Elevated CO_2 Modifies N Acquisition of *Medicago truncatula* by Enhancing N Fixation and Reducing Nitrate Uptake from Soil

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Abstract

The effects of elevated CO_2 (750 ppm vs. 390 ppm) were evaluated on nitrogen (N) acquisition and assimilation by three *Medicago truncatula* genotypes, including two N-fixing-deficient mutants (*dnf1-1* and *dnf1-2*) and their wild-type (Jemalong). The proportion of N acquisition from atmosphere and soil were quantified by ^{15}N stable isotope, and N transportation and assimilation-related genes and enzymes were determined by qPCR and biochemical analysis. Elevated CO_2 decreased nitrate uptake from soil in all three plant genotypes by down-regulating nitrate reductase (*NR*), nitrate transporter *NRT1.1* and NR activity. Jemalong plant, however, produced more nodules, up-regulated N-fixation-related genes and enhanced percentage of N derived from fixation (%Ndf) to increase foliar N concentration and N content in whole plant (Ntotal Yield) to satisfy the requirement of larger biomass under elevated CO_2. In contrast, both *dnf1* mutants deficient in N fixation consequently decreased activity of glutamine synthetase/glutamate synthase (GS/GOGAT) and N concentration under elevated CO_2. Our results suggest that elevated CO_2 is likely to modify N acquisition of *M. truncatula* by simultaneously increasing N fixation and reducing nitrate uptake from soil. We propose that elevated CO_2 causes legumes to rely more on N fixation than on N uptake from soil to satisfy N requirements.

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Introduction

Global atmospheric CO_2 concentrations have been increasing at an accelerating rate [1]. The concentration, which was 280 ppm before industrialization and was 394 ppm in December 2012 (Mauna Loa Observatory: NOAA-ESRL), is expected to reach at least 550 ppm by the year 2050 [1]. The effects of elevated CO_2 on C3 plants are generally characterized by increased photosynthesis, growth and yield in plant tissues [2]. Under elevated CO_2, the "extra C" is assimilated and transported from leaves and shoots to roots, and the C:N ratio is consequently increased [3]. Thus, plant responses to elevated CO_2 are likely to be limited by the availability of N.

Besides increases in biomass and productivity, a common characteristic of non-leguminous C3 plants in an elevated CO_2 environment is a 10–15% decrease in N concentration (g of N per g of plant tissue) [4]. Three major hypotheses have been proposed to explain this phenomenon [5]. According to the reduced uptake hypothesis, N content is reduced because decreased stomatal conductance and transpiration under elevated CO_2 reduces N uptake by roots [6]. The N loss hypothesis presumes that N losses increase under elevated CO_2 because of increasing NH_3 volatilization or increasing root exudation of organic N [7]. The dilution hypothesis, which has received the most attention, considers that N content is diluted under elevated CO_2 by accumulation of more total non-structural carbohydrates (TNC), which results in a greater biomass for a given quantity of N [8]. Depending on the species or genotype, these hypotheses may partially or largely explain the substantial reduction in the N content in non-leguminous plants under elevated CO_2 [9]. Furthermore, elevated CO_2 has little effect on the N content in legumes, which might be attributed to their unique ability to utilize atmospheric N_2 [4], but it still lacks the experimental evidence to address the physiological mechanism underlying N metabolism of legume plants under elevated CO_2.

Leguminous plants acquire N by three major pathways. First, legumes uptake ammonia (NH_4^+) from soil and incorporate it into organic compounds. Second, legumes uptake nitrate from soil and reduce it to NH_4^+. Third, legumes in symbioses with N-fixing bacteria can obtain N from the atmosphere by N fixation, i.e., by converting N_2 to NH_4^+ [10]. Among these three pathways, N fixation is most costly in terms of energy and resources. LaRue and Patterson (1981), for example, found that four legumes including *Glycine max*, *Vigna unguiculata*, *Phaseolus vulgaris*, and *Pisum sativum*, consume an average of 6.7 g of carbohydrate to obtain 1 g of N by symbiosis [11]. Acquiring N via uptake of nitrate or ammonia from soil required less carbohydrate C than acquiring N by symbiosis [12,13]. Nitrogenase activity, the most important

enzyme involved in N fixation, and nodule formation are often suppressed when nitrate or ammonia availability is sufficient to meet the requirements of plant growth [14]. Thus, it seems that legume plants preferentially obtain N via uptake from the soil rather than fixation from the atmosphere [13].

To sustain and maximize growth and biomass under elevated CO_2, legumes require additional N [8]. Owing to the high C consumption required for N fixation, elevated CO_2 helps legumes fix N from atmosphere [15]. After reviewing 127 studies, Lam *et al.*, (2012) concluded that the amount of N fixed from the atmosphere by legumes increased 38% under elevated CO_2, which was accompanied by increases in whole plant nodule number (+33%), nodule mass (+39%), and nitrogenase activity (+37%) [16]. Furthermore, enhancement of N fixation in legumes is essential for overcoming the N limitation under elevated CO_2 [17]. However, the relative contributions of N fixation and uptake from soil to the N content of legumes under elevated CO_2 are largely unknown. It is likely that legumes adjust their means of utilizing N resources to adapt to environmental changes [18], and a CO_2-enriched environment may affect the crosstalk between the different N acquisition pathways in legumes.

The current study examined N acquisition via N fixation and N uptake in N-fixing-deficient mutants *(dnf1)* and wild-type (Jemalong) of *M. truncatula*. We tested the hypothesis that *M. truncatula* plants regulate the relative contribution of N fixation and N uptake from soil to maximum the N assimilation rate to satisfy the higher N requirement under elevated CO_2. The specific objectives were to determine: (1) how elevated CO_2 affects N fixation from the atmosphere and N uptake from soil; and (2) whether elevated CO_2 affects N assimilation of the *M. truncatula* genotypes. To help meet these objectives, we measured the expression of key genes and the activity of key enzymes involved in N acquisition and assimilation (glutamine synthase/glutamate synthase, GS/GOGAT cycle) [19]. Meanwhile, ^{15}N stable isotope technique was used to determine N acquisition and partitioning, and estimate the proportion of N fixed from atmosphere/N uptake from soil [20].

