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Original Article

Elevated CO_2 alters the feeding behaviour of the pea aphid by modifying the physical and chemical resistance of *Medicago truncatula*

Huijuan Guo[1,2], Yucheng Sun[1], Yuefei Li[1], Xianghui Liu[1], Pingyan Wang[1], Keyan Zhu-Salzman[3] & Feng Ge[1]

[1]*State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China,* [2]*Graduate University of Chinese Academy of Sciences, Beijing 100049, China and* [3]*Department of Entomology, Texas A&M University, College Station, TX 77843, USA*

ABSTRACT

Elevated CO_2 compromises the resistance of leguminous plants against chewing insects, but little is known about whether elevated CO_2 modifies the resistance against phloem-sucking insects or whether it has contrasting effects on the resistance of legumes that differ in biological nitrogen fixation. We tested the hypothesis that the physical and chemical resistance against aphids would be increased in Jemalong (a wild type of *Medicago truncatula*) but would be decreased in *dnf1* (a mutant without biological nitrogen fixation) by elevated CO_2. The non-glandular and glandular trichome density of Jemalong plants increased under elevated CO_2, resulting in prolonged aphid probing. In contrast, *dnf1* plants tended to decrease foliar trichome density under elevated CO_2, resulting in less surface and epidermal resistance to aphids. Elevated CO_2 enhanced the ineffective salicylic acid-dependent defence pathway but decreased the effective jasmonic acid/ethylene-dependent defence pathway in aphid-infested Jemalong plants. Therefore, aphid probing time decreased and the duration of phloem sap ingestion increased on Jemalong under elevated CO_2, which, in turn, increased aphid growth rate. Overall, our results suggest that elevated CO_2 decreases the chemical resistance of wild-type *M. truncatula* against aphids, and that the host's biological nitrogen fixation ability is central to this effect.

Key-words: *Acyrthosiphon pisum*; legume plant; nitrogen fixation; resistance; trichome.

INTRODUCTION

Global atmospheric CO_2 concentrations have been increasing at an accelerating rate and are expected to reach 550–950 ppm by the end of this century (IPCC 2007). The typical effects of elevated CO_2 on plants include increases in the photosynthetic rate, biomass and carbon : nitrogen (C : N) ratio (Ainsworth *et al*. 2007). The assimilation and re-assignment of C and N resources within plant tissues under elevated CO_2 inevitably alters the primary and secondary metabolites of host plants, which, in turn, affects the performance of herbivorous insects (Couture *et al*. 2010).

The effects of elevated CO_2 on the fitness of herbivorous insects have been reviewed (Zavala *et al*. 2013). An emerging conclusion from the literature is that the decreased N concentration caused by elevated CO_2 prolongs developmental time and reduces fecundity and fitness of most leaf-chewing insects (Coll & Hughes 2008). For piercing-sucking insects such as aphids, however, recent reviews conclude that the effects are species-specific (Hughes & Bazzaz 2001; Himanen *et al*. 2008). Importantly, some aphid species exhibit increased fecundity, abundance and survival under elevated CO_2 (Pritchard *et al*. 2007; Robinson *et al*. 2012). A limited but growing number of studies have attempted to illuminate the underlying physiological mechanisms that explain the positive response of these aphid species to elevated CO_2 (Newman *et al*. 2003).

Aphid response to elevated CO_2 is largely determined by the bottom-up effect of host plant including nutritional quality and resistance (Guo *et al*. 2014). With respect to nutritional status, the response of the pea aphid to elevated CO_2 differed when fed on two *Medicago truncatula* genotypes, the N-fixing-deficient mutant *dnf1* and its wild-type control Jemalong. The increased biological N fixation in Jemalong under elevated CO_2 increases available N and thereby increases aphid abundance (Guo *et al*. 2013b). In contrast, the *dnf1* mutant, in which biological N fixation is artificially mutated, fails to meet the increased demand for N under elevated CO_2, leading to decreases in aphid abundance (Guo *et al*. 2013b).

Before passively feeding on phloem sap (and obtaining or not obtaining sufficient N), aphids must overcome considerable plant defences (Smith & Boyko 2007). These defences, which have developed as a consequence of co-evolution between plant and herbivore, include both physical and chemical components (Smith & Clement 2012). After arriving on a plant, the aphid must first struggle against physical defences, including trichomes and waxes (Wang *et al*. 2004), before it can insert its long, flexible stylet into the leaf; the stylet forms the channel that permits the delivery of saliva and the eventual removal of phloem sap (Will *et al*. 2007). Probing by the aphid stylet triggers the formation of reactive

Correspondence: Y. Sun. Fax: +86 10 6480 7099; e-mail: sunyc@ioz.ac.cn; and F. Ge. Fax: +86 10 6480 7099; e-mail: gef@ioz.ac.cn

oxygen species (ROS) in the leaf, which, in turn, activates phytohormone-dependent-induced defence (Giordanengo *et al*. 2010). Like pathogen infection, aphid attack typically induces salicylic acid (SA)-dependent resistance (Thomma *et al*. 2001). Chewing insects, in contrast, typically activate jasmonate (JA)/ethylene (ET)-dependent resistance (Ziegler *et al*. 2001). Large-scale transcriptional evidence, however, has shown that SA, JA and ET signalling pathways are all involved in plant–aphid interactions (Thompson & Goggin 2006).

Elevated CO_2 tends to alter the plant chemical defences that are induced by herbivorous insect (Casteel *et al*. 2008). For example, elevated CO_2 increased soybean susceptibility to two chewing insects, the Japanese beetle and the western corn rootworm, by suppressing the expression of the proteinase inhibitor gene and other downstream genes of the JA pathway (Zavala *et al*. 2009). For piercing-sucking insects, Sun *et al*. (2013) found that JA-regulated defence is more effective against green peach aphids than SA-regulated defence in *Arabidopsis*, and that elevated CO_2 tends to enhance the ineffective SA signalling pathway and reduce the effective JA signalling pathway against aphids. Unlike *Arabidopsis*, *M. truncatula* is a legume and therefore has biological N fixation ability. As noted earlier, the enhancement in biological N fixation in Jemalong under elevated CO_2 prevents decreases in plant N concentration (Guo *et al*. 2013a). The N available to plants could affect their accumulation of defensive phytohormones and induced defence responses (Stout *et al*. 1998; Schmelz *et al*. 2003). We suspected that the difference in N availability between wild-type *M. truncatula* and *dnf1* mutants would lead to differences in the allocation of primary metabolites (C and N) to secondary metabolites and therefore to differences in resistance against aphids under elevated CO_2.

