Decreased Biosynthesis of Jasmonic Acid via Lipoxygenase Pathway Compromised Caffeine-Induced Resistance to *Colletotrichum gloeosporioides* Under Elevated CO₂ in Tea Seedlings

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ABSTRACT

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Caffeine, the major purine alkaloid in tea has long been known for its role in plant defense. However, its effect on *Colletotrichum gloeosporioides* that causes brown blight disease in tea is largely unknown especially under elevated CO₂. Here we show that elevated CO₂ reduced endogenous caffeine content in tea leaves, but sharply increased susceptibility of tea to *C. gloeosporioides*. The expression of *C. gloeosporioides actin* gene was gradually increased during the postinoculation period. In contrast, foliar application of caffeine decreased the *C. gloeosporioides*-induced necrotic lesions and the expression of *C. gloeosporioides actin*. Analysis

of endogenous jasmonic acid (JA) content revealed that exogenous caffeine could induce JA content under both CO₂ conditions in absence of fungal infection; however, in presence of fungal infection, caffeine increased JA content only under elevated CO₂. Furthermore, exogenous caffeine enhanced lipoxygenase (LOX) activity and its biosynthetic gene expression under both CO₂ conditions, indicating that increased JA biosynthesis via LOX pathway by caffeine might strengthen plant defense only under elevated CO₂, while caffeine-induced defense under ambient CO₂ might be associated with JA-independent LOX pathway in tea. These results provide novel insights into caffeine-induced plant defense mechanisms that might help to develop an eco-friendly approach for disease control.

Additional keywords: *Camellia sinensis*, climate change, foliar disease.

Tea is an important cash crop, tremendously popular as a nonalcoholic beverage around the world. Although green tea has been consumed as a pleasant drink since ancient times, elucidation of its health benefits has markedly increased overall consumption of tea in recent years (Tounekti et al. 2013). To meet the increasing demand, tea cultivation has been expanding in many countries of tropical and subtropical regions including China. Nonetheless, insect and disease incidence limits tea production to a great extent (Ashihara et al. 2008). In China, there are more than one hundred kinds of tea diseases that affect tea cultivation. Among major tea diseases, brown blight disease is considered as one of the serious foliar diseases, because of its potential to significantly reduce both tea yield and quality (Chen et al. 2015). Brown blight disease is caused by *Colletotrichum gloeosporioides* on tea during July to September in the Yellow Mountain region (latitude: 30°18' N, longitude: 118°17' E) in Anhui Province of China (Guo et al. 2014). Initially symptoms appear as small, water-soaked lesions on young leaves and twigs that are coalesced at latter stage to form larger dark brown necrotic lesion. In the Yellow Mountain region, about 30 to 60% of the tea fields are affected by brown blight disease every year (Guo et al. 2014). *C. gloeosporioides* is also responsible for causing typical anthracnose and die back disease of tea under favorable weather conditions in various geographical locations. However,

symptoms for each disease may vary remarkably. In tea oil camellia (*Camellia oleifera*), *C. gloeosporioides* causes anthracnose that leads to severe fruit and bud drop (Zhang et al. 2012). Thus, beyond doubt, *C. gloeosporioides* has emerged as a destructive fungal pathogen that causes significant yield losses in tea. Although chemical pesticides are still effective for disease control, the use of large amounts of chemical fungicides threatens human health due to its residual toxicity (Saha et al. 2012). Moreover, development of fungicide resistance is a rising concern in the tea industry that hampers productivity of tea plantations (Kretschmer and Hahn 2008; Saha et al. 2012). In these circumstances, to ensure fungicide-free tea, tea research has been focused on identification and development of plant products that are capable of inhibiting the growth of fungal phytopathogens.

