Elevated CO₂ Influences Nematode-Induced Defense Responses of Tomato Genotypes Differing in the JA Pathway

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Abstract

Rising atmospheric CO₂ concentrations can affect the induced defense of plants against chewing herbivores but little is known about whether elevated CO₂ can change the induced defense of plants against parasitic nematodes. This study examined the interactions between the root-knot nematode Meloidogyne incognita and three isogenic tomato (Lycopersicon esculentum) genotypes grown under ambient (390 ppm) and elevated (750 ppm) CO₂ in growth chambers. In a previous study with open-top chambers in the field, we reported that elevated CO₂ increased the number of nematode-induced root galls in a JA-defense-dominated genotype but not in a wild-type or JA-defense-recessive genotype. In the current study, we tested the hypothesis that elevated CO₂ will favor the salicylic acid (SA)-pathway defense but repress the jasmonic acid (JA)-pathway defense of plants against plant-parasitic nematodes. Our data showed that elevated CO₂ reduced the JA-pathway defense against M. incognita in the wild-type and in a genotype in which defense is dominated by the JA pathway (a JA-defense-dominated genotype) but up-regulated the SA-pathway defense in the wild type and in a JA-defense-recessive genotype (jasmonate-deficient mutant). Our results suggest that, in terms of defense genes, secondary metabolites, and volatile organic compounds, induced defense of nematode-infected plants could be affected by elevated CO₂ and that CO₂-induced changes of plant resistance may lead to genotype-specific responses of plants to nematodes under elevated CO₂. The changes in resistance against nematodes, however, were small relative to those reported for chewing insects.

Introduction

Global atmospheric CO₂ concentration has increased by approximately 40% from a pre-industrial value of 280 ppm to 387 ppm in 2009, and is anticipated to double by the end of this century [1]. Along with its direct effect on plant physiology and growth, elevated CO₂ typically reduces the quality of plants by increasing the C:N ratio, and causes plants to re-allocate assimilates to the synthesize secondary metabolites, thereby altering the interactions between host plants and herbivores [2,3].

Generally, elevated CO₂ does not trigger or alter the induced-defense processes of undamaged plants but may modify the induced defense of plants damaged by herbivores [4,5]. For example, elevated CO₂ increased the susceptibility of soybean to Japanese beetle and western corn rootworm by down-regulating the expression of genes related to the jasmonic acid (JA) pathway [6,7]. Although elevated CO₂ impairs the JA defense response against these chewing insects, the effect of elevated CO₂ on defense responses induced by plant-parasitic nematodes (i.e., JA, salicylic acid, antioxidant) has not been investigated.

The root-knot nematode Meloidogyne incognita is an obligate endoparasite that feeds exclusively on the cytoplasm of living plant cells [8]. M. incognita infects a large number of crops and causes severe losses in yield. The disease symptoms on infected plants include galls on the roots, stunted growth, wilting, and increased susceptibility to other pathogens [9]. Effects of elevated CO₂ on nematode densities as mediated by the host plant are “plant species-specific” and include negative effects [10], positive effects [11], and no significant effects [12]. Most of these studies proposed that changes in root biomass and C/N ratio were the main factors responsible for the effects of elevated CO₂ on nematode abundance [10–12]. However, the mechanisms underlying the effect of elevated CO₂ on the interaction between plant-parasitic nematodes and their host plants are poorly understood.

Systemic acquired resistance (SAR) is considered to be the major induced plant defense that confers long-lasting protection against nematodes [13]. SAR depends on the salicylic acid (SA) pathway and is associated with accumulation of pathogenesis-related proteins, which are considered to contribute to resistance. Researchers have recently suggested, however, that the JA pathway is also an indispensable component of plant resistance to nematodes [14,15]. The JA pathway is associated with expression of proteins including proteinase inhibitors, phenylalanine ammonia-lyase, and lipoygenase, up-regulation of secondary
metabolites, and induction of plant volatile organic compounds (VOC). Cooper et al. (2005) reported that the artificial induction of JA-pathway defenses reduced reproduction of root-knot nematodes on tomato plants [16]. Our previous research also found that a tomato genotype (35S::Prosystemin) in which induced defense was dominated by the jasmonic acid (JA) pathway (hereafter referred to as “JA defense-dominated genotype”) has stronger resistance to nematodes than a JA-defense-recessive genotype (spr2, a jasmonate-deficient mutant) and that the specific responses of these isogenic tomato genotypes to elevated CO2 requires our further investigation [17].