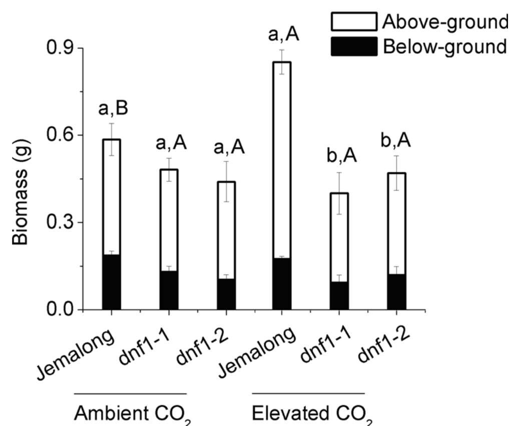

Figure 1. Above- and below-ground biomass of *M. truncatula* plants as affected by CO_2 level and plant genotype: *dnf1-1* and *dnf1-2* are deficient in N fixation, and Jemalong is their wild type. Each value represents the average (±SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences among genotypes within the same CO_2 treatment as determined by Tukey's multiple range test at $P<0.05$.
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Materials and Methods

Atmospheric CO_2 Concentration Treatments

This experiment was performed in eight octagonal open-top field chambers (OTCs) (4.2 m diameter and 2.4 m height) at the Observation Station of the Global Change Biol Group, Institute of Zoology, Chinese Academy of Science in Xiaotangshan County, Beijing, China (40°11′N, 116°24′E). The atmospheric CO_2 concentration treatments were: (1) current atmospheric CO_2 levels (390 μl/L), and (2) elevated CO_2 levels (750 μl/L, the predicted level in about 100 years) (IPCC, 2007). Four blocks were used, and each block contained one OTC with ambient CO_2 and one with elevated CO_2. From seedling emergence to the harvesting of *M. truncatula* plants (27 August to 15 October 2011, a total of 50 days), CO_2 concentrations were 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 391±23 ppm in the ambient CO_2 chambers and 743±32 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 and Ge (2005) [21]. 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 (24.9±3.4°C in OTCs with ambient CO_2 vs. 26.2±3.9°C in OTCs with elevated CO_2).

M. Truncatula Mutants and Rhizobium Inoculation

Three *M. truncatula* genotypes were studied: the N-fixation-deficient mutants *dnf1-1* and *dnf1-2* as well as their wild-type Jemalong. These three genotypes were obtained from the laboratory of Sharon Long, Department of Biology, Stanford University. The nodules of these *dnf1* mutants are small and white and are blocked at an intermediate stage of development [22]. The *dnf1-1* mutant allele has a large deletion of at least 20 kb around TC121074 locus, and the *dnf1-2* mutant allele has an independent disruption of the TC121074 locus [20]. Although both mutants can be infected in the inner cortex, both lack acetylene reduction activity and *Nodulin31* expression and have only a small level of *nifH* expression in the symbiotic nodule [23].

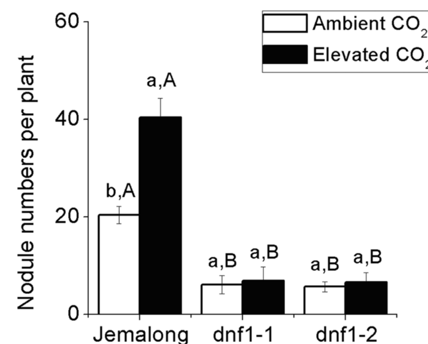

Figure 2. Nodule number per root of *M. truncatula* plant as affected by CO_2 level and plant genotype: *dnf1-1* and *dnf1-2* are deficient in N fixation, and Jemalong is their wild type. Each value represents the average (±SE) of four replications. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences among *M.* genotypes within the same CO_2 treatment as determined by Tukey's multiple range test at $P<0.05$.
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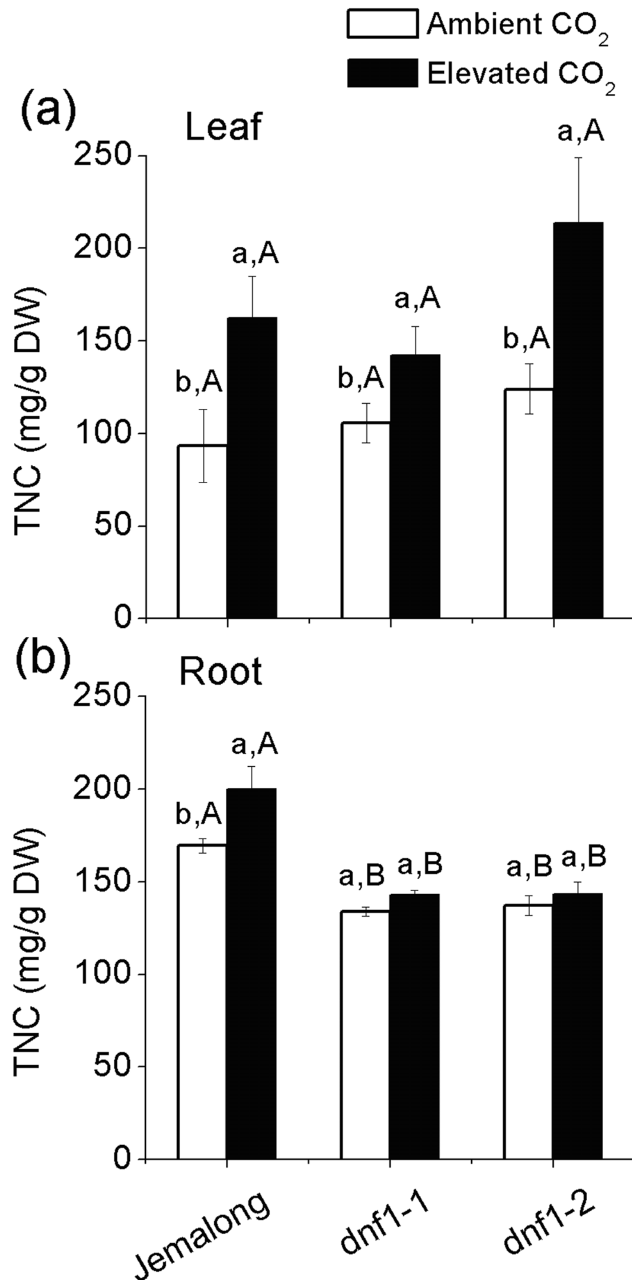

Figure 3. Total non-structural carbohydrate (TNC) content in leaves and roots of *M. truncatula* plants as affected by CO_2 level and plant genotype: *dnf1-1* and *dnf1-2* are deficient in N fixation, and Jemalong is their wild type. Each value represents the average (±SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences among genotypes with the same CO_2 treatment as determined by Tukey's multiple range test at $P<0.05$.
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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* Rm1021 [23], which was kindly 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 August 2011. Each OTC contained 30 plants (10 each per genotype) with 240 plants in total.

Plants were maintained in the OTCs for 50 days. Pot placement was re-randomized within each OTC once every week to avoid any effects from the position of pots in each OTC. No chemical fertilizers and insecticides were used. Water was added to each pot once every 2 days.

Plant Sampling and Preparation

All the plants of *M. truncatula* were randomly harvested on 13–15 October 2011. Root of each plant were carefully removed from soil and washed. A stereomicroscope was used to count the nodules on the entire root system of 6 plants from each *M. truncatula* genotype per OTC (= 24 plants from each genotype at each CO_2 level and 144 in total). After nodules were counted, the shoots and roots of each plant were collected, oven-dried (65°C) for 72 h, and weighed. The leaves and root tissues were then ground to a fine powder (approx. 0.85 mm size) and analyzed for total non-structural carbohydrates (TNCs), N concentration and ^{15}N isotopic analysis. Another three plants from each *M. truncatula* genotype per OTC (9 plants per OTC and 72 plants in total) were randomly selected for enzyme analysis and real-time PCR. 50 mg of mature leaves and 100 mg of lateral roots from each plant were stored in freezing tubes at −75°C until used for real-time PCR. 0.5 g of mature leaves and 1.0 g of lateral roots from the same plants were frozen for enzyme analysis as described in the following paragraph.