Caffeine is an important purine alkaloid occurring in various plant species including members of *Camellia* and *Coffea*. It is synthesized in planta from purine nucleotides as a secondary metabolite. The concentration of caffeine in tea and coffee plants is high in young leaves and flowers compared with other plant parts (Ashihara et al. 2008; Filho and Mazzafera 2000). Transcript analysis of *TEA CAFFEINE SYNTHASE 1 (TCSI)* that encodes caffeine synthase was found to be threefold higher in young tea leaves compared with mature leaves, implying that increased caffeine biosynthesis in young leaves is dependent on enhanced expression of the *TCSI* gene (Li et al. 2008). Previous studies have indicated a potential involvement of caffeine in chemical defense and allelopathy (Ashihara et al. 2008). Accumulation of alkaloids such as caffeine is boosted by insect and pathogen attack which plays a protective biological role in plant defense against pathogens and insect herbivory (Filho and Mazzafera 2000). *Coffea arabica* genotypes with higher caffeine content showed enhanced resistance to *Colletotrichum coffeanum* that causes coffee berry disease (Biratu et al. 1996). Furthermore,

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transgenic tobacco plants that produce high level of caffeine (5 µg per gram fresh weight of leaves) showed increased resistance to a range of pests such as tobacco cutworms, *Tobacco mosaic virus*, and *Pseudomonas syringae*. Enhanced disease resistance of these transgenic tobacco plants was associated with higher expression of *PATHOGENESIS-RELATED PROTEIN 1a* (*PR-1a*) and *PR-2* genes (Kim and Sano 2008). In addition, exogenous application of caffeine onto leaves of wild-type tobacco plant also increased disease resistance against those virus and bacteria. However, the role of caffeine in plant defense against fungal pathogens particularly against *C. gloeosporioides*, in tea is largely unknown. It is to be noted that caffeine biosynthesis showed dramatic seasonal variation in field condition which is limited to young tea leaves of new shoots during the spring (Ashihara et al. 2008). Therefore, exogenous application of caffeine might supplement caffeine content to overcome seasonal limitation and thereby fortifying plant defense against various pests.

Rising atmospheric CO₂ is a major concern for crop production (including tea) in the face of climate change (Ahmed et al. 2014). Studies have shown that increasing CO₂ concentrations could boost crop yield; however, it might differentially modulate host–pathogen interaction and related disease epidemics. In general, severity of diseases caused by necrotrophic pathogens is triggered by elevated CO₂, while plant defenses against biotrophic pathogens are fortified under elevated CO₂ (Zhang et al. 2015). Nonetheless, research relating to effect of elevated CO₂ on plant disease abundance often produced conflicting results that limit generalization of climate change effect on plant–pathogen interaction (Yáñez-López et al. 2012). In *Stylosanthes scabra*, aggressiveness of *C. gloeosporioides* that causes anthracnose disease was dramatically increased under elevated CO₂ (Chakraborty and Datta 2003). A study conducted on tomato plants showed that elevated CO₂ generally stimulated the salicylic acid (SA) signaling pathway, but repressed the jasmonic acid (JA) pathway, which eventually increased plant susceptibility to necrotrophic *Botrytis cinerea* (Zhang et al. 2015).

JA, a crucial signaling molecule in plant defense, is produced through lipoxygenase (LOX) pathway (Blée 2002). It is well recognized that LOX pathway plays an essential role in plant defense. A LOX gene namely *CsLOX1* has been identified in tea plants which plays a critical role in the JA-related defense response to phloem feeders such as *Empoasca vitis* (Liu and Han 2010). In general, LOX activity is dramatically increased following wounding and pathogen infection. Although LOX-derived fatty acid hydroperoxides serve as precursor for JA synthesis, exogenous application of methyl jasmonate (MeJA) could also induce *LOX1* gene expression and activity of LOX, implying that jasmonate itself might be a positive regulator of JA synthesis (Melan et al. 1993). Activation of JA signaling is essential for plant resistance against necrotrophic pathogens (Zhang et al. 2015). However, studies have revealed that LOX pathway could produce other important phyto-oxylipins through JA-independent pathways that trigger plant defense and stress tolerance (Blée 2002). Oxylipins comprise a large family of lipid-derived metabolites that are mainly produced enzymatically through LOX pathways. Pretreatment with 9-LOX and α-dioxygenase (α-DOX)-derived oxylipins protect *Arabidopsis* plants against *P. syringae* pv. *tomato* DC3000-induced infection (Vicente et al. 2012). An earlier study has shown that elevated CO₂ reduces tomato plant resistance to cotton bollworm (*Helicoverpa armigera*) by suppressing JA level as well as LOX activity (Guo et al. 2012). However, it is still unclear how caffeine and/or elevated CO₂ influence LOX upon *C. gloeosporioides* infection in tea plants. Understanding plant defense mechanisms against *C. gloeosporioides* under elevated CO₂ is essential to develop efficient disease control strategies in tea in the face of climate change. Considering the chemical defense property of caffeine in plants, the present study was conducted to elucidate the role of caffeine in tea plant defense against *C. gloeosporioides* both under ambient and elevated CO₂ conditions. This study demonstrated that caffeine fortified host defense systems against *C. gloeosporioides* through increasing JA biosynthesis via

LOX pathway under elevated CO₂. Thus, application of caffeine could be potentially useful for the control of *C. gloeosporioides* in tea.

