

# Elevated CO<sub>2</sub> 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<sub>2</sub> (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 <sup>15</sup>N stable isotope, and N transportation and assimilation-related genes and enzymes were determined by qPCR and biochemical analysis. Elevated CO<sub>2</sub> 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<sub>2</sub>. 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<sub>2</sub>. Our results suggest that elevated CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> on C3 plants are generally characterized by increased photosynthesis, growth and yield in plant tissues [2]. Under elevated CO<sub>2</sub>, 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> reduces N uptake by roots [6]. The N loss hypothesis presumes that N losses increase under elevated CO<sub>2</sub> because of increasing NH<sub>3</sub> volatilization or increasing root exudation of organic N [7]. The dilution hypothesis, which has received the most attention,

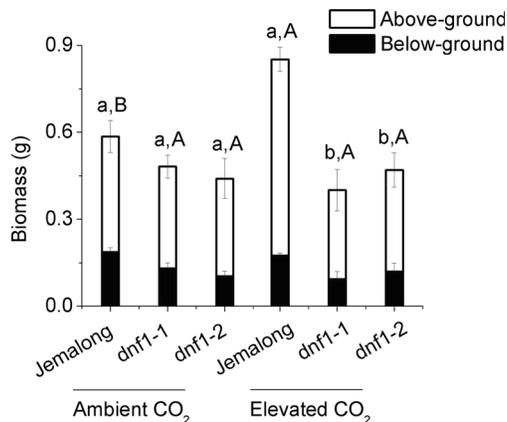
considers that N content is diluted under elevated CO<sub>2</sub> by accumulation of more total non-structural carbohydrates (TNC), which results in a greater biomass for a given quantity of N [8]. Depending on the species or genotype, these hypotheses may partially or largely explain the substantial reduction in the N content in non-leguminous plants under elevated CO<sub>2</sub> [9]. Furthermore, elevated CO<sub>2</sub> has little effect on the N content in legumes, which might be attributed to their unique ability to utilize atmospheric N<sub>2</sub> [4], but it still lacks the experimental evidence to address the physiological mechanism underlying N metabolism of legume plants under elevated CO<sub>2</sub>.

Leguminous plants acquire N by three major pathways. First, legumes uptake ammonia (NH<sub>4</sub><sup>+</sup>) from soil and incorporate it into organic compounds. Second, legumes uptake nitrate from soil and reduce it to NH<sub>4</sub><sup>+</sup>. Third, legumes in symbioses with N-fixing bacteria can obtain N from the atmosphere by N fixation, i.e., by converting N<sub>2</sub> to NH<sub>4</sub><sup>+</sup> [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<sub>2</sub>, legumes require additional N [8]. Owing to the high C consumption required for N fixation, elevated CO<sub>2</sub> 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<sub>2</sub>, 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<sub>2</sub> [17]. However, the relative contributions of N fixation and uptake from soil to the N content of legumes under elevated CO<sub>2</sub> are largely unknown. It is likely that legumes adjust their means of utilizing N resources to adapt to environmental changes [18], and a CO<sub>2</sub>-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<sub>2</sub>. The specific objectives were to determine: (1) how elevated CO<sub>2</sub> affects N fixation from the atmosphere and N uptake from soil; and (2) whether elevated CO<sub>2</sub> 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, <sup>15</sup>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<sub>2</sub> 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 ( $\pm$ SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> within the same genotype. Different uppercase letters indicate significant differences among genotypes within the same CO<sub>2</sub> treatment as determined by Tukey's multiple range test at  $P < 0.05$ . doi:10.1371/journal.pone.0081373.g001