TNCs, N Concentration and $\delta^{15}N$ Analysis

TNCs, mainly starch and sugars, in leaves and roots were quantified by acid hydrolysis following the method of Tissue & Wright (1995). N concentrations in leaves and roots were measured by Kjeltec N analysis (Foss automated Kjeltec™ instruments, Model 2100) [24].

$\delta^{15}N$ were determined from approximately 3 mg plant sample with an isotope-ratio mass spectrometer (IRMS; Delta^plus XP and Delta C prototype Finnigan MAT, respectively, Finnigan MAT, Bremen, Germany; 0.1‰ precision). The $\delta^{15}N$ values represent nitrogen isotopic composition of the sample relative to that of atmospheric dinitrogen in ‰:

$$\delta^{15}N_{sample} = \left(R_{sample} - R_{standard}\right)/R_{standard} \times 100$$

Where $R_{standard}$ is the $^{15}N/^{14}N$ ratio of atmospheric N_2 and R_{sample} is the $^{15}N/^{14}N$ ratio of the sample plant. The repeated measurement precision was 0.2‰.

Percentage of N derived from atmosphere and N uptake from soil

Percentage of N fixed from atmosphere is a yield-independent parameter and was calculated according to Pausch *et al.*, (1996) [25]:

Table 1. CO_2 effects on N characteristics (%Ndf, Nf Yield, Ns Yield) of wild-type Jemalong plant.

	Jemalong		
	%Ndf[1]	Nf Yield[2] (mg/plant)	Ns Yield[3] (mg/plant)
Ambient CO_2 (390 ppm)	39.8±0.7 b	9.1±0.7 b	13.8±0.4 a
Elevated CO_2 (750 ppm)	65.3±1.2 a	25.3±1.1 a	11.5±1.0 b

Each value represents the average (±SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 as determined by independent *t*-test at $P<0.05$.
[1]percentage of N derived from atmosphere.
[2]N derived from N-fixation per plant.
[3]N derived from soil per plant.
doi:10.1371/journal.pone.0081373.t001

$$\%\mathrm{Ndf} = \left(1-\delta^{15}\mathrm{N}_{M.truncatula}/\delta^{15}\mathrm{N}_{reference}\right)\times 100$$

Where % Ndf is the percentage of N derived from atmosphere. $\delta^{15}N_{M.\ truncatula}$ is the $\delta^{15}N$ of wild-type Jemalong, $\delta^{15}N_{reference}$ is the average value of $\delta^{15}N$ of *dnf1-1* and *dnf1-2* in the same OTC. *dnf1-1* and *dnf1-2* have similar N uptake and rooting patterns as Jemalong but are deficient in nitrogen fixation, and therefore served as the reference plant for analyzing the N fixation of wild-type *M. truncatula*.

In order to evaluate changes in N source (i.e. as derived from N-fixation or soil) for *M. truncatula* plants, Nf Yield (N derived from N-fixation per plant) and Ns Yield (N derived from soil per plant) of Jemalong estimates were calculated as follows:

$$\mathrm{NtatalYield(mg/plant)} = \mathrm{Biomass}_{\text{above-ground}}\times \mathrm{N}_{\text{leaf}} + \mathrm{Biomass}_{\text{under-ground}}\times \mathrm{N}_{\text{root}}$$

$$\mathrm{NfYield(mg/plant)} = \mathrm{NtotalYield}\times\%\mathrm{Ndf}$$

$$\mathrm{NsYield(mg/plant)} = \mathrm{NtotalYield} - \mathrm{NfYield}$$

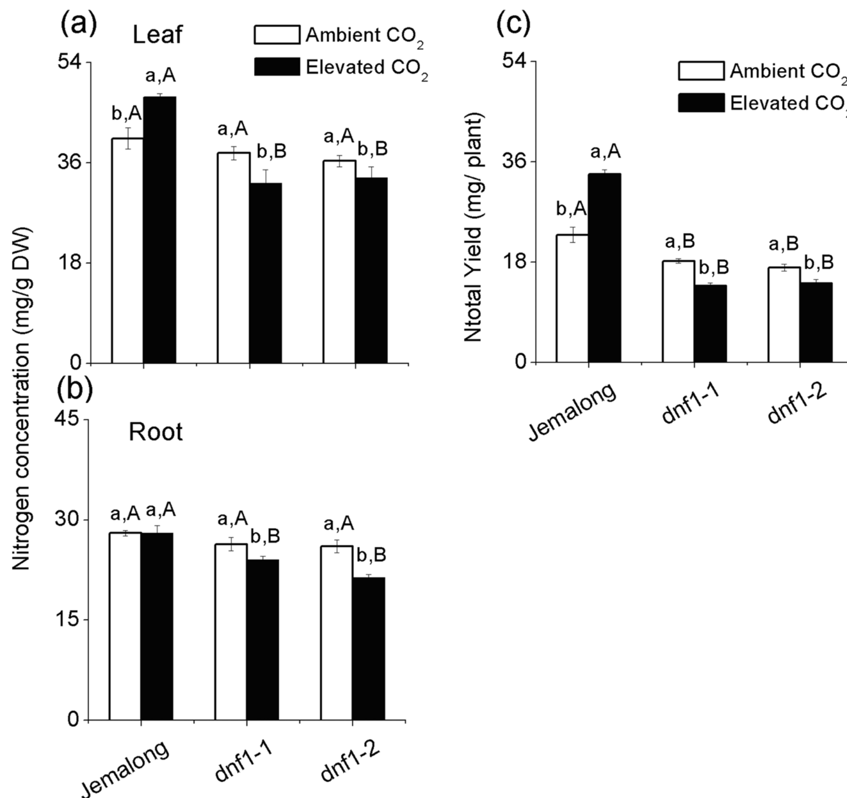

Figure 4. N concentrations in leaves and roots as well as Ntotal Yield (N content in per plant) of *M. truncatula* plants as affected by CO_2 level and plant genotype: *dnf1-1* and *dnf1-2* are deficient in N fixation, and Jemalong is their wild type. Each value represents the average (±SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO_2 and elevated CO_2 within the same genotype. Different uppercase letters indicate significant differences among genotypes within the same CO_2 treatment as determined by Tukey's multiple range test at $P<0.05$.
doi:10.1371/journal.pone.0081373.g004