The current study determined how elevated CO_2 affects the physical and chemical defences against the pea aphid in an N-fixing-deficient mutant (*dnf1*) of *M. truncatula* and in its wild-type control (Jemalong). We tested two hypotheses: (1) because elevated CO_2 stimulates photosynthesis and enhances biological N fixation in Jemalong, the sufficient N and excess photosynthate should support an increase in the structural and chemical defence of Jemalong plants against the pea aphid; and (2) because elevated CO_2 decreases the N concentration in the *dnf1* mutant, the limited N availability should constrain both the physical and the chemical defences against the pea aphid. To test these hypotheses, we determined (1) whether elevated CO_2 affects the resistance traits of the two *M. truncatula* genotypes; and (2) how pea aphid feeding behaviour and growth are affected by the changes in resistant traits of the two genotypes under elevated CO_2.

MATERIALS AND METHODS

Open-top field chambers and CO_2 levels

Experiments were performed in eight octagonal, open-top field chambers (4.2 m diameter and 2.4 m height) at the Observation Station of the Global Change Biology Group, Institute of Zoology, Chinese Academy of Sciences in Xiaotangshan County, Beijing, China (40°11′N, 116°24′E). The CO_2 concentrations were set at the current atmospheric CO_2 level (~390 μL L^{-1}) and at an elevated level (750 μL L^{-1}, the predicted level at the end of this century) (IPCC 2007). Four blocks were used for the CO_2 treatment, and each block contained paired open-top chambers, one with ambient and one with elevated CO_2. CO_2 concentration in each open-top chamber was monitored and adjusted with an infrared CO_2 analyzer (Ventostat 8102, Telaire Company, Goleta, CA, USA) once every minute to maintain relatively stable CO_2 concentrations. The measured CO_2 concentrations throughout the experiment (mean ± SD per day) were 395 ± 22 ppm in the ambient CO_2 chambers and 752 ± 33 ppm in the elevated CO_2 chambers. The auto-control system for maintaining the CO_2 concentrations and open-top chamber specifications have been previously described in detail (Chen *et al*. 2005). Air temperatures, which were measured three times per day throughout the experiment, did not differ significantly between the two treatments (21.1 ± 1.1 °C in open-top chambers with ambient CO_2 versus 21.9 ± 1.2 °C in open-top chambers with elevated CO_2).

Host plants and *Rhizobium* inoculation

Wild-type *M. truncatula* plants (cv. Jemalong) and N-fixing-deficient mutant (*dnf1*) plants were kindly provided by Professor Sharon Long, Department of Biology, Stanford University. The nodules of the *dnf1* mutant are small and white, and are blocked at an intermediate stage of development (Wang *et al*. 2010). Moreover, the *dnf1* mutant allele has an independent disruption of the TC121074 locus (Wang *et al*. 2010). The mutant lacks acetylene reduction activity and *Nodulin31* expression, and has only a low level of *nifH* expression in the symbiotic nodule (Mitra *et al*. 2004; Starker *et al*. 2006).

After the seeds were chemically scarified and surface sterilized by immersion in concentrated H_2SO_4 for 5 min, they were rinsed with sterilized water several times. The seeds were placed in Petri dishes containing 0.75% agar, kept in the dark at 4 °C for 2 d, and then moved to 25 °C for 2 d to germinate. The germinated seeds were sown on sterilized soil and inoculated 2 d later with the bacterium *Sinorhizobium meliloti* 1021, which was provided by Professor Xinhua Sui (Department of Microbiology, College of Biological Sciences, Chinese Agricultural University). *S. meliloti* was cultured on YM medium (H_2O 1000 mL, yeast 3 g, mannitol 10 g, KH_2PO_4 0.25 g, K_2HPO_4 0.25 g, $MgSO_4{\cdot}7H_2O$ 0.1 g, NaCl 0.1 g, pH 7.0–7.2) for 3 d at 28 °C to obtain an approximate cell density of 10^8 mL^{-1}. At sowing, each seedling was inoculated with 0.5 mL of this suspension. After they had grown in sterilized soil for 2 weeks, the *M. truncatula* seedlings were individually transplanted into plastic pots (35 cm diameter and 28 cm height) containing sterilized loamy field soil and placed in the open-top chambers; this was on 21 August 2012. Each open-top chamber contained 18 plants for each genotype.

M. truncatula plants were maintained in the open-top chambers for 40 d from seedling emergence to harvest

(from 31 August 2012 to 8 October 2012). Pot placement was re-randomized within each open-top chamber once each week. No chemical fertilizers and insecticides were used. Water was added to each pot every 2 d.

Pea aphid infestation

The pea aphid (*Acyrthosiphon pisum*) was obtained from the laboratory of Dr. Feng Cui (Institute of Zoology, Chinese Academy of Sciences). Nymphs from the same parthenogenetic female were reared on *Vicia faba* under a 14 h light (25 °C)/10 h dark (22 °C) photoperiod in chambers (Safe PRX-450C, Ningbo, China).

After 5 weeks in open-top chambers (6 October 2012), four plants of each genotype per open-top chamber (64 plants in total) were randomly selected for aphid infestation. Each plant was infested with aphids by placing a total of 50 apterous fourth instar nymphs on the fourth and fifth trifoliate leaves (counting from the base), and the leaves, which were terminal and mature, were caged (80 mesh gauze). Another four plants of each genotype per open-top chamber (64 plants in total) were selected as control plants, and their corresponding leaves were caged in the same way but without aphids. After 24 h, the pea aphids were removed with a soft Chinese writing brush and were weighed, as described later in the Materials and Methods section. The damaged leaves from two plants in each genotype of each open-top chamber and the corresponding control leaves were harvested and immediately frozen and stored in liquid N for analysis of the expression of resistance-related genes. As described later in the Materials and Methods section, trichome density was determined on three mature leaves and three young leaves on each of four control plants of each genotype per open-top chamber.

