Antagonism between phytohormone signalling underlies the variation in disease susceptibility of tomato plants under elevated CO₂

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Abstract
Increasing CO₂ concentrations ([CO₂]) have the potential to disrupt plant–pathogen interactions in natural and agricultural ecosystems, but the research in this area has often produced conflicting results. Variations in phytohormone salicylic acid (SA) and jasmonic acid (JA) signalling could be associated with variations in the responses of pathogens to plants grown under elevated [CO₂]. In this study, interactions between tomato plants and three pathogens with different infection strategies were compared. Elevated [CO₂] generally favoured SA biosynthesis and signalling but repressed the JA pathway. The exposure of plants to elevated [CO₂] revealed a lower incidence and severity of disease caused by tobacco mosaic virus (TMV) and by Pseudomonas syringae, whereas plant susceptibility to necrotrophic Botrytis cinerea increased. The elevated [CO₂]-induced and basal resistance to TMV and P. syringae were completely abolished in plants in which the SA signalling pathway nonexpressor of pathogenesis-related genes 1 (NPR1) had been silenced or in transgenic plants defective in SA biosynthesis. In contrast, under both ambient and elevated [CO₂], the susceptibility to B. cinerea highly increased in plants in which the JA signalling pathway proteinase inhibitors (PI) gene had been silenced or in a mutant affected in JA biosynthesis. However, plants affected in SA signalling remained less susceptible to this disease. These findings highlight the modulated antagonistic relationship between SA and JA that contributes to the variation in disease susceptibility under elevated [CO₂]. This information will be critical for investigating how elevated CO₂ may affect plant defence and the dynamics between plants and pathogens in both agricultural and natural ecosystems.

Key words: Botrytis cinerea, nonexpressor of pathogenesis related genes 1 (NPR1), elevated CO₂, jasmonic acid, plant disease, Pseudomonas syringae, salicylic acid, Solanum lycopersicum (tomato), tobacco mosaic virus (TMV).

Introduction
Global climate change due to increasing anthropogenic emissions is markedly affecting natural ecosystems (Kerr, 2007). Rising CO₂ levels, among other factors, are thought to be responsible for climate change (IPCC, 2007). Furthermore, concentrations of carbon dioxide ([CO₂]) have increased markedly since the inception of the industrial
revolution, reaching current levels of 380 μmol mol⁻¹, and they will continue climbing to 730–1020 μmol mol⁻¹ by the end of the twenty-first century (Meehl et al., 2005). Additionally, the rise in CO₂ is often projected to increase the production and quality of agroecosystems, particularly in C₃ greenhouse vegetable crops (Uprety, 1998; Dion et al., 2013). Many studies have investigated the likely impacts of rising CO₂ concentration on crop growth and production (Tubiello et al., 2000; Leakey et al., 2006; Soares et al., 2008; Aranjuelo et al., 2013), and there has been general agreement on the beneficial effects of elevated [CO₂] on yield, probably due to increased photosynthesis, C:N ratio, and water-use efficiency, from the CO₂ “fertilization effect” (Drake et al., 1997; Ainsworth and Long, 2005; Slattery et al., 2013). However, yield-limiting factors such as pathogens have been ignored in most of these studies (Pangga et al., 2011; Juroszek and von Tiedemann, 2013). Disease symptoms are influenced by three main components: (i) host, (ii) pathogen, and (iii) environmental conditions (McElrone et al., 2005). Thus, the altered environmental conditions associated with elevated [CO₂] will potentially modify plant disease susceptibility. However, knowledge of the effects of climate change on diseases and related plant responses is still lacking. Pathogens reduce plant productivity worldwide, and billions of dollars in plant yield are lost to diseases each year. Therefore, more work is needed to elucidate how plant diseases will respond to the interacting factors of elevated [CO₂] climatic conditions (McElrone et al., 2010; Runion et al., 2010). Understanding such relationships is essential for predicting disease pressure and managing agricultural and natural ecosystems under changing climatic conditions.

