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Pea aphid promotes amino acid metabolism both in *Medicago truncatula* and bacteriocytes to favor aphid population growth under elevated CO_2

Short title: Aphid enhanced use of amino acid under ECO_2

List of authors:

Huijuan Guo[1,2], Yucheng Sun[1,*], Yuefei Li[1], Bin Tong[1,2], Marvin Harris[3], Keyan Zhu-Salzman[3] and Feng Ge[1,*]

[1] State Key Laboratory of Integrated Management of Pest and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China;

[2] Graduate School, Chinese Academy of Sciences, Beijing 100039, People's Republic of China;

[3] Department of Entomology, Texas A&M University, College Station, TX 77843, USA.

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* Corresponding authors:

Tel: 8610 6480 7130; Fax: 8610 6480 7099; Email: sunyc@ioz.ac.cn (Y. Sun)

Tel: 8610 6480 7123; Fax: 8610 6480 7099; Email: gef@ioz.ac.cn (F. Ge)

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Abstract

Rising atmospheric CO_2 levels can dilute the nitrogen (N) resource in plant tissue, which is disadvantageous to many herbivorous insects. Aphids appear to be an exception that warrants further study. The effects of elevated CO_2 (750 ppm vs. 390 ppm) were evaluated on N assimilation and transamination by two *Medicago truncatula* genotypes, a N-fixing-deficient mutant (*dnf1*) and its wild-type control (Jemalong), with and without pea aphid (*Acyrthosiphon pisum*) infestation. Elevated CO_2 increased population abundance and feeding efficiency of aphids fed on Jemalong but reduced those on *dnf1*. Without aphid infestation, elevated CO_2 increased photosynthetic rate, chlorophyll content, nodule number, biomass and pod number for Jemalong, but only increased pod number and chlorophyll content for *dnf1*. Furthermore, aphid infested Jemalong plants had enhanced activities of N assimilation-related enzymes (GS, GOGAT) and transamination-related enzymes (GOT, GPT), which presumably increased amino acid concentration in leaves and phloem sap under

elevated CO_2. In contrast, aphid infested *dnf1* plants had decreased activities of N assimilation-related enzymes and transmination-related enzymes and amino acid concentrations under elevated CO_2. Furthermore, elevated CO_2 up-regulated expression of genes relevant to amino acid metabolism in bacteriocytes of aphids associated with Jemalong, but down-regulated those associated with *dnf1*. Our results suggest that pea aphids actively elicit host responses that promote amino acid metabolism in both the host plant and in its bacteriocytes to favor the population growth of the aphid under elevated CO_2.

Introduction

Global atmospheric CO_2 concentrations have been increasing at an accelerating rate from 280 ppm before industrialization to 393 ppm in November 2012 (Mauna Loa Observatory: NOAA-ESRL), and are anticipated to reach at least 550 ppm by the year 2050 (IPCC, 2007). The effects of elevated CO_2 on C3 plants are generally characterized by increased photosynthetic rate, biomass, and carbon:nitrogen (C:N) ratios in plant tissues (Ainsworth & Long, 2005; Ainsworth *et al.* 2007). The assimilation and re-assignment of C and N resources within plant tissues under elevated CO_2 would therefore alter the nutritional quality and palatability of host plants, which in turn affects the performance of herbivorous insects (Couture *et al.*, 2010).

Decrease in N concentration (g of N per g of plant tissue, 10-15% in average) is the most remarkable characteristic of non-leguminous C3 plants response to elevated CO_2 (Taub *et al.* 2008), which in turn prolongs developmental time and reduces fecundity and population

fitness of most leaf-chewing insects (Coll and Hughes, 2008). N concentrations of legumes, however, due to an unique ability of utilizing atmospheric N_2, were hardly affected by elevated CO_2 (Taub *et al.* 2008). Typically, elevated CO_2 increases nodule number (+33%), nodule mass (+39%) and nitrogenase activity in legumes (+37%), and eventually leads to a 38% increase in N fixed from the atmosphere (Lam *et al.*, 2012). The enhanced biological N fixation (BNF) can compensate for decreases in plant N under elevated CO_2 and maintain the equivalent C/N of those plants under ambient CO_2, which partially or even completely counteracts the adverse effect of elevated CO_2 on leaf-chewing insects (Karowe *et al.*, 2007; 2011). Thus, compared with those on non-legume plants, enhanced BNF is favorable to leaf-chewing insects fed on legume plants under elevated CO_2. It remains unclear, however, whether the enhanced BNF under elevated CO_2 would similarily be beneficial to other feeding guilds of insects i.e. sap-sucking insects on legume plants.

Host plant suitability for leaf-chewing insects is generally achieved at a 0.8-1.5 protein/carbohydrate ratio (w/w) (Behmer, 2009). Very different from the leaf-chewing insects, the sap-sucking insects (i.e. aphids) with stylet-like mouthparts feed exclusively upon phloem sap of plants for their nutrient intake (Douglas, 2003); this substrate has a protein/carbohydrate ratio (mainly amino acid/sugar in phloem sap, w/w) as low as 0.1 (Nowak *et al.*, 2010). Aphid success is attributed to dozens of specialized cells (bacteriocytes) in the haemocoel, which harbor a primary endosymbiont *Buchnera* (Nakabachi *et al.*, 2005). *Buchnera* utilizes nonessential amino acids in the aphid to provide up to 90% of the essential

amino acids for the aphid (Douglas 2006). Conversely, aphid metabolism supports a substantial flux of nonessential amino acids from the insect to *Buchnera*, mostly via phloem sap from host plants (Wilson *et al*., 2010). Presumably, this is why aphids prefer host plants with relatively higher N and amino acid concentrations (Nowak *et al.*, 2010). Thus, even though aphids could develop without one or even several kinds of amino acids (Gunduz and Douglas, 2008), total N and especially the amino acid concentration of the host plant is the most important determinant of aphid fitness.

