and accumulation of JA signaling and JA-induced defense responses. Drought not only amplified the expression of a key gene involved in JA biosynthesis (*lox7*; Fig 4.1a), but it also amplified the expression of a gene involved in the synthesis of ET (*acc1*; Fig. 4.1b), known to act synergistically with JA to up-regulated defense responses. Furthermore, drought enhanced expression of *hpl*, which is involved in the synthesis of volatiles that can then be used as an indirect defense attracting natural enemies of herbivores (Fig. 4.1c).

Unexpectedly, exposure to elevated CO₂ and drought together intensified the induction of JA/ET signaling transcripts by herbivory in soybean plants (Fig. 4.1a, b, c). Consistent with transcriptional changes, the combination of elevated CO₂ and drought increased JA-regulated defense accumulation after herbivory (Fig. 4.3b) raising the possibility that palatability may be altered. We observed that soybean plants exposed to elevated CO₂ had increased susceptibility to JB (Fig. 4.4), consistent with previous studies (Zavala *et al.* 2008, 2009; Chapter 3). However, the susceptibility was removed in plants exposed to elevated CO₂ and drought (Fig. 4.4), likely as a result of the increased defenses from up-regulated JA signaling (Fig. 4.1a, b, c; Fig. 4.3b). Considering the positive influence of drought on JA signaling and related defenses and the fact that ABA can act synergistically with JA to induce defense responses (Reinbothe *et al.* 1992; Wasternack & Parthier 1997; Chao *et al.* 1999; Adie *et al.* 2007; Fan *et al.* 2009), ABA-related signaling may be involved. Although ABA was not measured in this study, previous studies demonstrate that levels are elevated in drought-stressed plants (Walton 1980; Zeevaart & Creelman 1988; Reinbothe *et al.* 1992; Wasternack & Parthier 1997; Chao *et al.* 1999; Adie *et al.* 2007; Fan *et al.* 2009). Further work is needed to identify the mechanisms by which drought intensifies the JA/ET signaling alone and in combination with elevated CO₂ in soybean and the possible involvement of other phytohormones such as ABA.

Differences in JA abundance were not resolved following exposure to elevated CO₂ or drought, singly or in combination (Fig. 4.2b). We speculate that the variation in JA may be related to the fact that these plants were grown in the field without protection from damage. Individual leaves used in these experiments were free of visible damage, but this was not the case for all trifoliates on an individual plant, as all field plants had some degree of damage

(Castele, personal observation). Damage to an adjacent leaf can cause JA to accumulate to levels 30 times that of undamaged leaves and elevated levels can persist for extended periods (Glauser *et al.* 2008; Koo *et al.*, 2009). In addition, pathogens may not have been visible in undamaged leaves chosen in field environments where infection is common. Pathogens induce JA (De Vos *et al.* 2005; Truman *et al.* 2007) and could contribute to the large variation observed in the field. Schmelz *et al.* (2009) found JA induction in soybean to be more transient than in other plant species, with no sharp peak in accumulation within 3 hrs of damage or after induction with most elicitors, further contributing to the difficulty in resolving significant differences. The apparent lack of JA induction is consistent with previous field data in soybean (Chapter 3) and likely stems from chronic induction in the field compared to controlled growth chamber experiments where exposure to elevated CO₂ down-regulates JA. This highlights the importance of examining plants under field conditions, which more accurately reflect the conditions plants experience and may not reflect previous findings using growth chambers.

Changes in nutritional quality of the host plant may not be as critical as secondary chemistry in determining beetle preference. In many species, exposure to elevated CO₂ decreases foliar nitrogen content (Long *et al.* 2006), a change that can be reversed by drought (White 1984; Mattson & Haack 1987; Huberty & Denno 2004). In our study, elevated CO₂ and drought together had no impact on nitrogen content (Table 4.1). The lack of a nitrogen response to elevated CO₂ in soybean may be related to its capacity for nitrogen fixation so that it is not generally nitrogen-limited in the field (Ainsworth *et al.* 2002). Exposure to elevated CO₂ increased constitutive levels of leaf sugars, but drought had no impact on carbohydrates (fructose, sucrose, and glucose; Table 4.1). Sugars are known feeding stimulants for Japanese

beetles (Ladd 1986) and increased sugar content in leaves grown under elevated CO₂ (Table 4.1) may explain the increased preference for and feeding on well-watered plants (Fig. 4.4).

However, increased sugars observed in the drought-stressed plants grown under elevated CO₂ did not result in higher leaf area removed (Fig. 4.4). Our results suggest that other aspects of host plant quality that are altered by drought, such as secondary chemistry (Fig. 4.3b; Inbar *et al.* 2001), may be more important in determining insect preference and future plant-insect dynamics.

Rising atmospheric CO₂ concentration will not be the only aspect of global change plants face in the future (Meehl *et al.* 2007). Rising CO₂ increases susceptibility of soybean to herbivory (Zavala *et al.* 2008, 2009; Chapter 3), but the effect of other factors in concert are largely unknown. In this study we demonstrate that drought attenuates the effects of rising CO₂ and allows soybean plants to defend themselves more effectively against JB herbivory. However, increased investment in defense responses might result in higher costs that in natural systems could be expected to exert selection on the evolution of plant signaling pathways and defenses in the future. In conclusion, the interactions between different aspects of global change may play a more important role in determining plant-insect interactions than previously hypothesized.

METHODS

Site description

Research was performed at the Soybean Free Air gas Concentration Enrichment (SoyFACE) experiment at the University of Illinois, Urbana-Champaign (40°02' N, 88°14' W, 228-m above sea level; [http:// soyface.illinois.edu](http://soyface.illinois.edu); Long *et al.* 2004), where large plots of soybean are exposed ('Pioneer 93B15') to elevated concentrations of CO₂. The experiment

consists of eight 20-m-diameter octagonal plots (total area 282.8-m²) distributed within four randomized blocks of soybean. Soybean was rotated annually with corn and no N fertilizer was added, according to standard agronomic practice in this region. Soybeans had been growing for 45 days at the beginning of the experiment and were in the vegetative stage.

Soybean plots either were at current ambient CO₂ concentration (ambient = 386 μmol mol⁻¹) or fumigated to a target CO₂ concentration of 550 μmol mol⁻¹ (elevated = 553 μmol mol⁻¹ for the 2008 season) the level predicted by 2050 (Forester *et al.* 2007). Within each plot, one 8 × 4 m² subplot was exposed to ambient levels of rainfall and one 8 × 4 m² subplot was exposed to reduced levels of rainfall. The reduction in rainfall was achieved with retractable awnings that were deployed manually at dusk when nighttime rainfall was predicted. The awnings were retracted at dawn the following day. In the second two weeks of July, 2008 when this experiment was conducted, the drought treatment significantly reduced soil volumetric water content in the top 25 cm by 12-30% in ambient CO₂ plots and by 0-24% in elevated CO₂ plots (S.B Gray, unpublished data). This reduction in soil water content represents a mild drought (~13-17 % reduction in soil water content; Erice *et al.* 2010). Soil volumetric water content at 25-105 cm depths was not affected by drought treatment.

In each plot the first fully expanded trifoliolate was enclosed in a mesh bag (1 x 4 mm mesh) of 24 undamaged plants to prevent movement of insects (12 in the well-watered area and 12 in the drought area in each plot). Five adult Japanese beetles [JB] were enclosed in each of nine bags in both drought and well-watered areas of each plot. Using commercially available traps JB were collected adjacent to the SoyFACE site and starved for 24 hrs prior to infestation. Starved beetles were allowed to feed until 10% of the leaf area was consumed (~1 hr). Following

damage, JB's were removed and three locally damaged trifoliates from separate plants were collected. Trifoliates were pooled separately at 2, 6, and 48 hrs after removal of beetles (total plants 9) and the remaining undamaged trifoliates were collected and pooled as an undamaged control. Tissue was frozen in liquid nitrogen, ground to a fine powder, and stored at -80°C. The complete experiment was replicated four times (4 plots for each treatment, 8 samples each (4 drought and 4 well-watered), resulting in 64 tissue samples ($n = 4$).

RNA preparation

Total RNA was extracted from the 32 frozen tissue samples (0 hrs and 2hrs) using Trizol reagent (Invitrogen, Carlsbad, CA). The early time points only (0 hrs and 2hrs) were examined for transcriptional changes based on previous observations of induction patterns in soybean (Chapter 3). Using a 1.2% formaldehyde agarose gel (Sambrook *et al.* 1989) and a microfluidic visualization tool (Bioanalyzer, Agilent Technologies, USA, <http://www.agilent.com>) RNA integrity was determined.

Quantitative real time RT-PCR (qRT-PCR)

The expression levels of genes coding for *aos* (allene oxide synthase), *hpl* (hydrogen peroxide lyase), *acc1* (1-aminocyclopropane-1-carboxylate) synthase and *cystPI* (cystiene proteinase inhibitor) were analyzed with quantitative real-time RT-PCR (qRT-PCR), with *con6* (contig 6) as an internal standard according to Casteel, (2010). Contig 6 was identified as constitutively expressed (Libault *et al.* 2008) and stable expression was verified across samples using qRT-PCR Total RNA (1 µg) was DNase treated (RQ1 DNase, Promega, Madison, WI) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo-dT₁₂₋₁₈ as a primer. Primers used for qRT-PCR were designed using Primer-Blast

(http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=NcbiHomeAd) according to Casteel *et al.*, unpublished. Primer sequences can be found in supplemental material (S1). Reactions were carried out using 5 µl of the ‘Syber green PCR master mix’ (Applied Biosystems, Foster City, CA), with 800nM of primer, in the ‘7500’ instrument (Applied Biosystems, Foster City, CA). The PCR conditions were run according to Casteel, (2010) and analyzed with the standard curve method (http://www.biotech.uiuc.edu/centers/Keck/Functional_genomics/taqman/Guide%20to%20relative%20quantitation.pdf). Applied Biosystems software was used for averaging the technical replicates that were run in triplicate on the plate for each sample.

Phytohormones

JA and SA were measured 2 hrs and 6 hrs, respectively, after the beetles were removed and in undamaged plants for both; these times represent maximum post-damage induction in soybean (Schmelz *et al.* 2009). According to Wu *et al.* (2007), approximately 150 mg from each frozen tissue sample transferred to FastPrep tubes (Qbiogene, Carlsbad, CA) containing 900 mg of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ, USA). Using a bead beater, samples with 1 ml of ethyl acetate and internal standards were then homogenized for 2 min (100 ng of D₂-JA and 100 ng D₄-SA per 1 ml of ethyl acetate). After centrifugation at 12000 g for 20 min at 4°C, 500 µl were removed and transferred to a new tube. An additional 1 ml of ethyl acetate without internal standards was added to the remaining tissue was then re-extracted and centrifuged as described. The supernatants were combined (500 from the first extraction and 250 µl from the re-extraction) and then dried by evaporation on a vacuum concentrator. Samples

were then dissolved in 500 μ l 70% (v/v) methanol, vortexed for 10 min and subsequently centrifuged at 12000 g for 10 min.

Measurements were conducted on a liquid chromatograph-mass spectrometry system (Shimadzu 2010 EV, Shimadzu, Columbia, MD, US) according to Wu *et al.* (2007). A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) was used in a gradient mode for the separation. The mass spectrometer was operated in a negative electro-spray ionization mode. The hormones (JA and SA) were quantified by comparing their peak areas with those from internal standards

Plant defenses

Measurements were made 48 hrs after the beetles were removed as this represents the time of maximum induction in soybean (Chapter 3). In summary for CystPI activity, 200 mg of frozen leaf powder was extracted in 1 ml of a 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 2.0 mM EDTA. Samples were vortexed for 10 sec and centrifuged at 12,000 g for 15 min. CystPI activity was measured in samples on a plate reader at 37°C for up to 20 min at 410 nm against a papain standard by following the release of p-nitroaniline after the addition of a synthetic substrate p-Glu-Phe-Leu-pNA.