MATERIALS AND METHODS

Plant material and growth conditions. Two-year-old tea seedlings of Longjing 43 (*Camellia sissensis* L.) were exposed to either ambient CO₂ (380 µmol mol⁻¹) or elevated CO₂ (800 µmol mol⁻¹) for 2 weeks in controlled-environment growth chambers (Convion, Winnipeg, Canada). The growth conditions were as follows: the photosynthetic photo flux density (PPFD) 600 µmol m⁻² s⁻¹, photoperiod 14/10 h (day/night), day/night air temperature 26/22°C, and relative humidity 85%. After 2 weeks of acclimation, plants exposed to both ambient and elevated CO₂ were subjected to inoculation with *C. gloeosporioides*. Plants were also mock-inoculated following the same inoculation procedure. The pot placement within each CO₂ condition was randomized every 2 days. In the experiments employing a combined treatment of *C. gloeosporioides* and exogenous treatment of caffeine (0.1, 1, and 10 mM) or MeJA (0.02, 0.2, and 1 mM), caffeine or MeJA solution was applied to the tea seedlings 6 h prior to *C. gloeosporioides* inoculation; distilled water (ddH₂O) was sprayed as control. Based on the preliminary experiments and previous literature, concentrations of 1 mM caffeine and 200 µM MeJA were selected for the rest of the experiments (Kim and Sano 2008). It is to be noted that such concentrations of caffeine and MeJA did not inhibit fungal growth in vitro (Supplementary Fig. S1). All plants were watered duly and fertilized with Hoagland's solution every 3 days. The experiments were replicated independently three times.

Culture of fungi and inoculation. The *C. gloeosporioides* was initially collected from diseased tea leaves showing typical disease symptom from tea garden of Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China. Small pieces of those tea leaves were surface-sterilized in 2% NaClO for 3 min followed by rinsing twice in distilled water and plated on potato dextrose agar. The culture was incubated at 28°C for 5 days. In addition to microscopic observation of fungal structure, the identity of the fungus was confirmed as *C. gloeosporioides* by DNA sequence analysis of the internal transcribed spacer (ITS4 and ITS5), which was 100% identical to other *C. gloeosporioides* isolates (GenBank Accession KC122769) (Guo et al. 2014). The fungus was subcultured using the method described by El Oirdi et al. (2010). To study the infection behavior of cultured *C. gloeosporioides*, all the leaves of tea plants were inoculated by spraying with a *C. gloeosporioides* spore suspension at a density of 2 × 10⁵ spores per ml.

Determination of caffeine content. Tea plants grown under ambient and elevated CO₂ were used for the quantification of caffeine content. Tea leaves samples were dried at 80°C for 24 h. Caffeine was extracted and purified from dried tea leaves. The content of caffeine was quantified using an HPLC-based method as described previously (Kim and Sano 2008).

Disease symptom assay. Disease symptoms were assayed by trypan blue staining of tea leaves as described by Bai et al. (2012). Tea leaves were boiled for 10 min in the lactophenol-trypan blue solution (10 ml of lactic acid, 10 ml of glycerol, 9.3 g of phenol, 10 mg of trypan blue, dissolved in 10 ml of distilled water) and then decolorized in chloral hydrate (25 g of chloral hydrate dissolved in 10 ml of distilled water) for 12 h. The dark-blue spots were photographed to evaluate cell death.

Quantification of JA content. Frozen leaf tissue (about 100 mg) was homogenized in 1 ml of ethyl acetate spiked with labeled internal standards. Homogenates were centrifuged at 13,000 × g for 20 min at 4°C and resulting supernatants were transferred to fresh 2-ml tubes. Then liquid portion was evaporated using a vacuum concentrator (Eppendorf). The residues were resuspended in 0.5 ml of 70% methanol (vol/vol) and centrifuged for 10 min at 4°C (13,000 × g). After pipetting the supernatants to

glass vials and JA content was quantified by HPLC-MS/MS following the method described by Wu et al. (2007).