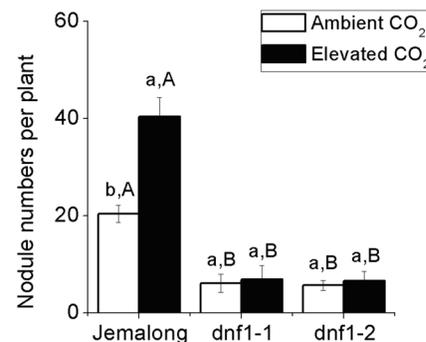
## Materials and Methods

### Atmospheric CO<sub>2</sub> Concentration Treatments

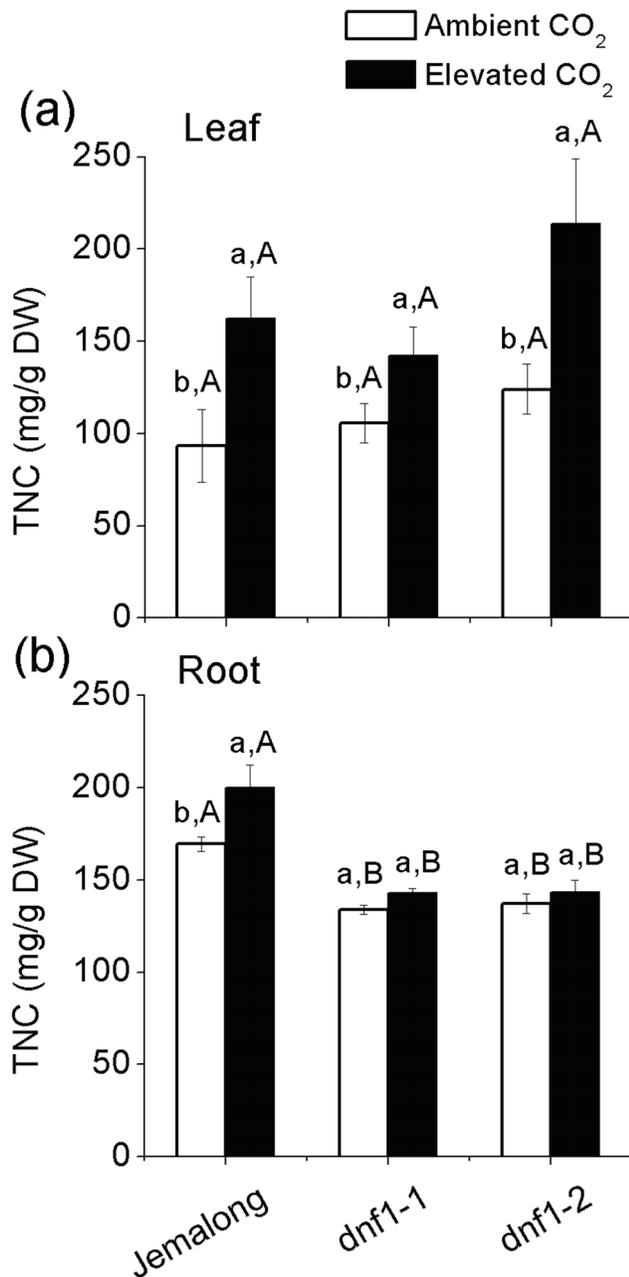
This experiment was performed in eight octagonal open-top field chambers (OTCs) (4.2 m diameter and 2.4 m height) at the Observation Station of the Global Change Biol Group, Institute of Zoology, Chinese Academy of Science in Xiaotangshan County, Beijing, China (40°11'N, 116°24'E). The atmospheric CO<sub>2</sub> concentration treatments were: (1) current atmospheric CO<sub>2</sub> levels (390  $\mu$ l/L), and (2) elevated CO<sub>2</sub> levels (750  $\mu$ l/L, the predicted level in about 100 years) (IPCC, 2007). Four blocks were used, and each block contained one OTC with ambient CO<sub>2</sub> and one with elevated CO<sub>2</sub>. From seedling emergence to the harvesting of *M. truncatula* plants (27 August to 15 October 2011, a total of 50 days), CO<sub>2</sub> concentrations were monitored and adjusted with an infrared CO<sub>2</sub> analyzer (Ventostat 8102, Telaire Company, Goleta, CA, USA) once every minute to maintain relatively stable CO<sub>2</sub> concentrations. The measured CO<sub>2</sub> concentrations throughout the experiment (mean  $\pm$  SD per day) were 391 $\pm$ 23 ppm in the ambient CO<sub>2</sub> chambers and 743 $\pm$ 32 ppm in the elevated CO<sub>2</sub> chambers. The auto-control system for maintaining the CO<sub>2</sub> 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 $\pm$ 3.4°C in OTCs with ambient CO<sub>2</sub> vs. 26.2 $\pm$ 3.9°C in OTCs with elevated CO<sub>2</sub>).