Limited research on the influence of elevated [CO₂] on plant pathogens and diseases shows that the severity and/or incidence of disease may increase, decrease, or remain unaffected (Lake and Wade, 2009; Newton et al., 2011; Pugliese et al., 2011; West et al., 2012). Free-air CO₂ enrichment (FACE) facilities allow for an assessment of the effects under field conditions. Such studies have found that rice plants grown under elevated [CO₂] conditions showed an increased susceptibility to both rice blast and sheath blight (Kobayashi et al., 2006), whereas in Solidago rigida, the disease incidence of leaf spot was reduced by half under similar FACE conditions (Strengbom and Reich, 2006). Another FACE study investigating crown rot on wheat found that elevated [CO₂] resulted in increased biomass of the necrotrophic fungal pathogen Fusarium pseudograminearum and increased stem browning (Melloy et al., 2010). Climate chamber-based studies also report conflicting results. The anthracnose pathogen Colletotrichum gloeosporioides increased in aggressiveness over 25 sequential infection cycles in the host Stylosanthes scabra under elevated [CO₂] (Chakraborty and Datta, 2003). However, investigations into the systemic responses of tomato to tomato yellow leaf curl virus (TYLCV) and of tobacco to potato virus Y found that elevated [CO₂] decreased disease incidence and severity (Matros et al., 2006; Huang et al., 2012). These FACE and chamber studies support earlier findings that plant disease responses to elevated [CO₂] vary with the host–pathogen system. In some cases, predictions of higher disease levels have been verified, especially for necrotrophic pathogens (Eastburn et al., 2010; Melloy et al., 2010; Eastburn et al., 2011). In contrast, plant defences against (hemi) biotrophic pathogens, including viruses, were generally more efficient under elevated [CO₂], although there were exceptions. Plants have evolved complex signalling networks to sense and respond to pathogen attacks, and it is generally accepted that the salicylic acid (SA) signalling pathway is mainly activated in response to biotrophic or hemibiotrophic pathogens, whereas resistance to necrotrophic pathogens requires the activation of the jasmonic acid (JA) signalling pathway, which incorporates ethylene (ET)-dependent responses in some cases (Tsuda and Katagiri, 2010; Xin and He, 2013). It was thus hypothesized that SA/JA cross talk was modulated under elevated [CO₂], which may create a flexible signalling network that is vital for defence responses to different types of invaders. Indeed, elevated [CO₂] typically increases the C/N ratio and causes plants to re-allocate resources to synthesize secondary metabolites, leading to a shift in leaf chemistry components (Matros et al., 2006). Previous studies demonstrated that elevated [CO₂] down-regulated the expression of genes related to the JA pathway in soybeans (Zavala et al., 2008; Zavala et al., 2013). In an investigation into tomato plants, elevated [CO₂] increased the SA level upon uninfected and TYLCV-infected treatments (Huang et al., 2012). Thus, whether the flexible SA/JA cross talk is associated with the elevated [CO₂]-induced alteration in plant defence strategies needs to be tested in a biological context using the same system, which might account for the highly specific nature of host–pathogen interactions under elevated [CO₂].

In this study, the spectrum of plant–pathogen interactions were compared under elevated [CO₂] using fungal, bacterial, and viral pathogens on tomato plants. These pathogens are common, have a wide host range, cause destructive foliar disease, and are widely distributed throughout the world. They have been widely investigated, and plants primarily defend against them through either SA-dependent basal resistance, as observed with Pseudomonas syringae and tobacco mosaic virus (TMV), or JA/ET-dependent basal resistance, as observed with necrotrophic Botrytis cinerea (Ton et al., 2002; El Oirdi et al., 2011). Here, it was found that elevated [CO₂] generally favoured the SA signalling pathway and repressed the JA pathway, which was accompanied by enhanced resistance to P. syringae and TMV, and susceptibility to B. cinerea. Silencing genes in the SA or JA signalling pathways or using plant lines defective in SA or JA biosynthesis overturned the [CO₂]-induced resistance or susceptibility. This work highlights SA/JA cross talk in specific host–pathogen interactions under elevated [CO₂]. This information is important for making proper predictions of disease pressure and for designing strategies to improve plant pathogen resistance under changing agricultural conditions.

**Materials and methods**

Plant material and growth conditions

Tomato (Solanum lycopersicum L. cv. Zheza 205) seeds were purchased from the Zhejiang Academy of Agricultural Sciences, China and were sown approximately 0.5 cm deep in sterilized soil
and germinated at 25 °C. Fifteen days after germination, the seedlings were transplanted into plastic pots (diameter, 10.5 cm; depth, 17.5 cm; one plant per pot) containing soil and perlite (1:3, v:v) in controlled-environment growth chambers (Conviron, Winnipeg, Canada). The growth conditions were as follows: the photosynthetic photon flux density (PPFD) was 600 μmol m$^{-2}$ s$^{-1}$, the photoperiod was 14/10h (day/night), the day/night air temperature was 26/22 °C, and the relative humidity was 85%. When the seedlings were at the four-to-five-leaf stage, they were exposed to atmospheric [CO$_2$] at either 380 μmol mol$^{-1}$ or 800 μmol mol$^{-1}$, corresponding to the "ambient [CO$_2$]" and "elevated [CO$_2$]" treatments, respectively. After 4 d of acclimation, plants exposed to both ambient and elevated [CO$_2$] were subjected to inoculation with TMV, *P. syringae*, or *B. cinerea*. Plants were also mock inoculated to control for tissue damage caused by the inoculation procedure. The petal placement within each [CO$_2$] condition was randomized every 2 d; all plants were watered and fertilized with Hoagland’s solution every 2–3 d as necessary. The experiments were conducted independently three times.

**Pathogen inoculation**

For TMV inoculation, two fully developed leaves were inoculated with TMV (U1 strain) suspensions using cotton tips on adaxial surfaces previously dusted with carbonbund powder (Liao et al., 2012).

