

# Photosynthetic characteristics and nitrogen allocation in the black locust (*Robinia pseudoacacia* L.) grown in a FACE system

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## Abstract

**Key message** The black locust is adapted to elevated [CO<sub>2</sub>] through changes in nitrogen allocation characteristics in leaves.

**Abstract** The black locust (*Robinia pseudoacacia* L.) is an invasive woody legume within Japan. This prolific species has a high photosynthetic rate and growth rate, and undergoes symbiosis with N<sub>2</sub>-fixing micro-organisms. To determine the effect of elevated CO<sub>2</sub> concentration [CO<sub>2</sub>] on its photosynthetic characteristics, we studied the chlorophyll (Chl) and leaf nitrogen (N) content, and the leaf structure and N allocation patterns in the leaves and acetylene reduction activity after four growing seasons, in *R. pseudoacacia*. Our specimens were grown at ambient [CO<sub>2</sub>] (370 μmol mol<sup>-1</sup>) and at elevated [CO<sub>2</sub>] (500 μmol mol<sup>-1</sup>), using a free air CO<sub>2</sub> enrichment (FACE) system. Net photosynthetic rate at growth [CO<sub>2</sub>] ( $A_{\text{growth}}$ ) and acetylene reduction activity were significantly higher, but maximum carboxylation rate of RuBisCo ( $V_{\text{cmax}}$ ), maximum rate of electron transport driving RUBP regeneration ( $J_{\text{max}}$ ), net photosynthetic rate under enhanced CO<sub>2</sub> concentration and light saturation ( $A_{\text{max}}$ ), the N concentration in leaf, and in leaf mass per unit area (LMA) and ribulose-1,5-bisphosphate carboxylase oxygenase

(RuBisCo) content were significantly lower grown at elevated [CO<sub>2</sub>] than at ambient [CO<sub>2</sub>]. We also found that RuBisCo/N were less at elevated [CO<sub>2</sub>], whereas Chl/N increased significantly. Allocation characteristics from N in leaves to photosynthetic proteins, N<sub>L</sub> (Light-harvesting complex: LHC, photosystem I and II: PSI and PSII) and other proteins also changed. When *R. pseudoacacia* was grown at elevated [CO<sub>2</sub>], the N allocation to RuBisCo ( $N_{\text{R}}$ ) decreased to a greater extent but N<sub>L</sub> and N remaining increased relative to specimens grown at ambient [CO<sub>2</sub>]. We suggest that N remobilization from RuBisCo is more efficient than from proteins of electron transport ( $N_{\text{E}}$ ), and from N<sub>L</sub>. These physiological responses of the black locust are significant as being an adaptation strategy to global environmental changes.

**Keywords** Free air CO<sub>2</sub> enrichment · Elevated CO<sub>2</sub> · RuBisCo · LMA · Acetylene reduction activity

## Abbreviations

$A_{\text{growth}}$	Net photosynthetic rate at growth CO <sub>2</sub> concentration
$A_{\text{max}}$	Net photosynthetic rate under enhanced CO <sub>2</sub> concentration and light saturation
ANOVA	Analysis of variance
ARA	Acetylene reduction assay
CBB	Coomassie brilliant blue
Chl <i>a</i>	Chlorophyll <i>a</i>
Chl <i>b</i>	Chlorophyll <i>b</i>
Ci	Intercellular CO <sub>2</sub> concentration
C/N ratio	Carbon to nitrogen ratio
[CO <sub>2</sub> ]	CO <sub>2</sub> concentration
C*	CO <sub>2</sub> compensation point in the absence of Rd
FACE	Free air CO <sub>2</sub> enrichment
<i>J</i>	The potential electron transport rate