Aphids have evolved to use this nutrient poor substrate and also to manipulate phloem sap compositions to achieve high reproductive rates (Goggin *et al.*, 2007). Elevated CO_2 negatively affects leaf-chewing insects primarily through a decrease in plant N; this has led to speculation that the performance of aphid would also decrease since the amino acid content in the plant phloem has decreased (Sun *et al*., 2009). However, recent reviews conclude that aphids have idiosyncratic response to elevated CO_2 (Hughes *et al.*, 2001, Himanen *et al.*, 2008). Furthermore, many aphid species exhibit increased fecundity, abundance and survival rate under elevated CO_2 (Pritchard *et al*., 2007; Robinson *et al.*, 2012). It appears that aphids are able to overcome N decrease in host plants by increasing the N sink strength of host plant in some cases (Giordanengo *et al*., 2010; Sandström *et al*., 2000). Thus, two possible explainations were proposed to address how aphids overcome N decrease in plants held under elevated CO_2: 1), aphids may enhance the N metabolism of plants to satisfy nutrition requirements under elevated CO_2. Most previous studies have focused on the single effect of

elevated CO_2 on plant nutrition without aphid infestation, but little information is available in terms of aphid infested plant nutritional responses to elevated CO_2. 2), decreased N contents in plants caused by elevated CO_2 may alter aphid interaction with endosymbiont *Buchnera*, which could enhance the amino acid metabolism in bacteriocytes. Nevertheless, it still lack of experimental evidence to address the physiological mechanism underlying why some aphids respond positively to N-dilution in plants growing under elevated CO_2.

The current study uses an N-fixing-deficient mutant (*dnf1*) of *Medicago truncatula* and its wild-type control to determine how elevated CO_2 affects the nutritional interactions among host plant, pea aphid and its endosymbiont *Buchnera*. We test two hypotheses here: (1) since elevated CO_2 stimulates BNF and thereby maintains the equivalent C:N ratio to legumes grown under ambient CO_2, aphid performance and its interaction with *Buchnera* would be unaffected by elevated CO_2; (2) elevated CO_2 would decrease N content and its related metabolic processes in N-fixing-deficient (*dnf1*) mutant, and therefore would increase reliance on *Buchnera*. To experimentally test these hypotheses, we specifically investigate: (1) how elevated CO_2 affects population abundance and feeding behavior of pea aphids when feeding on *dnf1* mutant and wild-type plants; (2) whether elevated CO_2 affects N assimilation and transamination of two *M. truncatula* genotypes infested by pea aphids, and the downstream expression of specific amino acid concentrations in both the host plant and the pea aphid; and (3) whether elevated CO_2 affects expression of key genes involved in amino acid metabolism of bacteriocytes hosted by pea aphids.

Materials and methods

Treatments under different CO_2 concentrations

Experiments were performed in eight octagonal, open-top field chambers (OTCs) (4.2m diameter and 2.4m height) at the Observation Station of the Global Change Biology Group, Institute of Zoology, Chinese Academy of Science in Xiaotangshan County, Beijing, China (40°11´N, 116°24´E). The CO_2 concentrations were set at: (1) current atmospheric CO_2 levels (~390 μL/L), and (2) elevated CO_2 levels (750 μL/L, predicted level at the end of this century) (IPCC, 2007). Four blocks were used for the CO_2 treatment, and each block contained paired OTCs, one with ambient and one with elevated CO_2. CO_2 concentration in each OTC 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, as well as specifications for the OTCs, is detailed in Chen *et al*. (2005). The tops of the OTCs were covered with nylon net to exclude insects. Air temperatures were measured three times per day throughout the experiment and did not differ significantly between the two treatments (22.7 ± 1.9 ºC in OTCs with ambient CO_2 vs. 24.2 ± 2.0 ºC in OTCs with elevated CO_2).

Host plants and rhizobium inoculation

Wild-type *M. truncatula* plants (cv. Jemalong) and the N-fixation-deficient *dnf1-1* mutant

(*dnf1*) 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 have only a small level of *nifH* expression in the symbiotic nodule (Mitra et al, 2004, Starker *et al.* 2006).

After 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 filled with 0.75% agar, kept in the dark at 4°C for 2 days, and then moved to 25°C for 2 days to germinate. The germinated seeds were sown on sterilized soil and inoculated 2 days 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 (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 days 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 (organic carbon 75 g/kg; N 500 mg/kg; P 200 mg/kg; K 300 mg/kg) and placed in OTCs on 27 March 2012. Each OTC contained 40 plants and 320 plants in total.

M. truncatula plants were maintained in the OTCs for 75 days from seedling emergence

to harvest (27 March to 7 June 2012). Pot placement was re-randomized within each OTC once a week. No chemical fertilizers and insecticides were used. Water was added to each pot every 2 days.