Based on several works referring to plant induced defense under elevated CO2 and our previous work [6,7,17], we hypothesized that elevated CO2 would reduce the resistance of a JA defense-dominated genotype against M. incognita by altering the JA pathway but enhance the SA-pathway defense of a JA-defense-recessive genotype infected by M. incognita. In this study, we determined whether elevated CO2 affects the regulation of genes and the production of secondary metabolites and the emission of VOC associated with the JA pathway of isogenic tomato genotypes. We also determined whether elevated CO2 affects the regulation of genes associated with the SA pathway. Finally, we determined whether the changes in these pathways and genes are associated with the performance of M. incognita under elevated CO2.

Results

**Temporal gene expression in leaves**

Elevated CO2 increased PAL, GST, PR1, and BGL2 levels of uninfected spr2 plants (Figure 1, Table S1). In contrast, elevated CO2 reduced the PAL level only of uninfected 35S plants (F1,6 = 9.16, P = 0.023). Regardless of CO2 level, uninfected 35S plants had the highest PI1 and PR1 levels among the genotypes. Furthermore, regardless of CO2 level, the 14-dpi treatment (nematodes added 14 days before sampling) increased PAL, PR1, and BGL2 levels and reduced the PI1 and RUBISCO level of spr2 plants, and increased the PI1, PAL, GST, PR1, and BGL2 levels of 35S plants. The 14-dpi treatment increased the PI1 and PAL levels of Wt plants under ambient CO2 and the GST, PR1, and BGL2 levels under elevated CO2. Elevated CO2 reduced the PI1 and LOX levels of Wt plants but increased the GST, PR1, and BGL2 levels of spr2 and Wt plants at 14-dpi. Elevated CO2 decreased the PI1 and PAL levels of 35S plants at 7- and 14-dpi (Figure 1).

**Levels of proteins, amino acids, and secondary metabolites**

Elevated CO2 increased the protein level of 35S plants and the foliar TNC:N ratio of all three genotypes. In contrast, elevated CO2 reduced the total phenolics and flavonoids of all the genotypes and the condensed tannins level of spr2 plants (Figure 2, Table S2). Regardless of CO2 level, uninfected Wt plants had the highest foliar TNC:N ratio among the genotypes. Furthermore, regardless of CO2 level, the 7-dpi treatment reduced whereas the 14-dpi treatment increased amino acid level of spr2 plants. The 14-dpi treatment increased the protein level of all the genotypes and the TNC:N ratio of all the genotypes under ambient CO2. Regardless of CO2 level, the 14-dpi treatment reduced total phenolics and flavonoids of Wt plants and flavonoids of 35S plants but increased condensed tannins of spr2 plants (Figure 2).

**Volatile emission rate**

CO2 level, tomato genotype, nematode infection, and the interaction between CO2 and nematode significantly affected the total amount of VOC (Table S3). In the absence of nematodes, elevated CO2 reduced the total amount of VOC released by spr2 plants. The jasmonate-deficient spr2 and Wt plants released less VOC than 35S plants under both ambient and elevated CO2 (Figure 3). Elevated CO2 reduced emission of ocimene and β-phellandrene in uninfected spr2 plants and hexenal in uninfected 35S plants. In the 14-dpi treatment under elevated CO2, spr2 plants emitted less of each volatile terpene than 35S plants (Table S4).

Galls resulting from nematode infection

CO2 level, tomato genotype, and their interactions affected the number of nematode-induced galls per gram of dry root (Figure 4). The number of galls on 35S roots was greater under elevated CO2 than under ambient CO2 (F1,16 = 78.3, P<0.001). Regardless of CO2 level, there were fewer galls on 35S plants than on Wt or spr2 plants (Figure 4). Under elevated CO2, galls were more abundant on spr2 roots than on the roots of the other two genotypes (F2,21 = 50.7, P<0.001).

Discussion

Although numerous studies have focused on the evolution of inducible defenses against herbivory, much less emphasis has been placed on how CO2 and other aspects of the abiotic environment affect these inducible responses [18]. Bidart-Bouzat et al. (2005) reported that elevated CO2 increased induced defense (i.e., increased glucosinolate levels) of Arabidopsis thaliana against diamondback moths [19]. Moreover, Zavala et al. (2009) showed that elevated CO2 down-regulated the gene expression and activity of cysteine proteinase inhibitors, which are the principal defenses of soybean against insect herbivores [7]. An interaction between CO2 level and herbivory, however, has seldom been detected in host plants [20]. Our results show that in the jasmonate-deficient mutant spr2, elevated CO2 up-regulated the induced defense at 14-dpi based on the SA pathway, including PR1 and BGL2 genes, but did not up-regulate the induced defense based on the JA pathway. Conversely, in the JA defense-dominated genotype 35S, elevated CO2 decreased induced defense based on the JA pathway (i.e., CO2 reduced the PI1 level) but did not increase induced defense at 7- and 14-dpi based on the SA pathway. Thus, our results support the hypothesis that, under elevated CO2, JA defense-dominated genotypes tend to express reduced JA-pathway-induced defense and JA-defense-recessive genotypes tend to amplify the SA-signaling pathway. To the best of our knowledge, this is the first study demonstrating that nematode-induced defense based on the JA or SA pathway in plants can be modified by CO2 level (significant CO2×nematode interactions for PI1 and PR1 genes, Table S1), and that these changes can differ among three isogenic tomato genotypes (significant CO2×nematode×genotype interaction).