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$J_{\max}$	Maximum rate of electron transport driving RUBP regeneration
LED	Light emitting diode
LHC	Light-harvesting complex
LMA	Leaf mass per unit area
N	Nitrogen
Narea	Nitrogen content per unit leaf area
$N_E$	Nitrogen allocation to electron transport proteins except $N_L$ and the carbon cycle proteins except RuBisCo
$N_L$	Nitrogen allocation to LHC, PSI and PSII
Nm	Nitrogen content per unit leaf mass
$N_R$	Nitrogen allocation to RuBisCo
$P_c$	Photosynthetic rate limited by RuBisCo activity
$P_i$	Inorganic phosphate
$P_j$	Electron transport-limited gross carboxylation rate
$P_N$	Net photosynthetic rate
$P_N/C_i$ curve	Net photosynthetic rate/intercellular $CO_2$ concentration curve
PNUE	Photosynthetic nitrogen use efficiency
$P_{\max}$	Maximum mass-based net $CO_2$ assimilation rate
PPFD	Photosynthetic photon flux density
PSI	Photosystem I
PSII	Photosystem II
Rd	Day respiration
RuBP	Ribulose-1,5-bisphosphate
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS–PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
$Q$	Photosynthetic photon flux density of light saturation
$V_{\max}$	Maximum carboxylation rate of RuBisCo
WUE	Water-use efficiency
$\Gamma^*$	$CO_2$ compensation point

## Introduction

The atmospheric  $[CO_2]$  rose dramatically in the previous century (from  $\sim 280$  ppm in the 19th century to  $\sim 396$  ppm in 2013) and is currently increasing at  $c. 0.4\%$  year $^{-1}$ , due mainly to the combustion of fossil fuels and deforestation (IPCC 2014; WMO 2014). Atmospheric  $[CO_2]$  is predicted to rise by a further 50% by 2050 (Meehl et al. 2007). Many plant species increase their photosynthesis and growth under elevated  $[CO_2]$  when other environmental resources do not limit these changes. Knowledge of long-term enhancing effects of  $[CO_2]$  on plant growth is still limited today (e.g. Norby and Zak 2011; Koike et al. 2015). There is much

evidence of decreased photosynthetic activity under elevated  $[CO_2]$ , and this correlates with less RuBisCo protein and a decline in leaf N concentration (Tissue et al. 1993; Rogers et al. 1996; Zhang et al. 2006; Madhu and Hatfield 2014). The  $V_{\max}$  and/or the  $J_{\max}$  are reduced in plants undergoing photosynthetic down-regulation at elevated  $[CO_2]$  (Sage 1994; Onoda et al. 2005; Norby and Zak 2011; Koike et al. 2015). The value of  $V_{\max}$  limits the photosynthetic rate at low  $[CO_2]$ , and  $J_{\max}$  limits the photosynthetic rate at high  $[CO_2]$ . Changes in the ratio  $J_{\max}/V_{\max}$  affect the photosynthetic rate; a decrease in  $J_{\max}/V_{\max}$  slows the photosynthetic rate at elevated  $[CO_2]$ . The reduction in  $V_{\max}$  is usually ascribed to lower amounts of RuBisCo or low RuBisCo activity (Sage et al. 1989; Jacob et al. 1995; Tissue et al. 1999; Onoda et al. 2005). Negative photosynthetic response often occurs at elevated  $[CO_2]$ , especially because of decline in leaf N content that often occurs under conditions of low soil N availability (Norby et al. 1986; Choi et al. 2005b, 2009; Sanz-S  ez et al. 2010; Watanabe et al. 2010).

In contrast, photosynthesis and growth of some plants in a symbiotic relationship with mycorrhizae do not decrease under elevated  $[CO_2]$  even where there is low soil N availability. In such situations there may be greater carbon allocation to roots, and consequently increased mycorrhizal symbiosis or activity (Choi et al. 2005a, b, 2009; Smith and Read 2008). In particular,  $N_2$ -fixing trees respond more strongly to elevated atmospheric  $[CO_2]$  than other species. These  $N_2$ -fixing trees are able to increase both C assimilation and plant growth under elevated  $[CO_2]$ , because of their ability to maintain a high internal nutrient supply, furthermore, symbiotic  $N_2$  fixation should increase as atmospheric  $[CO_2]$  increases (Norby et al. 1986; Norby 1987; Saxe et al. 1998; Evans et al. 2000; Schortemeyer et al. 2002; Feng et al. 2004; Nguyen et al. 2006; Sanz-S  ez et al. 2010; Hoosbeek et al. 2011). Various studies suggest that total  $N_2$  fixation and total plant N increase in several  $N_2$ -fixing trees grown at  $CO_2$  concentrations greater than the ambient value (Vogel et al. 1997; Atkin et al. 1999; Feng et al. 2004; Sanz-S  ez et al. 2010). Of these, *Robinia pseudoacacia* L. is the best known (Olesniewicz and Thomas 1999; Schortemeyer et al. 2002; Feng et al. 2004). *R. pseudoacacia* is native to North America and is now planted worldwide as a pioneer during the early stage of succession because of its adaptability to environmental stresses, high photosynthetic rate and rapid growth (Kurokuchi et al. 2010). It is also known to be an invasive tree species and has a high  $N_2$ -fixing capacity (Barrett et al. 1990; Feldhake 2001; Lee et al. 2004; Rice et al. 2004; Karaki et al. 2012; Watanabe et al. 2014). This species was introduced into Japan in 1873 as a revegetation plant and has been planted in degraded areas and roadsides for greening (Maekawa and Nakagoshi 1997; Kurokuchi et al. 2010). In forest ecosystems, *R. pseudoacacia* can enhance