Pea aphid infestation

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

After 6 weeks fumigation in OTCs (18 May, 2012), plants were arranged for three different treatments with pea aphids. 6 plants of each genotype per OTC (96 plants in total) were randomly selected and each plant was infested with 5 apterous 4^{th} instar pea aphid nymphs. The nymphs caged with 80 mesh gauze developed and reproduced offspring freely on each plant for 23 days (to 7 June 2012). In the second part, 6 plants of each genotype per OTC (96 plants in total) were randomly selected and each plant was infested with 1 apterous adult pea aphid for 8 h to record feeding behavior. In the third part, 2 plants of each genotype per OTC (32 plants in total) were randomly selected and each plant was infested with 50 apterous 4^{th} instar nymphs for 48h. The nymphs were transferred to two mature trifoliate leaves with a fine paint brush, and were caged on each plant for 48h with 80 mesh gauze. Another 2 plants of each genotype per OTC serving as the uninfested control were caged in the same way. The 48h-damaged leaves of infested plants and uninfested controls were

harvested and immediately frozen in liquid nitrogen for analyzing enzymatic activities and free amino acid concentrations.

Another 6 plants of each genotype per OTC were also caged as uninfested controls from March 27 until 7 June 2012. Plants were then harvested for measurement of photosynthetic rate, chlorophyll content, biomass, pod and nodule numbers.

Population abundance and feeding behavior of aphids

Aphid population abundance was recorded on each plant at days 7, 11, 15, 19 and 23. At the end of the investigation, the aphids were brushed from each plant; 30 adult aphids were collected per plant to analyze the amino acid concentrations and gene expression as described later.

The electrical penetration graph (EPG) method is a powerful means of discerning, in real time, the locations and activities of aphid stylets during probing, including their salivation into sieve elements and passive uptake of phloem sap (Walker, 2000). The feeding behavior of pea aphids on the plants of Jemalong and *dnf1* was studied as described in Gao *et al.* (2007) with some modifications. Twenty-four biological replicates were included for each Jemalong and *dnf1* genotype under each CO_2 level. A single apterous adult pea aphid was placed on a single trifoliate leaf and their feeding behavior was monitored. An eight-channel amplifier simultaneously recorded eight individual aphids on separate plants for eight hours by monitoring the aphids feeding on two Jemalong under ambient CO_2, two Jemalong under elevated CO_2, two *dnf1* under ambient CO_2 and two *dnf1* under elevated CO_2 during each trial.

Waveform patterns in this study were scored according to categories described by Tjallingii and Esch (1993): Nonpenetration (np); pooled pathway phase activities (C); salivary secretion into sieve elements (E1); phloem ingestion (E2); Derailed stylets (F) and xylem ingestion (G).

Plant photosynthesis and growth traits

Net photosynthetic rate was determined according to Guo *et al*., (2012) with some modification. Net photosynthetic rate of each plant was measured with a Li-Cor 6400 gas exchange system (Li-Cor Inc., Lincoln, NE, USA). The fourth terminal mature trifoliate leaf from the base of the shoot was selected for measurement. All measurements took place between 9:00 am and 12:00 pm. The CO_2 concentration of the incoming air was adjusted to 400 μmol mol^{-1} CO_2 or 750μmol mol^{-1}. Relative humidity corresponded to ambient conditions. Before gas exchange was measured, photosynthetic active radiation (PAR) for the leaf in the measuring cuvette was increased in steps to 1200 μmol m^{-2} s^{-1}.When the CO_2 assimilation rate was stable for at least 2 min, a measurement of photosynthetic rate was recorded. Leaf chlorophyll content was determined by using a Minolta SPAD-502DL (Konica Minolta Sensing Inc., Osaka, Japan), which measures leaf transmittance at two wavelengths: red (approximately 660 nm) and near infrared (approximately 940 nm). SPAD readings were taken on the fourth terminal mature trifoliate leaf from the base of the shoot. The SPAD sensor was placed randomly on leaf mesophyll tissue.

Roots of each plant were carefully removed from the soil and washed. A

stereomicroscope aided counting the nodules on the entire root system of each plant. This was followed by counting the pod numbers and then the shoots and roots of each plant were collected, oven-dried (65°C) for 72 h, and weighed for biomass.

Enzymes and amino acid determination

Activity of key enzymes glutamine synthetase/ glutamate synthase (GS/GOGAT), glutamate oxalate transaminase (GOT) and glutamine phenylpyruvate transaminase (GPT) involved in N assimilation and transamination were determined (Schoenbeck *et al.* 2000; Andrews *et al.* 2004) and quantified using frozen leaf tissue (approximately 0.5g per plant). Once the tissue was ground to a fine powder, leaves from three plants of the same treatment were combined to form one sample from each OTC. The unit of replication for statistical analyses was the OTC (n=4): An extract was obtained by grinding each leaf sample in 50 mM Tris HCl buffer (pH 7.8, 3 mL/g of leaf tissue) containing 1 mM $MgCl_2$, 1 mM EDTA, 1 μM β-mercaptoethanol, and 1% (w/v) polyvinylpolypyrro-lidone. This extract was immediately frozen for later use. For assays, the thawed extract was centrifuged at 13,000 *g* for 10 min, and the enzymatic activities were measured in the supernatant as described by Kaiser *et al*. (1984) for GS, and by Suzuki *et al*. (2001) for GOGAT. GOT and GPT according to Asthir *et al*. (2011). Protein concentrations of leaves and roots were measured using bovine serum albumin as a standard (Bradford, 1976).