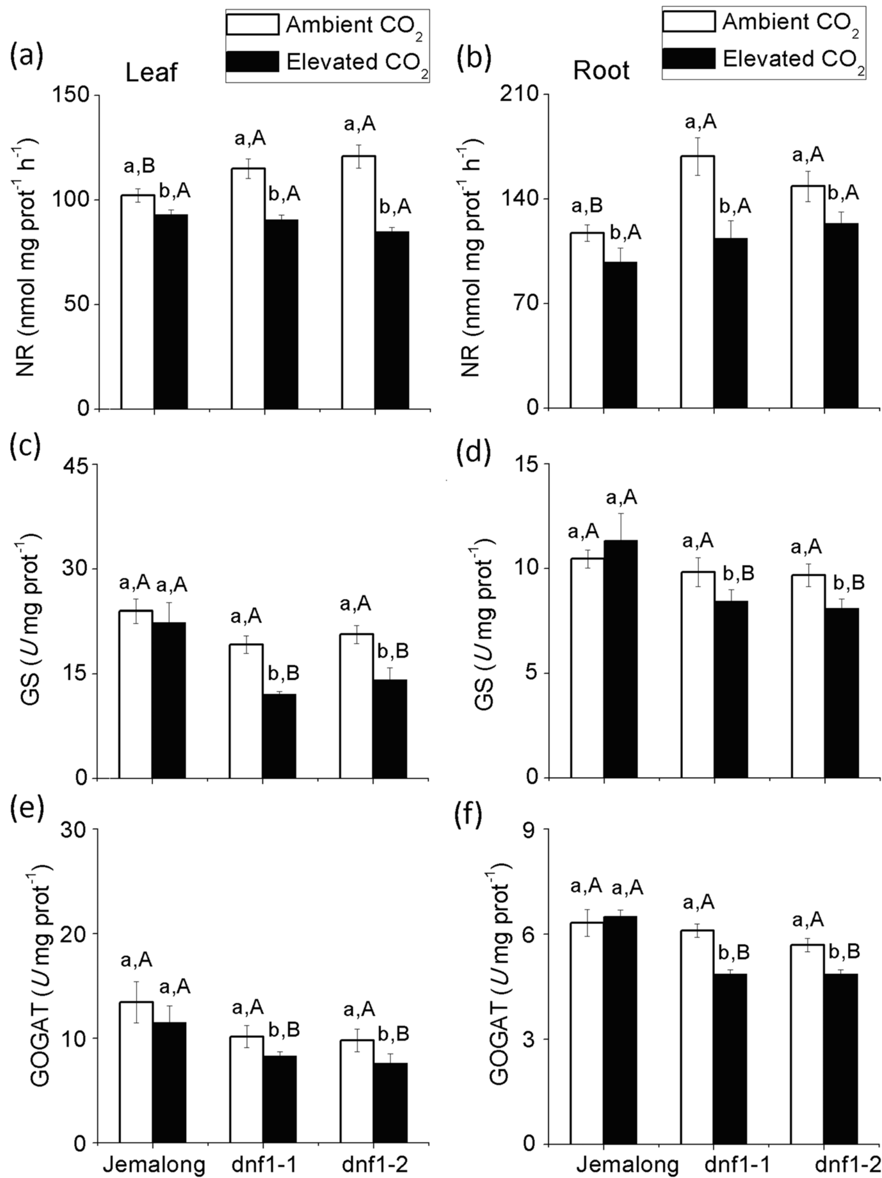

Figure 5. Activities of the enzymes involved in N reduction (NR) and in N assimilation (GS and GOGAT) in the leaves and roots of *M. truncatula* plants as affected by CO_2 level and plant genotype: *dnf1-1* and *dnf1-2* are deficient in N fixation, and Jemalong is their wild type. Each value represents 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 among genotypes within the same CO_2 treatment as determined by Tukey's multiple range test at $P<0.05$.
doi:10.1371/journal.pone.0081373.g005

Where Ntotal Yield is the N content in per whole plant, $Biomass_{above-ground}$ is the biomass of above-ground tissue in *M. truncatula* plants, $Biomass_{under-ground}$ is the biomass of under-ground tissue, N_{leaf} is the N concentration of leaves and N_{root} is the N concentration of root.

Activities of Enzymes Involved in N Uptake and Assimilation

The activities of nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthase (GOGAT) in leaves and roots were determined using frozen tissue (approximately 0.5 g leaf tissue and approximately 1.0 g root tissue per plant). Once the tissue was ground to a fine powder, leaves or roots from three plants of the same genotype within each OTC 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 or root 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 enzyme activities were measured in the supernatant as described by Geiger *et al.* (1998) for NR [26], by Glévarec *et al.* (2004) for GS [27], and by Suzuki *et al.* (2001) for GOGAT [28]. Protein concentrations of leaves and roots were measured using bovine serum albumin as a standard. One unit (*U*) of GS/GOGAT activities are defined as the amount of the GS or