Increased tissue levels of reactive oxygen species like H2O2 and O2- and the metabolism of glutathione induced by nematode infection are linked to defensive/secondary metabolism and cell differentiation of plant roots [21]. The results presented in this study show that, under ambient CO2, nematode infection up-regulated the GST gene in leaves only in the JA defense-dominated genotype 35S and that elevated CO2 increased levels of the GST gene in spr2 and Wt plants infected by nematodes at 14-dpi. Furthermore, for all three genotypes, nematode infection up-regulated the foliar PAL gene but only increased condensed tannins levels. Regardless of nematode infection, elevated CO2 decreased the level of the PAL gene only in 35S plants but unexpectedly reduced levels of total phenolics and flavonoids in all three genotypes. These results can probably be explained by...
elevated CO2 inhibiting the activity of the PAL enzyme at the post-transcriptional level rather than at the transcriptional level. Interestingly, the results of research investigating the effect of elevated CO2 on plant volatiles emissions are controversial [22,23]. Our finding of reduced VOC (including monoterpens and sesquiterpenes) emissions from spr2 plants is consistent with the results of Loreto et al. [22], who demonstrated that, because of down-regulation of terpene synthase activity, elevated CO2 reduced emission of monoterpenes from Quercus ilex leaves by approximately 68%. The authors suggested that VOC is probably limited by the availability of photosynthetic carbon. In addition, Vuorinen et al. (2004) also reported that elevated CO2 decreased the emission of JA-regulated terpene volatiles in cabbage [24].

In accordance with previous studies and because of photosynthetic acclimation, down-regulation of the mRNA level of the RUBISCO gene in Wt plants was observed under elevated CO2 (Figure 1D). Furthermore, as predicted by the Carbon Nutrient Balance (CNB) hypothesis [25], accumulated ‘extra’ carbon (relative to nitrogen) increases the plant C/N ratio and consequently would increase carbon-based defenses. Our data, however, showed that most carbon-based secondary metabolites including total phenolics, flavonoids, monoterpens, and sesquiterpenes volatiles were decreased by elevated CO2. It seems likely that the ‘extra’ carbon might be allocated to the formation of other secondary metabolites and that the response of different biosynthetic pathways to elevated CO2 could be species-specific or dependent on the developmental stage of plants. Moreover, the induction of a higher C/N ratio and lower nitrogen content by elevated CO2 often results in lower plant quality (i.e., reduced quantities of amino acids and protein) [26]. In contrast, our relatively short-term study found no evidence that elevated CO2 reduced the levels of amino acids and protein, which is not consistent with results from our previous, relatively long-term study in open-top chambers (OTC) in the field [17]. Perhaps long-term cumulative effects of elevated CO2 contributed to these differences.

In plants, JA and SA are ubiquitous signals of induced resistance against many if not most herbivores and pathogens, respectively [14]. Although the widespread acceptance that the accumulation of SA is the major signaling pathway of plant response to nematode infection [27], JA has been recently proven to be another efficient plant defense against nematodes [28]. For example, Cooper et al. (2005) reported that foliar application of JA suppressed the reproduction of the nematode Meloidogyne javanica on tomatoes [16]. In our current growth chamber study and in our previous field OTC study, a JA defense-dominated genotype exhibited higher resistance to nematodes than wild types or JA defense-recessive genotypes [17]. Typically, nematode infection primarily induces SA-mediated defense responses because nematode infection generates only minor trauma. Our data show that, under ambient CO2 level, nematode infection triggers the SA pathway at 14-dpi and involves the up-regulation of PR1 and BGL2 genes in jasmonate-deficient spr2 mutants but not in Wt plants. These results suggest that induction of SA-mediated defense did not confer resistance to nematodes. In contrast, 35S transgenic plants over-express prosystemin, which can constitutively activate the JA pathway in unwounded plants and result in stronger and quicker induced resistance in response to damage by herbivores. Nematode infection consequently triggered the up-regulation of the JA pathway (PR1) and the SA pathway (PR1 and BGL2) at 14-dpi in 35S plants. Likewise, we found that, regardless of CO2 level, the JA defense-dominated genotype 35S plants had the strongest resistance to nematodes among three genotypes. Accordingly, nematodes may tend to activate the ineffective defense pathway (SA-mediated defense) but suppress effective induced defense (JA-mediated defense) of plants.