Assay of lipoxygenase (LOX) activity. For lipoxygenase (LOX, EC 1.13.11.1) activity assay, tea leaf samples were collected at 4 dpi. Extraction of LOX enzymes and subsequent quantification of its activity were performed following the description of Nascimento et al. (2014). Briefly, 0.3 g of leaf tissue was ground into a fine powder with liquid nitrogen. Resulting fine powder was homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 6.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2% (wt/vol) polyvinylpyrrolidone (PVP) in an ice bath. After centrifugation at

$12,000 \times g$ for 15 min at 4°C , the resulting supernatant was used to determine the LOX activity. The reaction mixture containing the enzyme extract, 50 mM sodium phosphate buffer (pH 6.5) and 50 μM sodium linoleate was incubated at 25°C . The absorbance of the product released by LOX for 1 min was measured at 234 nm. LOX activity was calculated by using the extinction coefficient of $25,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Axelrod et al. 1981).

RNA isolation and transcript analysis. Total RNA from tea leaves was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. A purifying column was used to remove genomic DNA in RNA samples. Reverse transcription was done using Superscript II (Invitrogen)

Fig. 1. Effects of elevated CO_2 ($800 \mu\text{mol mol}^{-1}$) on endogenous caffeine and jasmonic acid (JA) content, and *Colletotrichum gloeosporioides* virulence on tea seedlings. Tea seedlings were acclimated at either ambient ($380 \mu\text{mol mol}^{-1}$) or elevated ($800 \mu\text{mol mol}^{-1}$) CO_2 for 2 weeks. **A**, Endogenous caffeine content in tea leaves. **B**, Endogenous JA content in tea leaves. **C**, Trypan blue staining showing cell death on tea leaves at 4 days postinoculation (dpi). Leaves of respective tea plants were inoculated by spraying with a *C. gloeosporioides* spore suspension at a density of 2×10^5 spores per ml. **D**, *C. gloeosporioides actin* gene expression at different postinoculation periods. Relative comparison was made considering the expression of *C. gloeosporioides actin* in 0 dpi-ambient as 1. Means denoted by different letters indicate significant differences between the treatments ($P < 0.05$).

following the manufacturer's protocol. qRT-PCR analysis was carried out using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems). Transcript abundance was normalized to *actin*, and relative gene expression was calculated following formulae of Livak and Schmittgen (2001). For analysis of *C. gloeosporioides actin* gene transcription, 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. The primers for *C. gloeosporioides actin* gene transcription assay have been shown in Supplementary Table S1.

Statistical analysis. At least four independent replicates were conducted for each determination. The data were subjected to analysis of variance using SAS 8.0 software package (SAS Institute, Cary, NC), and the means were compared using Tukey's test at the $P < 0.05$ level.

RESULTS

Elevated CO₂ favors *C. gloeosporioides* virulence on tea by repressing caffeine and JA accumulation. In order to understand the biological significance of caffeine in plant defense under altered CO₂ conditions, we first determined the endogenous content of caffeine both under ambient and elevated CO₂ conditions. Preliminary dose trial of CO₂ showed that although caffeine content decreased with increasing CO₂ concentration, the

reduction was only significant ($P < 0.05$) when it reached 800 $\mu\text{mol mol}^{-1}$ CO₂ (Supplementary Fig. S2). For instance, caffeine content was significantly decreased by 23.19% in tea leaves under elevated CO₂ (800 $\mu\text{mol mol}^{-1}$ CO₂) compared with that under ambient CO₂ (Fig. 1A).

In accordance with caffeine content, JA content was sharply decreased by elevated CO₂ (Fig. 1B). To assess the effect of elevated CO₂ on susceptibility of tea to *C. gloeosporioides*, we examined cell death by trypan blue staining in inoculated tea leaves (Fig. 1C). In mock-inoculated tea leaves, no sign of dead cells was noticed at 4 dpi. In contrast, occurrence of cell death was prevalent following *C. gloeosporioides* inoculation at 4 dpi, which was more obvious in tea plants grown under elevated CO₂, indicating that elevated CO₂ conditions might make tea plants susceptible to *C. gloeosporioides*. We also analyzed the expression of *C. gloeosporioides actin* gene at different postinoculation period. As shown in Figure 1D, *C. gloeosporioides actin* expression was gradually increased following fungal inoculation both under ambient and elevated CO₂ conditions. These results indicate a potential involvement of endogenous caffeine in chemical defense against *C. gloeosporioides* in tea plants.