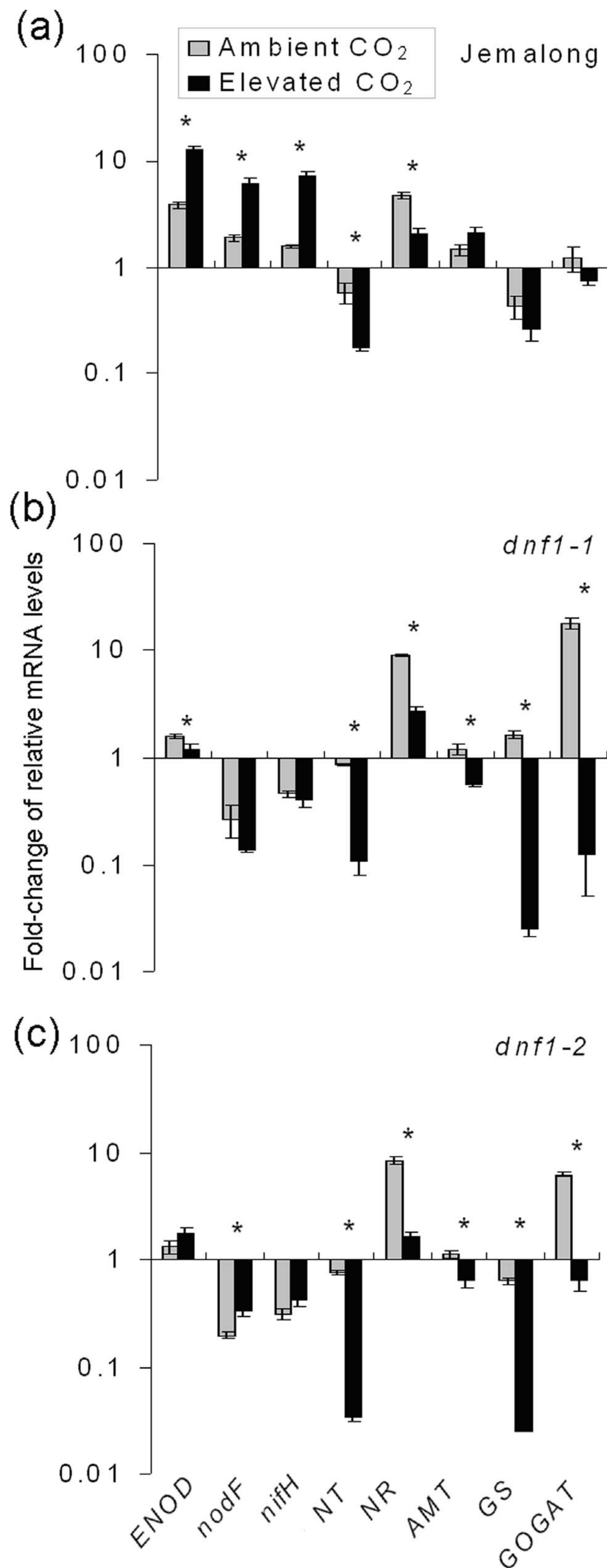

Figure 6. Expression of genes involved in N fixation (*ENOD*, *nodF*, and *nifH*), nitrate transportation and reduction (*NT* and *NR*), ammonium uptake (*AMT*), and N assimilation (*GS* and *GOGAT*) in leaves of *M. truncatula* plants as affected by CO_2 level and plant genotype: *dnf1-1* and *dnf1-2* are deficient in N fixation, and Jemalong is their wild type. 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 ($P<0.05$).
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GOGAT that catalyzes 1 nmol of glutamine or glutamate per minute in the homogenate.

Expression of Genes Associated with N Fixation, Uptake, and Assimilation as Determined by Quantitative RT-PCR

Each treatment combination was replicated four times for biological repeats, and each biological repeat contained three technical repeats. The RNAeasy Mini Kit (Qiagen) was used to isolate total RNAs from *M. truncatula* leaves and roots, and 1 μg of RNA was used to generate the cDNAs. The mRNAs of the following nine target genes were quantified by real-time quantitative PCR: early nodule-specific protein 40 (*ENOD*) (maintenance of nodule symbiosis) [29], nodulation gene (*nodF*) (*nodF* genes are required for nodulation) [29], nitrogen-fixing gene (*nifH*) (*nifH* genes control synthesis of nitrogenase) [30], nitrate transporter *NRT1.1* (*NT*) [31], nitrate reductase (*NR*), ammonium transporter protein (*AMT*) [32], glutamine synthetase 2 (*GS*) [33], and glutamate synthetase (*GOGAT*) [33] (Figure S1 in File S3). Specific primers for each gene were designed from the *M. truncatula* EST sequences using PRIMER5 software (Table S1 in File S1). The PCR reactions were 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) 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. This PCR protocol produced the melting curves, which can be used to judge the specificity of PCR products. A standard curve was derived from the serial dilutions to quantify the copy numbers of target mRNAs. *β*-actin and *pnp* were used as internal qPCR standards for the analysis of plant and bacterial gene expression, respectively [29]. The relative level of each target gene was standardized by comparing the copy numbers of target mRNA with copy numbers of *β*-actin or *pnp* (the house-keeping gene), which remain constant under different treatment conditions. The levels of *β*-actin or *pnp* mRNAs in the control were examined in every PCR plate to eliminate systematic error. The fold-changes of target genes were calculated using the $2^{-\Delta\Delta Ct}$ normalization method.

Statistical Analysis

Statistical analyses were performed with SPSS 13.0 software (SPSS Inc., Chicago, IL). Two-way analyses of variance (ANOVA) were used to analyze the effect of CO_2 and plant genotype on *M. truncatula* growth traits, TNC, N concentration, Ntotal Yield and enzyme activities. If an ANOVA was significant, Tukey's multiple range test was used for mean separation ($P<0.05$). Significance of the effect of CO_2 on %Ndf, Nf Yield, Ns Yield of Jemlaong and genes regulating N metabolism were determined by independent *t*-tests.

Results

Plant Biomass and Nodule Number

CO_2 level, genotype and their interaction significantly affected the above-ground biomass, below-ground biomass and total biomass (Table S2 in File S2). Total biomass did not

significantly differ among the genotypes under ambient CO_2 but was greater for the wild-type Jemalong than for the mutants under elevated CO_2 (Fig. 1). In response to elevated CO_2, above-ground biomass increased 37.1% and total biomass increased 41.9% for Jemalong plants but the biomass of *dnf1* mutant plants was not significantly affected by the CO_2 treatments (Fig. 1). CO_2 level and genotype significantly affected the nodule numbers (Table S2). Regardless of CO_2 level, nodule number was greater for Jemalong than for the *dnf1* mutants (Fig. 2). Elevated CO_2 increased nodule numbers of Jemalong but not of the mutants.

TNC and N Characteristic in Plant

CO_2 level significantly affected the foliar TNC, and all factors significantly affected the root TNC (Table S2). Elevated CO_2 increased the TNC content in leaves and roots of Jemalong but only in leaves of *dnf1-1* and *dnf1-2* (Fig. 3). Foliar TNC content did not differ among the three *M. truncatula* genotypes (Fig. 3). Regardless of CO_2 level, Jemalong had the highest root TNC content (Fig. 3).

Genotype was significant for the foliar N concentration, and all factors significantly affected the root N concentration and Ntotal Yield (Table S2). Elevated CO_2 increased the foliar N concentration and Ntotal Yield in Jemalong but reduced in both *dnf1* mutants (Fig. 4). Elevated CO_2 reduced N concentration in the roots of both *dnf1* mutants but not in Jemalong (Fig. 4). Under ambient CO_2, foliar N and root N concentrations were not significantly different among three genotypes. Under elevated CO_2, however, N concentration in leaves and roots were higher in Jemalong than in the mutants (Fig. 4). Regardless of CO_2 level, Jemalong had higher Ntotal Yield than *dnf1-1* and *dnf1-2* mutants (Fig. 4). Furthermore, elevated CO_2 increased %Ndf and Nf Yield but decreased Ns Yield of Jemalong (Table 1).

Activities of the Enzymes NR, GS and GOGAT

CO_2 level was significant for the activities of foliar NR and root NR. (Table S2). Elevated CO_2 reduced NR activity in the leaves and roots of all three genotypes (Table S2, Fig. 5). Under ambient CO_2, NR activity in both leaves and roots were higher in both *dnf1* mutants than in Jemalong (Fig. 5). Under elevated CO_2, however, NR activity did not differ among the three genotypes (Fig. 5).

Genotype and the interaction between CO_2 and genotype significantly affected the foliar GS and root GS. All factors significantly affected the foliar GOGAT and root GOGAT (Table S2). Elevated CO_2 decreased GS and GOGAT activities in the two *dnf1* mutants but not in Jemalong (Fig. 5). GS and GOGAT activities in Jemalong leaves and GOGAT in roots were higher than *dnf1-1* and *dnf1-2* mutant in both CO_2 levels. GS activity in roots did not differ among the three genotypes under ambient CO_2 but were higher in Jemalong than in the mutants under elevated CO_2 (Fig. 5).