For polyphenol oxidase activity (PPO), 50 mg of frozen leaf powder was extracted according to Anderson and Morris (2001). Briefly, tissue was extracted in a 50 mM MOPS buffer (pH 6.5, Sigma Aldrich) with 10 mM L-DOPA as a phenolic substrate (L-DOPA solution was made fresh daily). Tubes were constantly rotated for 0.5 hrs at room temperature and then were centrifuged for 5 min at 12000 g. The change in absorbance at 475 nm was determined and

compared with a substrate-only control. One unit of PPO activity was defined as a change of 0.001 absorbance unit/min/ml. All reactions were conducted at room temperature (about 20°C).

Protein concentration was determined (Bradford 1976) using a commercial kit (BCA protein assay kit; Thermo scientific; Rockford, IL, USA) with BSA (bovine serum albumin) as a standard. Total CystPI and PPO calculations were adjusted to control for differences in protein loading.

Leaf nutrients

To measure carbohydrates and protein content, ground leaf tissue was extracted according to Jones *et al.* (1977). Briefly, tissue was extracted five times in 80% (v/v), buffered (2 mM HEPES, pH 7.8) ethanol at 80 °C for 20-mins. Glucose, fructose, and sucrose concentrations were determined using a continuous enzymatic substrate assay (Jones *et al.* 1977). For protein determination, pellets of the ethanol extraction were dissolved at 95 °C in 0.1 M NaOH. The pH of the NaOH solution was then adjusted to 4.9 and protein concentration determined as described.

Choice experiment

The first fully expanded soybean trifoliolate was collected from 4 plants that had been growing for 45 days in the following four treatments: 1) ambient CO₂, well-watered; 2) elevated CO₂, well-watered; 3) ambient CO₂, drought; 4) elevated CO₂, drought. Leaf disks (2.5-cm diameter) were harvested from each treatment and placed in Petri dishes (15-cm diameter) on filter paper moistened with distilled water equidistant from the center. JB were collected and starved as described previously. Beetles were placed in the center of the Petri dish and allowed to

feed for 24 hrs after which they were removed. Photographs were taken of the leaf disks to analyze leaf area removed with image analysis software (Image J, <http://rsbweb.nih.gov/ij/>).

Statistical analyses

The transcript and metabolite values were analyzed with a 2 x 2 x 2 (CO₂ exposure x water supply x herbivory) factorial mixed model analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) post hoc comparisons in all experiments. For the comparisons, CO₂ exposure, water supply and herbivory was considered as fixed effects and block as a random effect. Leaf area removed in the choice experiment was analyzed using a 2 x 2 ANOVA (CO₂ exposure and water supply), followed by Fisher's protected LSD post hoc comparison. Data were analyzed using SAS, version 9.0 (SAS Institute).

TABLE 4.1. Nutritional changes in soybean grown under ambient and elevated CO₂ concentration with or without drought at SoyFACE in undamaged plants and after herbivory by Japanese beetles (mean ± SEM). All values are expressed in μmol/g of tissue F.W.

Nutritional Component	Undamaged				48 hours post infestation				Sig*
	Amb CO ₂	Elev CO ₂	Amb CO ₂ + Drought	Elev CO ₂ + Drought	Amb CO ₂	Elev CO ₂	Amb CO ₂ + Drought	Elev CO ₂ + Drought	
Protein	34.0 (+/- 2.9)	29.0 (+/- 3.7)	33.6 (+/-3.3)	31.8 (+/- 1.4)	37.6 (+/- 3.5)	33.6 (+/- 2.4)	32.9 (+/- 2.3)	30.7 (+/- 4.9)	ns.
Fructose	0.50 (+/- 0.08)	1.06 (+/- 0.08)	0.51 (+/- 0.06)	0.90 (+/- 0.08)	0.59 (+/- 0.10)	0.53 (+/- 0.07)	0.63 (+/- 0.01)	0.60 (+/- 0.04)	C, H, CxH
Glucose	2.0 (+/- 0.2)	3.4 (+/- 0.2)	1.3 (+/- 0.09)	3.0 (+/- 0.09)	1.2 (+/- 0.1)	1.4 (+/- 0.3)	2.0 (+/- 0.3)	1.5 (+/- 0.3)	C, H, CxH
Sucrose	1.9 (+/- 0.6)	3.0 (+/- 0.8)	2.7 (+/- 0.3)	4.8 (+/- 1.1)	1.6 (+/- 0.8)	2.0 (+/- 0.2)	2.0 (+/- 0.3)	1.8 (+/- 0.2)	H

Values represent the mean ± SEM. A statistically significant (P< 0.05) main effect of CO₂, drought or herbivory is indicated by C, D or H, respectively.

Interactions terms are included where significant. “ns” indicates no significant differences were observed.

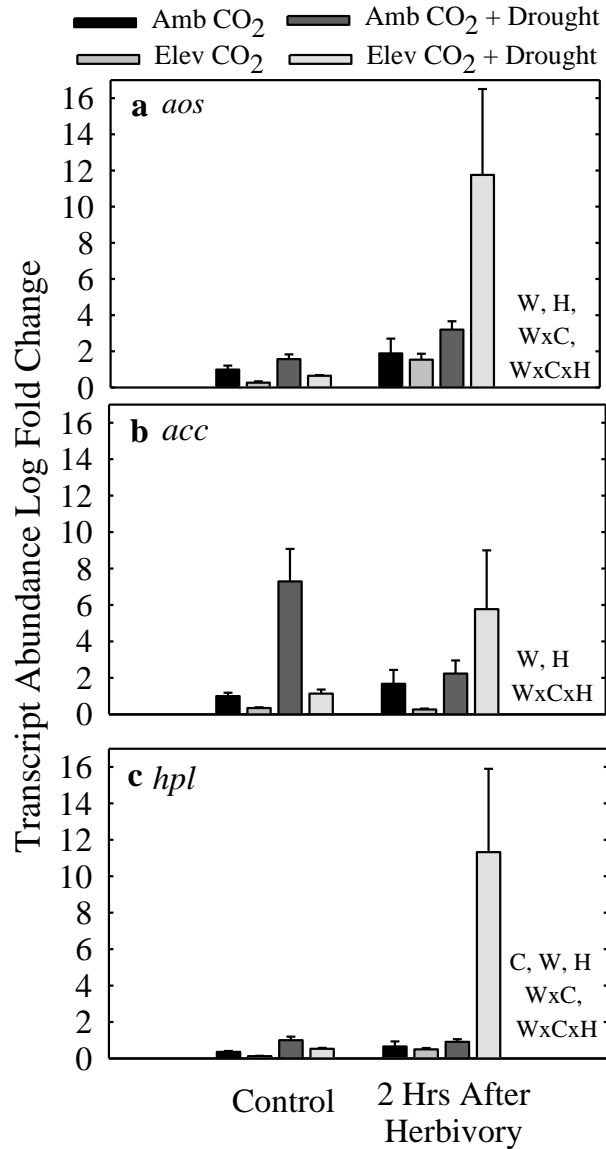


Figure 4.1. Transcript abundance of three genes from fully expanded leaves of soybean grown either under elevated or ambient CO₂ concentration with or without drought after a burst of Japanese beetle feeding: (a) allene oxide synthase (*aos*), (b) 1-aminocyclopropane-1-carboxylate synthase (*acc1*), (c) hydrogen peroxide lyase (*hpl*). A statistically significant ($P < 0.05$) main effect of CO₂ exposure, water supply or herbivory is indicated by C, W or H, respectively. Interaction terms are included where significant. Values represent the mean \pm SEM.

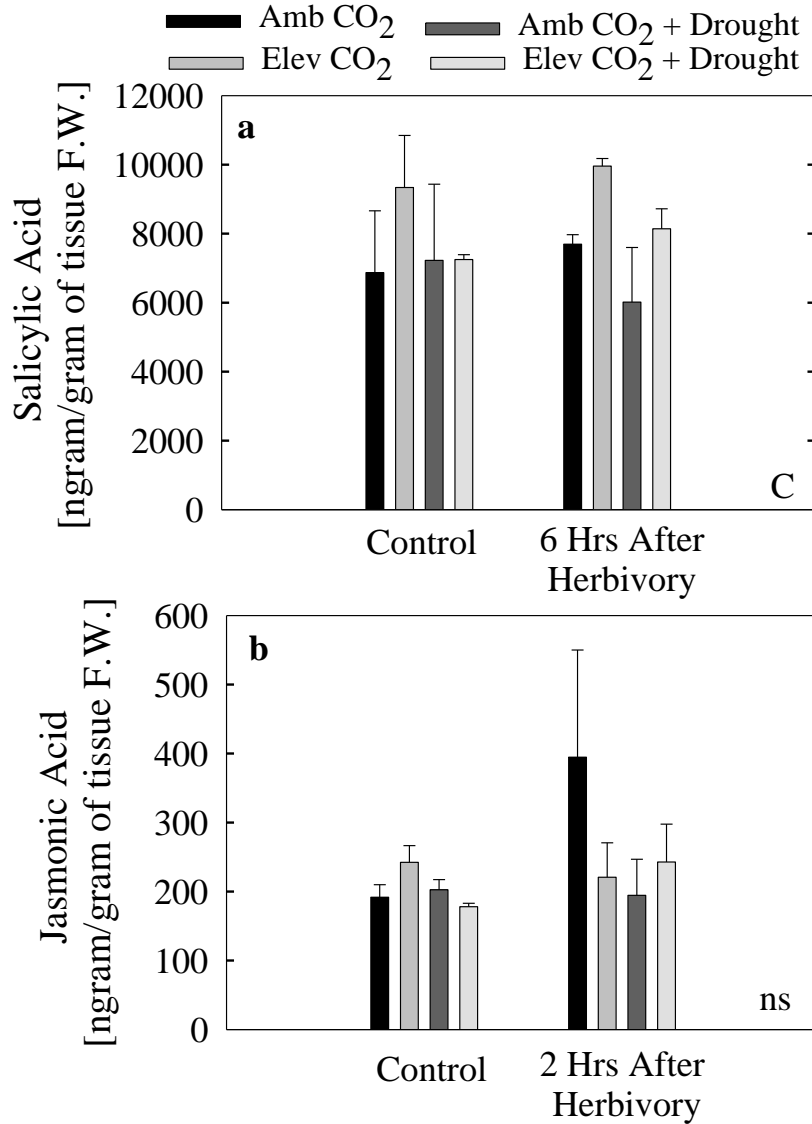


Figure 4.2. Accumulation of (a) jasmonic acid (JA) and (b) salicylic acid (SA) in soybean grown under ambient and elevated CO₂ concentration with or without drought in undamaged plants (control) and after a burst of herbivory by Japanese. A statistically significant ($P < 0.05$) main effect of CO₂ exposure, water supply or herbivory is indicated by C, W or H, respectively. Interaction terms are included where significant. “ns” indicates no significant differences were observed. Values represent the mean \pm SEM.

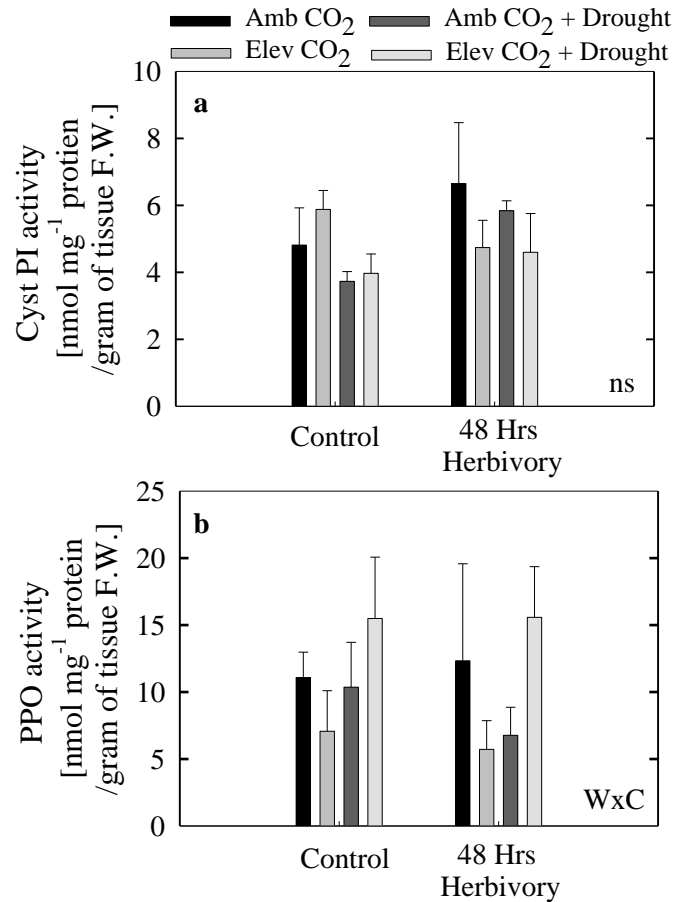


Figure 4.3. Abundance of (a) cysteine proteinase inhibitor (CystPI) and (b) polyphenol oxidase (PPO) activity from fully expanded soybean leaves grown under ambient or elevated CO₂ concentration with or without drought in undamaged plants (control) and after a burst of herbivory by Japanese beetles. A statistically significant ($P < 0.05$) main effect of CO₂ exposure, water supply or herbivory is indicated by C, W or H, respectively. Interaction terms are included where significant. “ns” indicates no significant differences were observed. Values represent the mean \pm SEM.