For quantifying amino acid concentrations in phloem, phloem exudates were obtained from three trifoliate per plant by using EDTA exudation technique of Douglas (1993). In

addition, amino acid concentrations of whole leaves and aphids were obtained from 0.5 g leaf samples and 0.3 g fresh weigh aphid samples, ground in liquid nitrogen and were extracted with 2.5 mL of cold 5% acetic acid for 1 h with gentle agitation on a shaker (C. Gerhardt GmbH & Co. KG, Germany) at room temperature. Homogenates were centrifuged at 4000 rpm for 15 min, and the supernatants were subjected to free amino acid analyses. The amino acids in each sample were analyzed by reverse-phase HPLC with pre-column derivatisation using *o*-phthaldialdehyde (OPA) and 9-fluorenylmethyloxycarbonyl (FMOC). Amino acids were quantified by comparison with the AA-S-17 (Agilent, PN: 5061-3331) reference amino acid mixture, supplemented with asparagine, glutamine and tryptophan (sigma-Aldrich Co., St.Louis, MO). Standard solutions were prepared from a stock solution by diluting with 0.1 M HCl. Free amino acid concentrations of the 5 standard solutions are 250 pmol/µl, 100 pmol/µl, 50 pmol/µl, 25 pmol/µl, 10 pmol/µl. Before injected to the HPLC, 10ul amino acid sample, 20ul sodium borate buffer (0.4N, pH 10.4), 10ul OPA, 10ul FMOC and 50ul water were mixed. The analysis was performed using Agilent 1100 HPLC systems (Agilent Technologies, Palo Alto, CA). A reverse phase Agilent Zorbax Eclipse C18 column AAA (5 µm, 250 mm×4.6 mm) and fluorescence detector were used for the chromatographic separation. The column was maintained at 35°C with a gradient (1 ml min^{-1} flow) programmed as follows: 98/2 (1 min) to 43/57 (25 min) to 0/100 (34 min) to 98/2 (42 min hold) of Eluent A / Eluent B. Eluent A was a 40 mM disodium phenyl phosphate buffer (pH 7.8 adjusted with sodium hydroxide). Eluent B was a 45% acetonitrile, 45% methanol and 10% water. Chemstation Plus Family for LC software was used for data acquisition and

analysis. Amino acid concentrations were quantified by comparison of sample peak areas to standard curves of 20 reference amino acids (Agilent Chemical Co.).

Expression of genes associated with amino acid metabolism in bacteriocytes as determined by quantitative RT-PCR

Bacteriocytes were isolated by initially homogenizing whole insects in Buffer A-250 (250 mM EDTA pH 8.0, 35 mM Tris pH 8.0, 25 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose) with a micropipette and filtering through a 100 μm nylon filter by methods of Nakabachi *et al*. (2005). In each sample, 1,500-2,000 bacteriocytes collected from 50 adult aphids were used for RNA extraction. Each treatment combination was replicated four times for biological repeats, and each biological repeat contained three technical replicates. The RNAeasy Mini Kit (Qiagen) was used to isolate total RNAs from bacteriocytes, and 1 μg of RNA was used to generate the cDNAs. The mRNAs of the following 5 target genes were quantified by real-time quantitative PCR: glutamine synthetase (GS), glycine cleavage system T protein (GCVT: an aminomethyl transferase, catalyzing the degradation of glycine), phenylalanine hydroxylase (Henna: an enzyme with activities of phenylalanine 4-monooxygenase and tryptophan 5-monooxygenase, which are involved in degradation of L-phenylalanine and L-tryptophan, respectively), glutaryl-CoA dehydrogenase (GCDH: an enzyme that catalyzes the oxidative decarboxylation of glutaryl-CoA, which is involved in L-tryptophan metabolism and degradative pathways of L-lysine and L-hydroxylysine), Phosphoserine

aminotransferase (PSAT: an enzyme that is involved in serine biosynthesis). Relative expression levels of target genes were normalized to mRNA for ribosomal protein (Rp)L7. PCR primer design, real-time quantitative PCR conditions, and analyses were performed according to Nakabachi *et al*. (2005) with modifications. The fold-changes of target genes were calculated using the $2^{-\Delta\Delta Ct}$ normalization method.

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. For the multivariate tests of amino acids, we performed a PCA on the correlations among the 20 response variables, and then performed factor rotation using the Varimax method (Rasmussen *et al*., 2008). A split-split plot design was used to analyze the univariate responses of the enzyme activities and rotated factors of amino acids in plants (ANOVA, PASW, 2009). In the following ANOVA model, CO_2 and block (a pair of OTCs with ambient and elevated CO_2) were the main effects, *M. truncatula* genotype was the subplot effect, and aphid infestation level 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 error because of the smaller scale differences between samples and variability within blocks (ANOVA, SAS institute). 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 presented so as to simplify the presentation. Tukey's multiple range tests were used to separate means when ANOVAs were significant. For quantifying the growth traits (biomass, pod numbers, photosynthetic rate, chlorophyll content and nodule numbers) of plants, feeding behavior, rotated factors of amino acids and gene expression of pea aphids on different *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. The population abundance of aphid was analyzed by repeated measures of ANOVA.