soil N concentration and N cycling by producing N-rich litter (Rice et al. 2004; Malcolm et al. 2008; Lopez et al. 2014). The effect of elevated atmospheric  $[\text{CO}_2]$  on its physiological responses and on N allocation patterns in leaves is not well known, however, and its  $\text{N}_2$ -fixing capacity is not always clear (Koike et al. 2007, 2015).

More than half of the N absorbed in roots goes into photosynthetic proteins in the leaves: into LHC, RuBisCo for  $\text{CO}_2$  fixation, proteins involved in electron transport, and other proteins (Evans and Terashima 1987; Kitaoka and Koike 2004). RuBisCo is the main enzyme involved in photosynthesis and is the most plentiful protein in the planet, accounting for approximately 50% of the soluble protein in most leaves (Hopkins and Hüner 2009). Although carboxylation reaction of RuBisCo carboxylation reaction, approximately three times faster than the oxygenation reaction in the air, RuBisCo is not saturated at current ambient  $[\text{CO}_2]$  (Buchanan et al. 2015). Photosynthesis under elevated  $[\text{CO}_2]$  is limited usually by the availability of inorganic phosphate (Pi) in leaves or by the electron transport capacity, and RuBisCo is substantially down-regulated (Farquhar et al. 1980; Sharkey 1985; Sage 1990; Buchanan et al. 2015). Plants grown under elevated  $[\text{CO}_2]$  are likely to have greater N-use efficiency and less N allocated to RuBisCo (Makino et al. 2000; Koike et al. 2015). As a result, elevated  $\text{CO}_2$  has negative effects not only on  $V_{\text{cmax}}$  and RuBisCo activity but also on the amounts of RuBisCo and soluble protein (Makino et al. 2000; Pérez et al. 2011). The  $\text{N}_2$ -fixing activity of *R. pseudoacacia* is expected to increase under elevated  $[\text{CO}_2]$ , and its N allocation to RuBisCo may increase.

We focus in this study on the relation between physiological activities in *R. pseudoacacia*, LMA, leaf N content, acetylene reduction activity of *Frankia* sp., RuBisCo content and N allocation patterns in leaves at ambient and elevated  $[\text{CO}_2]$ . We first investigated the effect of  $[\text{CO}_2]$  during four growing seasons upon leaf biometric changes of *R. pseudoacacia* in connection with acetylene reduction activity of *Frankia* sp., the photosynthetic nitrogen use efficiency (PNUE), and water use efficiency (WUE) at each  $[\text{CO}_2]$ . Second, we looked into the photosynthetic and physiological parameters relating to RuBisCo content and leaf N content grown under ambient and elevated  $[\text{CO}_2]$ . Third, we examined the RuBisCo, Chl, leaf N content and the N allocation patterns in the leaves of *R. pseudoacacia* grown at ambient and elevated  $[\text{CO}_2]$ .