### *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<sub>2</sub> 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 ( $\pm$ SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> within the same genotype. Different uppercase letters indicate significant differences among *M.* genotypes within the same CO<sub>2</sub> treatment as determined by Tukey's multiple range test at  $P < 0.05$ . doi:10.1371/journal.pone.0081373.g002



**Figure 3. Total non-structural carbohydrate (TNC) content in leaves and roots of *M. truncatula* plants as affected by CO<sub>2</sub> 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 ( $\pm$ SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> within the same genotype. Different uppercase letters indicate significant differences among genotypes with the same CO<sub>2</sub> treatment as determined by Tukey's multiple range test at  $P < 0.05$ . doi:10.1371/journal.pone.0081373.g003

After seeds were chemically scarified and surface sterilized by immersion in concentrated H<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub>O 1000 ml, yeast 3 g, mannitol 10 g, KH<sub>2</sub>PO<sub>4</sub> 0.25 g, K<sub>2</sub>HPO<sub>4</sub> 0.25 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 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<sup>8</sup> ml<sup>-1</sup>. 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<sub>2</sub> 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 <sup>15</sup>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 $\delta^{15}\text{N}$ 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 Kjeltac N analysis (Foss automated Kjeltac<sup>TM</sup> instruments, Model 2100) [24].

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

$$\delta^{15}\text{N}_{\text{sample}} = (\text{R}_{\text{sample}} - \text{R}_{\text{standard}}) / \text{R}_{\text{standard}} \times 100$$

Where  $\text{R}_{\text{standard}}$  is the <sup>15</sup>N/ <sup>14</sup>N ratio of atmospheric N<sub>2</sub> and  $\text{R}_{\text{sample}}$  is the <sup>15</sup>N/ <sup>14</sup>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<sub>2</sub> effects on N characteristics (%Ndf, Nf Yield, Ns Yield) of wild-type Jemalong plant.

	Jemalong		
	%Ndf <sup>1</sup>	Nf Yield <sup>2</sup> (mg/plant)	Ns Yield <sup>3</sup> (mg/plant)
Ambient CO <sub>2</sub> (390 ppm)	39.8±0.7 b	9.1±0.7 b	13.8±0.4 a
Elevated CO <sub>2</sub> (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<sub>2</sub> and elevated CO<sub>2</sub> as determined by independent *t*-test at *P*<0.05.

<sup>1</sup>percentage of N derived from atmosphere.

<sup>2</sup>N derived from N-fixation per plant.

<sup>3</sup>N derived from soil per plant.

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$$\%Ndf = (1 - \delta^{15}N_{M.truncatula} / \delta^{15}N_{reference}) \times 100$$

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*.

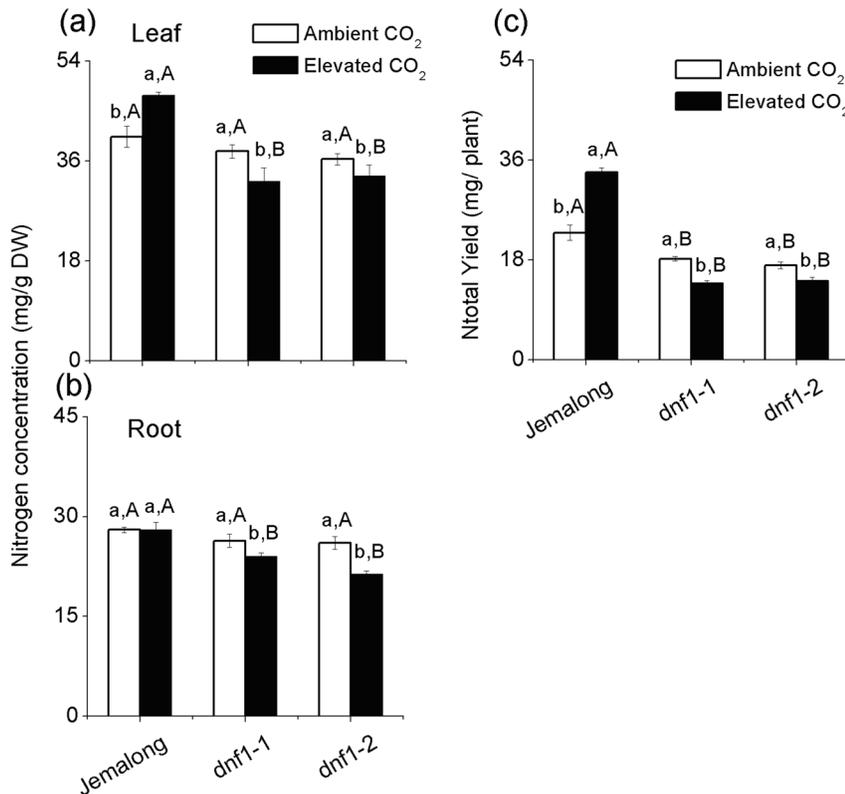
In order to evaluate changes in N source (i.e. as derived from N-fixation or soil) for *M. truncatula* plants, Nf Yield (N derived from N-fixation per plant) and Ns Yield (N derived from soil per plant) of Jemalong estimates were calculated as follows:

$$NtotalYield(mg/plant) = Biomass_{above-ground} \times N_{leaf}$$

$$+ Biomass_{under-ground} \times N_{root}$$

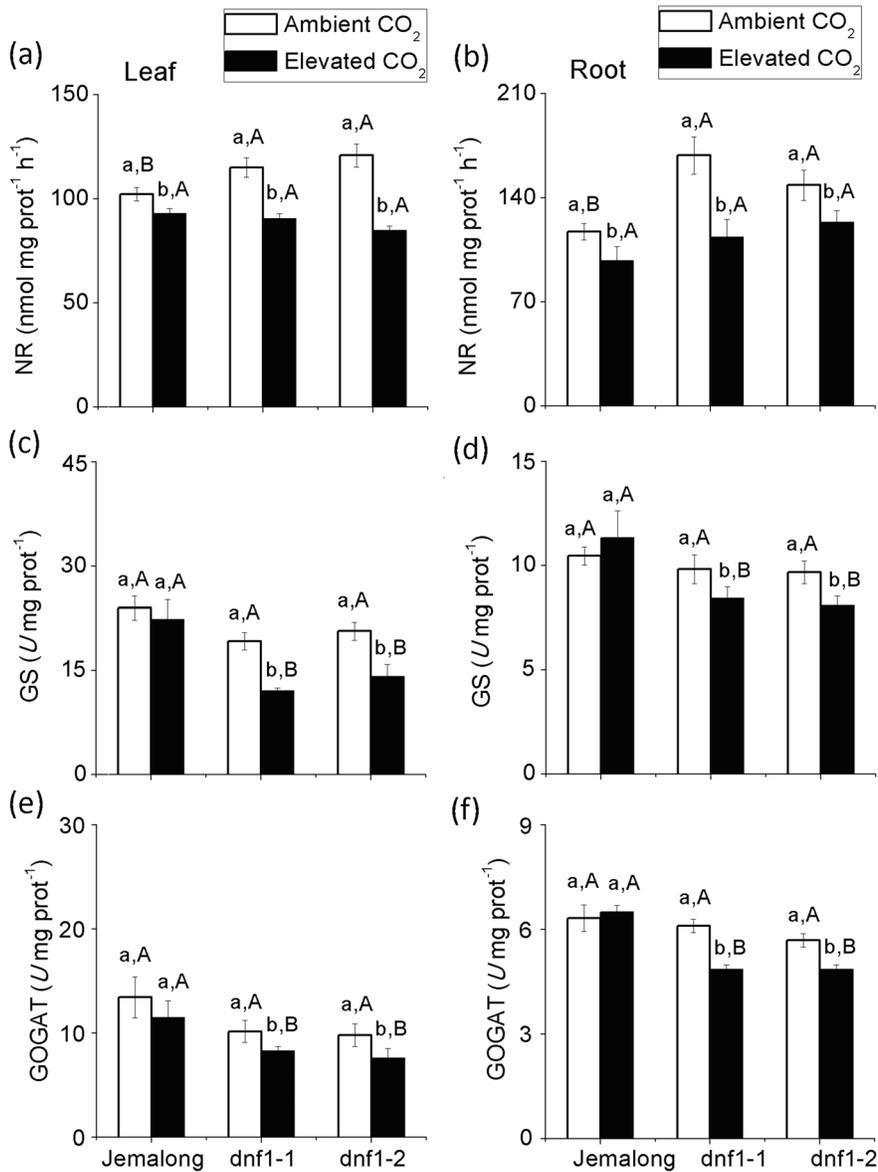
$$NfYield(mg/plant) = NtotalYield \times \%Ndf$$