Results

Aphid population abundance and feeding behavior

Elevated CO_2 favors the population growth of pea aphids on Jemalong, but decreases those on *dnf1* plants beginning 15 days post infestation (Fig. 1a). Pea aphids reared on Jemalong exhibit a dramatically increased rate of population growth compared to those reared on *dnf1* mutant under elevated CO_2, while remaining treatments under ambient CO_2 did not significantly differ from one another during the test period.

Elevated CO_2 increased the portion of time aphids spent on salivation in the sieve elements (E1 phase) and phloem sap ingestion (E2 phase) when fed on Jemalong, but did not affect those fed on *dnf1* plants (Fig. 1b). The relatively longer E1 and E2 phase and shorter nonpathway phase suggests higher feeding efficiency of aphids on Jemalong than those fed

on *dnf1* plants under elevated CO_2. The genotype difference, on the other hand, was not apparent under ambient CO_2.

Plant photosynthesis and growth traits

Elevated CO_2 increased photosynthetic rate by 31.7%, chlorophyll content by 37.5%, nodule numbers by 38.4%, biomass by 63.3% and pod number by 1.1-fold for Jemalong. In contrast, for *dnf1* mutant, elevated CO_2 increased pod number by 0.8-fold and chlorophyll content by 18.4% (Fig. 2).

N assimilation and transamination enzymes of plants

Among uninfested plants: Jemalong and *dnf1* at ambient CO_2 significantly differed in enzyme activities of GS, GOGAT, GPT and GOT compared to uninfested *dnf1* plants under elevated CO_2, which were lower (Fig. 3). Among infested plants: elevated CO_2 increased all enzyme activities of N assimilation and transamination we measured in the infested Jemalong, while decreased activities were observed for all enzymes in the infested *dnf1* plants (Fig. 3).

A comparison of uninfested to infested plants shows, for Jemalong plants, aphid infestation decreased activities of GOT under ambient CO_2, but increased GOT and GPT under elevated CO_2. In contrast, aphid infestation decreased activities of GS, GOGAT and GOT under ambient CO_2 as well as decreased all enzymes we measured involved in N assimilation and transamination under elevated CO_2 of *dnf1* plants (Fig. 3).

Amino acid concentration in plants

The uninfested treatments showed elevated CO_2 did not significantly affect foliar total amino acid concentration of Jemalong (Fig. 4), but decreased foliar total amino acid concentration by 20.5%, of *dnf1* plants (Fig. 4). Infested treatments showed elevated CO_2 increased the concentration of foliar total amino acids by 60.9%, while a decreased concentration of foliar total amino acid by 43.3% of *dnf1* plants.

Aphid infestation did not affect foliar total amino acid concentration in Jemalong In contrast, aphid infestation increased 58.7% total amino acid in leaves under elevated CO_2. For the *dnf1* mutant, aphid infestation did not affect total amino acids in leaves under ambient CO_2, however, aphid infestation decreased 37.0% total amino acids in leaves under elevated CO_2.

The EDTA technique was used to measure the relative composition of amino acids in the phloem sap of *M. truncatula*. Since the responses of 20 amino acids we measured are non-independent, we then used a principal components analysis (PCA) to reduce the number of phloem amino acid response variables to a new set of composite variables (Rasmussen *et al*., 2008). In order to facilitate interpretation of principal components, we subjected the first three principal components to factor rotation with the most common form of factor rotation, *varimax* rotation, retained three rotated factors (RF1, RF2 and RF3 represented 72% of the total variance). As the values of the rotated factor increase, those variables that load heavily and positively (loading $\geq$ +0.5) also increase, while those variables that load heavily but

negatively (loading ≤ -0.5) decrease. The standardized univarite responses of these variables are shown in Fig. 6b,d,f in support of the interpretation of the multivariate responses and to allow a closer inspection of those variables loading heavily onto RF1, RF2 and RF3, respectively.

11 individual amino acids consisting of Val, Asn, Tyr, Arg, His, Ser, Asp, Lys, Gln, Glu and Cys loaded heavily and positively onto RF1 (Fig. 5a). In RF1, nonessential amino acids comprised an average of 80%. We subjected RF1 to the split-plot ANOVA model and found all factors, with the exception of the interaction between CO_2 level and aphid infestation, had significant effects on RF1 (Table S3). The uninfested treatments showed elevated CO_2 did not significantly affect RF1 in the phloem of both Jemalong and *dnf1* plants. Infested treatments showed elevated CO_2 increased RF1 in the phloem of Jemalong, while RF1 decreased in the phloem of *dnf1* plants (Fig. 6a). Ala, Gly, Phe, Ile, Pro and Met loaded heavily and positively in RF2 (Fig. 5b). All factors, with the exception of the interaction between genotype and aphid infestation, as well as the interaction among CO_2 level, genotype and aphid infestation, had significant effects on RF2 (Table S3). The uninfested treatments showed elevated CO_2 decreased RF2 in the phloem of both Jemalong and *dnf1* plants. Infested treatments showed elevated CO_2 did not significantly affect RF2 in the phloem of both Jemalong and *dnf1* plants (Fig. 6c,d).Thr and Trp loaded heavily and positively in RF3 (Fig. 5c). The interaction between CO_2 level and aphid infestation significantly affected RF3 (Table S3). The uninfested treatments showed elevated CO_2 increased RF3 in the phloem of Jemalong. Infested treatments showed elevated CO_2 decreased RF3 in the phloem of *dnf1*

plants (Fig. 6e,f).