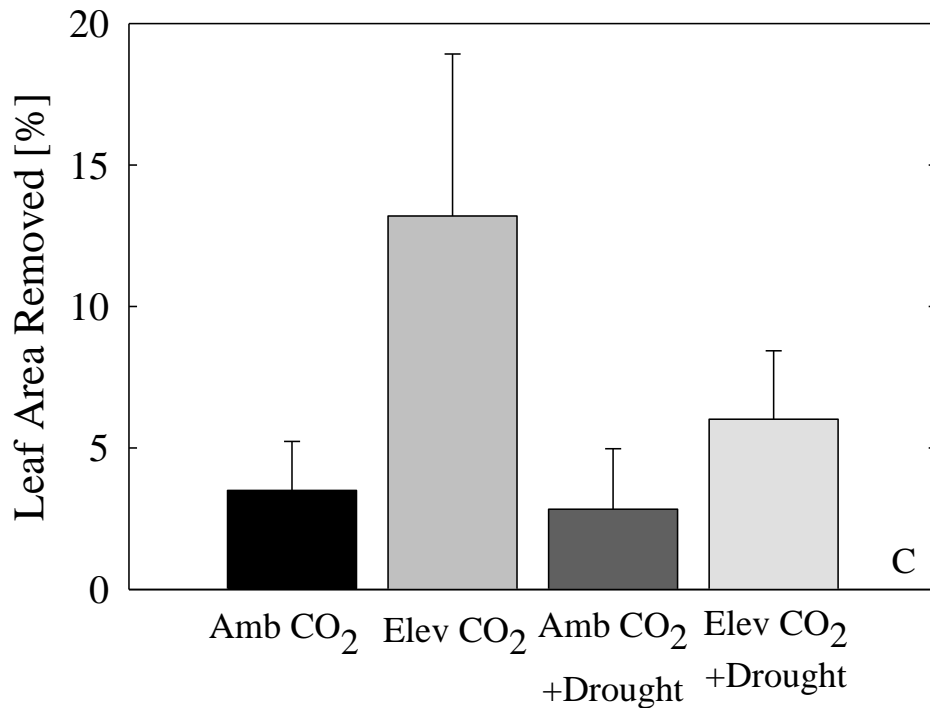


Figure 4.4. Leaf area removed of fully expanded soybean leaves grown under ambient or elevated CO₂ concentrations with or without drought after exposure to Japanese beetles for 24 hours. Asterisks indicate significant differences between ambient and elevated CO₂ treatments ($P < 0.05$). A statistically significant ($P < 0.05$) main effect of CO₂ exposure, water supply or herbivory is indicated by C, W or H, respectively. Interactions terms are included where significant. “ns” indicates no significant differences were observed. Values represent the mean \pm SEM.

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CHAPTER 5

IMPACT OF ELEVATED CARBON DIOXIDE ON PHYTOHORMONE SIGNALING: VARIATION ACROSS SIX CULTIVARS OF *Glycine max* AND SIX DIFFERENT PLANT SPECIES

ABSTRACT

Human activities are increasing the concentration of CO₂ in the atmosphere, affecting leaf chemistry and resistance to herbivores and pathogens in soybean (*Glycine max*). Modulation of soybean herbivore resistance under elevated CO₂ is a consequence of reconfigured phytohormone signaling pathways that coordinate defense responses to stress. It is not known if the impact of elevated CO₂ on phytohormones and induced defenses is a generalized response among soybean varieties or among other plant species. We examined jasmonic acid [JA], salicylic acid [SA], and ethylene [ET] signaling under ambient and elevated CO₂ concentrations across six soybean cultivars (HS93-4118, Pana, IA 3010, Loda, LN97-15076, and Dwight), and JA and SA were examined in six additional plant species (*Populus tremuloides*, *Triticum aestivum*, *Nicotiana tabacum*, *Solanum lycopersicum*, *Arabidopsis thaliana* and *Zea mays*). Constitutive and induced levels of transcripts and metabolites related to the phytohormones signaling pathways were measured following a common damage and sampling protocol. Elevated CO₂ reduced constitutive levels of JA and related transcripts in some but not all soybean cultivars. Similar to JA, constitutive and induced ET signaling varied significantly among soybean cultivars. In contrast to the variation in JA and ET, constitutive levels of SA were increased universally among soybean cultivars grown under elevated CO₂. In most species examined other than soybean, elevated CO₂ generally reduced constitutive JA signaling transcripts. However, in contrast to soybean there were no impacts of elevated CO₂ on

constitutive or induced levels of SA across species. Elevated CO₂ affected phytohormones associated with defense against herbivores but, with the exception of uniformly elevated constitutive levels of SA in soybean, the direction and magnitude of the response was cultivar and species-specific. Variation in hormonal signaling may underpin observed variation in the response of insect herbivores to plants grown under elevated CO₂. Determining genotypic variation in soybean and interspecific variation in other species to the effects of elevated CO₂ will facilitate breeding programs in the future and assist with development of suitable management strategies.

INTRODUCTION

The CO₂ concentration in the atmosphere is predicted to double from current levels by 2050 (Forster *et al.* 2007). Recent studies established that phytohormone signaling pathways are reconfigured in one cultivar of soybean (*Glycine max*) grown under predicted CO₂ concentrations (Casteel *et al.* 2008; Chapter 3; Chapter 4). Phytohormones are universally employed by plants to coordinate responses to abiotic and biotic stressors, such as biosynthesis of chemical defenses. Defensive traits can be constitutively present, but plants can also induce a defense response after attack (Kessler & Baldwin 2002; Howe & Jander 2008). Defense responses to biotic stressors, such as pathogens and herbivores, may involve one or more of three phytohormone signaling pathways: jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) (Glazebrook 2005; Howe & Jander 2008). In previous studies with soybean, induction of transcripts and metabolites related to JA and ET signaling was dampened while SA abundance was amplified under elevated CO₂, resulting in increased susceptibility to beetle herbivores (Casteel *et al.* 2008; Zavala *et al.* 2008, 2009; Chapter 3; Chapter 4) and greater resistance to

pathogens (Eastburn *et al.* 2010). These findings suggest that elevated CO₂ may alter plant interactions with other organisms in the future through changes in phytohormone signaling networks and induced defenses.

Genotypic differences in constitutive and induced phytochemistry are common within plant species (Maddox & Root 1990; Gols *et al.* 2008). Previous studies have demonstrated variation in transcription of defense-related genes and individual plant secondary metabolites correlates with variation in subsequent resistance to herbivores (Kusnierczyk *et al.* 2007; Gao *et al.* 2008; Wu *et al.* 2008). In addition, intraspecific variation in plant traits, such as resistance, may influence the composition of the herbivore and plant community (Wimp *et al.* 2005; Whitham *et al.* 2006; Poelman *et al.* 2009; Broekgaarden *et al.* 2010). Although it is well known that individual traits express natural variation within a species, few studies examine the impact of elevated CO₂ on plant-herbivore interactions across genotypes of plants (Castells *et al.* 2002; Holton *et al.* 2003; Bidart-Bouzat *et al.* 2004, 2005).

The relative plasticity of soybean signaling and induction of defenses under projected CO₂ concentrations will play a major role in determining the crop's future potential to resist herbivores. Soybean has been shown to express variation in the response to elevated CO₂ exposure, with cultivar-specific increases in yield (Ziska *et al.* 1998; Ziska & Bunce 2000). In addition, intraspecific variation in herbivore resistance is established in the literature (Dadson *et al.* 2007; McPherson & Buss 2007). To date, however, no information is available on how different soybean cultivars vary their phytohormone defense signaling in response to elevated CO₂ alone or in combination with herbivory. In addition, it is not currently known if elevated CO₂ affects phytohormone signaling and induced defenses uniformly across plant species. Only one cultivar of a single plant species has been examined (Casteel *et al.* 2008; Chapter 3; Chapter

4). Examining intra-specific and inter-specific variation in phytohormone signaling and induced defenses to the effects of elevated CO₂ will provide information for future breeding programs and assist the development of suitable management practices.

The first objective of this study was to evaluate variation in phytohormone signaling under elevated CO₂ among six cultivars of soybean. To address this objective, constitutive and induced levels of transcripts and metabolites related to JA, ET and SA were examined under ambient and elevated CO₂ concentrations in soybean cultivars at the Soybean Free Air Concentration Enrichment (SoyFACE) facility. Cultivars were chosen that vary in sensitivity to CO₂ exposure (HS93-4118, Pana, IA 3010, Loda, LN97-15076, and Dwight; R. Nelson, pers. comm.). The second objective of this study was to determine if alterations in JA and SA signaling under elevated CO₂ (Casteel *et al.* 2008; Chapter 3) are unique to soybean, or if these are a more generalized responses across various plant species. Diverse species were chosen, for economic importance and availability of sequence information (*Populus tremuloides*, *Triticum aestivum*, *Nicotiana tabacum*, *Solanum lycopersicum*, *Arabidopsis thaliana* and *Zea mays*). To aid in comparison of different species a common damage and sampling protocol was utilized across all experiments.

RESULTS

Intraspecific variation among soybean cultivars

To determine if the expression of genes related to phytohormone signaling and production of cysteine proteinase inhibitors (*cystPI*), an important soybean defense compound, vary among soybean cultivars grown under elevated CO₂, transcripts related to ET (1-aminocyclopropane-1-carboxylate synthase (*acc1*)), JA (lipoxygenase 7 (*lox7*), allene oxide

synthase (*aos*) and *cystPI*) were analyzed in six different soybean cultivars that vary in their sensitivity to elevated CO₂ (cultivars: HS93-4118, Pana, IA 3010, Loda, LN97-15076, and Dwight; R. Nelson, pers. comm.).

Jasmonic acid signaling and defenses

Soybean cultivars varied in constitutive expression of *lox7* (significant main effect of cultivar, ANOVA, $P = 0.0007$; Fig. 5.1), and exposure to elevated CO₂ reduced constitutive expression of *lox7* in 5 of 6 cultivars (significant CO₂ x cultivar interaction, $P = 0.07$; Fig. 5.1). Similarly, there was a trend for reduced expression of *aos* (main effect CO₂ exposure, $P = 0.07$; Fig. 5.2), downstream of *lox7* in the octadecanoid pathway leading to the production of JA. Averaged across cultivars, exposure to elevated CO₂ reduced constitutive levels of JA by 23% (main effect of CO₂, $P = 0.02$; Fig. 5.3), although there was significant variation in the impact of elevated CO₂ on individual cultivars (significant CO₂ x cultivar interaction, $P = 0.04$; Fig. 5.3).

Accumulation of JA in plants up-regulates the biosyntheses of herbivore specific defenses, such as cysteine proteinase inhibitors and related transcripts (Kessler & Baldwin 2001; Howe & Jander, 2008). There was no main effect of CO₂ exposure on constitutive *cystPI* expression among cultivars (main effect of CO₂, $P > 0.05$; Fig. 5.4). Constitutive expression of *cystPI* was cultivar-specific as was the impact of CO₂ (significant main effect of cultivar, $P = 0.0002$; Fig. 5.4; significant CO₂ x cultivar interaction, $P < 0.0001$; Fig. 5.4).