Aphid feeding behaviour and mean relative growth rate (MRGR)

The electrical penetration graph (EPG) method is useful for discerning, in real time, the locations and activities of aphid stylets, including probing, salivation into sieve elements and passive uptake of the phloem sap (Tjallingii & Esch 1993). The feeding behaviour of pea aphids on Jemalong and *dnf1* plants was studied as described by Alvarez *et al.* (2006), with some modifications. One apterous adult pea aphid was placed on a single trifoliate leaf, and its feeding behaviour was monitored. An eight-channel amplifier simultaneously recorded eight individual aphids on separate plants for 6 h: two aphids feeding on Jemalong under ambient CO_2, two on Jemalong under elevated CO_2, two on *dnf1* under ambient CO_2 and two on *dnf1* under elevated CO_2. Waveform patterns in this study were scored according to categories described by Tjallingii & Esch (1993): non-penetration (NP); pooled pathway phase activities (C); salivary secretion into sieve elements (E1); phloem ingestion (E2); derailed stylets (F); and xylem ingestion (G). According to Alvarez *et al.* (2006), six EPG parameters were selected to be relevant with respect to resistance to aphids: (1) the time spent before the first probe (a prolonged period before the first probe is thought to reflect the effects of surface resistance); (2) the minimum duration of waveform C within a probe before E1; (3) the number of probes shorter than 3 min (test probes) that occur before the first E1 wave (these short probes likely reflect the role of surface/epidermis factors); (4) the duration to the first E1; (5) the time required to reach the first sustained E2 (period of E2 longer than 10 min), which indicates the ease of phloem access and acceptance; and (6) the average duration of E2 periods (total time spent in E2 divided by the number of E2 events per aphid), which indicates phloem suitability as well as general plant suitability. The EPG analysis was based upon data collected from 20 aphids for each combination of plant genotype and CO_2 level.

Pea aphids were weighed with an automatic electrobalance before and after feeding on *M. truncatula* plants. The MRGR was calculated as previously described (Leather & Dixon 1984): $\text{MRGR} = (\ln W_2 - \ln W_1)/t$, where W_1 is the initial weight, W_2 is the final weight and t is the time in days between weighings.

Scanning electron microscopy

For determination of trichome density, leaves were fixed in 2.5% glutaraldehyde in 1 mM sodium phosphate buffer (pH 6.8) for 12 h, and then were washed in 50, 60, 70, 90, 100 and 100% ethanol (15 min for each wash). The samples were subjected to critical point drying in CO_2 and were coated with gold using an Eiko 1B.5 sputter coater (Eiko, Tokyo, Japan). The samples were then examined with a Hitachi s570 scanning electron microscope (Tokyo, Japan) at an accelerating voltage of 5–15 kV, and numbers of glandular and non-glandular trichomes per unit of leaf surface area were determined (Brlansky & Raju 1981).

Expression of genes associated with induced resistance as determined by quantitative RT-PCR

The RNA Easy Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate total RNA from *M. truncatula* leaves, and 1 μg of RNA was used to synthesize cDNAs. Messenger RNAs (mRNAs) of the following nine target genes were quantified by real-time quantitative PCR: pathogenesis-related protein (*PR*), β-1,3-glucanase (*BGL*), endochitinase (*CHTN*), *lipoxygenase* (*LOX*), cysteine proteinase inhibitor (*PI*) and 1-aminocyclopropane-1-carboxylate oxidase (*ACC*). Specific primers for each gene were designed from the *M. truncatula* EST sequences using PRIMER5 software (Supporting Information Table S1). The PCR was performed in 20 μL reaction volumes that included 10 μL of 2× SYBRs Premix EX TaqTM (Qiagen) master mix, 5 mM of each gene-specific primer and 1 μL of cDNA template. Reactions were carried out on the Mx 3500P detection system (Stratagene, La Jolla, CA, USA) as follows: 2 min at 94 °C; followed by 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. The melting curves were used to determine the specificity of the

PCR products. A standard curve was derived from the serial dilution to quantify the copy numbers of target mRNAs. The housekeeping gene *β*-actin was used as the internal qPCR standard to analyse plant gene expression. The relative level of each target gene was standardized by comparing the copy numbers of target mRNA with copy numbers of *β*-actin, which is supposed to remain constant under different treatment conditions. The levels of *β*-actin transcripts in the control were examined in every PCR plate to eliminate systematic error. The fold changes of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ normalization method. Each combination of aphid infestation (+), plant genotype and CO_2 level was represented by four biological replicates, and each biological replicate contained four technical repeats.

Statistical analyses

All data were checked for normality and equality of residual error variances and appropriately transformed (log or square root) if needed to satisfy the assumptions of analysis of variance (ANOVA). A split-split plot design was used to analyse the univariate responses of gene expression in plants (ANOVA, PASW Statistics 18.0, SPSS Inc., Chicago, IL, USA). In the following ANOVA model, CO_2 and block (a pair of open-top chambers with ambient and elevated CO_2) were the main effects, *M. truncatula* genotype was the subplot effect and aphid infestation (±) was the sub-subplot effect:

$$X_{ijklm} = \mu + C_i + B(C)_{j(i)} + G_k + CG_{ik} + GB(C)_{kj(i)} + H_l + CH_{il} + HB(C)_{lj(i)} + GHB(C)_{klj(i)} + e_{m(ijkl)}$$

where C is the CO_2 treatment (i = 2), B is the block (j = 4), G is the *M. truncatula* genotype (k = 2) and H is the aphid infestation treatment (l = 2). X_{ijklm} represents the average response at CO_2 treatment i, in block j, genotype k and infestation level l. $e_{m(ijkl)}$ represents the error because of the smaller scale differences between samples and variability within blocks (ANOVA, SAS Institute, Inc., Cary, NC, USA). Effects were considered significant if $P < 0.05$. The effect of block and the interactive effects of block and other factors were not significant ($P > 0.45$), and the effect of block and its interaction with other factors are not described so as to simplify the presentation. Tukey's multiple range tests were used to separate means when ANOVAs were significant. For quantifying the feeding behaviour and MRGR of pea aphids as well as the trichomes of the two *M. truncatula* genotypes under two CO_2 levels, a split-plot design was also applied, with CO_2 and block as the main effects and *M. truncatula* genotype as the subplot effect.

