

# Anthropogenic increase in carbon dioxide compromises plant defense against invasive insects

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Elevated levels of atmospheric carbon dioxide (CO<sub>2</sub>), a consequence of anthropogenic global change, can profoundly affect the interactions between crop plants and insect pests and may promote yet another form of global change: the rapid establishment of invasive species. Elevated CO<sub>2</sub> increased the susceptibility of soybean plants grown under field conditions to the invasive Japanese beetle (*Popillia japonica*) and to a variant of western corn rootworm (*Diabrotica virgifera virgifera*) resistant to crop rotation by down-regulating gene expression related to defense signaling [lipoygenase 7 (*lox7*), lipoygenase 8 (*lox8*), and 1-aminocyclopropane-1-carboxylate synthase (*acc-s*)]. The down-regulation of these genes, in turn, reduced the production of cysteine proteinase inhibitors (CystPIs), which are specific deterrents to coleopteran herbivores. Beetle herbivory increased CystPI activity to a greater degree in plants grown under ambient than under elevated CO<sub>2</sub>. Gut cysteine proteinase activity was higher in beetles consuming foliage of soybeans grown under elevated CO<sub>2</sub> than in beetles consuming soybeans grown in ambient CO<sub>2</sub>, consistent with enhanced growth and development of these beetles on plants grown in elevated CO<sub>2</sub>. These findings suggest that predicted increases in soybean productivity under projected elevated CO<sub>2</sub> levels may be reduced by increased susceptibility to invasive crop pests.

*Diabrotica virgifera* | global change | *Glycine max* | plant–insect interactions | *Popillia japonica*

At present rates of anthropogenic emissions, the atmospheric carbon dioxide (CO<sub>2</sub>) concentration of 381 μmol mol<sup>-1</sup> is predicted to rise to 550 μmol mol<sup>-1</sup> by 2050 (1). The CO<sub>2</sub> increase is projected to increase the productivity of agroecosystems by enhancing photosynthesis and water use efficiency, particularly in C<sub>3</sub> crops (2), although precise estimates of the magnitude of the enhancement vary with experimental approach (3). Such projections, however, generally do not take into account the potential for interactions between plants and herbivorous insects to modify the fertilization effect of elevated CO<sub>2</sub> on plant production (4). Impacts of herbivory on plant responses to elevated CO<sub>2</sub>, however, can be readily measured in Free Air Gas Concentration Enrichment (FACE) experiments, where the movement of insects into field plots is unrestricted.

Typically, elevated CO<sub>2</sub> diminishes plant host quality by increasing leaf C:N ratio, specific leaf mass and thickness, the proportion of nonstructural carbohydrates, and allocation to phenolic compounds (5–7). However, other compounds, such as proteinase inhibitors (PIs), can play a role in plant defense, affecting the digestibility of proteins and decreasing the availability of free amino acids required by insects for growth, development, and reproduction (8). Cysteine proteinases are common in the slightly acidic midgut (pH 5–7) of many coleopterans (9, 10), and cysteine PIs (CystPIs) in plant tissues decrease growth and development by inhibiting these proteinases (10, 11). Induction of the jasmonate signaling pathway in plants by herbivore damage leads to increased synthesis of CystPIs (12).

Soybean (*Glycine max*), the world's most widely grown seed legume, has one constitutive *CystPI* gene (*L1*) and two inducible *CystPI* genes (*N2* and *R1*) (13, 14). Soybean CystPIs, as well as the synthetic CystPI, transepoxy succinyl-L-leucyl-amido (4-guanidino)

butane (E-64), inhibit gut cysteine (cathepsin L-like) activity, growth, and survival of larval and adult western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte: Coleoptera) (10, 13, 15, 16). Although WCR normally causes economic injury by damaging roots of corn (*Zea mays*), a variant of WCR feeds on soybean foliage and lays eggs in soybean fields (17). The Japanese beetle (JB; *Popillia japonica* Newman: Coleoptera), a broadly polyphagous species introduced into the United States in 1916 and now expanding its range throughout the Midwest, feeds on ≈300 species of wild and cultivated plants in 79 families; soybeans are among its many host plants (18). Elevated CO<sub>2</sub> increased herbivory and oviposition by both JB and WCR in soybean grown in FACE experiments (19–21). Although sugars can stimulate feeding in JB (18), higher carbohydrate levels in leaves did not account for changes in preference or fecundity of this species (21), leaving open the possibility that plant chemical defenses, such as CystPIs, may mediate these changes.