No main effect of elevated CO₂ on induction of *lox7* or *aos* transcripts was detected 2 hrs after damage (main effect CO₂, $P > 0.05$; Fig. 5.1 and 5.2, respectively). However, the induction of both transcripts varied among cultivars as did the impact of elevated CO₂ (significant cultivar main effect, $P < 0.01$; Fig. 5.1 and 5.2; significant CO₂ x cultivar interaction, $P \leq 0.01$; Fig. 5.1 and 5.2), indicating some cultivars responded differently on the transcriptional level. There was

no significant main effect of elevated CO₂ on induction of JA or *cystPI* (main effect of CO₂, $P > 0.05$; Fig. 5.3 and 5.4), nor was there significant variation in induced levels of JA among cultivars (main effect of cultivars and CO₂ x cultivar interaction, $P > 0.05$; Fig. 5.3). However, induction of *cystPI* varied among cultivars (main effect of cultivar, $P < 0.0001$; Fig. 5.4). Despite the lack of significance of elevated CO₂ on *cystPI*, the end product of the pathway, patterns were consistent for some cultivars throughout the pathway: elevated CO₂ reduced induced JA signaling and *cystPI* levels in HS93 and Pana, and increased induced levels in Dwight.

Ethylene signaling

In contrast with the expression of genes leading to the synthesis of JA, as well as JA itself, when averaged among cultivars elevated CO₂ increased the constitutive levels of *acc1* by 164% (significant main effect of CO₂, $P = 0.007$; Fig. 5.5), although this response was driven by the large increases in two cultivars (HS93 and IA3010; Fig. 5.5). There was considerable variation in the constitutive levels of *acc1* among cultivars examined (significant main cultivar effect, $P = 0.003$; Fig. 5.5). Because of the dynamic nature of ethylene, metabolite levels were not measured.

Consistent with constitutive levels, induced levels of *acc1* varied considerably with cultivar examined (significant main cultivar effect, $P < 0.0001$; Fig. 5.5). Overall, accumulation of *acc1* 2 hrs after damage was significantly increased in cultivars exposed to elevated CO₂ compared to ambient-grown plants (77% increase; significant main effect of CO₂, $P = 0.002$; Fig. 5.5), but this response was driven largely by two cultivars (HS93 and IA3010; Fig. 5.5)

Salicylic acid signaling

In previous studies (Chapter 3), elevated CO₂ increased SA abundance, although SA signaling transcripts examined were not altered, so for this study transcript abundance was not

examined. When averaged across cultivars, elevated CO₂ significantly increased the constitutive levels of SA by 50% (significant main effect of CO₂, $P = 0.008$; Fig. 5.6), and abundance of SA was not cultivar specific (main effect of cultivar, $P > 0.05$). Exposure to elevated CO₂ seemed to affect cultivars uniformly (CO₂ exposure x cultivar, $P > 0.05$). There was no significant main effect or interaction with CO₂ exposure or cultivar on induced levels of SA ($P > 0.05$).

Variation among plant species

To determine if the expression of genes related to phytohormone signaling varied among species exposed to elevated CO₂, JA and a related transcript (lipoxygenase (*lox*)) and SA were analyzed in six different species (*Populus tremuloides*, *Triticum aestivum*, *Nicotiana tabacum*, *Solanum lycopersicum*, *Arabidopsis thaliana* and *Zea mays*). These species were chosen because of their economic importance and the availability of relevant sequence information (<http://www.ncbi.nlm.nih.gov/>). Because of the dynamic nature of ethylene and difficulty obtaining sequence information and designing primers for all species, metabolite and transcript levels were not measured.

Jasmonic acid signaling

The constitutive levels of *lox* varied among species (significant main effect of species, ANOVA, $P = 0.0002$; Fig. 5.7), and when averaged across all species, elevated CO₂ caused a 154% decrease in constitutive levels (significant main effect of CO₂, $P = 0.02$; Fig. 5.7). Constitutive expression of *lox* was lower under elevated than ambient CO in five of six species and there was no significant CO₂ x species interaction ($P > 0.05$). However, these changes leveling transcription did not result in a significant decrease in JA across species (main effect of CO₂, $P > 0.05$; Fig. 5.8). Consistent with *lox*, JA abundance overall depended on species examined (main effect species, $P = 0.0006$; Fig. 5.8) and there was no significant interaction in

the constitutive level JA attributed to the interaction between elevated CO₂ and species (CO₂ x species, $P > 0.05$; Fig. 5.8).

While there was considerable variation in the induced levels of *lox* and JA among species (significant main effect of species, $P < 0.001$; Figs. 5.7 and 5.8), exposure to CO₂ had no effect on *lox* or JA accumulation 2 hrs after damage (main effect CO₂, $P > 0.05$), and there was no significant CO₂ x species interaction ($P > 0.05$).

Salicylic acid signaling

Abundance of SA was species-specific (significant main effect of species, $P = 0.0003$; Fig. 5.9). In contrast to soybean cultivars, elevated CO₂ exposure had no effect on constitutive levels of SA across species (main effect CO₂ exposure, $P > 0.05$; Fig. 5.9). Accumulation of SA after damage was not altered by elevated CO₂ exposure (main effect CO₂, $P > 0.05$), but induced levels of SA varied among species (significant main effect of species, $P < 0.0001$).

DISCUSSION

Examining genotypic variation in phytohormone defense signaling in response to elevated CO₂ is fundamental for predicting potential consequences of global change and community composition (Whitham *et al.* 2006). Our results suggest individual signaling pathways will respond distinctly to global change within a species and across species. We observed large variation in JA and ET signaling pathways and little variation in SA within soybean, while SA varied among different species. Intra-specific variation implies there is potential for specific signaling pathways to evolve in future enriched CO₂ environments, while others pathways might have less flexibility. While this study demonstrated that elevated CO₂

affects hormonal defense signaling in plants, the magnitude and direction of the response can be cultivar- and species-specific.

The impact of elevated CO₂ on JA and ET signaling appears to exhibit significant plasticity across soybean cultivars. Previous studies demonstrated that constitutive and induced expression of JA and ET signaling transcripts are reduced in soybean grown under elevated CO₂ conditions (Pioneer 93B15 cv.; Casteel *et al.* 2008; Chapter 3; Chapter 4). In this study, however, constitutive and induced expression of the JA and ET signaling transcripts (*acc1*, *lox*) increased in response to elevated CO₂ in some soybean cultivars (Fig. 5.5). While the cultivar previously examined was not utilized in this study (Pioneer 93B15 cv.; Casteel *et al.* 2008; Zavala *et al.* 2008, 2009; Chapter 2; Chapter 3), similar expression patterns were observed for *lox* and *acc1* (e.g. HS93, Ln97 cv. respectively; Fig. 5.1; Fig. 5.5). This highlights the importance of examining the most appropriate plants and genotypes that accurately reflect future populations to make predictions about how climate change will affect crop species.

Few studies have examined phytohormone levels in different plant species grown under elevated CO₂ exposure. Changes in an individual phytohormone may be species-specific. Salicylic acid levels were elevated across soybean cultivars with little variation in the response (Fig. 5.6), consistent with previous studies in soybean (Chapter 3; Chapter 4). However, elevated CO₂ did not consistently increase SA abundance across the other species examined (Fig. 5.9). Despite the few studies examining phytohormones or induced defenses under elevated CO₂, patterns observed for individual species were consistent with previous results for both JA (induced defenses; Bidart-Bouzat *et al.* 2005) and SA (Jwa & Walling 2001).

Because changes in SA and JA levels can strongly influence plant resistance to pathogens (Vlot *et al.* 2009) and insects (Howe & Jander 2008), these results suggest that some species and

cultivars will experience increased losses to pests while other may be more resistant under future CO₂ concentrations. Changes in phytohormone signaling networks represent possible changes in induced defenses, which can alter plant fitness in the presence of subsequent herbivore and pathogen attack (Agrawal 1999; Kessler *et al.* 2004). For instance, increased levels of SA may confer greater resistance to pathogens in soybean under elevated CO₂ (Glazebrook 2005), while aspen and tobacco may be more vulnerable (Fig. 5.9). Recent studies have demonstrated lower pathogen populations in soybean grown under elevated CO₂ (Eastburn *et al.* 2010), further supporting our results. Thus, natural selection acting in future environments may favor those plant species and cultivars showing enhanced signaling and defenses which, in turn, can alter plant–insect interactions and community dynamics. However, variation in other species response should be investigated further to make accurate predictions.

In conclusion, this study demonstrates that phytohormone signaling could be affected by global atmospheric CO₂ changes across soybean cultivars and other species of plants, although a large range of variation exists in the response. Herbivores can be an important selective agent in plant evolution because they can reduce plant fitness and growth. The existence of variation in the response to elevated CO₂ suggests a potential for phytohormone signaling and defenses to evolve under future environmental challenges. Thus elevated CO₂ may affect interactions of plants with herbivores and other biotic factors in the future. In addition, these results indicate there is significant potential for directed selection in future breeding programs to mitigate the impacts of global change on plant resistance. This work will be critical for motivating further research on how future CO₂ enrichment may affect plant signaling and defenses and the dynamics between plants and their insect herbivores.

METHODS

Plant material and growth conditions

This study was conducted using leaf tissue exposed to ambient and elevated CO₂ concentrations either in Free Air gas Concentration Enrichment (FACE) experiments or in growth chambers when FACE sites were not available. The FACE experiments included SoyFACE and CornFACE, located at the University of Illinois, Urbana-Champaign (40°02' N, 88°14' W; <http://www.soyface.uiuc.edu>) and Aspen FACE, located near Rhineland, WI, USA (45°67' N, 89°66' W; www.aspenface.mtu.edu). The SoyFACE, CornFACE and Aspen FACE sites encompassed two diverse ecosystems: a soybean (*Glycine max*) and corn (*Zea mays*) agro-ecosystem, and a mixed aspen (*Populus*) community, respectively (Dickson *et al.* 2000; Long *et al.* 2004).

Each FACE facility comprised multiple 20-30 m diameter open-air plots (SoyFACE = 20 m; CornFACE = 20 m; Aspen FACE = 30 m). In these plots, CO₂ concentration was maintained at one of two levels: current ambient and elevated concentrations forecasted for the middle of this century (SoyFACE and CornFACE = ~550 μmol mol⁻¹; AspenFACE = ambient + 200 μmol mol⁻¹; Miglietta *et al.* 2001). The plots were replicated 3-4 times for each treatment at each site (SoyFACE and CornFACE N = 4; Aspen FACE N = 3). Complete descriptions of these individual facilities have been previously published (Long *et al.* 2004; Dickson *et al.* 2000).

Species utilized in the experiment grown under FACE conditions included soybean (*G. max*), corn (*Z. mays*) and trembling aspen (*Populus tremuloides*). Six soybean cultivars were utilized that varied in sensitivity to elevated CO₂: HS93-4118, Pana, IA 3010, Loda, LN97-15076, and Dwight. Cultivars were selected based on their varying sensitivity to elevated CO₂ (R. Nelson, pers. comm.). At the start of this experiment in 2008, aspen had been growing in the

treatments since the beginning of 1997. Soybeans and corn had been growing for ~35 and ~60 days respectively.

Additional species that were not grown in FACE experiments were examined in supplemental growth chamber experiments. These species, including wheat (*Triticum aestivum*), tobacco (*Nicotiana tabacum*), tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana*, were chosen because of their economic importance and the availability of relevant sequence information (<http://www.ncbi.nlm.nih.gov/>). All plants except *Arabidopsis* were grown from seeds in 3.8 liter pots containing soil-less mix (Sunshine Mix LC1, Sun Gro Horticulture Inc., Bellevue, WA). Plants were grown in controlled environment chambers (Egc, Chagrin Falls, OH) under a 10/14 h day/night cycle with a temperature regime of 22/18 °C and ~200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance. *Arabidopsis* was grown from bleach treated seeds in 8-cm pots under the same growth conditions following 4 day cold treatment at 4 °C in the dark. CO₂ concentrations were 400 $\mu\text{mol mol}^{-1}$ (ambient N = 4) or 600 $\mu\text{mol mol}^{-1}$ (elevated N = 4). At the beginning of the experiment species had been growing in chambers for ~ 28 days.