Exogenous caffeine reduces susceptibility of tea plants to *C. gloeosporioides*. To investigate the potential role of exogenous caffeine in *C. gloeosporioides* infection, we first performed dose trials of caffeine and JA on the growth of the *C. gloeosporioides* under ambient CO₂ condition and analyzed

Fig. 2. Effect of different concentrations of caffeine and methyl jasmonate (MeJA) on endogenous contents of caffeine and jasmonic acid (JA) as influenced by *Colletotrichum gloeosporioides* under ambient (380 $\mu\text{mol mol}^{-1}$) CO₂. **A**, Trypan blue staining showing cell death on tea leaves at 4 days postinoculation (dpi) following pretreatment with different levels of caffeine. **B**, Trypan blue staining showing cell death on tea leaves at 4 dpi following pretreatment with different levels of MeJA. **C**, Changes in endogenous concentration of JA by different levels of exogenous caffeine treatment. **D**, Changes in endogenous concentration of caffeine as influenced by different levels of MeJA treatment. Means denoted by different letters indicate significant differences between the treatments ($P < 0.05$).

disease symptoms by trypan blue staining for cell death. Results showed that 0.1 mM caffeine was incapable of suppressing *C. gloeosporioides*-induced disease symptoms, while both 1 and 10 mM caffeine could strongly reduce *C. gloeosporioides*-induced cell death in tea leaves (Fig. 2A). Similarly, a dose trial of JA showed that MeJA at a concentration of 200 μ M or above could suppress disease symptom (Fig. 2B). When we analyzed endogenous JA content upon caffeine treatment, we found that 1 mM and 10 mM caffeine significantly ($P < 0.05$) increased endogenous concentration of JA (Fig. 2C). Based on these observations, we chose 1 mM caffeine for rest of the experiments. Although MeJA at a concentration of 200 μ M or above could suppress disease symptom, it did not alter endogenous concentration of caffeine, indicating that JA perhaps functions downstream of caffeine and there is less probability of feed-back regulation of caffeine biosynthesis by exogenous JA (Fig. 2D).

Next, we treated tea leaves with 1 mM caffeine and 200 μ M MeJA at 6 h prior to *C. gloeosporioides* inoculation and analyzed the dead cells at 4 dpi in tea leaves both under ambient and elevated CO_2 . Treatment with caffeine noticeably reduced occurrence of *C. gloeosporioides*-induced cell death under both CO_2 conditions (Fig. 3A). To confirm possibility of direct effect of elevated CO_2 on *C. gloeosporioides* growth, we also cultured the same fungus on petri dishes under both CO_2 conditions. Observation up to 8 dpi revealed that elevated CO_2 did not have any direct effect on *C. gloeosporioides* growth. Similarly, we examined the effect of exogenous caffeine and MeJA on *C. gloeosporioides* growth. We found that 0.1 and 1 mM concentrations of caffeine did not inhibit fungal growth in vitro, implying that 1 mM caffeine reduced *C. gloeosporioides* growth through stimulating the tea plant defense pathway. However, exogenous MeJA at any concentration did not show any growth inhibitory effect on *C. gloeosporioides* in vitro. Caffeine-induced reduction in cell death in tea leaves was consistent with the down-regulation of *C. gloeosporioides actin* gene under

Fig. 3. Effect of caffeine and methyl jasmonate (MeJA) application on susceptibility of tea plants to *Colletotrichum gloeosporioides* under ambient (380 $\mu\text{mol mol}^{-1}$) or elevated (800 $\mu\text{mol mol}^{-1}$) CO_2 conditions. **A**, Trypan blue staining showing cell death on tea leaves at 4 days postinoculation (dpi). **B**, *C. gloeosporioides actin* gene expression at 4 dpi. Relative comparison was made considering the expression of *C. gloeosporioides actin* in caffeine-ambient treatment as 1. Means denoted by different letters indicate significant differences between the treatments ($P < 0.05$). Concentrations of caffeine and MeJA used for exogenous application were 1 mM and 200 μ M, respectively.

aforesaid conditions (Fig. 3B). More precisely, caffeine down-regulated expression of *C. gloeosporioides actin* by 92.71 and 72.20% in inoculated leaves under ambient and elevated CO_2 grown tea plants, respectively. These data further confirm the requirement of optimal level of caffeine in tea leaves for defense against *C. gloeosporioides* under elevated CO_2 .