To examine the role of cysteine proteinases in altered resistance to coleopteran herbivores, soybeans were grown at the SoyFACE facility established at the University of Illinois at Urbana–Champaign. The SoyFACE facility, which elevates CO<sub>2</sub> under fully open-air field conditions without any barriers, allowed us to investigate the mechanism whereby elevated CO<sub>2</sub> increases susceptibility of soybean to naturally occurring herbivores. Specifically, we examined whether growth in elevated CO<sub>2</sub> down-regulates the expression of genes associated with signaling hormones that regulate *CystPI* and other defense genes, whether CystPI activity in soybean foliage is reduced under elevated CO<sub>2</sub>, and whether JB and WCR display higher levels of digestive cysteine proteinase activity in their guts when consuming soybean leaves grown under elevated CO<sub>2</sub>. A reduction in the ability to mobilize defenses in response to herbivory is a potential mechanism to account for the enhanced performance of beetles consuming foliage of soybean grown under elevated CO<sub>2</sub> and for the increased amount of damage sustained by soybean plants under elevated CO<sub>2</sub> under field conditions.

## Results

To determine the effect of elevated CO<sub>2</sub> on the expression of soybean defense genes, five genes from fully expanded leaves grown either under ambient or elevated CO<sub>2</sub> were analyzed by semiquantitative RT-PCR. We analyzed the expression of 1-aminocyclopropane-1-carboxylate synthase (*acc*), which is the key regulatory point in the biosynthesis of the signaling hormone ethylene, two genes related to the signaling hormone jasmonic acid (JA), lipoygenase 7 (*lox7*) and 8 (*lox8*), and the

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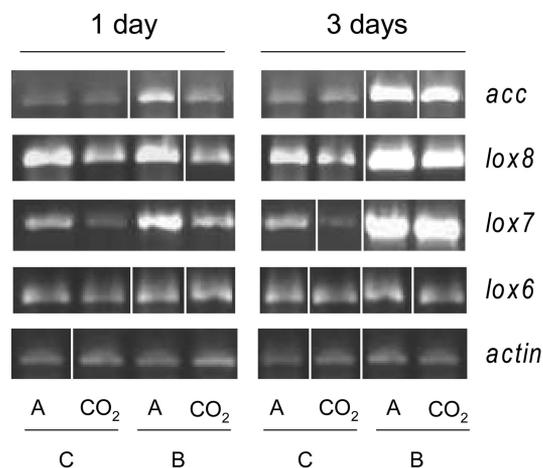
The authors declare no conflict of interest.

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**Fig. 1.** Expression analysis of genes related with JA and ethylene biosynthesis. Quantitative RT-PCR of five genes from fully expanded leaves of soybean grown either under elevated [CO<sub>2</sub>] (CO<sub>2</sub>) or ambient [CO<sub>2</sub>] (A): 1-aminocyclopropane-1-carboxylate synthase (*acc*), lipoxygenase 7 (*lox7*), 8 (*lox8*) and 6 (*lox6*). RNA was extracted from four replicates (one replicate per plot) of either unattacked (C) or attacked leaves by Japanese beetles (B) for 1 or 3 days and reverse-transcribed to cDNA. PCR reactions were replicated from four independent cDNA samples for all primers. Prior to statistical analysis, the spot intensity values generated by image analysis software for each gene was normalized to the intensity of actin to correct for differences in amplification of cDNA. The figure is a composite of multiple experiments and contains images spliced into place.