Experimental Design

A common wounding protocol was applied and all tissue was collected at the same time points following damage. Six undamaged plants were selected in each treatment and the first fully expanded leaf on three of the plants was damaged five times with a pattern wheel transverse to the major veins. For aspen, the first fully expanded leaf from the end of the branch was utilized. Three locally damaged leaves from separate plants were collected and pooled 2 hours after damage. The three remaining undamaged leaves were collected as an undamaged control. This complete block design was conducted in ambient and elevated grown tissue. The experiment was repeated four times (blocks: rings in field or chambers) for all herbaceous

species and three times for trees, resulting in 16 tissue samples per herbaceous species/cultivar and 12 for aspen (188 samples total). Tissue was frozen in liquid nitrogen, ground to a fine powder, and stored at -80°C.

RNA preparation

Total RNA was extracted from frozen leaf tissue for herbaceous species using Trizol (Invitrogen, Carlsbad, CA). Total RNA from poplar was extracted according to a modification of a Cetyl trimethylammonium bromide (CTAB) method (Chang *et al.* 1993). For this method, tissue was re-ground into an extra fine powder twice after which 3 ml of hot CTAB extraction buffer per 800 mg was ground into each sample. Samples were then incubated at 65°C for 10 min, were centrifuged and then had the supernatant removed. The supernatant was re-incubated with a hot 5% CTAB solution for an additional 20 minutes. Following centrifugation, the supernatant was extracted with chloroform-isoamyl alcohol (24:1 [v/v]) three times and precipitated overnight with 10M LiCl. RNA integrity was verified using a 1.2% formaldehyde agarose gel (Sambrook *et al.* 1989) and microfluidic visualization tool (Bioanalyzer, Agilent Technologies, USA, <http://www.agilent.com>).

qRT-PCR

The expression level of genes coding for *lox7* (lipoxygenase), *aos* (allene oxide synthase) and *acc* (1-aminocyclopropane-1-carboxylate) synthase were analyzed with quantitative real-time RT-PCR (qRT-PCR), with *con6* (contig 6) as an internal standard for soybean genotypes. Contig 6 was identified from a microarray study as constitutively expressed (Libault *et al.* 2008) and stable expression was verified across samples using qRT-PCR. After treatment with RQ1 DNase (Promega, Madison, WI), 1 µg of total RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo-dT₁₂₋₁₈ as a primer. Gene specific

primers used for qRT-PCR were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=NcbiHomeAd) with the following criteria: TM of 60°C, PCR amplicon lengths of 90 to 150 bp yielding primer sequences with lengths of 18 to 24 nucleotides with an optimum at 21 nucleotides, and guanine-cytosine contents of 40% to 60%. Primer sequences for the selected genes can be found in supplemental Table B (Appendix A). Reactions were carried out using 5 µl of the 'Syber green PCR master mix' (Applied Biosystems, Foster City, CA), with 800nM of primer, in the '7500' instrument (Applied Biosystems, Foster City, CA). The PCR was initiated with incubation at 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 analyzed according to the standard curve method (http://www.biotech.uiuc.edu/centers/Keck/Functional_genomics/taqman/Guide%20to%20relative%20quantitation.pdf).

For each species the expression level of genes coding for wound inducible *lox* and appropriate controls was examined as described, except for tobacco and corn where the annealing temperature was adjusted to 55 °C. Primer information for all species and genotypes can be found in supplemental Table B (Appendix A)

Phytohormone analysis

Approximately 150 mg from each frozen tissue sample were extracted for analysis of JA and SA according to Wu *et al.* (2007). Plant material was transferred to FastPrep tubes (Qbiogene, Carlsbad, CA) containing 900 mg of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ, USA). One milliliter of ethyl acetate mixed with 100 ng of D₂-JA and 100 ng D₄-SA, used as internal standards, was added to each sample. Samples were then homogenized

in a bead beater for 2 min. After centrifugation at 12000 g for 20 min at 4°C, 500 µL were removed and transferred to a new tube. The remaining tissue was then re-extracted with 1 mL ethyl acetate without internal standard and centrifuged as described. The supernatants were combined (250 µL from the re-extraction) and then dried by evaporation on a vacuum concentrator. The dried residue was dissolved in 500 µL 70% (v/v) methanol, vortexed for 10 min and subsequently centrifuged at 12000 g for 10 min.

Measurements were conducted on a liquid chromatograph-mass spectrometry system (Shimadzu 2010 EV, Shimadzu, Columbia, MD, US). A mobile phase composed of solvent A (0.05% formic acid) and solvent B (0.05% formic acid in methanol) was used in a gradient mode for the separation. The mass spectrometer was operated in a negative electro-spray ionization mode. The most abundant and characteristic fragment ions were quantified by comparing their peak areas with those from internal standards (Wu *et al.* 2007).

Statistics

The qRT-PCR and hormone metabolite values were analyzed with a 6 x 2 (cultivars/species x CO₂ exposure) factorial analysis of variance ANOVA for constitutive and induced experiments separately. We analyzed constitutive and induced treatments separately for simplicity; when included as an effect in a combined ANOVA, herbivory, and herbivory by cultivar or species interactions were highly significant. A subsequent Fisher's protected least significant differences LSD post hoc test was performed for all individual comparisons in all experiments. Data were analyzed using SAS, version 9.0 (SAS Institute).

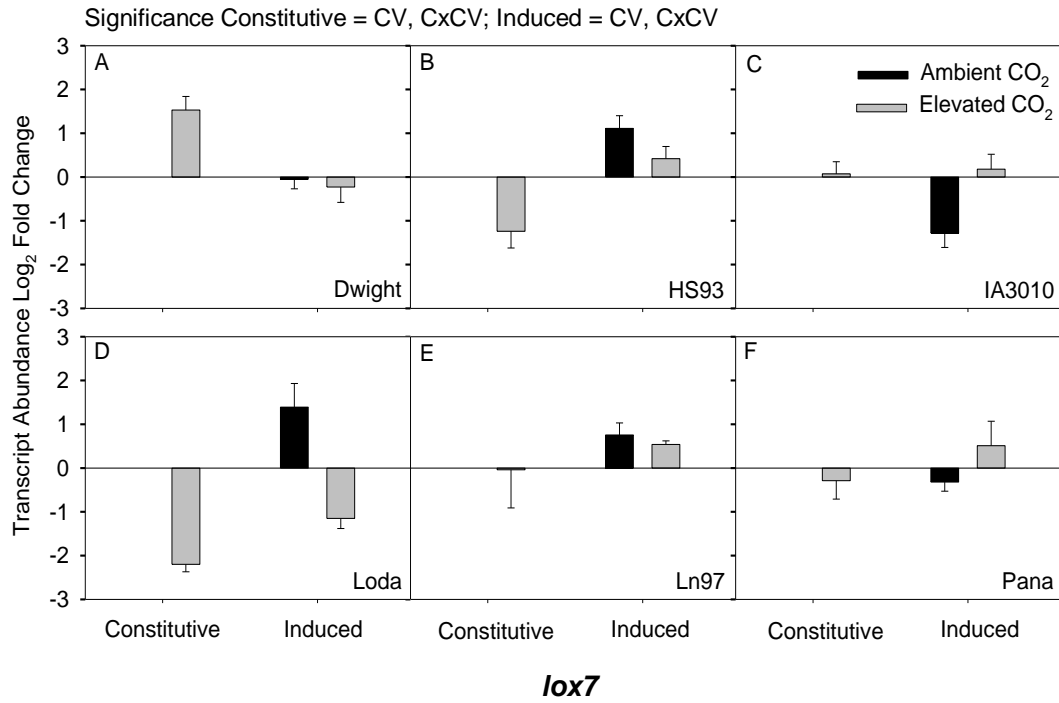


Fig. 5.1 Transcript abundance (mean \pm SEM) of *lox7* from leaves of six cultivars of soybean grown under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) Dwight, (B) HS93-4118, (C) IA31010, (D) Loda, (E) LN97-15076, and (F) Pana. Significant main effect of CO₂ exposure and Cultivar are indicated by C and CV, respectively, while significant interactions are indicated by CxCV (ANOVA; $P < 0.05$).

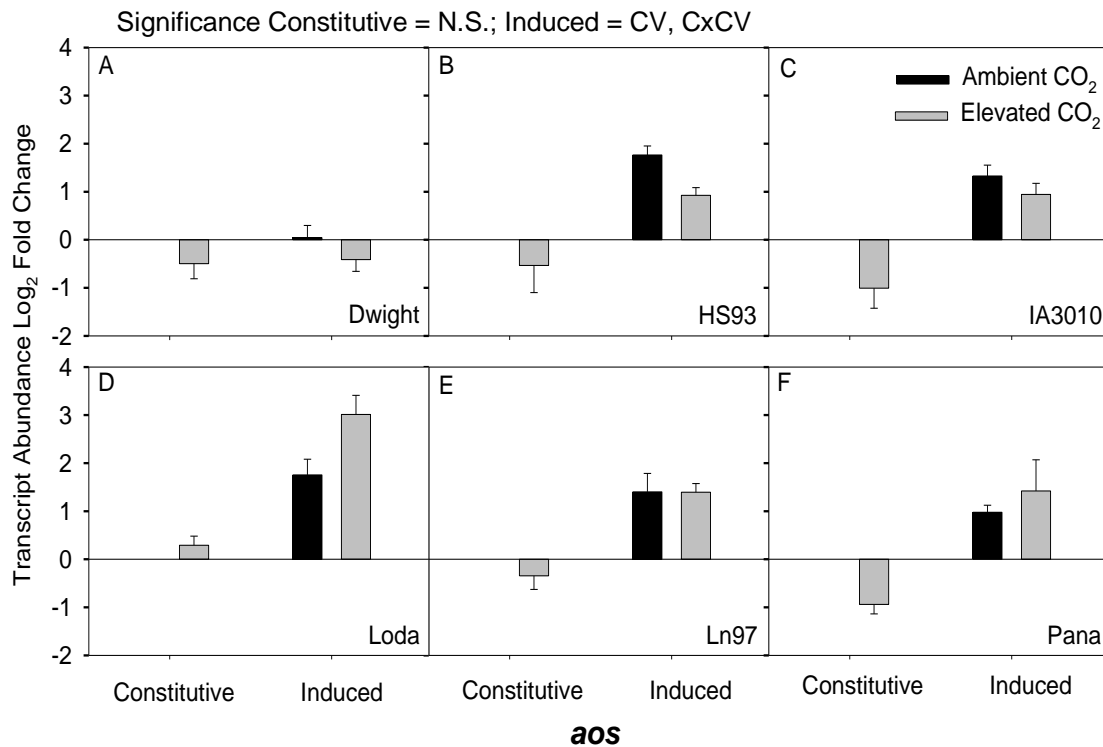


Fig. 5.2 Transcript abundance (mean \pm SEM) of *aos* from leaves of six cultivars of soybean grown under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) Dwight, (B) HS93-4118, (C) IA31010, (D) Loda, (E) LN97-15076, and (F) Pana. Significant main effect of CO₂ exposure and Cultivar are indicated by C and CV, respectively, while significant interactions are indicated by CxCV (ANOVA; $P < 0.05$).