Expression of Genes Associated with N Fixation, Uptake, and Assimilation as Determined by Quantitative RT-PCR

Elevated CO_2 up-regulated the expression of N fixation related genes including *ENOD*, *nodF*, and *nifH*, but down-regulated the expression of nitrate uptake and transport related genes including *NR* and *NT* in Jemalong plants (Fig. 6). For *dnf1-1* and *dnf1-2*, elevated CO_2 down-regulated the gene expression of *NR* and *NT*, and ammonia transport related genes *AMT*, and N assimilation related gene including *GS* and *GOGAT* (Fig. 6).

Discussion

The notion that elevated CO_2 can increase plant biomass and TNC content in plant tissues is widely accepted [8]. Although this concept was further supported by the current report, our results also indicate that the key element in the increase in biomass of *M. truncatula* under elevated CO_2 is the availability of N (Fig. S2). Using *dnf1-1* and *dnf 2* mutants, we demonstrated that *M. truncatula* is able to adjust different N partitioning pathways to ensure a sufficient N supply under ambient CO_2. Elevated CO_2, however, reduced N uptake from soil by suppressing N uptake related gene and increased the reliance on fixation of atmospheric N_2.

N availability is one of the key factors limiting plant growth and production. Elevated CO_2 stimulating plant growth would increase the N demand of plants [34]. The extent of the CO_2 response at the plant level could consequently be limited by N availability [35,36]. In current study, since elevated CO_2 increased foliar N concentration and Ntotal yield (Table 1; Fig. 4), Jemalong plants were able to produce more biomass under elevated CO_2 (Fig. 1). Moreover, although TNC content in leaves and roots of *dnf1-1* and *dnf1-2* mutants were increased (Fig. 3), N concentration in leaves and roots as well as the Ntotal yield of *dnf1* mutant plants were decreased by elevated CO_2 (Table 1; Fig. 4). It may suggest that *dnf1* mutants were unable to provide sufficient N to support the enhancement of biomass under elevated CO_2 (Fig. 1). Thus, our results demonstrated that the symbiotic N_2 fixation provided legumes an incomparable advantage in producing larger amounts of biomass under elevated CO_2 [37].

The results of the current study show that legumes are very flexible in their utilization of N from soil and atmosphere under ambient CO_2. Although the *dnf1* mutants are unable to fix atmospheric N_2, GS/GOGAT activities involved in N assimilation and N concentration in leaves and roots did not differ from those of the wild-type Jemalong under ambient CO_2. As indicated by increased gene expression (*NR*, *NT*) and enzyme activities (NR) of essential components of the alternate N acquisition pathways (Table 1; Fig. 5, 6), the *dnf1* mutants compensated for the loss of N fixation by enhancing their uptake of N from soil under ambient CO_2. However, the GS/GOGAT activities and N concentration in *dnf1* plants were lower than in Jemalong plants under elevated CO_2 (Fig. 4, 5), which indicated that elevated CO_2 limited the N availability for both *dnf1* mutants. Furthermore, Lüscher *et al.* (2000) found even in the high soil N treatment, ineffectively nodulating lucerne were unable to increase the N concentration and biomass under elevated CO_2 [38]. Our results confirmed that soil N is insufficient to meet the increasing N demand of *M. truncatula* which can fully transform increased C assimilation into biomass [39].

The soil N availability appears to be suppressed by elevated CO_2 for all three *M. truncatula* genotypes, as reflected in decreased Ns Yield in Jemalong and Ntotal Yield in both *dnf1* mutants under elevated CO_2. Furthermore, the enzyme activity of NR and the expression of *NT* and *NR* genes of all three genotypes were also down-regulated by elevated CO_2 (Fig. 5). This indicates that elevated CO_2 suppresses N uptake of *M. truncatula* from soil. This is consistent with the the finding that N uptake from soil by *Trifolium repens* were decreased under elevated CO_2 grown in a grassland ecosystem [40]. In addition, our results showed that elevated CO_2 down-regulated *NR* and *NT* but was not significant for ammonia transporter *AMT* (Fig. 6). It seems that the decreases of N uptake from soil were mainly associated with the decreases of nitrate uptake rather than ammonia uptake.

The decreased nitrate uptake under elevated CO_2 could be explained by two factors: lower soil N availability and plant NO_3^-

reduction. Elevated CO_2 reduced the soil N availability by increasing N immobilization and denitrification in soil. For example, elevated CO_2 increased microbial community composition in rhizosphere soil of white clover, and subsequently increased N immobilization into the expanded microbial biomass [41]. Additionally, elevated CO_2 increased the emission of N_2O from soil [42], and this increase of N loss caused decreases of nitrate availability in soil. On the other hand, lower plant photorespiration induced by elevated CO_2 could decrease the nicotinamide adenine dinucleotide (NADH) [43], which provides the energy required to convert NO_3^- to NO_2^- in the cytoplasm of leaf mesophyll cells [44]. Moreover, elevated CO_2 increased HCO_3^-, and in turn inhibited NO_2^- transportation from cytosol into the chloroplast [45], which led to a decrease in plant nitrate reduction.

Insufficient soil N uptake was considered to be one of the reasons for the increased contribution of N_2 fixation under elevated CO_2 [39]. In agreement with higher foliar N concentration and Ntotal Yield in the Jemalong plants, there was a strong increase in the %Ndf, nodule numbers and up-regulation of N fixation-related genes (ENOD, *nodF* and *nifH*) under elevated CO_2. Furthermore, elevated CO_2 decreased N concentration and Ntotal Yield in both *dnf1* mutants, suggesting that the increased N concentration and Ntotal Yield in Jemalong was solely the result of elevated CO_2-induced increases of N_2 fixation. In addition, the fixation of N_2 required substantial amount of C resource, and the respiration measurements showed the costs of C for N assimilation from nitrate seem to be lower than those for N_2 fixation [14]. Elevated CO_2, however, provided the sufficient C to satisfy the energy demand for N_2 fixation, and decreased soil N availability under elevated CO_2 accelerated N_2 fixation in Jemalong plants [46].

Although elevated CO_2 tends to increase the N concentration and modify N acquisition patterns of legumes, there is little evidence that elevated CO_2 can affect the key enzymes involved in N assimilation [19]. GS and GOGAT are critical enzymes involved in the assimilation of ammonia, which is not only derived from nitrate reduction and N_2 fixation but also from some secondary metabolism processes, i.e. photorespiration or amino acid catabolism [47]. Photorespiration is one of the most important physiological process in which high amounts of ammonium are released [48], which was likely to be suppressed by elevated CO_2 [45]. This is probably the reason why GS and GOGAT activities were unaffected even though *M. truncatula* could acquire more N from fixation under elevated CO_2. Furthermore, elevated CO_2 decreased the enzyme activity and transcripts of GS and GOGAT in both *dnf1* mutants, and these decreases were accompanied by decreases in the N concentration of roots and leaves. Thus, it appears that *M. truncatula* and presumably other legumes require N fixation to maintain N assimilation under elevated CO_2.