RESULTS

Aphid feeding behaviour and MRGR

The EPG data were used to infer whether the aphids encountered (1) surface resistance; (2) epidermis/mesophyll resistance; (3) mesophyll/phloem resistance; or (4) phloem resistance (Fig. 1).

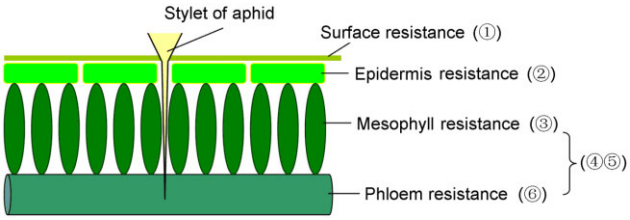

Figure 1. When inserting its long and flexible stylet into the phloem, the aphid must overcome a set of structural barriers and plant defences, which include surface resistance, epidermis resistance, mesophyll resistance and phloem resistance. The electrical penetration graph (EPG) parameters associated with particular resistance components include ① time to first probe indicates surface resistance; ② minimum C before E1 indicates epidermis resistance; ③ numbers of probes <3 min before first E1 indicates mesophyll resistance; ④ time to first E1 and ⑤ time to first E2 indicates mesophyll/phloem resistance; and ⑥ length of E2 indicates phloem resistance.

With respect to surface resistance, elevated CO_2 delayed the 'time to first probe' on Jemalong plants but not on *dnf1* plants (Fig. 2a). The phenotypic difference between the genotypes was not apparent under ambient CO_2, but the 'time to first probe' was longer on Jemalong than on *dnf1* plants under elevated CO_2 (Fig. 2a).

With respect to epidermis/mesophyll resistance, elevated CO_2 prolonged the minimum duration of C wave (pathway phase activities between mesophyll cells) and increased the number of probes <3 min before E1 wave (salivary secretion into sieve elements) on Jemalong but not on *dnf1* plants (Fig. 2b,c). These parameters did not differ between genotypes under ambient CO_2 but were greater on Jemalong than on *dnf1* plants under elevated CO_2 (Fig. 2b,c).

With respect to mesophyll/phloem resistance and phloem resistance, elevated CO_2 reduced the time to the first E1, reduced the time to the first E2 (phloem ingestion) and increased the length of E2 on Jemalong but not on *dnf1* plants (Fig. 2d–f). Under ambient CO_2, the time before the first E2 was shorter on Jemalong than on *dnf1* plants, but the time before the first E1 and the length of E2 did not differ between the genotypes; under elevated CO_2, however, the time before the first E1 and E2 was shorter and the length of E2 was greater on Jemalong plants than on *dnf1* plants (Fig. 2d–f).

Elevated CO_2 increased aphid MRGR on Jemalong plants but did not affect aphid MRGR on *dnf1* plants (Fig. 3). Aphid MRGR was higher on Jemalong than on *dnf1* plants under elevated CO_2, but MRGR did not differ between the genotypes under ambient CO_2 (Fig. 3).

Density of glandular and non-glandular trichomes

We also examined the density of non-glandular and glandular trichomes on young and mature leaves of Jemalong and *dnf1*

plants (Fig. 4a,d). For Jemalong plants, elevated CO_2 increased the density of non-glandular trichomes on both young and mature leaves and increased glandular trichomes on mature leaves (Fig. 4b,c). For *dnf1* plants, however, elevated CO_2 decreased the density of non-glandular trichomes on both young and mature leaves (Fig. 4b,c), and decreased the density of glandular trichomes on mature leaves (Fig. 4f). In comparison of genotypes under ambient CO_2, non-glandular trichome density on young leaves was greater for Jemalong than for *dnf1* plants (Fig. 4b); under elevated CO_2, non-glandular trichome density on both young and mature leaves was greater for Jemalong than for *dnf1* plants (Fig. 4b,c). Furthermore, glandular trichome density on mature leaves was greater on Jemalong than on *dnf1* plants under elevated CO_2 (Fig. 4f).

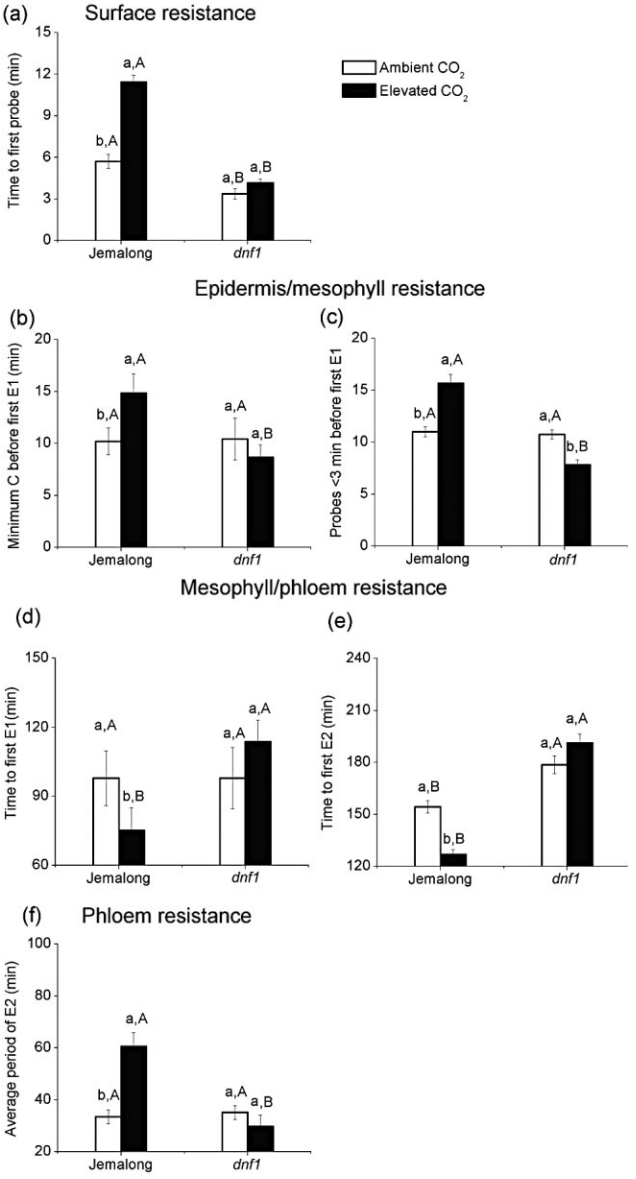

Figure 2. Electrical penetration graph (EPG) results for pea aphids feeding on Jemalong and the *dnf1* mutant during a 6 h of exposure to ambient CO_2 (ACO_2) and elevated CO_2 (ECO_2): (a) time to first probe; (b) minimum C before E1; (c) number of probes <3 min before first E1; (d) time to first E1; (e) time to first E2; and (f) length of E2. Each value is the mean (±SE) of 20 biological replicates. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences between genotypes within the same CO_2 treatment as determined by Tukey's multiple range test at $P < 0.05$.