$$NsYield(mg/plant) = NtotalYield - NfYield$$



**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<sub>2</sub> 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<sub>2</sub> and elevated CO<sub>2</sub> within the same genotype. Different uppercase letters indicate significant differences among genotypes within the same CO<sub>2</sub> treatment as determined by Tukey's multiple range test at *P*<0.05.

doi:10.1371/journal.pone.0081373.g004



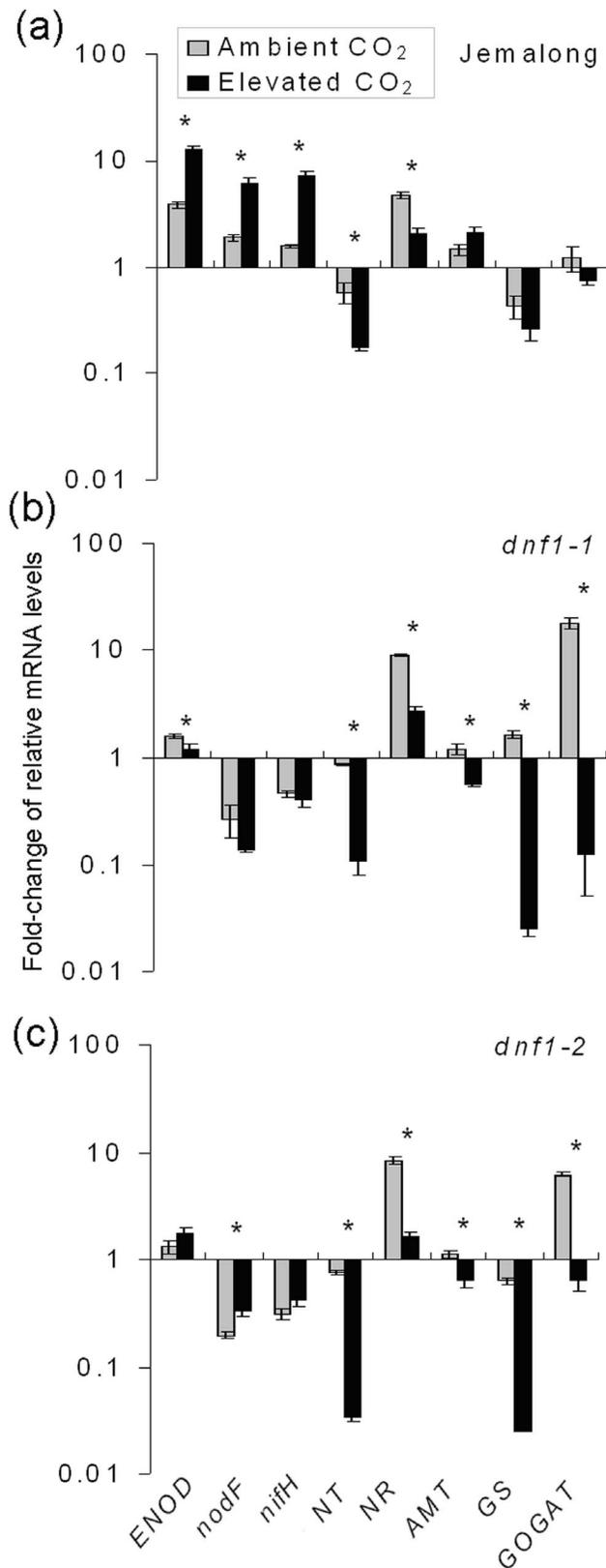
**Figure 5. Activities of the enzymes involved in N reduction (NR) and in N assimilation (GS and GOGAT) in the leaves and roots of *M. truncatula* plants as affected by CO<sub>2</sub> level and plant genotype: *dnf1-1* and *dnf1-2* are deficient in N fixation, and Jemalong is their wild type.** Each value represents the average ( $\pm$ SE) of four replicates. Different lowercase letters indicate significant differences between ambient CO<sub>2</sub> and elevated CO<sub>2</sub> within the same genotype. Different uppercase letters indicate significant differences among genotypes within the same CO<sub>2</sub> 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<sub>above-ground</sub> is the biomass of above-ground tissue in *M. truncatula* plants, Biomass<sub>under-ground</sub> is the biomass of under-ground tissue, N<sub>leaf</sub> is the N concentration of leaves and N<sub>root</sub> 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<sub>2</sub>, 1 mM EDTA, 1  $\mu$ M  $\beta$ -mercaptoethanol, and 1% (w/v) polyvinylpyrrolidone. 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<sub>2</sub> 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 ( $\pm$ SE) of four replicates. An asterisk above a column indicates a significant difference in gene expression under ambient vs. elevated CO<sub>2</sub> ( $P < 0.05$ ).