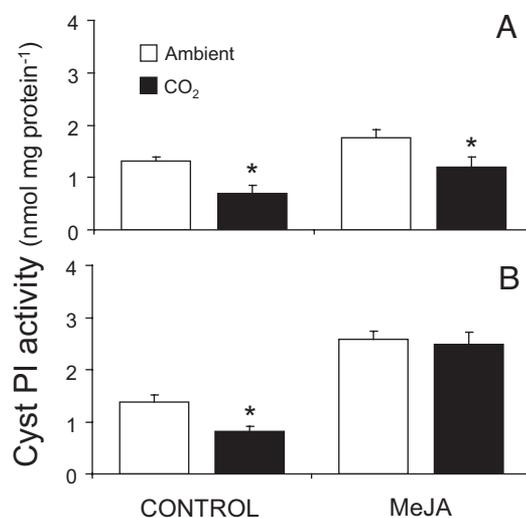
gene encoding vegetative lipoxygenase 6 (*lox6*), which is not related to plant signaling and served as an internal control (22–24).

There was no main effect of elevated CO<sub>2</sub> on the expression of *acc* ( $P = 0.92$ ). However, the significant interaction between elevated CO<sub>2</sub> and herbivory by JB ( $P \leq 0.01$ ) indicates that the induction of *acc* after herbivory was reduced in leaves grown under elevated CO<sub>2</sub>, compared with ambient CO<sub>2</sub> (Fig. 1). One day after damage by JB, *acc* was induced by 86% in leaves grown in ambient CO<sub>2</sub>, but this induction was only 54% in leaves grown in elevated CO<sub>2</sub>. The induction of *acc* increased to 222% in ambient leaves 3 days after attack, but only to 155% after 3 days in leaves grown in elevated CO<sub>2</sub>. Similar results were found in response to damage inflicted by WCRs (data not shown).

Elevated CO<sub>2</sub> inhibited constitutive levels of *lox7* and *lox8* by 30% and 28%, respectively ( $P \leq 0.01$ ). As with *acc*, the magnitude of induction after beetle attack was lower for plants grown under elevated than ambient CO<sub>2</sub> (Fig. 1). Averaged across both time points, the expression of *lox7* after herbivory increased under ambient CO<sub>2</sub> by 98%, but only by 77% under elevated CO<sub>2</sub>. Similarly, the expression of *lox8* after herbivory increased by 49% under ambient CO<sub>2</sub>, but only by 5% under elevated CO<sub>2</sub>. The expression level of both genes after induction by herbivory increased with time ( $P \leq 0.01$ ). As expected, there were no changes in the expression of *lox6* by either elevated CO<sub>2</sub> or beetle damage ( $P = 0.5$ ) (Fig. 1).

Our results indicate that elevated CO<sub>2</sub> not only decreased the expression of genes related to the signaling hormones JA and ethylene, but also decreased their induction after beetle damage. The positive interaction between JA and ethylene after wounding synergistically induces PI genes, such as *CystPI* genes in soybean (14). However, changes in the synthesis of JA by elevated CO<sub>2</sub> can affect constitutive and inducible *CystPI* activity levels.

Jasmonic acid (JA), a ubiquitous wound hormone known to increase the synthesis of diverse defense-related metabolites, is strongly implicated in activating *CystPI* synthesis (12). Endogenous levels of JA increase (5–500 ng per plant), in proportion to



**Fig. 2.** MeJA restored *CystPI* activity in elevated CO<sub>2</sub> treatments. (A and B) *CystPI* activity (mean  $\pm$  SEM) from fully expanded soybean leaves grown under ambient CO<sub>2</sub> (open bars) or elevated CO<sub>2</sub> (filled bars) 1 (A) or 3 (B) days after the application of 150  $\mu$ g of MeJA in lanolin paste or pure lanolin (control). Asterisks indicate the level of significant differences between ambient and elevated CO<sub>2</sub> treatments (\*,  $P < 0.05$ ; \*\*,  $P < 0.001$ ; \*\*\*,  $P < 0.0001$ ).

the amount of damage, within 90 min (25, 26). The addition of JA and its methyl ester, methyl jasmonate (MeJA), to leaves mimics leaf damage by increasing the synthesis of *CystPI*s (12).