JA is involved in caffeine-induced resistance against *C. gloeosporioides* in tea. To elucidate the potential mechanisms involved in caffeine-induced plant defense, we quantified the endogenous content of JA under different experimental treatments. As shown in Figure 4A, both caffeine and/or *C. gloeosporioides* infection significantly increased JA content under ambient CO_2 and elevated CO_2 conditions when compared with their respective Mock-ddH₂O (control) treatment. Compared with mock-control treatment, JA content was increased by 42.30, 46.15, and 48.08% in caffeine-, *C. gloeosporioides*-, and caffeine plus *C. gloeosporioides*-treated tea leaves under ambient CO_2 , respectively. Similarly, under elevated CO_2 , JA content was increased by 61.54, 52.31, and 113.84% following caffeine, *C. gloeosporioides*, and caffeine plus *C. gloeosporioides* treatments, respectively, compared with their respective mock-control treatment. In parallel contrast, JA content was decreased under elevated CO_2 upon ddH₂O, caffeine, and *C. gloeosporioides* only treatments compared with their respective ambient counter parts. This implies that reduction in JA content by elevated CO_2 might increase susceptibility of tea plants to *C. gloeosporioides*. Strikingly, we noticed that caffeine pretreatment increased JA content only in presence of *C. gloeosporioides* infection under elevated

Fig. 4. Endogenous jasmonic acid (JA) and caffeine content in tea plants grown under ambient (380 $\mu\text{mol mol}^{-1}$) or elevated (800 $\mu\text{mol mol}^{-1}$) CO_2 conditions with or without inoculation with *Colletotrichum gloeosporioides*. **A**, JA content as influenced by caffeine under above-mentioned conditions. **B**, Caffeine content as influenced by MeJA under aforementioned conditions. Leaf tissues were sampled at 4 days postinoculation (dpi) for JA and caffeine content analysis. The results are expressed as the mean values $\pm$ SD, $n = 4$. Means denoted by different letters indicate significant differences between the treatments ($P < 0.05$).

CO₂. This observation indicates a unique interaction between caffeine and *C. gloeosporioides* that only occurs under elevated CO₂, leading to a higher JA content for enhanced resistance to *C. gloeosporioides*. We also analyzed endogenous caffeine content in tea leaves following MeJA pretreatment with or without *C. gloeosporioides* inoculation under ambient and elevated CO₂. Results showed that although elevated CO₂ significantly decreased endogenous caffeine content in presence of *C. gloeosporioides* inoculation, exogenous MeJA could not reverse the effect of elevated CO₂ on caffeine content in tea plants (Fig. 4B). This observation implies that JA perhaps functions downstream of caffeine in tea plants defense against *C. gloeosporioides* and JA is not a positive regulator of caffeine synthesis in presence of *C. gloeosporioides* under elevated CO₂. These results further confirm that caffeine-induced accumulation of JA under elevated CO₂ could activate plant defense pathway that eventually reduced susceptibility of tea plants to *C. gloeosporioides*.

Caffeine triggers LOX pathway to enhance plant defense.

To understand the mechanism controlling caffeine-induced JA accumulation upon *C. gloeosporioides* infection, we analyzed LOX activity and its biosynthetic gene expression in tea leaves. Results showed that LOX activity and *CsLOX1* expression were decreased under elevated CO₂ conditions (Fig. 5). However, exogenous caffeine and/or *C. gloeosporioides* infection significantly increased LOX activity both under ambient and elevated CO₂. Compared with mock-control, caffeine, *C. gloeosporioides*, and caffeine plus *C. gloeosporioides* treatments increased LOX activity by 138.08, 68.20, and 158.57% under ambient CO₂, respectively, while under elevated CO₂, it was increased by 178.34, 77.07, and 169.42%, respectively. Likewise, exogenous MeJA and/or *C. gloeosporioides* infection significantly increased LOX activity both under ambient and elevated CO₂ (Fig. 5A). As caffeine also increased LOX activity and *CsLOX1* expression under ambient CO₂ without altering endogenous JA content in presence of *C. gloeosporioides* infection, a JA-independent defense pathway might function in caffeine-induced defense against *C. gloeosporioides* under ambient CO₂ in tea plants.