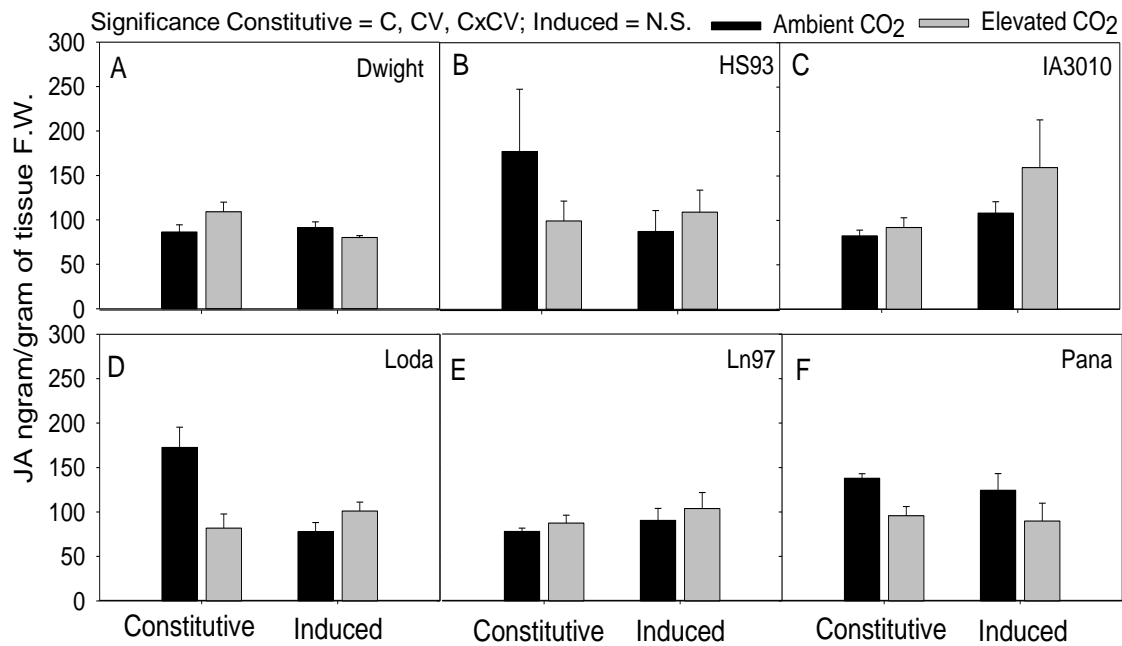


Fig. 5.3 Jasmonic acid (JA; mean \pm SEM) from leaves of six cultivars of soybean grown either under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) Dwight, (B) HS93-4118, (C) IA31010, (D) Loda, (E) LN97-15076, and (F) Pana. Significant main effect of CO₂ exposure and Cultivar are indicated by C and CV, respectively, while significant interactions are indicated by CxCV (ANOVA; $P < 0.05$).

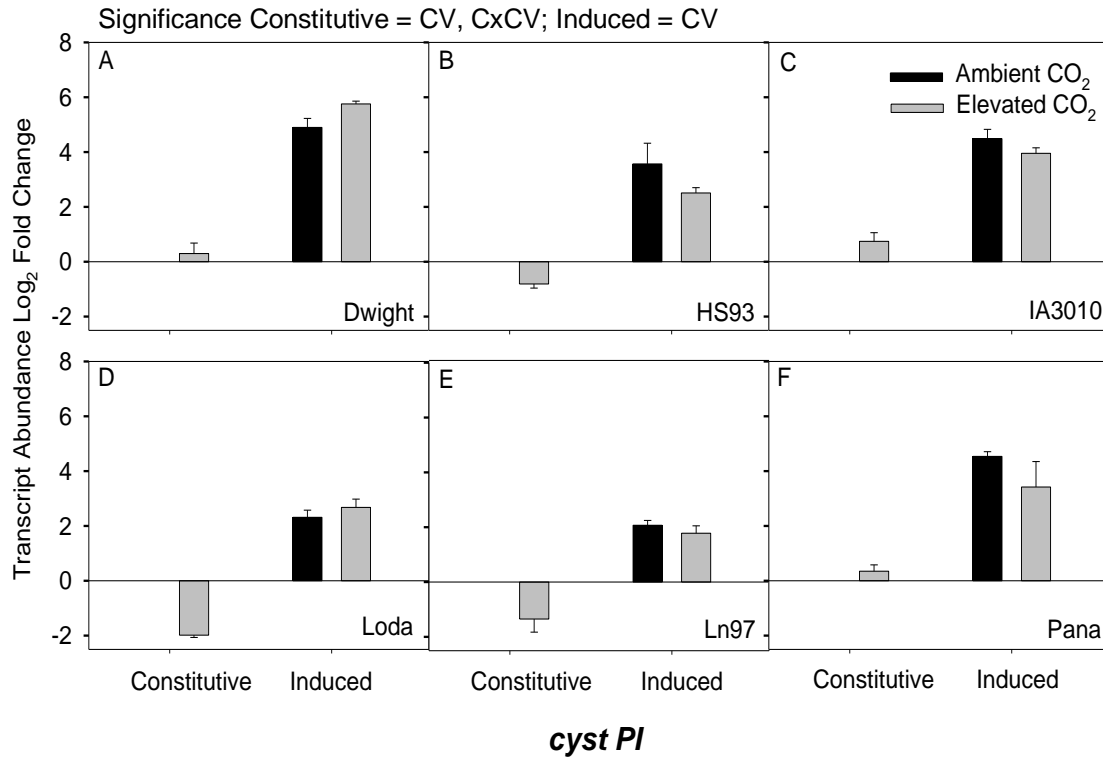
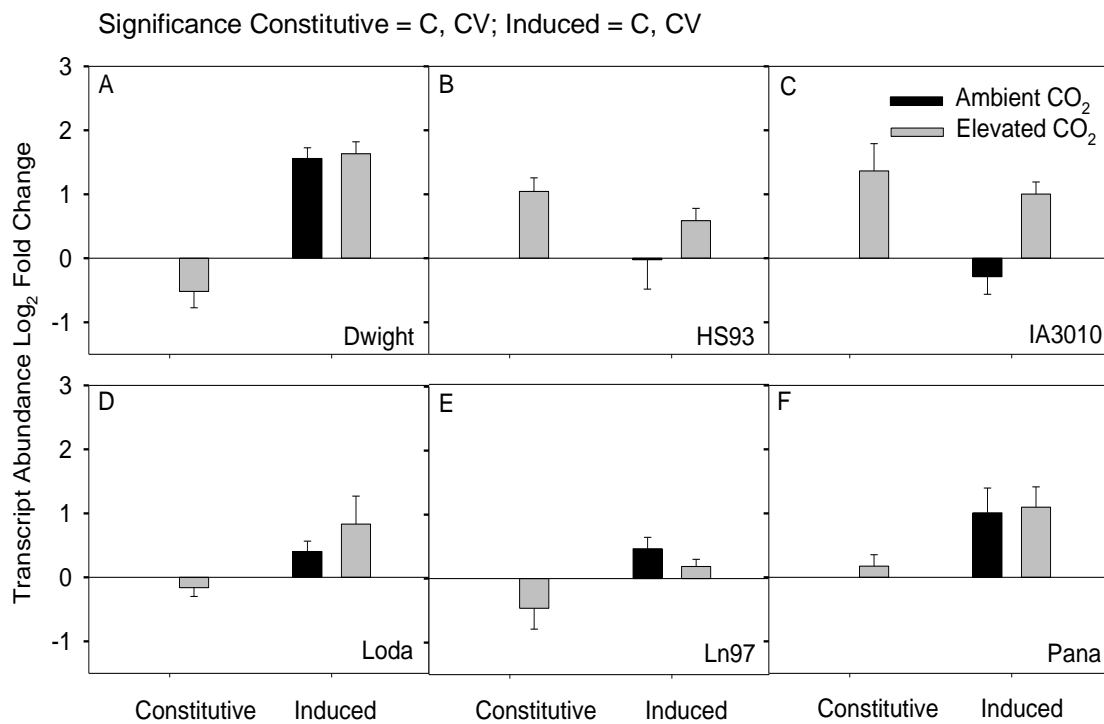


Fig. 5.4 Transcript abundance (mean \pm SEM) of *cystPI* from leaves of six cultivars of soybean grown either under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) Dwight, (B) HS93-4118, (C) IA31010, (D) Loda, (E) LN97-15076, and (F) Pana. Significant main effect of CO₂ exposure and Cultivar are indicated by C and CV, respectively, while significant interactions are indicated by CxCV (ANOVA, $P < 0.05$).



acc1

Fig. 5.5 Transcript abundance (mean \pm SEM) of *acc1* from leaves of six cultivars of soybean grown under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) Dwight, (B) HS93-4118, (C) IA31010, (D) Loda, (E) LN97-15076, and (F) Pana. Significant main effect of CO₂ exposure and Cultivar are indicated by C and CV, respectively, while significant interactions are indicated by CxCV (ANOVA, $P < 0.05$).

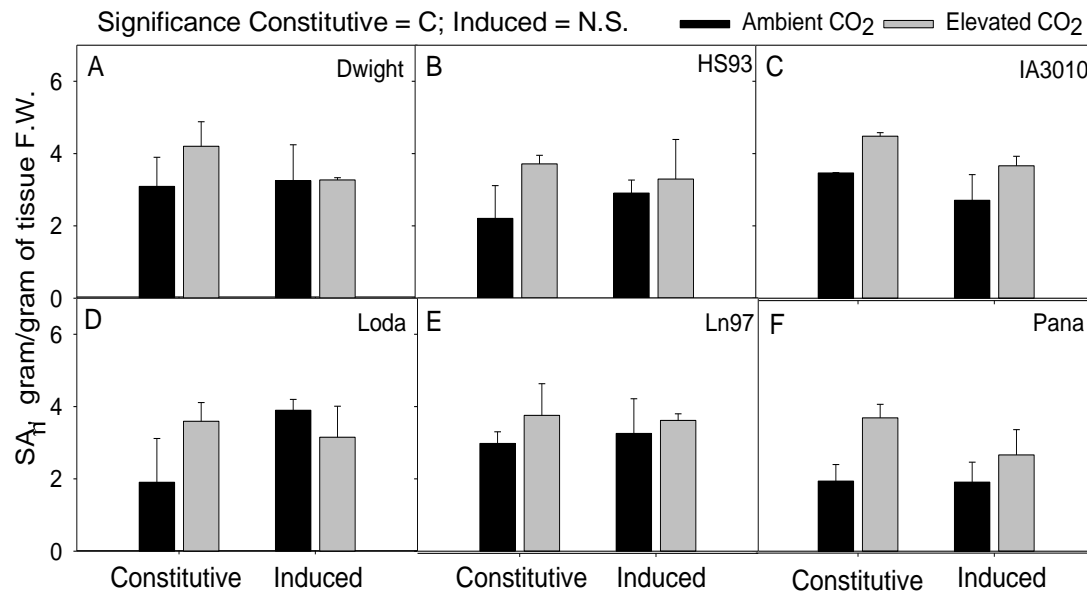


Fig. 5.6 Salicylic acid (SA; mean \pm SEM) from leaves of six cultivars of soybean grown either under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) Dwight, (B) HS93-4118, (C) IA31010, (D) Loda, (E) LN97-15076, and (F) Pana. Significant main effect of CO₂ exposure and Cultivar are indicated by C and CV, respectively, while significant interactions are indicated by CxCV (ANOVA, $P < 0.05$).

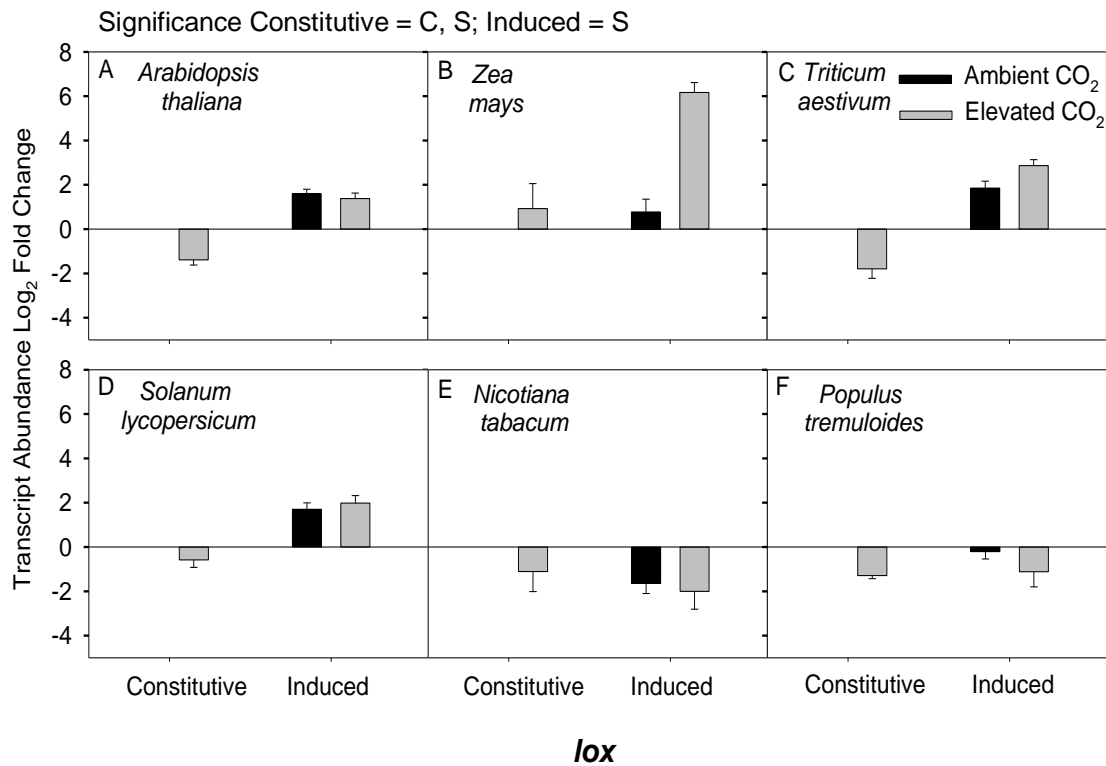


Fig. 5.7 Transcript abundance (mean \pm SEM) of *lox* from leaves of six different plant species grown either under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) *A. thaliana*, (B) *Z. mays*, (C) *T. aestivum*, (D) *S. lycopersicum*, (E) *N. tabacum*, and (F) *P. tremuloides*. Significant main effect of CO₂ exposure and Species are indicated by C and S, respectively, while significant interactions are indicated by CxS (ANOVA, $P < 0.05$).