## Materials and methods

### Study site

This study was conducted at the FACE system in Hokkaido University's Sapporo Experimental Forest, in northern Japan (43°06'N, 141°20'E). The FACE system consists of

six 5 m high circular experimental plots, having a diameter of 6 m. This arrangement is based on the system at Davos, Switzerland (Hättenschwiler et al. 2002). Elevated  $[\text{CO}_2]$  in three FACE rings was  $500 \pm 50 \mu\text{mol mol}^{-1}$  for 65% of the time, and  $500 \pm 100 \mu\text{mol mol}^{-1}$  for 88% for the time, which is adequate performance (Takagi et al. 2004; Eguchi et al. 2008a, b). After dawn,  $\text{CO}_2$  fumigation was stopped when irradiance was less than  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ , which is below the light compensation point of the study species (Eguchi et al. 2008b). During the growing seasons, from May to late November,  $\text{CO}_2$  was applied in these three FACE rings. The three control rings were left at ambient  $[\text{CO}_2]$  (ca.  $370 \mu\text{mol mol}^{-1}$ ). Long-term (1981–2010) annual precipitation in this area is  $1006.5 \text{ mm year}^{-1}$ , and the average temperature is  $8.9 \text{ }^\circ\text{C}$  (Japan Meteorological Agency 2016). The soil type at the FACE was fertile brown forest soil, which is native to Sapporo Experimental Forest and is distributed widely throughout Japan.

### Plant materials

One-year-old *R. pseudoacacia* L. (the black locust) was planted in 2003 at ambient  $[\text{CO}_2]$  (about 370 ppm as of 2003) as control and at elevated  $[\text{CO}_2]$  ( $500 \mu\text{mol mol}^{-1}$ , as has been estimated for the year 2040 by the IPCC), in the FACE system. Thirty *R. pseudoacacia* trees were planted randomly at six FACE rings: fifteen trees in elevated rings and fifteen trees in control rings, all approximately 30 cm tall. After four growing seasons under elevated  $[\text{CO}_2]$  or ambient  $[\text{CO}_2]$ , we selected twelve *R. pseudoacacia* trees, six from the three elevated  $[\text{CO}_2]$  rings and six from the three control rings. Sampling and measurements on the trees took place on clear rainless days. Leaves grown at each concentration of  $\text{CO}_2$  were collected randomly at a height of between 120 and 150 cm for measurement of physiological characters, chemical analysis (N, chl and RuBisCo content) and morphological measurements (e.g. LMA). These leaves were taken from sun-exposed branches of six trees from elevated  $[\text{CO}_2]$  and six from ambient  $[\text{CO}_2]$  in early September.

### Photosynthetic rate

Curves of the net photosynthetic rate versus intercellular  $\text{CO}_2$  concentration ( $P_N/C_i$ ) were examined for leaves that were exposed to the sun, using an open gas exchange system (LI-6400, Li-Cor, Lincoln, NE, USA) with a light-emitting diode (LED) light source (6400-02B, Li-Cor) between 09:00 and 13:00 h, when photosynthetic activity and stomatal conductance were stable to changes in environmental conditions. The measurement conditions were: leaf temperature,  $25 \text{ }^\circ\text{C}$ ; relative humidity, 70%; photosynthetic photon flux density (PPFD),  $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,

which is considered to be optimal for RuBisCo activity (Larcher 2003; Choi et al. 2005a, 2006; Kitao et al. 2009). The O<sub>2</sub> concentration in the leaf chamber was normal 21% and the water vapour deficit (VPD) in the leaf chamber was about 1.2 kPa.

The intercellular CO<sub>2</sub> concentration ( $C_i$ ) response curve of the net photosynthetic rate ( $P_N$ ), known as the  $P_N/C_i$  curve, was measured with a CO<sub>2</sub> mixer for the sequence of concentrations 370, 300, 220, 140, 60, 140, 220, 300, 370, 500, 700, 900, 1200 and 1500  $\mu\text{mol mol}^{-1}$  in the control specimens, and the sequence 500, 370, 220, 140, 60, 220, 300, 370, 500, 700, 900, 1200 and 1500  $\mu\text{mol mol}^{-1}$  for *R. pseudoacacia* grown in the FACE; measurements were recorded after equilibration to a steady state had taken place (coefficient of variation <2%).