In conclusion, regardless of wild-type and N fixation mutant, elevated CO_2 decreased N uptake from soil by down-regulating the expression of NR and NT of *M. truncatula*. Wild-type plants, however, are able to up-regulate N fixation related genes and increase nodule numbers under elevated CO_2 to maintain sufficient N concentration for plant growth. This suggests that as atmospheric CO_2 continues to rise, legumes may rely more on N fixation due to less on N uptake from soil. This could benefit agriculture because higher N fixation may compensate N depletion from soil, which would facilitate the growth of non-leguminous plants. Although our study has important implications for agriculture and for regional and global N budgets under predicted CO_2 conditions, the enhancement of leguminous N fixation by elevated CO_2 is environment-dependent [49]. N fixation can be limited by the availability of other soil nutrients (i.e., molybdenum, phosphorus, potassium) or by abiotic stresses (i.e., salinity, alkalinity, acidity, drought, fertilizer, metal toxicity) [50]. Moreover, since N uptake from soil is constrained by elevated CO_2, legumes are very likely to find it more difficult to maintain their growth under elevated CO_2 when they are subjected to stresses that reduce N fixation. Considering few studies have examined the interactive effects of elevated CO_2 and other abiotic stress on the N dynamics of legume, environmental variables in addition to atmospheric CO_2 concentrations should be considered when predicting future N dynamics of legumes. Besides, Understanding the N dynamics of legume plants and ensuring food security in the future also require a deeper understanding of interaction between legume plants and other organisms such as herbivorous insects.

Supporting Information

File S1 Table S1: Primer sequences used for real-time quantitative PCR.
(DOC)

File S2 Table S2: *P* values from two-way ANOVAs for the effects of CO_2 level, *M. truncatula* genotype, and their interaction on the growth traits and foliar chemical components of alfalfa plants.
(DOC)

File S3 Figure S1: The legume genes shown in this figure were tracked in the current study and are involved in N fixation, N uptake from soil, and N assimilation as indicated. The genes include: early nodule-specific protein 40 (*ENOD*), nodulation genes (*nodF*), nitrogen-fixing genes (*nifH*), nitrate transporter NRT1.1 (*NT*), nitrate reductase (*NR*), nitrate transporter NRT1.1 (*NT*), ammonium transporter protein (*AMT*), glutamine synthase 2 (*GS2*), and glutamate synthase (*GOGAT*).
(DOC)

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Author Contributions

Conceived and designed the experiments: HG YS FG. Performed the experiments: HG YL QR. Analyzed the data: HG. Contributed reagents/materials/analysis tools: XL KZ. Wrote the paper: HG YS.

References

1. IPCC-WGI (2007) Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL, editors. CambridgeUK: Cambridge University Press. 1009 p.
2. Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New phytol 165: 351–372.