Amino acid concentration in pea aphids

Principal component analysis (PCA), was also used to characterize amino acids in the pea aphid by subjecting the first four principal components to factor rotation. We retained four rotated factors (RF1, RF2, RF3 and RF4 account for 76% of the total variance). Phe, Asp, Val, Ser, Ile and Pro loaded heavily and positively onto RF1 (Fig. 7a). We subjected RF1 to the split-plot ANOVA model and found factors and their interactions had no significant effects on RF1 (Table S4). Gln, Asn, Arg, Glu and Leu and Thr loaded heavily and positively in RF2 (Fig. 7b). Factors and their interaction had significant effects on RF2 (Table S4). Elevated CO_2 increased RF2 in pea aphids associated with Jemalong plants, but did not affect RF2 in pea aphids associated with *dnf1* plants (Fig. 8 c,d). Pea aphids had higher RF2 associated with Jemalong than *dnf1* under both CO_2 levels (Fig. 8 c,d). Ala, Lys, Tyr and Met loaded heavily and positively in RF3 (Fig. 7c). The interaction between CO_2 level and genotype significantly affected RF3 (Table S4). Elevated CO_2 increased RF3 in pea aphids associated with Jemalong plants, but decreased RF3 in pea aphids associated with *dnf1* plants (Fig. 8 e,f). Pea aphids had lower RF3 associated with Jemalong than *dnf1* under ambient CO_2 but had higher RF3 associated with Jemalong than *dnf1* under elevated CO_2. Cys, Met, Trp and Leu loaded heavily and positively in RF4 (Fig. 7d). Factors and their interaction significantly affected RF4 (Table S4). Elevated CO_2 decreased RF4 in pea aphids associated with *dnf1* plants (Fig. 8g,h). Pea aphids had higher RF4 associated with Jemalong compared to *dnf1*

under elevated CO_2.

Expression of genes involved in amino acid metabolism of aphid bacteriocytes

For aphids fed on Jemalong plants, genes relevant to degradation of amino acids (i.e. Henna, GCDH and GCVT) and genes relevant to synthesis of nonessential amino acids (i.e., GS and PSAT) were up-regulated by elevated CO_2 in the bacteriocytes of pea aphids. Conversely, elevated CO_2 down-regulated Henna, GCVT and GS in the bacteriocytes of aphids associated with *dnf1* plants (Fig. 9).

Discussion

The performance of herbivorous insects relies primarily on plant nutritional quality, and the bottom-up effects of host plants has been challenged by the rising atmospheric CO_2 concentration (Awmack *et al*., 2002). Although elevated CO_2 typically dilutes the N concentration for most C3 plants, different feeding guilds of herbivorous insects exhibit diverse strategies to avoid N shortage under elevated CO_2 environments (Bezemer and Jones, 1998). Here, we reported that pea aphids are able to enhance amino acid metabolism both in host plants and bacteriocytes to increase their fitness (in terms of population abundance) under elevated CO_2.

Positive effect of elevated CO_2 on plant photosynthetic rate, biomass and seed yield in Jemalong are consistent with the results from previous work in leguminous plants (Ainsworth *et al.* 2012). The nodule number, as an important index for BNF, increased in Jemalong under

elevated CO_2 (Fig. 3). Similarly to our previous study, elevated CO_2 enhanced BNF, which compensated for the N decrease in Jemalong, but had no effect on BNF in the N-fixation deficient mutant *dnf1*, resulting in decreases of N assimilation and N concentration in *dnf1* under elevated CO_2 (Guo *et al*., unpublished data). Furthermore, decreases in N metabolism leads to reduced investment in Rubisco and other C assimilation related enzymes (Gleadow *et al*., 1998), which in turn reduces photosynthesis and biomass in *dnf1*. Thus, elevated CO_2 has a contrasting influence on the bottom-up effect of nutritional quality in two *M. truncatula* genotypes, exemplified by higher aphid density on Jemalong compared to that on *dnf1*.

The mathematic model constructed by Newman (2003) predicted that aphid populations tend to be larger under elevated CO_2 if host plants have higher N supplementation, that the nitrogen requirement of aphids is low and that the density dependent response of the aphid is weak. Our results support the first prediction of the model, namely that elevated CO_2 contributes to increased population abundance and feeding efficiency of pea aphids on Jemalong plants with enhanced BNF compared to the *dnf1* mutants, which are unable to fix N_2. We speculate that, over evolutionary time, pea aphids evolved (perhaps even co-evolved) to act as specialists that feed exclusively on some species of legumes where BNF renders N to be more available to the aphids than would be available on non-leguminous hosts (also see Mabry *et al.*, 1997). Arguably, the pea aphid has evolved to depend on BNF for optimal metabolic activities involving N, and thus may be more vulnerable to N deficiencies under elevated CO_2 if the BNF system is also compromised.