The bacteria *P. syringae pv. tomato* DC3000 were cultured in King’s B medium containing 25mg l$^{-1}$ rifampicin. An overnight culture was diluted 1:50 with fresh King’s B medium before the experiment and grown for another 2h at 28 °C. Bacterial cells were harvested by centrifugation (4 °C, 3000rpm, 10 min) and dissolved in 10 mM MgCl$_2$ to optical density (OD)=0.2 measured at 600nm, which corresponded to approximately 10$^8$ colony-forming units (cfu) ml$^{-1}$. Tomato plants were vacuum infiltrated with *P. syringae* suspended in 10 mM MgCl$_2$ at a final concentration of 10$^5$ cfu ml$^{-1}$ after serial dilution according to Katagiri et al. (2002). Bacterial leaf populations were measured according to the method described in Wolle et al. (2000). Trypan blue staining was carried out according to Bai et al. (2012).

The *B. cinerea* isolate used in this study is BOS-10, and was subcultured using the method described in El Oirdi et al. (2010). Two different inoculation methods were used in the current study. In the *in planta* inoculation method, all the leaves on the plants were inoculated by spraying them with a *B. cinerea* spore suspension at a density of 2 × 10$^8$ spores per ml. In the *in vitro* inoculation method, detached fully developed leaves were spot inoculated with a *B. cinerea* suspension (2 × 10$^8$ spores per ml) using a 2.5 μl droplet of *B. cinerea* spores on the upper surface of each leaf using a micropipette (El Oirdi et al., 2010). After inoculation, disease symptoms were assessed by trypan blue staining (Bai et al., 2012), quantification of *B. cinerea* gene transcription, or by analysis of chlorophyll fluorescence with an Imaging-PAM Chlorophyll Fluorometer (IMAG-MAXI, Heinz Walz, Effeltrich, Germany). For *B. cinerea* actin gene transcription assay, the primers used are shown in Supplementary Table S1. PCR conditions consisted of denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing at 58 °C for 45 s. For the chlorophyll fluorescence assay, in actinic light (300 μmol m$^{-2}$ s$^{-1}$), maximal fluorescence ($F_{m}^{\prime}$), and steady-state fluorescence before the flash ($F_{0}$) were measured, whereas saturated light flashes were applied every 20 s, and the quantum efficiency of light-adapted leaves ($F_{v}/F_{m}$) was calculated as $F_{m}^{\prime} - F_{0}$ (Genty et al., 1989).

**Virus-induced gene silencing in tomato**

Virus-induced gene silencing (VIGS) was performed using the bipartite tobacco rattle virus (TRV) vectors, pTRV1 and pTRV2, as previously described (Liu et al., 2002). Fragments from tomato non-expressor of pathogenesis-related genes 1 (NPR1), protease inhibitors I and II (PI I and PI II) cDNAs were PCR-amplified using the primers shown in Supplementary Table S1. Restriction sites were added to the 5’ ends of the forward and reverse primers for cloning into the pTRV2 vector. Amplification using these primers produced a 300-bp fragment. pTRV2 vectors containing the cDNA fragments were also described in El Oirdi et al. (2011). The pTRV-RNA2 empty vector (pTRV:0) was used as a control. The resulting plasmids were subsequently introduced into *Agrobacterium tumefaciens* strain GV3101, and a culture of *Agrobacterium tumefaciens* (OD$_{600}$=0.9) containing either the pTRV3 or the pTRV: target gene and pTRV-RNA1 (OD$_{600}$=0.9) in a 1:1 ratio was infiltrated into fully expanded cotyledons of tomato plants. It should be noted that pTRV:PI was an equal mix of pTRV:PI I and pTRV:PI II. The inoculated plants were grown under a 14-h photoperiod at 22 °C. After 3–4 weeks, the levels of targeted transcripts were analysed by qRT-PCR using the primers in Supplementary Table S1.

**RNA isolation and transcript analysis**

Total RNA from tomato leaves was prepared using TRIZol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s procedure. Genomic DNA was removed using a purifying column. Reverse transcription was performed using Superscript II (Invitrogen) following the manufacturer’s instructions. The primers are listed in Supplementary Table S1, and most of these primers have been described previously in El Oirdi et al. (2011). qRT-PCR analysis was performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was normalized to actin, and relative gene expression was calculated as described by Livak and Schmittgen (2001). For semi-quantitative RT-PCR analysis of TMV-coat protein (CP) gene, the PCR reaction was performed using the TaKaRa Ex Taq Hot Start Version (Takara Bio) with denaturing, annealing, and extension at temperatures of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, respectively. The PCR products were analysed by electrophoresis on a 2% agarose gel, and actin was used as a control.

**SA and JA quantification**

Frozen plant material (about 100 mg) was homogenized in 2-ml microcentrifuge tubes and then 1 ml of ethyl acetate spiked with labelled internal standards (D3-JA and D6-SA) was added to each sample. After centrifugation at 13 000 g for 20 min at 4 °C, supernatants were transferred to fresh 2 ml Eppendorf tubes and then evaporated to dryness on a vacuum concentrator (Eppendorf). The residue was resuspended in 0.5 ml of 70% methanol (v/v) and centrifuged for 10 min at 4 °C (13 000 g). The supernatants were pipetted to glass vials and then analysed by HPLC-MS/MS using the same method as described in Wu et al. (2007). Each treatment was biologically replicated five times.