3. Ainsworth EA, Rogers A (2007) The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. Plant Cell Environ 30: 258–270.
4. Taub DR, Miller B, Allen H (2008) Effects of elevated CO_2 on the protein concentration of food crops: a meta-analysis. Global Change Biol 14: 565–575.
5. Taub DR, Wang X (2008) Why are nitrogen concentrations in plant tissues lower under elevated CO_2? A critical examination of the hypotheses. J Integr Plant Biol 50: 1365–1374.
6. McDonald EP, Erickson JE, Kruger EL (2002) Can decreased transpiration limit plant nitrogen acquisition in elevated CO_2? Func Plant Biol 29: 1115–1120.
7. Pang J, Zhu JG, Xie ZB, Liu G, Zhang YL, et al. (2006) A new explanation of the N concentration decrease in tissues of rice (*Oryza sativa* L.) exposed to elevated atmospheric pCO_2. Environ Exp Bot 57: 98–105.
8. Ainsworth EA, Davey PA, Bernacchi CJ, Dermody OC, Heaton EA, et al. (2002) A meta-analysis of elevated [CO_2] effects on soybean (*Glycine max*) physiology, growth and yield. Global Change Biol 8: 695–709.
9. Sun Y, Cao H, Yin J, Kang L, Ge F (2010) Elevated CO_2 changes the interactions between nematode and tomato genotypes differing in the JA pathway. Plant Cell Environ 33: 729–739.
10. Keeney DR, Bremner JM (1966) A chemical index of soil nitrogen availability. Nature 211: 892–893.
11. LaRue TA, Patterson GT (1981) How much nitrogen do legumes fix? In *Advances in Agronomy*. (eds P.A. Sánchez, J.G. Salinas),pp.15–38. Academic Press. New York.
12. Silsbury JH (1977) Energy requirement for symbiotic nitrogen fixation. Nature 267: 149–150.
13. Voisin AS, Salon C, Jeudy C, Warembourg FR (2003) Symbiotic N_2 fixation activity in relation to C economy of *Pisum sativum* L. as a function of plant phenology. J Exp Bot 54: 2733–2744.
14. Barbulova A, Rogato A, D'Apuzzo E, Omrane S, Chiurazzi M(2007) Differential effects of combined N sources on early steps of the Nod factor-dependent transduction pathway in *Lotus japonicus*. Mol Plant Microbe Interact 20: 994–1003.
15. Munier-Jolain N, Salon C (2005) Are the carbon costs of seed production related to the quantitative and qualitative performance? An appraisal for legumes and other crops. Plant Cell Environ 28: 1388–1395.
16. Lam SK, Chen D, Norton R, Armstrong R, Mosier AR (2012) Nitrogen dynamics in grain crop and legume pasture systems under elevated atmospheric carbon dioxide concentration: A meta-analysis. Global Change Biol18: 2853–2859.
17. Rogers A, Gibon Y, Stitt M, Morgan PB, Bernacchi CJ, et al. (2006) Increased C availability at elevated carbon dioxide concentration improves N assimilation in a legume. Plant Cell Environ 29: 1651–1658.
18. Valentine AJ, Benedito VA, Kang Y (2010) Legume Nitrogen Fixation and Soil Abiotic Stress: From Physiology to Genomics and Beyond. In *Nitrogen Metabolism in Plants in the Post-Genomic Era* (eds C.H. Foyer, H. Zhang), 207–248. Oxford, UK.
19. Cabrerizo PM, González EM, Aparicio-Tejo PM, Arrese-Igor C (2001) Continuous CO_2enrichment leads to increased nodule biomass, carbon availability to nodules and activity of carbon-metabolising enzymes but does not enhance specific nitrogen fixation in pea. Physiol Plantarum 113: 33–40.
20. Gubsch M, Roscher C, Gleixner G, Habekost M, Lipowsky A, et al. (2011) Foliar and soil $\delta^{15}N$ values reveal increased nitrogen partitioning among species in diverse grassland communities. Plant Cell Environ 34: 895–908.
21. Chen FJ, Ge F, Su JW (2005) An improved top-open chamber for research on the effects of elevated CO_2 on agricultural pests in field-improved open-top chamber. Chinese J Ecol 24: 585–590.
22. Wang D, Griffitts J, Starker C, Fedorova E, Limpens E, et al. (2010) A nodule specific protein secretory pathway required for nitrogen-fixing symbiosis. Science 327: 1126–1129.
23. Starker CG, Parra-Colmenares AL, Smith L, Mitra RM, Long SR (2006) Nitrogen fixation mutants of *Medicago truncatula* fail to support plant and bacterial symbiotic gene expression. Plant Physiol 140: 671–680.
24. Tissue DT, Wright SJ (1995) Effects of seasonal water availability on phenology and the annual shoot carbohydrate cycle of tropical forest shrubs. Funct Ecology 9: 518–527.
25. Pausch RC, Mulchi CL, Lee EH, Meisinger JJ (1996) Use of ^{13}C and ^{15}N isotopes to investigate O_3 effects on C and N metabolism in soybeans. 2. Nitrogen uptake, fixation, and partitioning. Agriculture Ecosystems & Environment 60: 61–69.
26. Geiger M, Walch-Liu P, Engels C, Harnecker J, Schulze ED, et al. (1998) Enhanced carbon dioxide leads to a modified diurnal rhythm of nitrate reductase activity in older plants, and a large stimulation of nitrate reductase activity and higher levels of amino acids in young tobacco plants. Plant Cell Environ 21: 253–268.
27. Glévarec G, Bouton S, Jaspard E, Riou MT, Cliquet J, et al. (2004) Respective roles of the glutamine synthetase/glutamate synthase cycle and glutamate dehydrogenase in ammonium and amino acid metabolism during germination and post-germinative growth in the model legume *Medicago truncatula*. Planta 219: 286–297.
28. Suzuki A, Rioual S, Lemarchand S, Godfroy N, Roux Y, et al. (2001) Regulation by light and metabolites of ferredoxin-dependent glutamate synthase in maize. Physiol Plantarum 112: 524–530.
29. Vernié T, Moreau S, de Billy F, Plet J, Combier J, et al. (2008) EFD is an ERF transcription factor involved in the control of nodule number and differentiation in *Medicago truncatula*. Plant Cell 20: 2696–2713.
30. Curatti L, Brown CS, Ludden PW, Rubio LM (2005) Genes required for rapid expression of nitrogenase activity in *Azotobacter vinelandii*. Proc Natl Acad Sci USA 102: 6291–6296.
31. Bagchi R, Salehin M, Adeyemo OS, Salazar C, Shulaev V, et al. (2012) Functional assessment of the *Medicago truncatula* NIP/LATD protein demonstrates that it is a high-affinity nitrate transporter. Plant Physiol 160: 906–916.
32. Fei H & Vessey JK (2009) Stimulation of nodulation in *Medicago truncatula* by low concentrations of ammonium: quantitative reverse transcription PCR analysis of selected genes. Physiol Plantarum 135: 317–330.
33. Limami AM, Glévarec G, Ricoult C, Cliquet JB, Planchet E (2008) Concerted modulation of alanine and glutamate metabolism in young Medicago truncatula seedlings under hypoxic stress. J Exp Bot 59: 2325–2335.
34. Daepp M, Suter D, Almeida JPF, Isopp H, Hartwig UA, et al. (2000) Yield response of *Lolium perenne* swards to free air CO_2 enrichment increased over six years in a high N input system on fertile soil. Global Change Biol 6: 805–816.
35. Norby RJ, Warren JM, Iversen CM, Medlyn BE, McMurtrie RE (2010) CO_2 enhancement of forest productivity constrained by limited nitrogen availability. Proc Natl Acad Sci USA107: 19368–19373.
36. Stitt M, Krapp A (1999) The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell Environ 22: 583–621.
37. Rogers A, Fischer BU, Bryant JB, Frehner M, Blum H, et al. (1998) Acclimation of photosynthesis to elevated CO_2 under conditions of low N nutrition is affected by capacity for assimilate utilisation. Perennial ryegrass under Free Air CO_2 Enrichment (FACE). Plant Physiol 118: 683–689.
38. Lüscher A, Hartwig UA, Suter D, Nösberger J (2000) Direct evidence that symbiotic N_2 fixation in fertile grassland is an important trait for a strong response of plants to elevated atmospheric CO_2. Global Change Biol 6: 655–662.
39. Zanetti S, Hartwig UA, Van Kessel C, Lüscher A, Hebeisen T. et al. (1997) Does nitrogen nutrition restrict the CO_2 response of fertile grassland lacking legumes? Oecologia 112: 17–25.
40. Zanetti S, Hartwig UA, Lüscher A, Hebeisen T, Frehner M, et al. (1996) Stimulation of Symbiotic N_2 Fixation in *Trifolium repens* L. under Elevated Atmospheric pCO_2 in a Grassland Ecosystem. Plant Physiol 112: 575–583.
41. Montealegre CM, Kessel C van, Russelle MP, Sadowsky MJ (2002) Changes in microbial activity and composition in a pasture ecosystem exposed to elevated atmospheric carbon dioxide. Plant Soil 243: 197–207.
42. Baggs EM, Richter M, Hartwig UA, Cadisch G (2003) Nitrous oxide emissions from grass swards during eight years of elevated atmospheric pCO_2 (Swiss FACE). Global Change Biol 9: 1214–1222.
43. Rachmilevitch S, Cousins AB, Bloom AJ (2004) Nitrate assimilation in plant shoots depends on photorespiration. Proc Natl Acad Sci USA101: 11506–11510.
44. Bloom AJ, Burger M, Asensio JSR, Cousins AB (2010) Carbon dioxide enrichment inhibits nitrate assimilation in wheat and *Arabidopsis*. Science 328: 899–902.
45. Bloom AJ, Smart DR, Nguyen DT, Searles PS (2002) Nitrogen assimilation and growth of wheat under elevated carbon dioxide. Proc Natl Acad Sci USA 99: 1730–1735.
46. Zanetti S, Hartwig UA, Nösberger J (1998) Elevated atmospheric CO_2 does not affect *per se* the preference for symbiotic nitrogen as opposed to mineral nitrogen of *Trifolium repens* L. Plant Cell Environ 21: 623–630.
47. Betti M, García-Calderón M, Pérez-Delgado CM, Credali A, Estivill G, et al. (2012) Glutamine Synthetase in Legumes: Recent Advances in Enzyme Structure and Functional Genomics. Inter J Mol Sci 13: 7994–8024.
48. Keys AJ, Bird IF, Cornelius MJ, Lea PJ, Wallsgrove RM, et al. (1978) The photorespiratory nitrogen cycle. Nature 275: 741–743.
49. West JB, HilleRisLambers J, Lee TD, Hobbie SE, Reich PB (2005) Legume species identity and soil nitrogen supply determine symbiotic nitrogen-fixation responses to elevated atmospheric [CO_2]. New phytol 167: 523–530.
50. Van Groenigen KJ, Six J, Hungate B, Graaff MD, Breemen NV, et al. (2006) Element interactions limit soil carbon storage. Proc Natl Acad Sci USA103: 6571–6574.