Elevated CO2 tends to affect plant hormones and thereby could modify the induced defense against nematodes. Li et al. (2002b) demonstrated that elevated CO2 sharply increased the levels of several plant hormones including indole-3-acetic acid, gibberellins, isopentenyladenosine, and zeatin riboside [29]. Additionally, our data show that, at 14-dpi, elevated CO2 enhanced the induced defense based on the SA pathway in spr2 and Wt plants but suppressed the induced defense based on the JA pathway in 35S and Wt plants. This is probably because elevated CO2 affects plant defense responses through pathway cross-talk, amplifying the SA-signaling pathway to repress the JA-signaling pathway in Wt plants. Thus, an explanation of our results may be that elevated CO2 repressed the JA-signaling pathway but did not trigger the defense based on the SA pathway in 35S plants, making the 35S plants more susceptible to nematodes under elevated CO2 level.

Elevated CO2 down-regulates the gene expression of JA-dependent pathway defense, reduces the activity of PI, and in turn increases the susceptibility of soybean to both below-ground and above-ground chewing insects [6]. In contrast, although our study indicated that elevated CO2 had a genotype-specific effect on JA and SA-dependent pathway defense, the nematode resistances in the wild type and in the JA-defense-recessive genotype were not changed by elevated CO2, and the JA-defense-dominated genotype still had the strongest nematode resistance among genotypes under elevated CO2. Thus, the CO2-induced changes in systemic resistance against parasitic nematodes were substantially smaller than those reported for chewing insects.

In conclusion, this study demonstrates that, as indicated by secondary metabolites, VOC, and genes associated with defense against herbivores, induced defense of nematode-infected plants could be affected by global CO2 changes, and that CO2-induced changes in plant resistance may lead to genotype-specific changes in the response of plants to nematodes under elevated CO2. Theory predicts that plant defenses are costly [30,31]. In this respect, our work provides a foundation for further research on how elevated CO2 affects the tradeoff between resistance and tolerance of different isogenic genotypes. Finally, future research on the effects of elevated CO2 on induced-defense responses of plants should consider multiple signaling pathways.

Materials and Methods

Atmospheric CO2 concentration treatments

This experiment was performed in six closed-dynamic CO2 chambers (CDCC; Safe PRX-450B, 68 cm long, 68 cm wide and 185 cm high) [32]. The chambers were maintained at 26±0.8°C, 70±2% RH, and 14:10 (L:D)-h photoperiod with 12,000 LX of
active radiation supplied by 18 fluorescent lamps (60-W) in each chamber.

Two CO$_2$ levels, 390±30 ppm (current ambient level) and 750±30 ppm (predicted level at the end of this century), were applied. Three chambers were used for each CO$_2$ treatment. Elevated CO$_2$ concentrations were monitored and adjusted with an infrared CO$_2$ transmitter (Ventostat 8102, Telaire Company, Goleta, CA, USA) once every minute to maintain relatively stable CO$_2$ concentrations. The automatic-control system for maintaining CO$_2$ levels was described in detail by Chen and Ge [32].

**Host plants and nematodes**

Wild-type (Wt) tomato plants (L. esculentum cv. Castlemart), the jasmonate-deficient spr2 mutants (spr2), and the 35S::Prosystemin transgenic tomato plants (35S) were kindly provided by Professor C. Li of the Institute of Genetics and Developmental Biology, the Chinese Academy of Sciences. The JA-biosynthesis mutant, suppressor of prosystemin-mediated responses2 (spr2), reduces chloroplast ω3 fatty acid desaturase, which impairs the synthesis of JA [33]. In contrast, 35S::Prosystemin (35S) transgenic plants over-express prosystemin, which constitutively activates systemic defense in unwounded plants and results in stronger and quicker induced resistance [34].

Tomato (L. esculentum cv Castlemart) was the Wt parent for both the spr2 mutant and the 35S transgenic genotypes. Tomato seeds of the three genotypes were sown individually in plastic pots (15 cm diameter and 16 cm high) filled with 4:1 (v/v) loamy soil:earthworm feces. Tomato plants were exposed plastic pots (15 cm diameter and 16 cm high) filled with 4:1 (v/v) loamy soil:earthworm feces. Tomato plants were exposed to the CO$_2$ treatments after seedling emergence, and plants were randomly repositioned within each chamber weekly to minimize position effects. No chemical fertilizers and insecticides were used. Water was added to each pot once every 2 days.

The root-knot nematode, M. incognita, was cultured on Wt plants grown under ambient CO$_2$. To prepare nematode inoculum, nematode eggs were extracted from infected tomato roots by blending them in water containing 10% bleach (CaCl$_2$·Ca(OCl)$_2$·2H$_2$O). Eggs and root debris were collected on a 25-μm-pore sieve. The second-stage juveniles (J2) were hatched from the eggs and used as inoculum [35].