**Statistical analysis**

At least four independent replicates were conducted for each determination. The data were subjected to analysis of variance, and the means were compared using Tukey’s test at the 5% level.

**Results**

Effects of elevated [CO$_2$] on pathogen incidence and severity

TMV-inoculated plants in ambient and elevated [CO$_2$] conditions were compared first. As pathogen infection often results in a reduction in the operating efficiency of PSII, the chlorophyll fluorescence imaging method was used to analyse the response of photochemical quantum yield at photosystem II...
(ΦPSII) to TMV infection under elevated [CO₂] (Fig. 1A). TMV infection was significantly reduced under elevated [CO₂]. At 9 d post-inoculation (dpi), TMV inoculation decreased ΦPSII in the upper uninoculated systemic leaves, whereas ΦPSII remained significantly higher in plants under the elevated [CO₂] condition compared with those at ambient [CO₂]. Moreover, the accumulation of TMV-CP mRNA detected by semi-quantitative RT-PCR analysis correlated well with the change in ΦPSII (Fig. 1B). All tested uninfected tomato leaves were negative. Among TMV-inoculated plants, transcript levels of the gene encoding the TMV-CP increased steadily from 3–9 dpi in both ambient and elevated [CO₂]. However, the transcript level was always lower under elevated [CO₂] than under ambient [CO₂].

The effects of CO₂ enrichment were similar between _P. syringae_ and TMV inoculation. Under elevated [CO₂], treatment of the leaves with bacterial _P. syringae_ resulted in a significant reduction of disease symptoms (6 dpi) and cell death (4 dpi) (Fig. 2A, B). Growth analysis of the pathogen also showed that plants grown under elevated [CO₂] had significantly lower bacterial colony counts at 2 and 4 dpi, compared with plants under ambient [CO₂] (Fig. 2C).

To analyse whether elevated [CO₂] affects a necrotrophic fungus differently, detached tomato leaves from 5-week-old tomato plants were inoculated with spores of _B. cinerea_. In the comparative assay, elevated [CO₂]-treated leaves seemed to be much more susceptible to _B. cinerea_ than ambient [CO₂]-treated leaves because a considerably larger increase in the spread of _B. cinerea_ lesions was observed at 2 dpi (Fig. 3A). Moreover, a whole-plant inoculation protocol was set up to obtain a more reliable and reproducible system for infection studies than those based on detached leaves (Fig. 3B, C). An analysis of symptom appearance, ΦPSII, and _B. cinerea_-specific actin genes in the whole-plant inoculation experiment again revealed increased infection in elevated [CO₂]-treated plants.

**Induction of SA- and JA-dependent pathways in different plant–pathogen interactions under elevated [CO₂]**

Whether the tomato plant resistance to different pathogens under elevated [CO₂] was related to the alterations in the SA/JA defence pathway was then tested. The SA effect occurs mainly through the co-activator NPR1 and pathogenesis-related gene 1 (PRI) (Durrant and Dong, 2004; Pieterse and Van Loon, 2004), whereas the JA effect occurs mainly through two JA-dependent genes, _PI I_ and _PI II_ (AbuQamar et al., 2008). The only PRI gene in _Arabidopsis_ is a good marker for SA signalling, whereas there are several PRI genes in tomato plants; the expression induction of tomato PRI gene (accession AK324060.1) used in this study was SA-dependent and JA-independent (Supplementary Fig. S1). The expression levels of these SA- and JA-dependent genes were then detected under different pathogen inoculation and CO₂ treatment conditions using qRT-PCR (Fig. 4). In mock-inoculated plants, elevated [CO₂] increased the transcript level of NPR1 by approximately 117.1% in all three independent experiments with TMV, _P. syringae_, and _B. cinerea_. Similarly, PRI expression was also higher in mock-infected elevated [CO₂]-treated plants; however, no significant quantitative changes were observed. In contrast, the CO₂ concentration had no effect on

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**Fig. 1.** Effects of tobacco mosaic virus infection on tomato plants grown under elevated (Ele, 800 μmol mol⁻¹) or ambient [CO₂] (Amb, 380 μmol mol⁻¹). (A) The leaf photochemical quantum yield at photosystem II was measured after 9 d of different treatments. The circles in the images indicate the locations where the fluorescence measurements were performed, and the data are shown in each figure. The colour gradient scale below the figure indicates the magnitude of the fluorescence signal represented by each colour. (B) Time-course changes in the transcription of the gene encoding the TMV-coat protein (CP) in young, fully expanded leaves.
Phytohormone and disease variation in elevated CO₂

PI I and PI II transcript expression. Pathogen infection significantly increased the SA-dependent gene NPR1 regardless of the invader type; moreover, pathogen infection induced significantly higher NPR1 transcripts under elevated [CO₂] than under ambient [CO₂]. The PRI induction pattern was similar to that of NPR1. Conversely, TMV and P. syringae infection had little or no effects on JA-dependent PI I and PI II transcript expression, whereas B. cinerea greatly induced PI I and PI II transcript expression by 30.4- and 60.3-fold under ambient [CO₂] and by 15.7- and 22.5-fold under elevated [CO₂], respectively. It should be noted that the increases in B. cinerea-induced PI I and PI II transcript expression were much lower in plants under elevated [CO₂] than in those under ambient [CO₂].