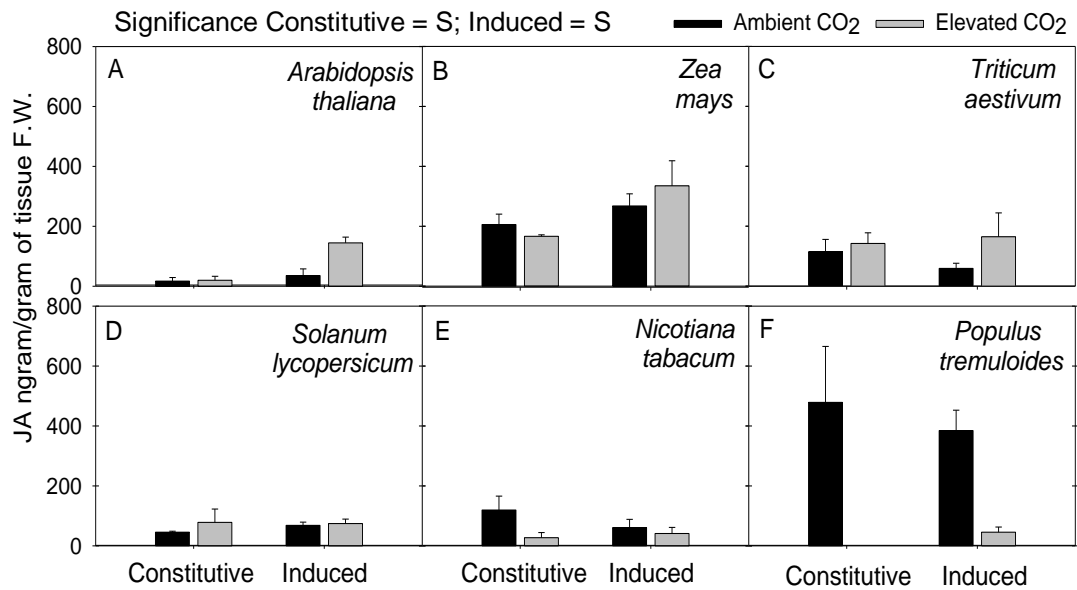


Fig. 5.8 JA (mean \pm SEM) from leaves of four different plant species grown either under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) *A. thaliana*, (B) *Z. mays*, (C) *T. aestivum*, (D) *S. lycopersicum*, (E) *N. tabacum*, and (F) *P. tremuloides*. Significant main effect of CO₂ exposure and Species are indicated by C and S, respectively, while significant interactions are indicated by CxS (ANOVA, $P < 0.05$).

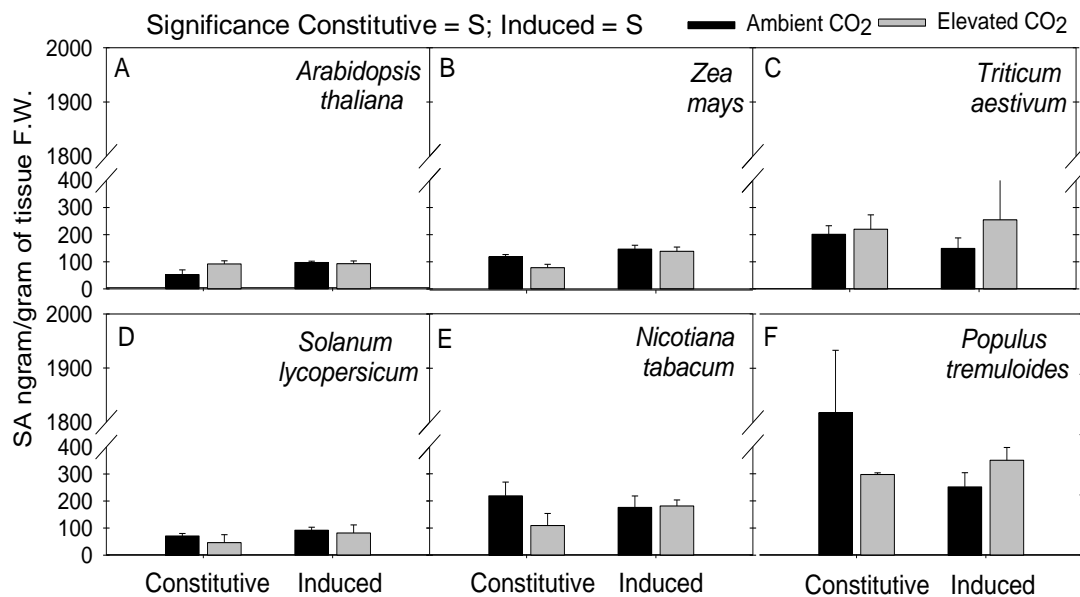


Fig. 5.9 Salicylic acid (SA; mean \pm SEM) from leaves of four different plant species grown either under elevated or ambient CO₂ in undamaged tissue (Constitutive) or two hours after artificial damage (Induced): (A) *A. thaliana*, (B) *Z. mays*, (C) *T. aestivum*, (D) *S. lycopersicum*, (E) *N. tabacum*, and (F) *P. tremuloides*. Significant main effect of CO₂ exposure and Species are indicated by C and S, respectively, while significant interactions are indicated by CxS (ANOVA, $P < 0.05$).

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CHAPTER 6

SUMMARY

Plants and insects comprise almost 50% of all identified species on Earth (Price 1997), so, understanding their responses to accelerated climate change from human activities is of undeniable importance in most ecosystems. In general, elevated CO₂ levels are predicted to enhance plant photosynthetic rates and growth resulting in increased productivity (Ainsworth & Long 2005; Long *et al.* 2006). However, it has been shown that potential benefits of CO₂ enrichment to plants may be offset by interactions with herbivores and other plants experiencing elevated CO₂ (Bazzaz & Miao 1993; Bazzaz *et al.* 1995; Andalo *et al.* 2001; Hamilton *et al.* 2005; DeLucia *et al.* 2008). In addition to elevated CO₂, models predict that plants and insects also will experience other environmental changes including global warming, changes in precipitation, rising ozone, and increases in extreme climatic events (Meehl *et al.* 2007). Despite this, the majority of studies focus on a single aspect of global change and a single species response, neglecting fundamental biotic and abiotic interactions that will occur in future ecosystem. My research investigated how plants respond to predicted increases in atmospheric CO₂, specifically considering the indirect effects on herbivores and interactions with other aspects of environmental change.

To conduct my research and examine these important interactions in the studies detailed in this thesis, I utilized the Soybean Free-air Concentration Enrichment (SoyFACE) experiment (<http://soyface.illinois.edu>) located in central Illinois, which increases atmospheric CO₂ under field conditions. At SoyFACE large plots of soybean (*Glycine max*) are fumigated with elevated CO₂ or O₃ but are otherwise unchanged from typical field conditions. This system provided a unique opportunity to evaluate the direct effects of global change on subsequent biotic and

abiotic challenges plants commonly face, such as insect herbivores, in an important agro-ecosystem.

In Chapter 2 of the thesis, I characterized the global transcriptional response of soybean to beetle herbivory in field conditions and determined how it is altered by elevated CO₂ or O₃, individually or in combination. Chapter 3 addresses the role of phytohormone signaling and related induced defenses upon soybean-beetle interactions previously observed under elevated CO₂ exposure, while Chapter 4 examines how drought, which will occur in combination with elevated CO₂ in some areas (Meehl *et al.* 2007), will modulate responses. While Chapters 2-4 examined impacts on one particular cultivar of soybean, the fifth chapter focused on the variation in the response of phytohormone signaling within soybean and across species under elevated CO₂ exposure.

The purpose of Chapter 2 was to determine the molecular mechanisms governing the response of soybean to herbivory under predicted CO₂ and O₃ atmospheres. I used microarray technology, which enabled the examination of over 35,000 soybean transcripts under field conditions at one time. I found that the number of transcripts in the leaves affected by Japanese beetle (JB; *Popillia japonica*) herbivory was greater when plants were grown under elevated CO₂, O₃ and the combination of both when compared to ambient atmosphere (Fig. 2.1). The effect of herbivory on transcription diminished strongly with time, and elevated CO₂ interacted more strongly with herbivory than elevated O₃ (Fig. 2.1). In addition, I found constitutive and JB induction of key transcripts related to phytohormone signaling, and defenses were down-regulated under elevated CO₂, suggesting that susceptibility may be altered. These results indicate that the majority of transcriptional regulation of plant responses to herbivory happens

relatively quickly after damage and that some aspects of global change may play a more important role in mediating plant-insect interactions in the future, possibly altering susceptibility.

In Chapter 3, I considered the impact of elevated CO₂ exposure on phytohormone signaling pathways and related defenses identified in the previous chapter. To determine if transcriptional changes result in alterations in plant chemistry and ultimately insect behavior, metabolites and JB preference were examined. Elevated CO₂ decreased the induction of JA and ET-related transcripts (Fig. 3.1) and metabolites (Fig. 3.2), resulting in decreased accumulation of defenses over time compared to ambient grown plants (Fig. 3.4). Moreover, elevated CO₂ exposure increased susceptibility of soybean to JB (Fig. 3.5). This finding indicates that predicted increases in soybean productivity under projected CO₂ levels may be reduced by alterations at the transcriptional level that leave soybean more susceptible to herbivory in the field. Elevated CO₂ exposure also increased salicylic acid (SA) levels in soybeans when compared to ambient-grown plants (Fig. 3.3), suggesting that interactions with pathogens may also be altered in the future. Elevated CO₂ exposure may cause increases in insect damage and reduction in pathogen infections in soybean in the future.

In addition to elevated CO₂, models predict that plants will also experience increased drought in the future (Meehl *et al.* 2007). Elevated CO₂ exposure will increase susceptibility of soybean to insect herbivores (Chapter 2 & 3); however, plant-insect dynamics under elevated CO₂ may be altered by the interaction with drought. In Chapter 4 I investigated the combined effects of drought and elevated CO₂ on plant-insect interactions and factors contributing to susceptibility in soybeans. The effect of elevated CO₂ exposure on phytohormone signaling was consistent with results reported in previous studies (Fig. 4.1; Fig. 4.2; Chapter 2 & 3). Exposure to mild drought stimulated the induction of the JA/ET signaling pathways but had no impact on

nutritional components (Fig. 4.1; Table 4.1). However, elevated CO₂ exposure in combination with drought amplified the induction of JA and ET signaling transcripts and the accumulation of related defenses in soybeans after beetle herbivory (Fig. 4.1; Fig. 4.3). The increased susceptibility of soybean to herbivores resulting from elevated CO₂ exposure was removed under drought conditions (Fig.4.4). This result suggests soybean in areas experiencing elevated drought will experience less herbivore damage compared to well-watered plants grown under elevated CO₂.