After plants had grown in the CDCCs for 4 weeks, 6 plants of each genotype in each CDCC were randomly selected (=18 plants per CDCC and 108 plants in total) and inoculated with freshly hatched M. incognita J2, and another 6 plants of each tomato genotype in each CDCC were treated with water as the control. One week later, 6 additional plants of each tomato genotype in each CDCC were inoculated with M. incognita as described above. All the nematode-treated pots received ≈3000 J2 in 5 ml of water applied with a pipette over the surface of the soil around the primary roots. One week after the second inoculation, the experiment was terminated and tomato plants were sampled. Thus, the experiment had two levels of CO$_2$, three tomato genotypes, and three nematode levels (uninfected control, nematodes added 7 days before sampling, and nematodes added 14 days before sampling). The latter two nematode treatments are referred to as the 7- and 14-dpi (days post inoculation) treatments, and the uninjected plants are referred to as controls.

**Assessment of plant traits and foliar chemical components**

Three plants from each combination of tomato genotype and nematode treatment in each chamber (=27 plants per CDCC and 162 plants in total) were randomly selected. Leaves and roots from each plant were collected and stored at −20°C until subjected to chemical analysis, except that a sample of fresh leaves (0.5 g) from each plant was removed and stored at −70°C for real-time PCR, as described later in this subsection.

The chemical components of the tomato leaves were analyzed. Protein concentrations were determined by the Bradford (1976) assay [36]. Total amino acids (TAA) were analyzed with a reagent kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

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**Figure 2.** Foliar chemical components of tomato genotypes grown under ambient and elevated CO$_2$ without (“−”) and with M. incognita plants with M. incognita were sampled 7 days post-inoculation (7 dpi) and 14 dpi. Each value represents the average (±SE) of three replicates. Different lowercase letters indicate significant differences among combinations of nematode and CO$_2$ level within the same tomato genotype (LSD test: d.f. = 5, 12; P<0.05). Different uppercase letters indicate significant differences among tomato genotypes within the same CO$_2$ and nematode treatment (LSD test: d.f. = 2, 6; P<0.05).

**Figure 3.** Emission rate of total volatile organic compounds (VOC) from tomato genotypes grown under ambient and elevated CO$_2$ without (“−”) and with M. incognita plants with M. incognita were sampled 7 days post-inoculation (7 dpi) and 14 dpi. Each value represents the average (±SE) of three replicates. Different lowercase letters indicate significant differences among combinations of nematode and CO$_2$ level within the same tomato genotype (LSD test: d.f. = 5, 12; P<0.05). Different uppercase letters indicate significant differences among tomato genotypes within the same CO$_2$ and nematode treatment (LSD test: d.f. = 2, 6; P<0.05). Emission rate represents ng of compound released by 10 g (fresh weight) of leaves per hour.

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significant differences among tomato genotypes within CO2 levels (LSD test: d.f. = 2,24, error.
were examined in every PCR plate to eliminate the systematic
different treatment conditions. The
house keeping gene) copy numbers, which remain constant under
mRNAs. The relative level of each target gene was standardized
the specificity of PCR products. A standard curve was derived
protocol produced the melting curves, which can be used to judge

| Parameter | Value |
|-----------|-------|
| Ambient | Elevated |
| sprw | a, A |
| WT | a, A |
| CO2 | a, A |
| CO2×genotype | a, B |
| CO2×genotype | b, C |

Provinces, China) [37]. Total non-structural carbohydrates (TNCs),
mainly starch and sugar, were assayed by acid hydrolysis following
the method of Tissue and Wright (1995) [38]. Nitrogen content
was assayed using Kjeltec nitrogen analysis (Foss automated
Kjeltec™ instruments, Model 2100). Total phenolics were
analyzed by the Folin-Ciocalteu method described by Kujala
et al. (2000) [39]. Flavonoids and condensed tannins were measured
using the methods of Jia et al. (1999) and Terrill et al. (1992),
respectively [40,41].