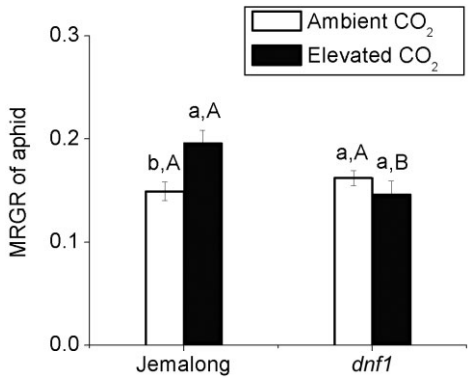

Figure 3. Mean relative growth rate (MRGR) of pea aphids feeding on Jemalong and *dnf1* mutant plants under ambient CO_2 and elevated CO_2. Each value is the mean (±SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences between genotypes with the same CO_2 treatment as determined by Tukey's multiple range test at $P < 0.05$.

Phytohormone-dependent-induced defences

A comparison between uninfested and infested plants of both plant genotypes indicated that aphid infestation up-regulated the expression of SA pathway-related genes, including *BGL*, *PR* and *CHTN*, regardless of the CO_2 level (Fig. 5). When plants were infested by aphids, elevated CO_2 increased *BGL*, *PR* and *CHTN* expression in Jemalong plants (Fig. 5a,c,e) but only increased *PR* expression in *dnf1* plants (Fig. 5d). Jemalong plants had higher SA-related gene expression than *dnf1* plants under elevated CO_2.

When plants were infested by aphids, elevated CO_2 decreased the expression of JA/ET pathway-related genes including *LOX*, *PI*, and *ACC* in Jemalong plants (Fig. 6a,c,e) but did not affect those genes in *dnf1* plants (Fig. 6b,d,f). Aphid infestation did not greatly affect the expression of *LOX* and *PI* in either genotype under ambient CO_2 (Fig. 6a–d). With elevated CO_2, however, aphid infestation down-regulated *LOX* and *PI* expression and up-regulated *ACC* expression in Jemalong plants (Fig. 6a,c,e) and did not affect *LOX* and *PI* expression in *dnf1* plants (Fig. 6b,d). Regardless of the CO_2 level, aphid infestation up-regulated *ACC* expression in both plant genotypes (Fig. 6b,d,f).

DISCUSSION

By altering the nutritional and resistant status of host plants, biological N fixation can affect the interaction between

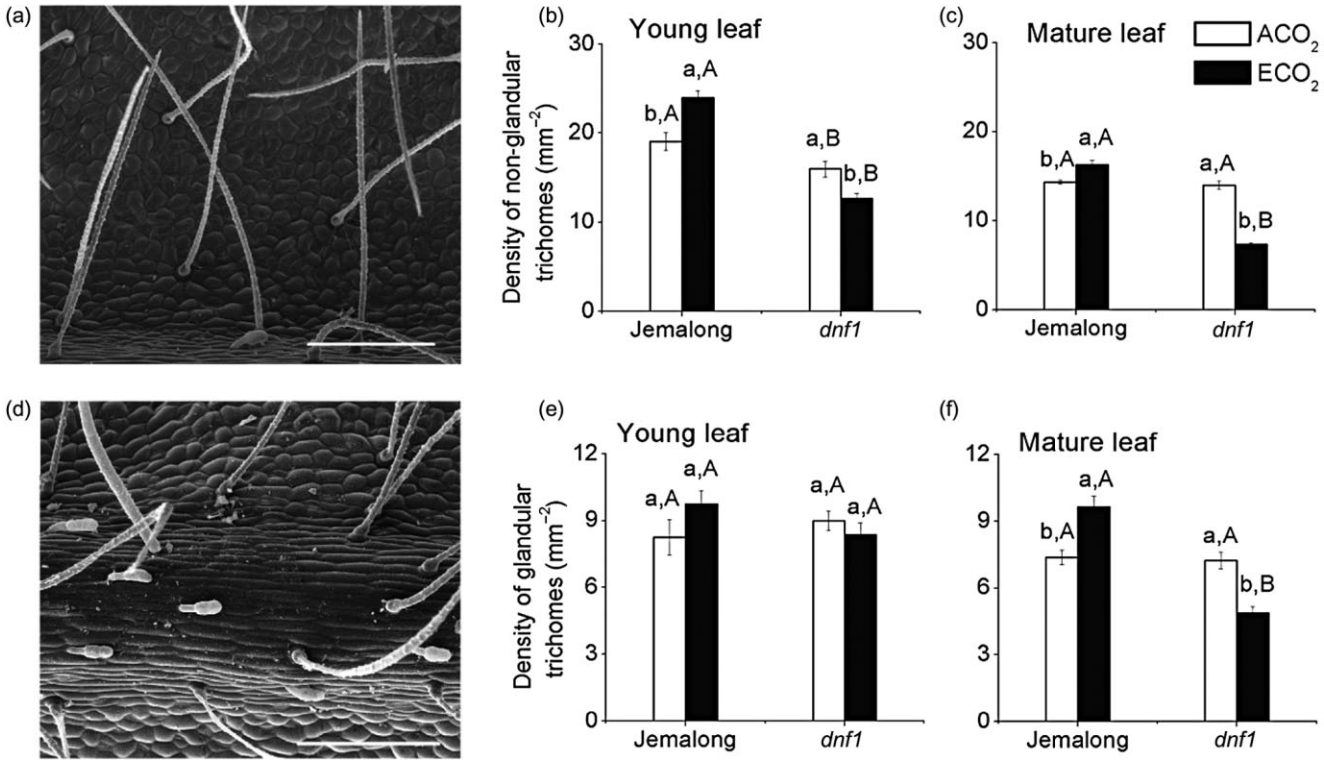

Figure 4. Trichome type and density on young and mature leaves of two *Medicago truncatula* genotypes grown under ambient CO_2 (ACO_2) and elevated CO_2 (ECO_2) without pea aphid infestation: (a) non-glandular trichomes; (b) density of non-glandular trichomes on young leaves; (c) density of non-glandular trichomes on mature leaves; (d) glandular and non-glandular trichomes; (e) density of glandular trichomes on young leaves; and (f) density of glandular trichomes for mature leaves. Scale bar: 200 μm. Each value is the mean (±SE) of four replicates.