Aphids possess various strategies to avoid N shortage when ingesting an imbalanced diet of phloem sap (Giordanengo *et al.*, 2010). For example, aphids maintained the quality of ingested nutrients by changing feeding sites as leaves age to feed on fresh leaves, or reducing the penetration attempts and increasing passive feeding to enhance feeding efficiency (Sun and Ge, 2010). In addition, some aphids may have adapted to alter phloem sap composition to better serve their growth requirements. *Myzus persicae* infestation on a potato plant strongly increased glutamine synthase and glutamate dehydrogenase activities, which promoted N concentration in phloem sap (Divol *et al.*, 2005). Moreover, pea aphids could ingest amino acids transferred from root nodules and leaves and also effect the export of N from sink tissues to source tissues (Girousse *et al*., 2005). Pea aphid infestation of Jemalong had no effect on total amino acids in whole leaves under ambient CO_2, but increased the concentration of total amino acid in leaves and most individual amino acids in the phloem by enhancing the enzyme activities of N transamination under elevated CO_2 (Fig. 3,4,6b). It seems that the changes of amino acid concentration can be manipulated more easily by pea aphids due to more nodules and stronger BNF under elevated CO_2.

Amino acid composition of phloem sap of Jemalong was dominated by the nonessential amino acids (70% concentration of total amino acids on average; Fig. S1). Furthermore, 7 nonessential amino acids loading onto RF1 represented an ~80% concentration of amino acids in RF1, which were significantly increased by aphid infestation under elevated CO_2. On the other hand, the amino acid profile of pea aphid associated with Jemalong revealed that

amino acids loadings onto RF2 and RF3 were significantly increased by elevated CO_2. Specifically, pea aphid infestation increased 6 amino acids (Asn, Tyr, Arg, Lys, Glu and Gln) in RF1 in the phloem under elevated CO_2, which correlated to corresponding increases of these 6 amino acids in aphids. In contrast, the 4 nonessential amino acids in RF1 of phloem sap increased by aphid infestation under elevated CO_2 were accompanied by increases of 3 essential amino acids in RF2 and RF3 in the aphids. Thus, the imbalanced increases of amino acids in Jemalong phloem sap and pea aphids strongly implicate *Buchnera* involvement in amino acid metabolism by pea aphids in response to elevated CO_2. By converting nonessential amino acids into essential amino acids, *Buchnera* provided aphids with essential amino acids needed for their growth (Nikoh *et al.*, 2010). The genome of *Buchnera* includes most genes required for essential amino acid biosynthesis, while lacking genes for amino acid degradation and the synthesis of most nonessential amino acids (Shigenobu *et al.*, 2000, Prickett *et al.*, 2006). Bacteriocytes are specialized cells in aphids for harboring *Buchnera* that provide *Buchnera* nonessential amino acids as substrate materials to synthesize essential amino acids (Nakabachi *et al.*, 2005). In the bacteriocytes, we found that genes relevant to the utilization of essential amino acids (Henna and GCDH), catabolism of nonessential amino acids (GCVT), as well as genes relevant to synthesis of nonessential amino acids (GS and PSAT) were up-regulated by elevated CO_2. It is indicated that aphids induced the increases in amino acid concentration in Jemalong plants and prolonged the passive feeding duration, which in turn provided more nonessential amino acids to *Buchnera*, and eventually allowed increased utilization of essential amino acids to maximize their fitness under elevated CO_2.

Thus, these results did not support our first hypothesis because pea aphid tend to enhance amino acid metabolism both in Jemalong plants and their endosymbiont *Buchnera* to increase population abundance.

BNF plays a key role in maintaining the growth of legumes and also regulates the interaction between legumes and herbivorous insects (Pineda *et al*., 2010). In our previous study, when BNF was artifically mutated, N concentration in *dnf1* was decreased by elevated CO_2, which was similar to outcomes observed in non-legume plants (Guo *et al.*, unpublished data). Furthermore, pea aphid infestations under elevated CO_2 reduced amino acid concentrations (RF1 and RF3; 7 nonessential and 6 essential amino acids) in *dnf1* plants by decreasing enzyme activities of N assimilation and transamination. Our second hypothesis proposed: pea aphids would rely more on *Buchnera* when exposed to conditions of decreased N nutrition in *dnf1* plants under elevated CO_2. Unexpectedly, elevated CO_2 down-regulated expression of henna, GCVT and GS genes of aphids fed on the *dnf1* mutant. Amino acid metabolism in aphid bacteriocytes depends largely on ingestion of nonessential amino acids from the host plant (Hansen and Moran, 2011). Thus, concentrations of 7 amino acids in pea aphids associated with *dnf1* that were decreased under elevated CO_2 may be due to decreased amino acid substrate in phloem sap that could not support *Buchnera* converting nonessential amino acids into essential amino acids (Fig.8, S2). Pea aphid infestation of the *dnf1* mutant showed that BNF played an important role in maintaining the nutritional condition of host plant and affected the downstream amino acid metabolism between aphids and *Buchnera* under elevated CO_2. In spite of this, our *dnf1* results inadequately explain why some aphids

are still successful under elevated CO_2 when fed on non-legume plants. Thus, a general explanation for the species-specific responses of aphids to elevated CO_2 remains to be elucidated.