Real-time quantitative PCR
Each treatment combination was replicated four times for biological
repeats, and each biological repeat contained three technical repeats. The RNeasy Mini Kit (Qiagen) was used to isolate total RNAs from tomato leaves (0.5 g from samples stored
at −70°C; see the first paragraph of the previous subsection), and
2 μg quantities of the RNAs were used to generate the cDNAs.
The mRNA amounts of 7 target genes were quantified by real-
time quantitative PCR; the target genes were proteinase inhibitor
(PHI), lipoygenase (LOX), ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), phenylalanine ammonia lyase (PAL),
glutathione-S-transferase (GST), pathogenesis-related protein
(PR), and β-1,3-glucanase (BGL2). Specific primers for each
gene selected were designed from the tomato EST sequences using
PRIMER5 software (Table S5). The PCR reactions were performed in a 20-μL total reaction volume including 10 μL of
2 × SYBRs Premix EX TaqTM (Qiagen) master mix, 5 mM each
of gene-specific primers, and 1 μL of cDNA templates. They were
carried out on the Mx 3000P detection system (Stratagene), and
the parameters were as follows: 2 min at 94°C; then 40 cycles of
20 s at 95°C, 30 s at 56°C, and 20 s at 68°C; and finally one cycle
of 30 s at 95°C, 30 s at 56°C, and 30 s at 95°C. This PCR
protocol produced the melting curves, which can be used to judge
the specificity of PCR products. A standard curve was derived
from the serial dilutions to quantify the copy numbers of target
mRNAs. The relative level of each target gene was standardized
by comparing the copy numbers of target mRNA with β-actin (the
house keeping gene) copy numbers, which remain constant under
different treatment conditions. The β-actin mRNAs of the control
were examined in every PCR plate to eliminate the systematic
error.

Collection and quantification of plant volatiles
Volatiles were collected from one randomly selected plant from
each combination of tomato genotype and nematode treatment in each chamber (= 9 plants per CDCC and 54 plants in total). The
headspace volatiles were collected according to Turlings et al. (1998) [42]. The shoots and leaves of each plant, except for the
stem extending 4 to 5 cm from the soil surface, were sealed in
a plastic bag (40 cm wide and 46 cm long). Purified air was
pumped (Beijing Institute of Labor Instruments, China) into the
bag through a freshly activated charcoal trap (Beijing Chemical
Company) and then withdrawn through a glass cartridge (3.0 mm
internal diameter and 12.6 cm long) packed with 100 mg of the
adsorbent Porapak Q (80–100 mesh, Supelco, Bellefonte, PA, USA); the flow rate was 0.25 L/min. Volatile compounds were
rinsed from the Porapak Q with 600 μL of n-hexane (HPLC grade,
Sigma-Aldrich, USA) containing internal standards (200 ng of
ethyl heptanoate) for quantification. The aeration extracts were
stored at −20°C until analyzed. Immediately after headspace
volatiles were collected, the fresh weights of the plant leaves were
measured.

Volatiles were quantified and identified using a gas chromato-
tography-mass spectrometry (GC-MS) system (Hewlett Packard
6890N GC model coupled with 5973 MSD) equipped with a HP-5MS column (60 m long, 0.25 mm inner diameter, and 0.25 μm
film thickness; Agilent Technologies, Palo Alto, CA, USA). The
initial oven temperature was kept at 50°C for 1 min and then
increased to 250°C at a rate of 5°C/min. Volatile compounds
were identified by comparing their retention times and spectra
with those of compounds in the NIST02 library (Scientific
Instrument Services, Inc., Ringoes, NJ, USA) and those of pure
standards.

Assessment of disease symptoms caused by the
nematode
When J2 of M. incognita infect roots, galls may occur, and galls
were quantified to estimate root infection. Roots of 14-dpi
nematode infected plants (3 plants from each combination of
tomato genotype and nematode treatment in each chamber, 27
plants per CDCC and 162 plants in total) were carefully removed
from soil and washed. A stereomicroscope was used to count
the number of galls produced on the entire root system of each plant.

Figure 4. Number of galls per gram of dry root infected by M. incognita 14 days post-inoculation on tomato genotypes grown under
ambient (390 ppm) and elevated CO2 (750 ppm). Each value represents the average (±SE) of four replicates. Different lowercase letters indicate
significant differences between CO2 levels within the same tomato genotype (LSD test: df = 1,16, P < 0.05); different uppercase letters indicate
significant differences among tomato genotypes within CO2 levels (LSD test: df = 2,24, P < 0.05).
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Table S3  *P* values from MANOVAs for the effect of CO2 level, tomato genotype, and nematode infection on plant volatiles. (DOC)

Table S4  Emission rate* of volatile organic compounds (VOC) from tomato genotypes grown under ambient (390 ppm) and elevated CO2 (750 ppm) without and with *M. incognita*. (DOC)

Table S5  Primer sequences used in the real-time quantitative PCR. (DOC)

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Author Contributions

Conceived and designed the experiments: LK FG CL. Performed the experiments: YS JY HC. Analyzed the data: YS HC. Contributed reagents/materials/analysis tools: YS JY CL. Wrote the paper: YS FG.