legumes and herbivorous insects (Dean *et al*. 2009). Our previous study showed that the nodule numbers and expression of biological N fixation-related genes in Jemalong, a wild-type cultivar of *M. truncatula*, were increased by elevated CO_2 (Guo *et al*. 2013a). Consequently, biological N fixation enabled the plants to maintain a balanced N metabolism under elevated CO_2 such that the plants provided more amino acids to aphids under elevated CO_2 than under ambient CO_2. Guo *et al*. (2013b) inferred that the increase in amino acids partially explained the increase in aphid abundance on Jemalong plants under elevated CO_2.

In the current study, we tested the hypothesis that because elevated CO_2 stimulates photosynthesis and enhances biological N fixation in Jemalong, the sufficient N and excess photosynthate should support an increase in the structural and chemical defence of Jemalong plants against the pea aphid. The results demonstrated that elevated CO_2 increased the structural defence but decreased the chemical defence of Jemalong plants against the pea aphid. We also tested the hypothesis that because elevated CO_2 decreases the N content in the *dnf1* mutant, the limited N availability should constrain both the physical and chemical defences against the pea aphid. The results demonstrated that elevated CO_2 constrained the structural defence but did not affect the chemical defence of the *dnf1* mutant against the pea aphid. In the following sections, we discuss how the increased biological N fixation in Jemalong but not in *dnf1* under elevated CO_2 affects the physical and chemical resistance against sap-sucking aphids.

Surface/epidermis resistance

Surface resistance is the first line of plant defence against aphid attack. The time that aphids spend between arriving on a leaf and making their first probe mainly reflects the physical barrier of the leaf surface. Plant trichomes have long been recognized as physical defences against some herbivorous insects and pathogens (Goffreda *et al*. 1989). Previous studies found that changes in trichome density in response to CO_2 are idiosyncratic. For example, trichome density increased in *Brassica rape* (Karowe & Grubb 2011) but decreased in *Arabidopsis* under elevated CO_2 (Lake & Wade 2009). In our study, elevated CO_2 increased the density of non-glandular trichomes on young leaves and mature leaves and of glandular trichomes on mature leaves of Jemalong plants. Conversely, it decreased the density of non-glandular trichomes on young leaves and mature leaves and of glandular trichomes on mature leaves of the *dnf1* mutant, in which biological N fixation is suppressed. Furthermore, the EPG results indicated that aphids spent more time before making their first probe, experienced a prolonged pathway phase, and increased the number of test probes before entering the E1