The  $P_N/C_i$  curve was fitted using the model of Farquhar et al. (1980). From the  $P_N/C_i$  curves,  $V_{\text{cmax}}$  was calculated by fitting the following equation to the initial slope the  $P_N/C_i$  curves ( $C_i < 300 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with the least square method with Kaleida Graph™ (Ver 4.11, Synergy Software, PA, USA) (Farquhar et al. 1980; von Caemmerer and Farquhar 1981; Sharkey 1985).

$$P_c = V_{\text{cmax}} \frac{C_i - \Gamma^*}{C_i + K_c \left(1 + \frac{O}{K_o}\right)} - R_d, \quad (1)$$

where  $P_c$  is the photosynthetic rate limited by RuBisCo activity,  $C_i$  is intercellular concentration of CO<sub>2</sub> ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ),  $\Gamma^*$  is the CO<sub>2</sub> compensation point (Pa),  $R_d$  denotes day respiration ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).  $O$  is the intercellular O<sub>2</sub> partial pressure and  $K_c$  and  $K_o$  are Michaelis–Menten constants of RuBisCo activity for CO<sub>2</sub> and O<sub>2</sub>, respectively.

$J_{\text{max}}$  was calculated from Farquhar et al. (1980) and Harley et al. (1992) to a near-plateau of  $P_N/C_i$  curves ( $C_i > 600 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) as follows:

$$P_j = J_{\text{max}} \frac{C_i - \Gamma^*}{4(C_i + 2\Gamma^*)} - R_d, \quad (2)$$

where  $P_j$  is the electron transport-limited gross carboxylation rate. We applied 40.4 Pa, 24.8 kPa, and 3.69 Pa for  $K_c$ ,  $K_o$ , and  $\Gamma^*$ , respectively (von Caemmerer et al. 1994).

The PNUE was calculated as the maximum mass-based net CO<sub>2</sub> assimilation rate ( $P_{\text{max}}$ ) divided by leaf N content per unit leaf area ( $N_{\text{area}}$ ) ( $\text{PNUE} = P_{\text{max}}/N_{\text{area}}$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1}/\text{g m}^{-2}$ ). WUE was taken as the ratio of  $P_N$  to the transpiration rate at PPFD saturation and constant water VPD (Field et al. 1983; Choi et al. 2014).

After measurement of the photosynthetic rate, six disk samples (each 1 cm<sup>2</sup>) were taken from the measured leaf for determination of the LMA (=1/SLA; specific leaf area), N content and Chl. The remaining leaves were placed

within 60 s into a deep freeze (−85 °C), prior to measurement of the RuBisCo content.

## N, Chl and RuBisCo content in leaves

Leaf N content was determined with a N–C analyzer (NC-900, Shimadzu, Kyoto, Japan), and Chl content in the leaves was extracted using dimethyl sulfoxide (DMSO) as according to Barnes et al. (1992) and Shinano et al. (1996). Chl (*a* and *b*) content was measured using a double-beam spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan), and was calculated as according to Barnes et al. (1992).

$$\text{Chl } a \ (\mu\text{g mg}^{-1}) = (14.85 \times A_{665} - 5.14 \times A_{648}) \times (a/b) \quad (3)$$

$$\text{Chl } b \ (\mu\text{g mg}^{-1}) = (25.48 \times A_{648} - 7.36 \times A_{665}) \times (a/b) \quad (4)$$

$A_{665}$ : absorbance at 665 nm,  $A_{648}$ : absorbance at 648 nm,  $a$ : DMSO solution (mL),  $b$ : dry mass of leaves (mg<sup>1</sup>).

The RuBisCo content was determined by SDS-PAGE (Makino et al. 1985; Hikosaka 1996; Kitaoka and Koike 2004). Disks (4 cm<sup>2</sup>) were punched out of the frozen leaves, and were homogenized in 100 mM sodium phosphate buffer (pH 7.5) containing 0.4 M sorbitol, 2 mM MgCl<sub>2</sub>, 10 mM NaCl, 5 mM iodoacetate acid, 1% (w/v)  $\beta$ -mercaptoethanol and 2% (w/v) polyvinylpyrrolidone, together with acid-washed quartz sand. The homogenate was centrifuged at 10,000g for 1 min at 0–4 °C. The resulting supernatant fluid was treated with SDS solution for 1 min at 100 °C. The gel was stained with Coomassie Brilliant Blue R-250 (CBB), and the RuBisCo large subunit was extracted with formamide, so as to facilitate spectrophotometric determination of RuBisCo. The RuBisCo content was determined spectrophotometrically by formamide extraction of the CBB Blue R-250-stained bands corresponding to the large and small subunits of RuBisCo separated by SDS–PAGE using calibration curves made with RuBisCo purified from *R. pseudoacacia* leaves. A calibration curve was made with bovine albumin (Sigma-Aldrich, St. Louis, MO).