References

1. Intergovernmental Panel on Climate Change (2007) Climate Change 2007: the physical science basis. Summary for policy makers. Report of Working Group I of the Intergovernmental Panel on Climate Change. http://www.ipcc.ch/pub/pdf/SPM/AR4_WG1_SPM.pdf.
2. Hartley SE, Jones CG, Couper GC, Jones TH (2000) Biosynthesis of plant phenolic compounds in elevated atmospheric CO2. Global Change Biol 6: 497–506.
3. Barbehrm RN, Chen Z, Karowe DN, Spickard A (2004) C3 grasses have higher nutritional quality than C4 grasses under ambient and elevated atmospheric CO2. Global Change Biol 10: 1565–1575.
4. Suling P, Cornelissen T (2007) How does elevated carbon dioxide (CO2) affect plant-herbivore interactions? A field experiment and meta-analysis of CO2-mediated changes on plant chemistry and herbivore performance. Global Change Biol 13: 1823–1842.
5. Li P, Ainsworth EA, Leakey ADB, Ulamov A, Lozovaya V, et al. (2008) Arabidopsis transcript and metabolite profiles: ecotype-specific responses to open-air elevated CO2. Plant Cell Environ 31: 1673–1687.
6. Zavala JA, Casteel CL, Delucia EH, Berenbaum MR (2008) Anthropogenic increase in carbon dioxide compromises plant defense against invasive insects. Proc Natl Acad Sci USA 105: 5129–5133.
7. Zavala JA, Casteel CL, Nabyt PD, Berenbaum MR, Delucia EH (2009) Role of cysteine proteinase inhibitors in preference of Japanese beetles (*Popillia japonica*) for soybean (* Glycine max*) leaves of different ages and grown under elevated CO2. Oecologia 161: 35–41.
8. Abad P, Favery B, Rosso MN, Castagnone-Sereno P (2003) Root-knot nematode parasitism and host response: molecular basis of a sophisticated interaction. Mol Plant Pathol 4: 217–224.
9. Williamson VM (1998) Root-knot nematode resistance genes in tomato and their susceptibility to root-knot nematodes requires an intact jasmonic acid signaling pathway. J Chem Ecol 34: 153–167.
10. Lau JA, Tiffin P (2009) Elevated carbon dioxide concentrations indirectly affect plant fitness by altering plant tolerance to herbivory. Oecologia 161: 401–410.
11. Melillo MT, Leonetti P, Bongiovanni M, Castagnone-Sereno P, Bleve-Zacheo T (2006) Modulation of reactive oxygen species activities and H2O2 accumulation during compatible and incompatible tomato-root-knot nematode interactions. New Phytol 170: 501–512.
12. Loreto F, Fuchschab RJ, Schnitzler JP, Ciccioli P, Brancucci E, et al. (2001) Monoterpene emission and monoterpene synthase activities in the Mediterranean evergreen oak *Quercus ilex* L. grown at elevated CO2 concentrations. Global Change Biol 7: 709–717.
13. Vuorinen T, Reddyb GVP, Nerga AM, Holopainen JK (2004) Monoterpenes and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO2 concentration. Atmos Environ 38: 675–682.
14. Vuorinen T, Nerg AM, Brahman MA, Reddy GVP, Holopainen JK (2004) Emission of *P. sylvestris*-induced compounds from cabbages grown at elevated CO2 and orientation behavior of the natural enemies. Plant Physiol 135: 1984–1992.
15. Hamilton JG, Zangerl AR, Delucia EH, Berenbaum MR (2003) The carbon-nutrient balance hypothesis: its rise and fall. Ecol Lett 4: 86–95.
16. Yin J, Sun Y, Wu G, Parajulee MN, Ge F (2009) No effects of elevated CO2 on the population relationship between cotton bollworm, *Helicoverpa armigera* (Hubner) (*Lepidoptera: Noctuidae*), and its parasitoid, *Mephisto inodorus Haliday* (*Hymenoptera: Braconidae*). Agri Ecosys Environ 132: 267–275.
17. Vasyukova NI, Zinovev’VA, Udalkova ZV, Panina YS, Ozeretskovskaya OL, et al. (2003) The Role of Sarcyclin in Systemic Resistance to Tomato (*Solanum lycopersicum*) and Tobacco (*Nicotiana tabacum*). J Appl Genet 42: 239–246.
18. Strauss SY, Rudgers JA, Lau JA, Erwin KE (2002) Direct and ecological costs of resistance to herbivory. Trends Ecol Evol 17: 276–281.
19. Bidart-Bezeat MG, Miltein R, Berenbaum MR (2005) Elevated CO2 influences herbivory-induced defense responses of *Arabidopsis thaliana*. Oecologia 145: 415–424.
20. Lau JA, Tiffin P (2009) Elevated carbon dioxide concentrations indirectly affect plant fitness by altering plant tolerance to herbivory. Oecologia 161: 401–410.