Growth of soybeans under elevated CO<sub>2</sub> resulted in a 47% reduction in constitutive *CystPI* activity in leaves ( $P = 0.006$ ), and treatment of plants grown under elevated CO<sub>2</sub> with MeJA restored *CystPI* activity to levels comparable to plants grown under ambient CO<sub>2</sub> (Fig. 2). These findings are consistent with the down-regulation of JA signaling by elevated CO<sub>2</sub>. After 1 day of MeJA elicitation, *CystPI* activity levels increased to a greater extent in plants grown under elevated CO<sub>2</sub> (42%) than under ambient CO<sub>2</sub> (25%;  $P = 0.003$ ) (Fig. 2). Similar results were found after 3 days of MeJA elicitation. *CystPI* activity levels increased to a greater extent in plants grown under elevated CO<sub>2</sub> (71%) than in those grown under ambient CO<sub>2</sub> (49%;  $P < 0.0001$ ), resulting in similar *CystPI* activity levels in both environments ( $P = 0.7$ ) (Fig. 2). Although elevated CO<sub>2</sub> decreased constitutive and inducible *CystPI* activity, inducible *CystPI* activity in plants grown under elevated CO<sub>2</sub> was restored by the application of MeJA.

The down-regulation of JA and ethylene by elevated CO<sub>2</sub> decreased both expression levels of *CystPI*-inducible genes *R1* and *N2* and constitutive and inducible *CystPI* activity levels in leaves ( $P < 0.0001$ ) (Fig. 3). Damage by JB induced *CystPI* genes *R1* and *N2* under ambient CO<sub>2</sub>, but not under elevated CO<sub>2</sub> (Fig. 3). Three days after insect damage, *R1* gene expression was up-regulated in both ambient and elevated CO<sub>2</sub>, but the magnitude of up-regulation was greater for plants grown in ambient atmosphere ( $P = 0.0003$ ) (Fig. 3). As expected, the treatments did not change the expression of the *L1* gene ( $P = 0.4$ ) (Fig. 3).

Elevated CO<sub>2</sub> not only decreased *CystPI* gene expression, but also affected *CystPI* activity levels. One day after beetle attack, *CystPI* activity levels were higher in plants grown under ambient CO<sub>2</sub> than in those grown under elevated CO<sub>2</sub> ( $P = 0.006$ ) (Fig. 3). Three days after beetle attack, *CystPI* elicitation was similar for both treatments (62% and 64%), resulting in higher activity levels in plants grown under ambient CO<sub>2</sub> (3.5 nmol mg protein<sup>-1</sup>) than in those grown under elevated CO<sub>2</sub> (2.2 nmol mg protein<sup>-1</sup>;  $P < 0.0001$ ) (Fig. 3). The potent inhibitory capacity and the effectiveness of these soybean *CystPI*s (*N2* and *R1*) against many coleopteran



including serine proteinase inhibitors (30), isoflavonoid content, or polyphenol oxidase activity (31, 32), potentially rendering plants more vulnerable to herbivores other than beetles and leading to even greater losses. As well, in other systems, elevated CO<sub>2</sub> has had diverse effects on insects other than coevolved specialist herbivores. Elevated CO<sub>2</sub> decreased the emission of JA-regulated terpene volatiles compounds in cabbage (*Brassica oleracea*), reducing the host-searching efficiency of the specialist parasitoid *Cotesia plutellae* (33). Impaired JA-signaling *Nicotiana attenuata* planted into native habitats was not only more vulnerable to adapted herbivores, but also was colonized at a higher rate by novel herbivore species, which fed and reproduced successfully (34). Thus, changes in defense-signaling systems of host plants produced by elevated CO<sub>2</sub> can be expected to affect the entire trophic structure of agroecosystems, potentially exacerbating pest problems by multiple mechanisms.