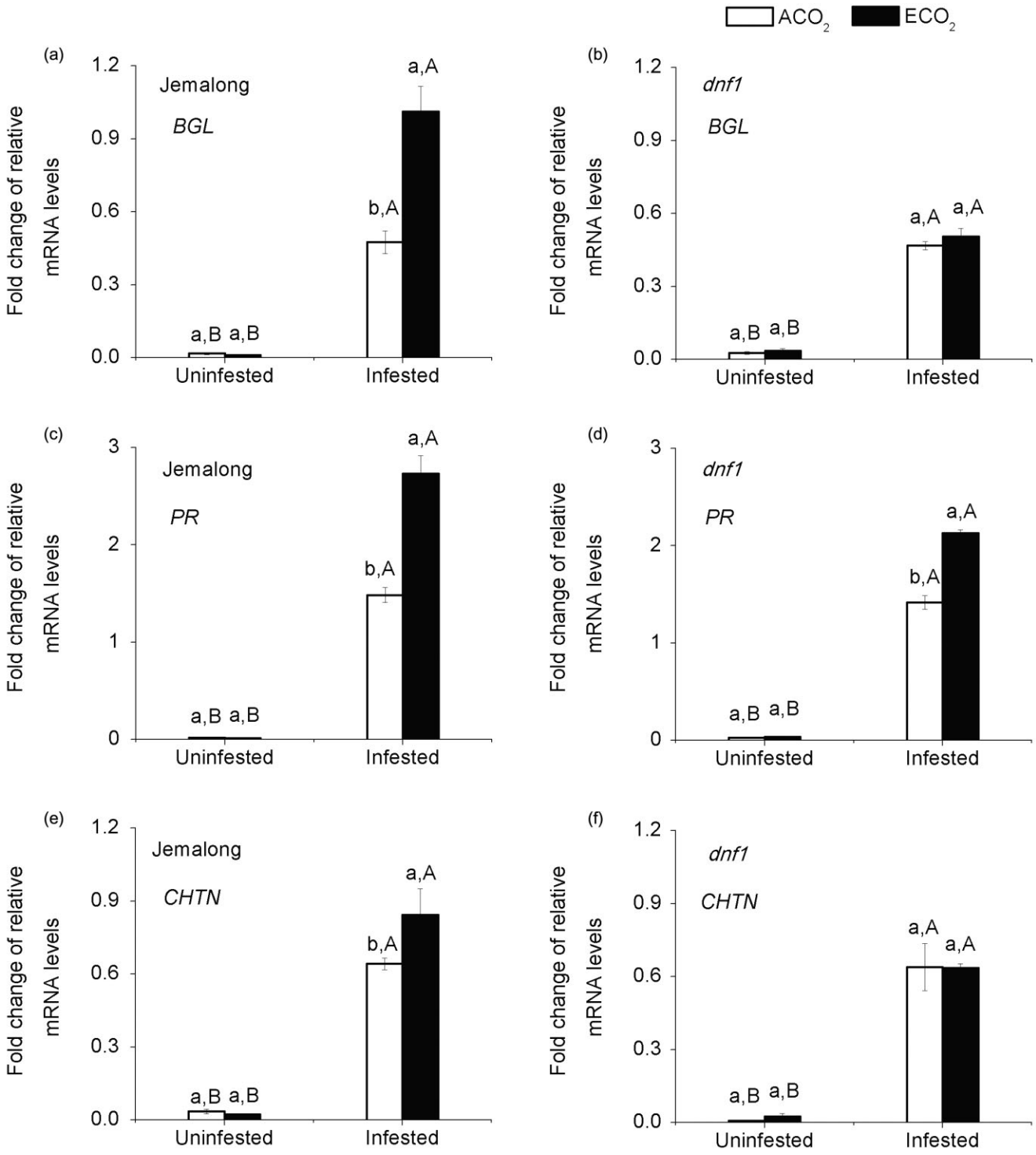

Figure 5. Expression of genes involved in the salicylic acid (SA) signalling pathway (*CHTN*, *PR* and *BGL*) in two *Medicago truncatula* genotypes grown under ambient CO_2 (ACO_2) and elevated CO_2 (ECO_2) with or without pea aphid infestation. Values indicate the fold change in expression based upon qPCR determination, and each value is the mean (±SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same aphid treatment. Different uppercase letters indicate significant differences between aphid treatment within the same CO_2 treatment as determined by Tukey's multiple range test at $P < 0.05$.

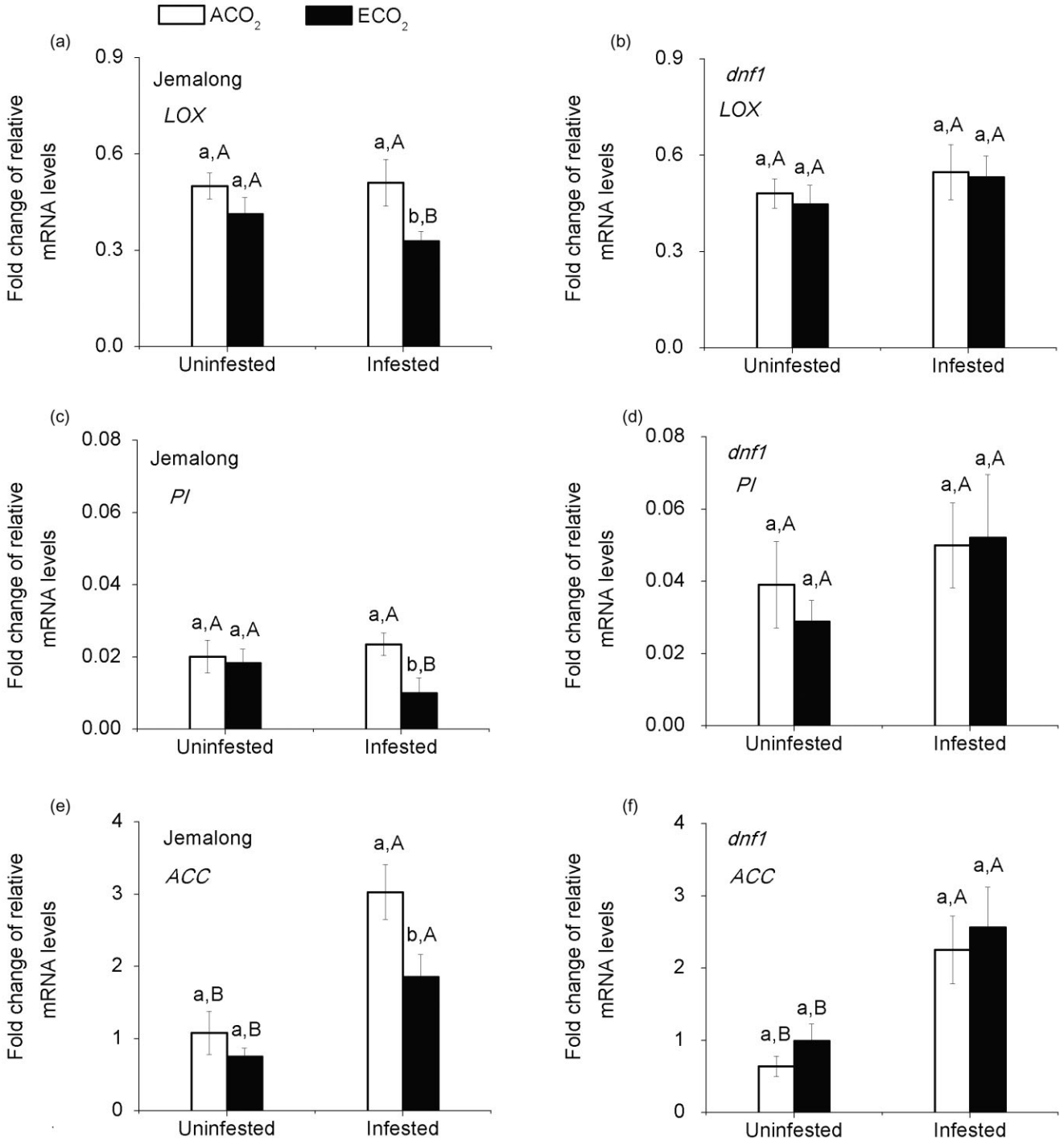

Figure 6. Expression of genes involved in the jasmonate (JA)/ethylene (ET) signalling pathway (*LOX*, *PI* and *ACC*) in two *Medicago truncatula* genotypes grown under ambient CO_2 (ACO_2) and elevated CO_2 (ECO_2) with or without pea aphid infestation. Values indicate the fold change in expression based upon qPCR determination, and each value represents the mean (±SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same aphid treatment. Different uppercase letters indicate significant differences between aphid treatment within the same CO_2 treatment as determined by Tukey's multiple range test at $P < 0.05$.

phase when feeding on Jemalong plants grown under elevated rather than under ambient CO_2. In contrast, the EPG results from aphids associated with *dnf1* plants indicated that surface and epidermis resistance decreased in response to elevated CO_2. The contrasting effects of CO_2 on trichome density of Jemalong and *dnf1* (Fig. 4) demonstrate that Jemalong but not *dnf1* plants can transform the elevated CO_2-induced primary metabolism into structural defence. As

a consequence, elevated CO_2 increases the time that aphids spend searching for a proper feeding site on Jemalong plants but reduces that time on *dnf1* plants.