The changes of SA and JA accumulation due to different pathogens under elevated [CO₂] were also determined through HPLC-MS/MS analysis using labelled internal standards (Fig. 5). Elevated [CO₂] caused a significant increase of SA accumulation in mock plants. TMV, P. syringae and B. cinerea inoculation raised SA content by 3.5-, 1.5-, and 2.0-fold respectively, under ambient [CO₂], which were further increased to 18.6-, 2.0-, and 2.7-fold respectively, under elevated [CO₂]. By contrast, JA content was not significantly affected under elevated [CO₂] in mock plants. There were also no changes of JA content in TMV or P. syringae-inoculated plants under ambient [CO₂]. Notably, B. cinerea inoculation induced JA accumulation by 36.9%, whereas this B. cinerea-induced JA content increase was only 25.5% under elevated [CO₂]. It was unexpected that under elevated CO₂ condition, P. syringae inoculation significantly induced JA content.

Impairment in SA or JA signalling and biosynthesis affected tomato–pathogen interactions under elevated [CO₂]

There were significantly different responses of SA- and JA-dependent genes and synthesis to pathogens and CO₂ conditions. To test whether these responses have a biological effect on plant–pathogen interactions under elevated [CO₂], VIGS experiments were performed using the TRV vectors. After 3–4 weeks, the NPR1, PRI, PI I, and PI II transcript expression levels were analysed in silenced plants (Fig. 6). The transcript expression levels for SA-dependent NPR1 and PRI but not JA-dependent PI I and PI II were again significantly increased in pTRV:0 plants grown under elevated [CO₂] compared with those grown under ambient conditions. pTRV:NPR1-silenced plants showed significantly lower levels of NPR1 and PRI transcripts in both ambient and elevated
[CO₂] conditions, whereas pTRV:PI-silenced plants exhibited significantly reduced PI I and PI II transcript expression compared with pTRV:0-silenced plants. The experiments were extended to ask whether NPR1 regulates the expression of PI I and PI II and vice versa. It was found that NPR1 gene silencing resulted in large and significant increases in PI I and PI II transcript expression under both ambient and elevated [CO₂] conditions. However, pTRV:PI-silenced plants did not exhibit altered levels of NPR1 and PR1 transcript expression compared with pTRV:0-silenced plants (Fig. 6).

Plants in which genes of interest were silenced were subjected to TMV challenge, and samples were harvested for RNA extraction at 9 dpi. In contrast with mock-inoculated pTRV:0 plants, TMV- and elevated [CO₂]-induced activation of both NPR1 and PR1 was abolished in NPR1-silenced plants, whereas these genes were expressed at similar levels in PI-silenced plants (Fig. 7A). In contrast, the expression levels of PI I and PI II did not change in response to TMV or elevated [CO₂] in pTRV:0 plants, although the expression levels were significantly reduced by silencing PI. These transcripts were significantly induced in NPR1-silenced plants compared with pTRV:0-silenced plants, suggesting that NPR1 induction suppresses the expression of the JA-dependent genes PI I and PI II.

The changes in SA and JA signalling were further investigated upon challenge with P. syringae and B. cinerea 2 dpi in response to CO₂ elevation (Figs 8A and 9A). The transcript abundance differences of SA- and JA-dependent genes were similar between pTRV:0 and gene-silenced plants regardless of the pathogen type. In pTRV:NPR1-silenced plants, NPR1 and PR1 transcripts were suppressed, whereas PI I and PI II transcripts were induced compared with those pTRV:0 plants (Figs 8A and 9A). In pTRV:PI-silenced plants, the expression levels of PI I and PI II were significantly reduced, without evident effect on NPR1 and PR1 expression (Figs 8A and 9A). Furthermore, regardless of the pathogen invader or the gene silencing constructs, NPR1 and PR1 transcript levels were generally higher, whereas PI I and PI II transcript levels...
Silencing NPR1 or PI affected tomato–pathogen interactions under elevated [CO2] in different ways (Figs 7–9). pTRV:NPR1-silenced plants accumulated much more TMV-CP RNA than pTRV:0 and pTRV:PI plants, regardless of the [CO2] conditions. The elevated [CO2]-induced TMV resistance was completely abolished in pTRV:NPR1-silenced plants (Fig. 7B). These results suggest that elevated [CO2]-induced tomato resistance against TMV was associated with SA-dependent NPR1 and PR1. The changes in disease development in response to elevated [CO2] and gene silencing were similar between plants inoculated with P. syringae and TMV. The symptoms of P. syringae infection were much more severe in pTRV:NPR1-silenced plants than in pTRV:0 plants, which showed classic symptoms of susceptibility at 6 dpi, such as chlorosis, water-soaked lesions, and necrotic pits (Fig. 8B). The elevated [CO2]-induced resistance was also abolished in these plants. In contrast, the pTRV:PI-silenced plants did not exhibit altered resistance to P. syringae in either ambient or elevated [CO2] conditions. Furthermore, bacterial colony numbers were also used to determine the bacterial growth after different treatments, and the P. syringae growth results at 4 dpi were in good agreement with the disease symptom (Fig. 8C). Tomato plants were then challenged with B. cinerea. As shown in Fig. 9B and C, elevated [CO2]-treated plants were more susceptible to B. cinerea compared with those grown under ambient conditions. NPR1 silencing induced resistance to B. cinerea, as the cell death and B. cinerea actin gene expression were significantly reduced in plants grown under elevated [CO2], although these genes did not show significant quantitative differences compared with their expression levels in plants grown under ambient conditions. In contrast, silencing PI greatly increased tomato susceptibility to B. cinerea, especially under elevated [CO2]. To verify the involvement of SA and JA in the variation of disease susceptibility under elevated [CO2], tomato genotypes were used that are impaired in SA biosynthesis, spr2 mutants affected in JA biosynthesis, and their respective wild-type MM and CM. The disease expression of these plants challenged with TMV, P. syringae, or B. cinerea were monitored, and similar trends were found as the results obtained from VIGS NPR1 and PI plants (Supplementary Fig. S3).