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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 RNaseasy Mini Kit (Qiagen) was used to isolate total RNAs from *M. truncatula* leaves and roots, and 1  $\mu$ g 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  $\mu$ L reaction volumes that included 10  $\mu$ L of 2 $\times$ SYBRs Premix EX Taq<sup>TM</sup> (Qiagen) master mix, 5 mM of each gene-specific primer, and 1  $\mu$ L 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.  $\beta$ -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  $\beta$ -actin or *pnp* (the house-keeping gene), which remain constant under different treatment conditions. The levels of  $\beta$ -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  $2^{-\Delta\Delta C_t}$  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<sub>2</sub> 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<sub>2</sub> on %Ndf, Nf Yield, Ns Yield of Jemalong and genes regulating N metabolism were determined by independent *t*-tests.

## Results

### Plant Biomass and Nodule Number

CO<sub>2</sub> 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<sub>2</sub> but was greater for the wild-type Jemalong than for the mutants under elevated CO<sub>2</sub> (Fig. 1). In response to elevated CO<sub>2</sub>, 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<sub>2</sub> treatments (Fig. 1). CO<sub>2</sub> level and genotype significantly affected the nodule numbers (Table S2). Regardless of CO<sub>2</sub> level, nodule number was greater for Jemalong than for the *dnf1* mutants (Fig. 2). Elevated CO<sub>2</sub> increased nodule numbers of Jemalong but not of the mutants.

### TNC and N Characteristic in Plant

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

### Activities of the Enzymes NR, GS and GOGAT

CO<sub>2</sub> level was significant for the activities of foliar NR and root NR. (Table S2). Elevated CO<sub>2</sub> reduced NR activity in the leaves and roots of all three genotypes (Table S2, Fig. 5). Under ambient CO<sub>2</sub>, NR activity in both leaves and roots were higher in both *dnf1* mutants than in Jemalong (Fig. 5). Under elevated CO<sub>2</sub>, however, NR activity did not differ among the three genotypes (Fig. 5).

Genotype and the interaction between CO<sub>2</sub> and genotype significantly affected the foliar GS and root GS. All factors significantly affected the foliar GOGAT and root GOGAT (Table S2). Elevated CO<sub>2</sub> 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<sub>2</sub> levels. GS activity in roots did not differ among the three genotypes under ambient CO<sub>2</sub> but were higher in Jemalong than in the mutants under elevated CO<sub>2</sub> (Fig. 5).

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

Elevated CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> is the availability of N (Fig. S2). Using *dnf1-1* and *dnf2* mutants, we demonstrated that *M. truncatula* is able to adjust different N partitioning pathways to ensure a sufficient N supply under ambient CO<sub>2</sub>. Elevated CO<sub>2</sub>, however, reduced N uptake from soil by suppressing N uptake related gene and increased the reliance on fixation of atmospheric N<sub>2</sub>.