## Methods

Soybeans (*Glycine max* cultivar 93B15; Pioneer Hi-Bred) were grown at the SoyFACE facility established at University of Illinois at Urbana-Champaign (40°02' N, 88°14' W, 228 m above sea level; <http://www.soyface.uiuc.edu>). SoyFACE consisted of eight 20-m-diameter octagonal plots distributed within four randomized blocks of soybean (for extended site and operation description, see ref. 35). Within each block, one control plot was at the current ambient CO<sub>2</sub> of 380 μmol mol<sup>-1</sup> and one plot was fumigated to a target CO<sub>2</sub> of 550 μmol mol<sup>-1</sup>. The experimental plots were separated by at least 100 m to prevent cross-contamination of CO<sub>2</sub>. 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. One-minute average CO<sub>2</sub> was ± 20% of the target for >95% of the time. At current rates of anthropogenic emissions, the targets for CO<sub>2</sub> represent predicted atmospheric levels in 2050 (1).

In each FACE plot, 38 days after emergence, 38 undamaged soybean plants at the vegetative stage were selected, and the uppermost fully expanded trifoliolate leaf on each of eight plants was treated with 150 μg of MeJA (Sigma-Aldrich) in 20 μl of lanolin paste. An additional 10 plants each were infested with five adult JB; 10 plants each were infested with five adult WCR, and 10 plants served as controls. Half of the treatment and control leaves were harvested for analysis 1 day after infestation, and the other half were harvested 3 days after infestation. To ensure that the control leaves remained undamaged and that the insects remained where they were placed, leaves were enclosed in 1 × 4-mm plastic mesh. Adult JB and WCR were collected from the SoyFACE site from plants outside the rings 24 h before infestation.

One and 3 days after the insects were placed on leaves, infested and control leaves were collected and flash-frozen in liquid nitrogen and ground to a fine powder. Control leaves (four at each time point) and those from each of the treatments (five at each time point) were combined to form one sample from each FACE plot; the unit of replication for statistical analyses was the individual FACE plot ( $n = 4$ ). To determine CystPI activity, leaf powder was extracted with 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 2.0 mM EDTA (4-ml extraction buffer g<sup>-1</sup> fresh weight of tissue). The samples were extracted by vortexing for 10 s and centrifuging at 12,000 × *g* for 15 min. CystPI activity in the leaves was measured against papain by following the release of *p*-nitroaniline (pNA; 37°C for up to 20 min at 410 nm) after adding the synthetic substrate *p*-Glu-Phe-Leu-pNA (36). Briefly, 30 μl of 28 μg/ml papain was incubated in a 96-microplate with 0–10 μl of supernatant of plant extracts at 37°C for 10 min before addition of the substrate. Protein concentrations were measured (37) by using BSA as a standard. The molar concentration of active papain in the commercial preparation (Sigma-Aldrich) was determined by titrating a known concentration of the inhibitor E-64 (1–100 μl of 2 μM) against papain until all activity had been inhibited (38). The inhibitory activity of extracted protein was lost after heating at 100°C for 30 min (39), indicating that no other compounds were involved in papain inhibition.

To determine the expression of genes related to CystPI and the signaling hormones jasmonic acid and ethylene in foliage from the treatments, total RNA was extracted from an aliquot of powder from each leaf with a guanidine thiocyanate-acid phenol-based method (<http://crosci.uiuc.edu/>