Mesophyll/phloem resistance

Infestation by herbivorous insects induces a series of phytohormone-dependent defences in plants (Goggin 2007). Interestingly, in compatible plant–aphid interactions, SA-dependent defence is activated (Giordanengo *et al*. 2010). In the current study, aphid infestation rapidly up-regulated SA-dependent gene expression in both genotypes under ambient CO_2 (Fig. 5), which is consistent with the results of Gao *et al*. (2008). Previous evidence indicated that elevated CO_2 tends to enhance the SA-signalling pathway (Casteel *et al*. 2012). When Jemalong plants were infested with aphids in our study, all of the analysed SA-regulated genes were up-regulated by elevated CO_2. For *dnf1* plants, in contrast, only *PR* was up-regulated by elevated CO_2. These results indicate that Jemalong plants generate a stronger SA-dependent defence response than *dnf1* plants against aphid infestation under elevated CO_2. This up-regulation of the SA-signalling pathway in Jemalong suggests that aphids might spend more time reaching the phloem sap in Jemalong than in *dnf1*, but this was not the case. Under elevated CO_2, the times to the first E1 and E2 were shortened and the average duration of E2 was longer on Jemalong plants than on *dnf1* plants. Mewis *et al*. (2005) also reported that the SA signalling was associated with increased susceptibility to aphids. The authors found that numbers of the green peach aphid were smaller on the SA-signalling pathway mutant *NahG* and *npr1* plants than on wild-type plants. Apparently, aphids are able to overcome or even benefit from the defences associated with increased activity of the SA-signalling pathway in Jemalong plants under elevated CO_2.

Although transcriptomics and functional genomics indicate that piercing-sucking insects mainly trigger the SA signalling pathway in plants (Thompson & Goggin 2006), many studies have demonstrated that JA signalling is more effective than SA signalling in plant defence against aphids (Zhu-Salzman *et al*. 2004; Walling 2008). In their long-term co-evolution with host plants, aphids have developed the ability to trigger the ineffective SA signalling pathway in order to suppress the effective JA signalling pathway (Mewis *et al*. 2005). Moreover, elevated CO_2 tends to change plant–insect interactions by modifying the JA-dependent pathway. Elevated CO_2 in previous studies decreased the JA level and suppressed key genes involved in the JA signalling pathway (such as *LOX*, *PI* and *PDF1.2*), which increased susceptibility to herbivorous insects (Zavala *et al*. 2008; Sun *et al*. 2013). In our study, elevated CO_2 decreased aphid-induced expression of genes in the JA/ET signalling pathway of Jemalong plants but did not affect the aphid-induced expression of those genes in *dnf1* plants. Similarly, the higher feeding efficiency of aphids in terms of increased length of E2 associated with Jemalong under elevated CO_2 suggests that the suppression of JA/ET signalling pathway defensive genes by elevated CO_2 helps aphids obtain nutrition from phloem sap. The SA and JA/ET signalling pathways are interconnected in a complex network that enables plants to rapidly adapt to biotic and abiotic stresses (Bruce & Pickett 2007). Elevated CO_2 potentially disrupts the homeostatic cross-talk between SA and JA/ET pathways by directly activating the NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) gene (DeLucia *et al*. 2012). NPR1-mediated suppression of JA signalling is regulated by glutathione biosynthesis (Spoel & Loake 2011). Elevated CO_2 changes the expression of genes that encode thioredoxins and glutathione S-transferase, which may activate the expression of NPR1 (DeLucia *et al*. 2012). However, Sun *et al*. (2013) found that when the NPR1 gene was knocked down, the JA-dependent defences of *Arabidopsis* were not enhanced by elevated CO_2, suggesting that the activation of NPR1 under elevated CO_2 may not be the only explanation for the antagonistic interaction between SA and JA signalling pathways. Thus, additional research is needed to determine how these regulatory molecules are coordinated in phytohormone signalling networks by elevated CO_2.

Aphid relative growth rate

In our previous study involving the long-term feeding of aphids on *M. truncatula* plants, aphid abundance increased on Jemalong but decreased on *dnf1* under elevated CO_2, and the difference was attributed to N nutrition (Guo *et al*. 2013b). When the feeding in that same study was short term, however, aphid growth on Jemalong was mainly limited by host resistance. The current findings indicate that the increased performance of aphids (the increase in MRGR) on Jemalong under elevated CO_2 results from decreases in particular components of host resistance. Although elevated CO_2 enhanced the physical defences on leaf surfaces, elevated CO_2 suppressed the chemical defences related to the JA/ET pathways in Jemalong plants. In contrast, elevated CO_2 had little effect on the feeding behaviour and MRGR of pea aphids associated with the N-fixing-deficient mutant *dnf1* plants because elevated CO_2 did not affect the efficient chemical defence related to the JA/ET pathway. Our results suggest that the chemical defence of *M. truncatula* is more effective than the physical barrier in altering aphid feeding behaviour and suppressing MRGR. With respect to *dnf1* under elevated CO_2, the results of the previous and current study seem inconsistent in that elevated CO_2 decreased short-term MRGR in current study but increased long-term population abundance in the previous study (Guo *et al*. 2013b). We suggest that it is important to distinguish between the performances of aphids based upon short-term and long-term feeding when host plants are exposed to elevated CO_2.

In summary, we investigated how the resistance of *M. truncatula* to aphids is affected by elevated CO_2. We detected genotype-specific responses in physical and chemical resistance, and connected the response to the different phases in aphid feeding behaviour. The results indicated that the chemical defence of *M. truncatula* is more effective than the physical barrier to prevent feeding behaviour and suppress growth rate of aphids. Moreover, elevated CO_2 reduces

the chemical resistance of a wild-type plant but not of an N-fixing-deficient mutant. This demonstrates that biological N fixation has an important role in the modification of plant resistance under elevated CO_2.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primer sequences used for real-time quantitative PCR.