**Discussion**

The assessment of plant disease under elevated [CO2] conditions is a key step in the development of plant–pathogen management, but experimental research results are often inconsistent (Chakraborty and Datta, 2003; Hoye et al., 2003; McElrone et al., 2005; Lake and Wade, 2009; Pangga et al., 2011; West et al., 2012; Juroszek and von Tiedemann, 2013). Here, reductions in plant disease caused by TMV and P. syringae but increases in the incidence and severity of disease caused by B. cinerea under elevated [CO2] in tomato plants were documented. Endogenous hormone biosynthesis, transcripts of genes involved signalling, and gene silencing/mutation experiments provided evidence that the variation in disease susceptibility is potentially related to flexibility in leaf

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**Fig. 4.** Expression levels of target genes in tomato plants grown under ambient (Amb, 380 μmol mol–1) or elevated [CO2] (Ele, 800 μmol mol–1) with or without inoculation with tobacco mosaic virus, Pseudomonas syringae, or Botrytis cinerea. Plants with TMV infections were sampled at 2 dpi. The results are expressed as the mean values±SD, n=4. Different letters indicate significant differences between the treatments with same pathogen (P<0.05).

**Fig. 5.** Endogenous phytohormone concentrations in tomato plants grown under ambient (Amb, 380 μmol mol–1) or elevated [CO2] (Ele, 800 μmol mol–1) condition with and without inoculation with tobacco mosaic virus, Pseudomonas syringae, or Botrytis cinerea. Plants with TMV infections were sampled 6 days post-inoculation (dpi), whereas plants with P. syringae or B. cinerea inoculation were sampled at 2 dpi. The results are expressed as the mean values±SD, n=4. Different letters indicate significant differences between the treatments with same pathogen (P<0.05).

were generally lower, in elevated [CO2]-treated plants compared with ambient-treated plants, even though no significant quantitative changes between treatments were observed in some cases (Figs 8A and 9A).
chemistry and cross talk between the SA and JA signalling pathways. Therefore, these results are critical for understanding the effects of elevated \([\text{CO}_2]\) on plant–pathogen microbe interactions and will help to ameliorate the negative effects and to use the benefits of elevated \([\text{CO}_2]\) in managed agricultural and natural ecosystems.

Fig. 6. Expression levels of target genes in gene-silenced tomato plants grown under elevated (Ele, 800 \(\mu\)mol mol\(^{-1}\)) or ambient \([\text{CO}_2]\) (Amb, 380 \(\mu\)mol mol\(^{-1}\)). Three weeks after seedling inoculation with pTRV:NPR1, pTRV:PI, or the empty vector pTRV:0, plants were subjected to elevated or ambient \([\text{CO}_2]\) for 5 d, and leaf samples were then collected for gene expression analysis. The results are expressed as the mean values\(\pm\)SD, \(n=4\). Different letters indicate significant differences between the treatments \((P<0.05)\).