N availability is one of the key factors limiting plant growth and production. Elevated CO<sub>2</sub> stimulating plant growth would increase the N demand of plants [34]. The extent of the CO<sub>2</sub> response at the plant level could consequently be limited by N availability [35,36]. In current study, since elevated CO<sub>2</sub> increased foliar N concentration and Ntotal yield (Table 1; Fig. 4), Jemalong plants were able to produce more biomass under elevated CO<sub>2</sub> (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<sub>2</sub> (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<sub>2</sub> (Fig. 1). Thus, our results demonstrated that the symbiotic N<sub>2</sub> fixation provided legumes an incomparable advantage in producing larger amounts of biomass under elevated CO<sub>2</sub> [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<sub>2</sub>. Although the *dnf1* mutants are unable to fix atmospheric N<sub>2</sub>, 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<sub>2</sub>. 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<sub>2</sub>. However, the GS/GOGAT activities and N concentration in *dnf1* plants were lower than in Jemalong plants under elevated CO<sub>2</sub> (Fig. 4, 5), which indicated that elevated CO<sub>2</sub> 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<sub>2</sub> [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<sub>2</sub> for all three *M. truncatula* genotypes, as reflected in decreased Ns Yield in Jemalong and Ntotal Yield in both *dnf1* mutants under elevated CO<sub>2</sub>. 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<sub>2</sub> (Fig. 5). This indicates that elevated CO<sub>2</sub> suppresses N uptake of *M. truncatula* from soil. This is consistent with the finding that N uptake from soil by *Trifolium repens* were decreased under elevated CO<sub>2</sub> grown in a grassland ecosystem [40]. In addition, our results showed that elevated CO<sub>2</sub> 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<sub>2</sub> could be explained by two factors: lower soil N availability and plant NO<sub>3</sub><sup>-</sup>

reduction. Elevated CO<sub>2</sub> reduced the soil N availability by increasing N immobilization and denitrification in soil. For example, elevated CO<sub>2</sub> increased microbial community composition in rhizosphere soil of white clover, and subsequently increased N immobilization into the expanded microbial biomass [41]. Additionally, elevated CO<sub>2</sub> increased the emission of N<sub>2</sub>O 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<sub>2</sub> could decrease the nicotinamide adenine dinucleotide (NADH) [43], which provides the energy required to convert NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup> in the cytoplasm of leaf mesophyll cells [44]. Moreover, elevated CO<sub>2</sub> increased HCO<sub>3</sub><sup>-</sup>, and in turn inhibited NO<sub>2</sub><sup>-</sup> 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<sub>2</sub> fixation under elevated CO<sub>2</sub> [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<sub>2</sub>. Furthermore, elevated CO<sub>2</sub> decreased N concentration and Ntotal Yield in both *dnyf1* mutants, suggesting that the increased N concentration and Ntotal Yield in Jemalong was solely the result of elevated CO<sub>2</sub>-induced increases of N<sub>2</sub> fixation. In addition, the fixation of N<sub>2</sub> 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<sub>2</sub> fixation [14]. Elevated CO<sub>2</sub>, however, provided the sufficient C to satisfy the energy demand for N<sub>2</sub> fixation, and decreased soil N availability under elevated CO<sub>2</sub> accelerated N<sub>2</sub> fixation in Jemalong plants [46].

Although elevated CO<sub>2</sub> tends to increase the N concentration and modify N acquisition patterns of legumes, there is little evidence that elevated CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> [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<sub>2</sub>. Furthermore, elevated CO<sub>2</sub> decreased the enzyme activity and transcripts of GS and GOGAT in both *dnyf1* 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<sub>2</sub>.

In conclusion, regardless of wild-type and N fixation mutant, elevated CO<sub>2</sub> 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<sub>2</sub> to maintain sufficient N concentration for plant growth. This suggests that as

atmospheric CO<sub>2</sub> 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<sub>2</sub> conditions, the enhancement of leguminous N fixation by elevated CO<sub>2</sub> 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<sub>2</sub>, legumes are very likely to find it more difficult to maintain their growth under elevated CO<sub>2</sub> when they are subjected to stresses that reduce N fixation. Considering few studies have examined the interactive effects of elevated CO<sub>2</sub> and other abiotic stress on the N dynamics of legume, environmental variables in addition to atmospheric CO<sub>2</sub> 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<sub>2</sub> 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.

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