21. Melillo MT, Leonetti P, Bongiovanni M, Castagnone-Sereno P, Bleve-Zacheo T (2006) Modulation of reactive oxygen species activities and H2O2 accumulation during compatible and incompatible tomato-root-knot nematode interactions. New Phytol 170: 501–512.
22. Loreto F, Fuchschab RJ, Schnitzler JP, Ciccioli P, Brancucci E, et al. (2001) Monoterpene emission and monoterpene synthase activities in the Mediterranean evergreen oak *Quercus ilex* L. grown at elevated CO2 concentrations. Global Change Biol 7: 709–717.
23. Vuorinen T, Reddy GVP, Nerga AM, Holopainen JK (2004) Monoterpenes and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO2 concentration. Atmos Environ 38: 675–682.
24. Vuorinen T, Nerg AM, Brahman MA, Reddy GVP, Holopainen JK (2004) Emission of *P. sylvestris*-induced compounds from cabbages grown at elevated CO2 and orientation behavior of the natural enemies. Plant Physiol 135: 1984–1992.
25. Hamilton JG, Zangerl AR, Delucia EH, Berenbaum MR (2003) The carbon-nutrient balance hypothesis: its rise and fall. Ecol Lett 4: 86–95.
26. Yin J, Sun Y, Wu G, Parajulee MN, Ge F (2009) No effects of elevated CO2 on the population relationship between cotton bollworm, *Helicoverpa armigera* (Hubner) (*Lepidoptera: Noctuidae*), and its parasitoid, *Mephisto inodorus Haliday* (*Hymenoptera: Braconidae*). Agri Ecosys Environ 132: 267–275.
27. Vasyukova NI, Zinovev’VA, Udalkova ZV, Panina YS, Ozeretskovskaya OL, et al. (2003) The Role of Sarcyclin in Systemic Resistance to Tomato (*Solanum lycopersicum*) and Tobacco (*Nicotiana tabacum*). J Appl Genet 42: 239–246.
28. Vuorinen T, Reddy GVP, Nerga AM, Holopainen JK (2004) Monoterpenes and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO2 concentration. Atmos Environ 38: 675–682.
29. Vuorinen T, Nerg AM, Brahman MA, Reddy GVP, Holopainen JK (2004) Emission of *P. sylvestris*-induced compounds from cabbages grown at elevated CO2 and orientation behavior of the natural enemies. Plant Physiol 135: 1984–1992.
30. Hamilton JG, Zangerl AR, Delucia EH, Berenbaum MR (2003) The carbon-nutrient balance hypothesis: its rise and fall. Ecol Lett 4: 86–95.
31. Yin J, Sun Y, Wu G, Parajulee MN, Ge F (2009) No effects of elevated CO2 on the population relationship between cotton bollworm, *Helicoverpa armigera* (Hubner) (*Lepidoptera: Noctuidae*), and its parasitoid, *Mephisto inodorus Haliday* (*Hymenoptera: Braconidae*). Agri Ecosys Environ 132: 267–275.
32. Chen FJ, Ge F (2004) An experimental instrument to study the effects of changes in CO2 concentrations on the interactions between plants and insects-CDCGC-1 Insect-Mediated VOLATILE MONOTERPENES. Entomol Knowl 41: 279–281.
33. Li C, Liu G, Xu C, Lee G, Bauer P, et al. (2003) The tomato suppressor of prosystemin-mediated responses2 gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression. Plant Cell 15: 1646–1661.
34. Li C, Williams MM, Loh YT, Lee GI, Howe GA (2002) Resistance of cultivated tomato to cell content-feeding herbivores is regulated by the octadecanoid-signaling pathway. Plant Physiol 130: 494–503.
35. Hussey RS, Barker KR (1973) A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Dis Rep* 57: 1025–1028.
36. Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Analy Biochem* 72: 248–54.
37. Sun Y, Su J, Ge F (2010) Elevated CO₂ reduces the response of *Sitobion avenae* (Homoptera: Aphididae) to alarm pheromone. *Agri Ecosys Environ* 135: 140–147.
38. Tissue DT, Wright SJ (1995) Effects of seasonal water availability on phenology and the annual shoot carbohydrate cycle of tropical forest shrubs. *Func Ecol* 9: 518–527.
39. Kujala TS, Loponen JM, Klika KD, Pihlaja K (2000) Phenolics and betacyanins in red beet root (*Beta vulgaris*) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds. *J Sci Food Agri* 48: 5338–5342.
40. Jia Z, Tang M, Wu J (1999) The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chem* 64: 555–559.
41. Tervill TH, Rescan AM, Douglas GB, Barry TN (1992) Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *J Sci Food Agri* 58: 321–329.
42. Turlings TCJ, Bernasconi M, Bertossa R, Bigler F, Caloz G, et al. (1998) The induction of volatile emissions in maize by three herbivore species with different feeding habits: possible consequences for their natural enemies. *Biol Control* 11: 122–129.