In summary, elevated CO_2 has the potential to increase pea aphid density on legume plants via bottom-up effects on nutritional quality, and a functional BNF system is crucial for obtaining this positive response. Results show that pea aphids are able to manipulate amino acid metabolism both in host plants and their bacteriocytes under elevated CO_2 when enhanced BNF is present. This study has generated several significant findings: First, our results support the view that amino acids imbalances increased under elevated CO_2 through the legume/aphid interaction, which also involved the aphid endosymbionts. Second, BNF provides increased N nutrition, especially via nonessential amino acids, to pea aphids, which enhances their population growth under elevated CO_2. Finally and perhaps most importantly, the results suggest that legumes may suffer greater damage from aphids if atmospheric CO_2 levels continue to increase. More research is needed to further elucidate the mechanisms underlying the synergistic or antagonistic effects of elevated CO_2 and aphids on plant N metabolism, and the role of *Buchnera* involved in the aphid adapation to future atmospheric environments.

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Figure legends

Fig. 1 Population abundance and feeding behavior of pea aphids when fed on wild-type Jemalong and N-fixing-deficient mutants *dnf1* grown under ambient CO_2 (ACO_2) and elevated CO_2 (ECO_2). (a) Population abundance (number per plant). Each value represents the average (±SE) of four replicates. Significant differences at $P<0.05$ are shown as *. (b) The percentage of time pea aphid spent in various activities during 8h. "Nonpenetration" indicates that stylets are outside the plant. "Pathway" indicates mostly intramural probing

activities between mesophyll or parenchyma cells. “Salivation” indicates that aphids are injecting watery saliva into the sieve element. “Phloem ingestion” indicates that aphids are ingesting the phloem sap. “Xylem” indicates stylet penetration of tracheary elements. “Derailed stylet” indicates penetration difficulties of stylets. Values are the mean (±SE) of 24 biological replicates. Means labeled with different lowercase letters are significantly different among the combinations of genotype and CO_2 levels (Tukey’s multiple range test; $P < 0.05$).

Fig. 2 Growth traits of two *M. truncatula* genotypes (Jemalong and *dnf1*) grown under ambient CO_2 (ACO_2) and elevated CO_2 (ECO_2) without pea aphid infestation. (a) Photosynthetic rate, (b) Chlorophyll content, (c) Nodule numbers per plant, (d) Biomass, (e) Pod numbers. Each value represents the average (±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 $P<0.05$.

Fig. 3 Enzyme activities involved in N metabolism of two *M. truncatula* genotypes grown under ambient CO_2 (ACO_2) and elevated CO_2 (ECO_2) with and without pea aphid infestation. (a) Glutamine synthetase (GS); (b) Glutamate synthase (GOGAT); (c) Glutamate oxalate transaminase (GOT); (d) Glutamine phenylpyruvate transaminase (GPT). Each value represents the average (±SE) of four replicates.

Fig. 4 Total amino acids in whole leaves of two *M. truncatula* genotypes grown under ambient CO_2 (ACO_2) and elevated CO_2 (ECO_2) with and without pea aphid infestation. Each value represents the average (±SE) of four replicates. Different lowercase letters indicate significant differences among the combinations of genotype and CO_2 levels within the same treatment by pea aphid. Different uppercase letters indicate significant differences between uninfested and infested plants within the same CO_2 treatment and aphid treatment as determined by Tukey's multiple range test at $P<0.05$.

Fig.5 The loadings for each individual amino acid of plant phloem sap onto the first three rotated factors. The individual amino acids loading heavily either positively (loading$\geqslant$0.5) or negatively (loading$\leqslant$-0.5) are highlighted in black. (a) Rotated factor 1; (b) Rotated factor 2; (c) Rotated factor 3. These multivariate responses can be interpreted as increasing as the positively loading variables increase and decreasing as the negatively loading variables increase.

Fig. 6 The mean response of rotated factors (a, c, e) and the standardized univariate response (b, d, f) of individual amino acids in plant phloem sap to CO_2 levels, genotypes, pea aphid infestation and their interactions. Different lowercase letters indicate significant differences among the combinations of aphid treatment and CO_2 levels within the same genotype. Different uppercase letters indicate significant differences between genotypes within the same CO_2 treatment and aphid treatment as determined by Tukey's multiple range test at

P<0.05. The underlined individual amino acids indicate these are essential amino acids for aphids.

Fig. 7 The loadings for each individual amino acid of pea aphid onto the first four rotated factors. The individual amino acids loading heavily either positively (loading$\geqslant$0.5) or negatively (loading$\leqslant$-0.5) are highlighted in black. (a) Rotated factor 1; (b) Rotated factor 2; (c) Rotated factor 3; (d) Rotated factor 4. These multivariate responses can be interpreted as increasing as the positively loading variables increase and decreasing as the negatively loading variables increase.

Fig. 8 The mean response of rotated factors (a, c, e, g) and the standardized univariate response (b, d, f, h) of individual amino acids in pea aphid to CO_2 levels, genotypes and their interaction. 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. The underlined individual amino acids indicate these are essential amino acids for aphids.

Fig. 9 Expression of genes of aphid involved in amino acid metabolism in the bacteriocyte (Henna, GCVT, GCDH, GS, PSAT) as affected by CO_2 level (ACO_2 vs. ECO_2) and plant genotype: Jemalong (J) vs. *dnf1* mutant (D). Values indicate fold-change in expression based

on qPCR determination, and each value represents the average (±SE) of four replicates. An asterisk above a column indicates a significant difference in gene expression under ambient vs. elevated CO_2 ($P < 0.05$).