DISCUSSION

Rising atmospheric CO₂ not only improves plant growth, but also modifies behavior of plant pathogens that eventually affect crop productivity (Ahmed et al. 2014; Runion et al. 2014; Zhang et al. 2015). Therefore, it is important to assess the virulence of major fungal pathogens of tea under elevated CO₂ in order to understand the mechanisms of plant defense in the face of climate change. In the current study, we found that elevated CO₂ increased susceptibility of tea plants to *C. gloeosporioides* which was closely associated with the reduction of endogenous concentration of

caffeine and JA in tea leaves (Figs. 1 and 4). Exogenous application of caffeine significantly decreased susceptibility of tea plant to *C. gloeosporioides* which was attributed to a significant ($P < 0.05$) increase in JA content under elevated CO₂ (Fig. 3 and 4). Further investigation showed that caffeine application increased LOX activity both under ambient and elevated CO₂ conditions in presence or absence of *C. gloeosporioides* inoculation. LOX activity is generally increased under pathogen attack as a response of amplified defense (Nascimento et al. 2014). Thus, these results indicate that under elevated CO₂ condition, caffeine induces disease resistance through increasing JA biosynthesis via 13-LOX pathway, while caffeine-promoted defense against *C. gloeosporioides* under ambient CO₂ might involve other phyto-oxylipins through 9-LOX pathway (Blée 2002).

The alteration in plant–pathogen interaction under elevated CO₂ is principally dependent on host plant's physiology (Matros et al. 2006; Zhang et al. 2015). In the current study, elevated CO₂ altered the physiology of the tea plant which was evidenced by a significant reduction in endogenous caffeine and JA content. Given that caffeine functions as a component of chemical defense in tea plants, its content is generally increased upon pest attacks (Ashihara et al. 2008). In this study, virulence of *C. gloeosporioides* on tea plants was promoted under elevated CO₂, implying that elevated CO₂-altered plant physiology might favor growth of *C. gloeosporioides* on tea leaves. It has been reported that virulence of necrotrophic fungus *Botrytis cinerea* on tomato is increased under elevated CO₂ which strongly supports our current observation. As transgenic tobacco plant with high caffeine content showed increased resistance to insects and pathogens (Kim and Sano 2008), it seems likely that reduction in caffeine content by elevated CO₂ might increase susceptibility of tea plants to *C. gloeosporioides* (Fig. 1).

Green tea is rich in caffeine which accounts for 2 to 5% dry mass of fresh leaves (Ashihara et al. 2008). Although high caffeine content is beneficial for plant defense, high level of caffeine in green tea is harmful for human health as it causes sleep deprivation, tachycardia, abortion and miscarriages (Ashihara et al. 2008; Bermejo et al. 2015). Therefore, decaffeination of green tea has become an important research focus that enabled researchers to develop efficient caffeine extraction procedure from tea leaves (Bermejo et al. 2015). Besides large scale utilization of caffeine in pharmaceutical industry, application of caffeine as pesticide might have great prospect. Previous studies have shown that foliar application of caffeine (1% solution) deters feeding by tobacco hornworms in tomato (Ashihara et al. 2008). Spraying cabbage leaves and orchids with 0.01 to 0.1% caffeine solutions kills or repels slugs and snails (Hollingsworth et al. 2002). Again, pre-treatment with 1 mM caffeine significantly increased resistance of tobacco plant against *P. syringae* pv. *glycinea* (Kim and Sano 2008). All these previous studies are in good agreement with our

Fig. 5. Interactive effects of caffeine, jasmonic acid (JA), fungal infection and elevated CO₂ on lipoxygenase (LOX) pathway in tea plants. **A**, Activity of LOX and **B**, expression of *CsLOX1* gene in tea leaves at 4 days postinoculation (dpi). The expression of gene was analyzed by qRT-PCR using gene-specific primer pairs. Relative comparison was made considering the expression of *CsLOX1* in mock-ambient treatment as 1. Means denoted by different letters indicate significant differences between the treatments ($P < 0.05$).

current observation that application of caffeine significantly decreased *C. gloeosporioides* infection on tea leaves (Fig. 3).