Intra- and inter- specific variation in plant traits, such as defense responses, can influence the composition of the herbivore and plant community (Chapin *et al.* 2000; Wimp *et al.* 2005; Whitham *et al.* 2006; Poelman *et al.* 2009; Broekgaarden *et al.* 2010). In addition, species richness and biodiversity can decline when one species has an advantage over others (Chapin *et al.* 2000). Therefore, it is of considerable importance to determine which species will respond most strongly to changes in atmospheric CO₂ concentrations. In Chapter 5, I considered how phytohormone signaling pathways will differentially respond to elevated CO₂ across six cultivars of soybean and six plant species. Significant variation in the response would provide the genetic flexibility needed to direct future breeding efforts for plants suited to a CO₂ enriched environment. If phytohormone signaling pathways and thus the ability to effectively mount a defensive response are altered distinctly in individual cultivars and species by elevated CO₂, widespread changes in species composition could be expected. Elevated CO₂ reduced constitutive levels of JA in some but not all soybean cultivars (Fig. 5.3). In addition, constitutive and induced ET signaling was increased in some cultivars, the response varied with cultivar examined (Fig. 5.5). In contrast to the variation seen in JA and ET, constitutive levels of SA were elevated universally across soybean cultivars grown under elevated CO₂ with little variation

in the response (Fig. 5.6). Across species examined, elevated CO₂ had a similar impact as with cultivars, reducing constitutive JA signaling transcripts in many but not all species (Fig. 5.7). However, in contrast to cultivars there was no impact of elevated CO₂ on constitutive or induced levels of SA across species (Fig. 5.9). This study suggests some pathways may experience generalized changes for a species with little variation (e.g. SA), while others may not (JA/ET). The existence of variation in response to elevated CO₂ suggests a potential for some phytohormone signaling and defenses to adapt under future environmental challenges, while others will have less flexibility. In addition, these results indicate there is significant potential for directed selection in future breeding programs to mitigate the impacts of global change on plant resistance.

Uncertainties in the response of phytohormone signaling to atmospheric and climate change reside in inconsistencies observed in field conditions. Differences in JA abundance were not resolved following exposure to elevated CO₂ or drought, singly or in combination in field conditions (Fig. 2.2B, Fig. 3.2B); however, transcripts consistently were down-regulated and differences in JA were resolved in growth chambers (Fig. 2.2A). In addition, we were not always able to resolve a significant difference in all defenses examined (Fig. 2.4; Fig. 3.3), although the patterns were similar to previous studies (Zavala *et al.* 2008, 2009). Despite this, insect preference was always for high CO₂ foliage (Fig. 2.5; Fig. 3.4), and this reflected changes in defense transcripts but not sugar increases (Fig. 3.1; Fig. 3.4; Table 3.1; O'Neill *et al.* 2008).

We speculate that the variation in JA and defenses may be related to the fact that plants were grown in the field without protection from damage. In addition, reduced sensitivity of methods utilized to measure hormone and defense compound levels compared to qPCR may further contribute to difficulties resolving significant differences. All field plants have some

damage (personal observation). Individual leaves used in these experiments were free of visible damage, but this was not the case for all trifoliates on an individual plant. Undamaged trifoliates may have been systemically induced from prior damage on adjacent leaves. Previous studies demonstrated that JA can accumulate to levels 30 times as high in undamaged leaves adjacent to damaged leaves and elevated levels can persist for extended periods (Glauser *et al.* 2008; Koo *et al.* 2009). In addition, pathogens, which can also alter plant hormones and defenses, may not have been visible in undamaged leaves chosen in field environments where infection is common. Finally, our study utilized a short bout of feeding or damage instead of continuous feeding utilized in previous studies, which possibly was not a strong enough treatment under field conditions. Thus, uncertainties likely stem from methods and chronic induction in the field compared to controlled growth chamber experiments where exposure to elevated CO₂ consistently regulated transcripts and metabolites in previous experiments.

The mechanism by which elevated CO₂ alters the hormonal response to herbivory is not known, although it may be related to changes in carbohydrate status. In plants, different sugar signals are generated by photosynthesis and carbon metabolism in source and sink tissues to modulate growth, development, and stress responses. Soybean leaves grown at elevated CO₂ have significantly increased photosynthesis rates (Rogers *et al.* 2004) and content of non-structural carbohydrates, including starch and sugars (sucrose, fructose and glucose; Sun *et al.* 2009). Previous studies have demonstrated there are extensive interactions between sugar and plant hormone signaling. Reports have also specifically connected glucose signaling to ET and SA signaling (Rolland *et al.*, 2006). In view of the fact that plant-specific sugar signaling mechanisms can interact and modify plant hormone signaling pathways (Rolland *et al.* 2006), elevated CO₂-induced accumulation of sugars may have an influence on the reconfiguration of

phytohormone signaling and defenses in soybean. However, the impact of elevated CO₂ on sugar signaling pathways and plant hormones needs to be further investigated.

It is certain elevated CO₂ will increase productivity of C₃ plants (Ainsworth & Long 2005; Long *et al.* 2006); however, predicted increases may be reduced due to interactions with insect herbivores in some plant species (DeLucia *et al.* 2008). For example, in soybean, elevated CO₂ may cause increases in insect damage and reduction in pathogen infections in the future through the modulation of hormone signaling and related defenses (Chapter 2 & 3). However, the effects of elevated CO₂ with different aspects of global change, such as drought, may alter plant responses in the future (Chapter 4). While considerable research has demonstrated that elevated CO₂ concentrations can alter plant-insect interactions, there is significant variation in the response (Coviella & Trumble 1999; Zvereva & Kozlov 2006). Variation in phytohormone signaling pathways in response to elevated CO₂ may partially explain these species-specific responses (Chapter 5), providing some species or cultivars a selective advantage under predicted CO₂ atmospheres. This selective advantage may affect species composition and biodiversity in the future. Thus, interactions among components of global climate change may play a more important role in determining plant-insect dynamics than previously hypothesized. To understand the impact of elevated CO₂ on plant-insect interactions and make accurate predictions about future environmental impacts upon them, research will need to focus on biotic and abiotic relationships and the changing environment they will face in the future, in addition to the interactions that occur within the sophisticated signaling systems plant possess to coordinate their responses.

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APPENDIX A

Supplementary information

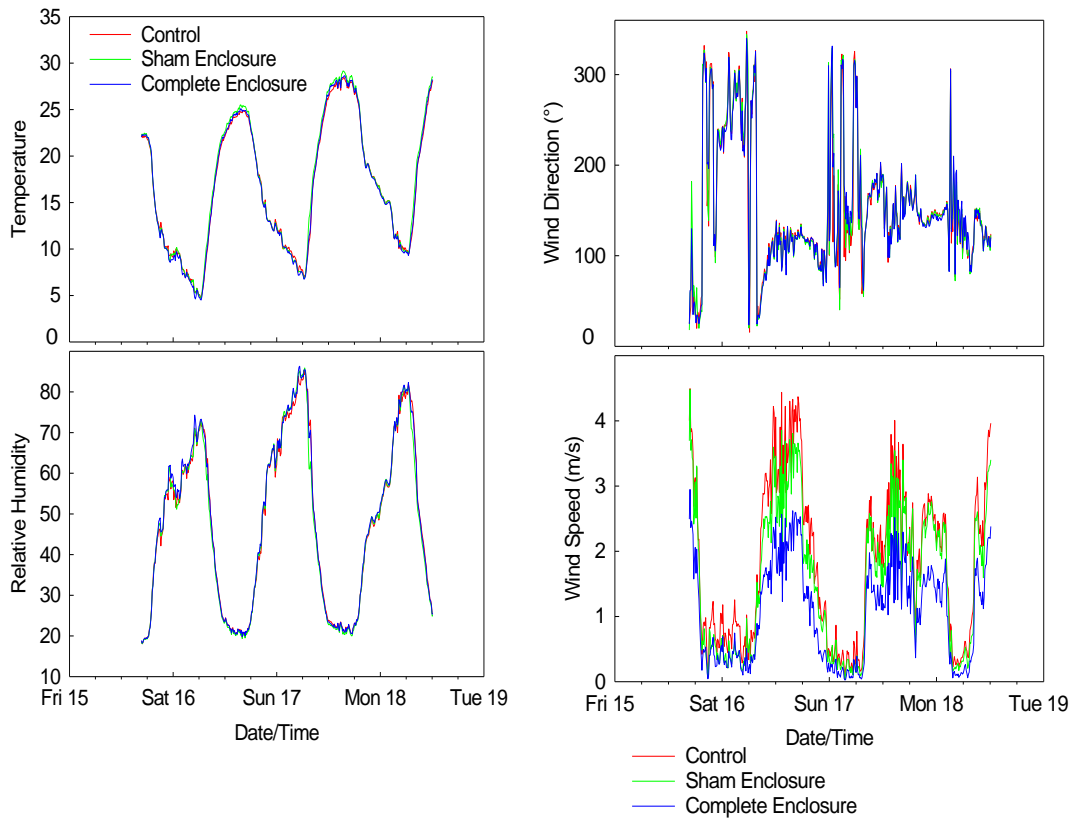
Table A. Total number of soybean transcripts regulated after 3 days beetle damage in ambient conditions compared to under elevated CO₂, elevated O₃, and the combination of gases with and without beetle damage, all relative to un-infested ambient atmospheric conditions. (P < 0.01).

Available on request.

Table B. Primers used for qPCR	
Primer	Primer Sequence
DB7 Actin F	TTGGATCTCGCTGGTCGTGA
DB8 Actin R	CGCTCAGCAGAGGTGGTGAA
CC1 lox F	AGCAACTTTGGTGATGGTAGTTTTGAATTTATT
CC2 lox R	TTCTCAACCCACGATTGTTCCACAT
CC13 acc F	GAGGCCTCCATTTGCACACC
CC14 acc R	TGAACTCGGGCAGACCATGA
soyAOS F	ATCACGCTCCCTCTTCAACT
soyAOS R	GTTCGGATGGTGGATTCTTC
soylox7 F	GAGGGATTGACTTTCAGAGGAATTC
soylox7 R	AACAGGCAGAGTAGTACAGCAACCT
soyPAL F	TGAATTGCAACCCAAGGAAGGTCTT
soyPAL R	AGCAAAAATAGCTGATAGAACTTCAGACAACACA
soyHPL F	AGCTCGTGGACACATGGCTTCTCT
soyHPL R	GCGGATTAGGAAGTCTTCGATGTAGTTG
cons6-F	AGATAGGGAAATGGTGCAGGT
cons6-R	CTAATGGCAATTGCAGCTCTC
poplox1-F	TTG ATG AAA GGA ATG CTG ACA
poplox1-R	GGC AAA TGC AAG AAG AAA CA
pop18Sb-F	TCA ACT TTC GAT GGT AGG ATA GTG
pop18Sb-R	CCG TGT CAG GAT TGG GTA ATT T
tabLOX-F	TGC TGA CCA TGC AGA ACC TGG A
tabLOX-R	ACT CCC ATG GAC TCA TCC CAG TCA
tobEF-1a-F	TGT GAT GTT TTT GTT CGG TCT TTA A
tobEF-1a-R	TCA AAA GAA AAT GCA GAC AGA CTC A
arbEF1-F	TGA GCA CGC TCT TCT TGC TTT CA
arbEF1-R	GGT GGT GGC ATC CAT CTT GTT ACA
arbLOX2-F	TGC ACG CCA AAG TCT TGT CA
arbLOX2-R	TCA GCC AAC CCC CTT TTG A
ZM18S-F	TCC TGA GTA ACG AAC GAG ACC
ZM18S-R	CAC GAT GAA ATT TCC CAA GAT
zmLOX10-F	GAG GTG AAG CAG CTC ACG TTC G
zmLOX10-R	TCT TGT TGA TGA GCA GCG GGC G

Table B. (Continued) Primers used for qPCR

wht18S-F	CGT CCC TGC CCT TTG TAC AC
wht18S-R	AAC ACT TCA CCG GAC CAT TCA
whtLOX-F	GGG CAC CAA GGA GTA CAA GGA
whtLOX-R	CGA TCA CCG ACA CTC CAA TG
tomloxD-F	ATGCAACACGCACCATTAT
tomloxD-R	TGCCACATCCAGTTACCAGT
ptom18s-F	GCCCGGGTAATCTTTGAAAT
ptom18s-R	GTACAAAGGGCAGGGACGTA



Supplemental Figure A. 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).