Fig. 7. Effects of tobacco mosaic virus infection on gene-silenced tomato plants grown under elevated (Ele, 800 \(\mu\)mol mol\(^{-1}\)) or ambient \([\text{CO}_2]\) (Amb, 380 \(\mu\)mol mol\(^{-1}\)). Three weeks after seedling inoculation with pTRV:NPR1, pTRV:PI, or the empty vector pTRV:0, the plants were subjected to elevated or ambient \([\text{CO}_2]\) with or without TMV inoculation. (A) Expression levels of target genes at 6 days post-inoculation \((\text{dpi})\). The results are expressed as the mean values\(\pm\)SD, \(n=4\). Different letters indicate significant differences between the treatments \((P<0.05)\). (B) Semi-quantitative analysis of the gene encoding the TMV-coat protein (CP) in young, fully expanded leaves at 9 dpi.
The effects of elevated [CO₂] on plant–pathogen interactions are expected to occur both directly through plant physiological responses and indirectly through effects on microbes that associate with plants (Malmstrom and Field, 1997; Rua et al., 2013). The in vitro pathogen microbe growth studies clearly showed that the bacteria *P. syringae* and the necrotrophic fungus *B. cinerea* were not affected by elevated [CO₂] (Supplementary Fig. S2). Similarly, based on the studies of plant pathogens *Erwinia* spp. and *Pseudomonas fluorescens*, Wells (1974) observed no inhibitory effects on cell growth in liquid culture from 0.03–3% CO₂. Thus, the most pronounced effects of elevated [CO₂] are on host physiology. We thus speculated that changes in plant physiology, i.e. biochemical profiles of pathogen-infected plants under elevated [CO₂], may result in increased resistance or susceptibility to specific pathogens (Matros et al., 2006). In the current study, the involvement of the phytohormones SA and JA was examined under elevated [CO₂] with and without pathogen inoculation. Regardless of the pathogen type, elevated [CO₂] generally increased constitutive levels of SA and SA-related transcripts in both uninfected and infected plants, especially in the additive treatments of elevated [CO₂] and pathogen infection (Figs 4 and 5). In contrast to the universal increase in SA, JA concentrations and the transcripts of genes involved in JA signalling were not increased by elevated [CO₂] in uninfected plants. TMV and *P. syringae* infection had little or no effects on the JA contents or transcripts of genes involved in JA signalling, although these genes were induced by *B. cinerea*. Furthermore, the *B. cinerea*-induced increases in JA contents as well as *PI I* and *PI II* transcript levels were much lower in plants grown under elevated [CO₂] compared with those grown under ambient [CO₂] (Figs 4 and 5). These results suggest that
elevated \([\text{CO}_2]\) favours the SA pathway but represses the JA pathway in plants. Similar results have been observed in other studies using tomato and soybean plants (Zavala et al., 2008; Sun et al., 2011; Huang et al., 2012; Zavala et al., 2013).

Thus, whether the elevated \([\text{CO}_2]\)-induced variation in hormonal signalling was associated with the observed variation in the tomato defence against different pathogens under elevated \([\text{CO}_2]\) was investigated. Specifically, in this study, under elevated \([\text{CO}_2]\), disease caused by TMV and hemibiotrophic \(P. \text{syringae}\) decreased, whereas plant disease incidence and severity caused by necrotrophic \(B. \text{cinerea}\) significantly increased compared with ambient \([\text{CO}_2]\) (Figs 1–3). It has been accepted that SA signalling is generally important for defence against biotrophs or hemibiotrophs such as \(P. \text{syringae}\), whereas JA signalling generally is relevant for defence responses directed against necrotrhophs, although there are exceptions (Tsuda and Katagiri, 2010; Kliebenstein, 2014).

In this study, the basal defence against TMV and the bacterial pathogen \(P. \text{syringae}\) was reduced in \(NPR1\)-silenced and \(NahG\) plants, which have altered SA signalling and biosynthesis, but not in \(PI\)-silenced or \(spr2\) plants. The converse trend was observed in plants treated with the fungal necrotrophic pathogen \(B. \text{cinerea}\) (Figs 7–9; Supplementary Fig. S3). These results verify the prediction made by previous studies that the basal defence against TMV and \(P. \text{syringae}\) is SA-dependent whereas the defence against \(B. \text{cinerea}\) is controlled by JA signalling (Thomma et al., 1998; Kunkel and Brooks, 2002; Ton et al., 2002). Furthermore, silencing \(NPR1\) not only abolished elevated \([\text{CO}_2]\)-induced TMV and \(P. \text{syringae}\) resistance but also alleviated elevated \([\text{CO}_2]\)-induced \(B. \text{cinerea}\) susceptibility. In contrast, silencing \(PI\) further enhanced \(B. \text{cinerea}\) susceptibility under both ambient or elevated \([\text{CO}_2]\), but it had no evident effects on resistance to TMV or \(P. \text{syringae}\) (Figs 7–9). These observations were in accordance with the experiment using \(NahG\) and \(spr2\) plants (Supplementary Fig. S3). These results suggest that elevated \([\text{CO}_2]\)-induced tomato defence against TMV, \(P. \text{syringae}\), and \(B. \text{cinerea}\) was associated with SA/JA signalling cross talk. Elevated \([\text{CO}_2]\) favours SA signalling, leading to resistance to TMV and \(P. \text{syringae}\), while dampening the JA-related defence against \(B. \text{cinerea}\). Accordingly, previous studies indicated that the accumulation of SA is often negatively correlated with the accumulation of