faculty/clough/protocol.htm). Total RNA (1 μg from each sample) was converted to cDNA by using a SuperScript first-strand synthesis system for RT-PCR according to the manufacturer's instructions (Invitrogen). PCRs were carried out in four replicates of cDNA for all primers. Two concentrations of cDNA (*viz.*, 50× and 250× dilutions of original cDNA) derived from control and induced leaves were used as a template to amplify the respective cDNA fragment with the right primer combinations and standardized independently for each pair of primers. After denaturing cDNAs at 94°C for 3 min, PCR was carried out for 25 cycles of 94°C for 60 s, followed by annealing at 56°C for 30 s and then extension at 72°C for 1.5 min. The amplified cDNA fragments were purified from agarose gel by using a GFX gel elution kit (Amersham). The intensity of the spots in the gel was determined with image analysis software (Photoshop 7.0; Adobe Systems). Actin and two non-wound-inducible genes, *lox6* and *L1*, in leaves served as internal standards to determine equal amplification of cDNA. The gene names, accession numbers, primer sequences, and size of amplified cDNA fragments are provided in Table S1.

After 3 days of feeding on leaves grown under ambient or elevated CO<sub>2</sub>, JB and WCR were removed for analysis of total gut proteinase activity and cysteine proteinase activity. For each species, midguts were removed from the five beetles on each leaf and combined with the midguts from beetles on the five replicate leaves to create one composite sample for each FACE plot. Midguts were stored at –20°C.

The effect of a synthetic cysteine inhibitor on gut proteinase activity was examined in a separate experiment. JB and WCR were fed an artificial diet of casein and wheat germ modified from ref. 40 with or without the synthetic cysteine inhibitor E-64 (50 nmol). A group of 15 JB or WCR was fed the control diet or the artificial diet with E-64. The midguts were removed, combined to form a composite sample for each species and treatment, and stored at –20°C. The experiment was repeated three times to generate three composite samples for the control and the treatment diet, respectively.

The composite samples of midguts of beetles from each field plot or from the artificial diet experiment were pulverized in liquid nitrogen with a mortar and pestle. Proteinases from midguts were extracted by homogenizing tissue with 30 mM Tri-K citrate (pH 6.0) 1:1 and incubated on ice for 30 min. The suspension was centrifuged at 12,000 × *g* for 15 min at 4°C, and the resulting supernatant was used as a source of either JB or WCR gut proteinase activity.

We used azocasein as a substrate to estimate total protease activity. Briefly, 10 μl of 2× diluted enzyme [gut proteinase in 30 mM Tri-K citrate and 125 μM dithioerythreitol (pH 6.0)] was added to 180 μl of 1% azocasein [in 30 mM Tri-K citrate (pH 6.0)] and incubated at 37°C for 2.5 h. The reaction was terminated by adding 300 μl of 10% trichloroacetic acid. After centrifuging at 10,000 × *g* for 10 min, an equal volume of 1 M NaOH was added to the supernatant, and absorbance was measured at 450 nm in both samples and controls. One protease unit was defined as the amount of enzyme that increases absorbance by 1 OD/min.

Cysteine proteinase activity was estimated by using the chromogenic substrate *p*-Glu-Phe-Leu-pNA (36). Then 10 μl of the 18× diluted enzyme was added to 20 μl of 0.38 mM *p*-Glu-Phe-Leu-pNA [in 0.1 M NaPhosphate, 0.3 M KCl, 0.1 mM EDTA, and 3 mM dithioerythreitol (pH 6.0)] and incubated at 37°C. Absorbance at 410 nm from wells on the microtiter plate was measured at 20-s intervals for 20 min with JB enzymes and for >30 min with WRC enzymes. Initial rates of hydrolysis were estimated from the slopes of the resulting absorbance versus time graphs. Assays were linear over the assay period. One cysteine activity unit was defined as the amount of enzyme required to produce 1 mM 4-nitroaniline per minute at 37°C using *p*-Glu-Phe-Leu-pNA as a substrate under given assay conditions.

Data were analyzed with Stat View, version 5.0 (SAS Institute). The intensity values of spots from the semiquantitative RT-PCR and CystPI activity values were analyzed with a 2 × 2 (time × treatment) repeated measures ANOVA, followed by Fisher's protected LSD post hoc comparisons in all experiments. Cysteine proteinase activity values were analyzed by ANOVA, followed by Fisher's protected LSD post hoc comparisons in all experiments.

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