Previous reports also suggest that caffeine stimulates plant defense mechanisms by activating the expression of defense-related genes that are typically induced by JA and SA (Ashihara et al. 2008). Nonetheless, it is still unclear how elevated CO₂ alters hormonal response of the plants to biotic stress (Zhang et al. 2015). Apart from the direct effect of elevated CO₂ on plant growth, resources are reallocated for the synthesis of secondary metabolites under elevated CO₂ that might contribute to the synthesis of SA and JA (Matros et al. 2006). It is to be noted that caffeine is a typical secondary metabolite abundant in tea leaves. Thus, above interpretation indicates potential relationship between caffeine and signaling molecules such as SA and JA that play vital role in plant defense. As JA pathway is repressed following necrotrophic fungal infection under elevated CO₂ (Zhang et al. 2015), we quantified the JA content under various experimental treatments in tea leaves (Fig. 4). Consistent with the reduction in disease symptoms, caffeine pretreatment significantly increased JA content under elevated CO₂. This result provides strong evidence that caffeine stimulates endogenous defense systems against *C. gloeosporioides* by increasing biosynthesis of JA in tea plant. Further investigation of LOX activity unveiled the mechanism controlling caffeine-induced JA accumulation under elevated CO₂ (Fig. 4 and 5). A close association between increased LOX activity and an effective defense response has been reported in various plant-pathogen systems. In Arabidopsis, rapid induction of *LOX1* gene and activity of LOX mediated a hypersensitive response for disease resistance (Melan et al. 1993). JA that acts as a signal molecule in plants, is synthesized through specific branch of the LOX pathway, the so called 13-LOX pathway (Blée 2002). Transgenic tomato plants with suppressed expression of the chloroplast targeted *Tomato lipoxygenase D (TomloxD)* genes showed diminished LOX activity and reduced content of endogenous JA in leaves (Hu et al. 2015). These plants also exhibited reduced expression of the defense genes such as *LeHSP90*, *LePRI*, and *LePR6* that eventually increased susceptibility to microbial pathogen *Cladosporium fulvum*. Thus, an increased JA accumulation via LOX pathway by caffeine treatment would preferentially activate plant defense system against *C. gloeosporioides* through regulation of various JA-responsive defense genes under elevated CO₂ (Zhang et al. 2015).

Another issue that remains unclear in the present study is how caffeine increased disease resistance under ambient CO₂ without altering endogenous JA level in tea leaves. This could be partially explained by the increased LOX activity in tea leaves upon exogenous caffeine and MeJA treatment (Fig. 5). LOX is considered essential for the resistance of tobacco plants to fungal infection (Rancé et al. 1998). It is to be noted that various oxylipins other than jasmonate also play essential role in plant resistance to pathogens. Moreover, precursors of JA and derivatives of JA also serve as effective regulators of defense responses. Phyto-oxylipins such as divinyl ethers or hydroxylated fatty acids are produced through the 9-LOX pathway. These oxylipins are fungitoxic and profusely accumulate in infested plants (Blée 2002; Göbel et al. 2001). In our study, expression of *CsLOX1* gene was significantly upregulated by caffeine treatment upon *C. gloeosporioides* inoculation both under ambient and elevated CO₂ conditions (Fig. 5). LOX might generate reactive molecules at the infection site, leading to hypersensitive cell death for plant resistance (Blée 2002).

In conclusion, the present study further confirms the biological role of caffeine in plant defense against *C. gloeosporioides*. It also unraveled important mechanisms involved in caffeine-promoted plant defense under elevated CO₂. Results of the current study showed that elevated CO₂ decreased caffeine and JA contents in tea leaves but increased susceptibility of tea to *C. gloeosporioides*. However, caffeine stimulated a specific branch of the LOX pathway that synthesizes JA to induce tea defense against *C. gloeosporioides* under elevated CO₂. These findings lead to an assumption that climate change will likely trigger diseases caused by *C. gloeosporioides*, and

that application of caffeine could be a potential solution for efficient disease control. As fungicide resistance and its residual toxicity are growing concerns in the tea industry, caffeine could serve as a probable alternative to synthetic fungicides for controlling foliar disease in tea plantations.

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