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**Fig. 9.** Effects of \(Botrytis \text{cinerea}\) infection on gene-silenced tomato plants grown under elevated (Ele, 800 \(\mu\)mol mol\(^{-1}\)) or ambient \([\text{CO}_2]\) (Amb, 380 \(\mu\)mol mol\(^{-1}\)). Three weeks after seedling inoculation with pTRV:NPR1, pTRV:PI, or the empty vector pTRV:0, the plants were subjected to elevated or ambient \([\text{CO}_2]\), with or without in planta \(B. \text{cinerea}\) spray inoculation. (A) Expression levels of target genes at 2 days post-inoculation (dpi). (B) Trypan blue staining for cell death was performed at 3 dpi. (C) \(B. \text{cinerea}\) actin gene expression at 3 dpi. The results in A and C are expressed as the mean values±SD, \(n=4\) in A and \(n=5\) in C. Different letters indicate significant differences between the treatments \((P<0.05)\).
Elevated [CO2]-induced accumulation of SA is related to the and JA signalling pathways, it might be expected that the pathogens infection to some extent, as expression induction of PI I and PI II by NPR1 silencing were generally lower in elevated [CO2] compared with ambient [CO2], in normal control, P. syringae-, or B. cinerea-inoculated plants (Figs 6, 8, 9). However, no such difference was observed in the different [CO2] when plants are TMV infected.

Previous studies have attributed SA/JA cross talk to elevated [CO2]-induced plant pathogen defences by investigating interactions between pathogens with different infection strategies in the same system. Whether the impact of elevated [CO2] on SA/JA cross talk and the associated pathogen defences is a general response is an open question. Many previous studies have reported that under elevated [CO2] conditions, lower levels of disease are caused by biotrophic pathogens such as downy mildew caused by Peronospora manshurica on soybean (Eastburn et al., 2010) and virus disease caused by potato virus Y on tobacco (Matros et al., 2006). Conversely, some studies have reported that higher levels of disease under elevated [CO2] conditions are caused by necrotrophic pathogens such as brown spot caused by Septoria glycines (Eastburn et al., 2010) and powdery mildew caused by Podosphaera xanthii on zucchini (Pugliese et al., 2011). However, there are also examples of necrotrophic, biotrophic, and hemibiotrophic pathogens having reduced, increased, or no effects on disease upon increased [CO2] (Lake and Wade, 2009; Eastburn et al., 2011; Oehme et al., 2013). It should be noted that B. cinerea disease was still higher in pTRV:NPR1 and pTRV:PI-silenced plants under elevated [CO2] than under ambient conditions (Fig. 9); NahG and spr2 plants also showed similar trend (Supplementary Fig. S3). These might be explained by the other hormone player(s). ET has been shown to act synergistically with JA in the response to B. cinerea in Arabidopsis (Thomma et al., 1999). In tomato plants, JA-mediated responses seem to act independently from ethylene-induced resistance against B. cinerea, and plants pre-treated with ethylene showed a decreased susceptibility toward B. cinerea, whereas pre-treatment with 1-methylocyclopropene (an inhibitor of ethylene perception), resulted in increased susceptibility (Diaz et al., 2002). Furthermore, previous studies also indicated that elevated CO2 suppresses the ethylene signalling pathway in soybean and Medicago truncatula (Zavala et al., 2009; Guo et al., 2014). Thus, ET might be involved in the susceptibility to B. cinerea under elevated [CO2]. Additionally, in a previous study with Arabidopsis, elevated [CO2] attenuated the SA-dependent runaway cell death in lesion simulating disease 1 (lsd1) mutant, which has been implicated in defence following avirulent or virulent pathogen challenge (Mateo et al., 2004). Given the complexity of the interactions between plants, plant pathogens, and the environment, it is not surprising that the understanding of how elevated [CO2] influences plant pests and disease agents is still incomplete and requires further study.

In conclusion, these results support the hypothesis that the variation in plant disease susceptibility under elevated [CO2] is

![Phytohormone and disease variation in elevated CO2](image-url)
related to cross talk between the SA and JA signalling pathways (Fig. 10). Elevated [CO2] up-regulated SA synthesis and signalling, and increased PRI and NPR1 expression, but it did not up-regulate components of the JA pathway. Thus, the altered SA/JA cross talk favours SA pathway-dependent defence but represses JA pathway-dependent defence, leading to a reduction in plant disease susceptibility to TMV and P. syringae but an increase in B. cinerea incidence and severity under elevated [CO2] in tomato plants. This work highlights the modulated antagonistic relationship between SA and JA that contributes to the variation in disease susceptibility under elevated [CO2]. These findings lead to the prediction that plants will experience increased resistance to some pathogens and increased susceptibility to others in the future when CO2 concentrations increase. Furthermore, the variation in the response to elevated [CO2] in plants suggests the potential for phytohormone signalling and defences to serve as targets for breeding efforts and disease management strategies upon climate change in elevated-[CO2] agronomic ecosystems.

Supplementary data
Supplementary data are available at JXB online

Figure S1. Effects of exogenous salicylic acid (SA) and methyl jasmonate (MeJA) application on PRI gene expression.

Figure S2. In vitro pathogen growth in elevated or ambient [CO2] concentrations.

Figure S3. Effects of pathogens inoculation on disease expression of wild-type, SA-, and JA-deficient tomato plants under elevated or ambient [CO2].

Table S1. Primers used in this study.

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