## N allocation patterns in the leaves

Allocation of N to  $N_L$  (LHC, PSI and PSII, even though the PSI and PSII were found as one unit of megacomplex; Yokono et al. 2015), to  $N_E$  (electron transport proteins except  $N_L$  plus the N content of carbon cycle proteins except  $N_R$ ) and to  $N_R$  (leaf N allocation to RuBisCo), as components of the total N concentration, was calculated using the formula of Kitaoka and Koike (2004):

$$N_L = [\text{Chl}] \times 37.1 \times 14, \quad (5)$$

where [Chl] is the calculated Chl *a* + *b* concentration ( $\text{mmol m}^{-2}$ ), 37.1 is mol N per mol Chl, and 14 is the molecular weight of N (Evans and Seemann 1989).

$$N_E = J_{\max}/156 \times 9.53 \times \text{LMA} \times \text{Nm}, \quad (6)$$

where 156 is the ratio of  $J_{\max}$  to the cytochrome *f* content ( $\text{mmol electrons (mol cyt } f)^{-1} \text{ s}^{-1}$ ) at 25 °C, 9.53 is N in bioenergetics per unit cytochrome *f* ( $\text{mol mmol}^{-1}$ ), LMA is the leaf dry mass per unit leaf area ( $\text{g m}^{-2}$ ), and Nm is the N content per unit leaf mass ( $\text{mg g}^{-1}$ ) (Hikosaka and Terashima 1995; Niinemets and Tenhunen 1997).

$$N_R = \text{RuBisCo (g m}^{-2}) \times 0.204, \quad (7)$$

where 0.204 is N content rate in the RuBisCo.  $N_R$  is calculated by the method of Evans and Seemann (1989).

### Acetylene reduction activity

The N fixation rate in *R. pseudoacacia* was determined by acetylene reduction assay (ARA) (Schöllhorn and Burris 1967; Hardy et al. 1968; Rivera-Ortiz and Burris 1975; Shah et al. 1975; Peterson and Burris 1976). Approximately 2.5 g of nodule cluster with subtending fine roots were harvested from the same trees used for photosynthetic rate measurement, and was placed immediately in the syringe. Then, 10% (v/v) of the air in the syringe was removed and replaced with an equal volume of acetylene ( $\text{C}_2\text{H}_2$ ) (99.9%). The nodules were maintained at ambient soil temperature (as measured by a digital thermometer) by placing the incubation syringe within the forest floor prior to assay. After 1, 5, 10 and 30 min, 10 ml of gas was removed from the syringe and injected into a gas chromatograph to measure the production of ethylene from the acetylene (G-3000, Hitachi, Tokyo, Japan).

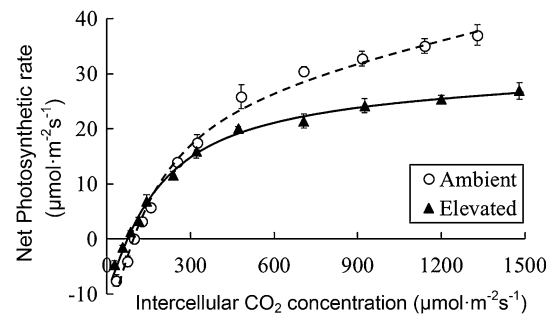
### Statistical analysis

To determine the effects of the FACE system, an analysis of variance (ANOVA) was carried out by StatView 5.0 (version 5.0, SAS Institute Inc., Cary, NC). Results are stated as mean  $\pm$  SD.

## Results

### Photosynthetic responses

The net photosynthetic rate/intercellular  $\text{CO}_2$  concentration curves at light saturation are shown in Fig. 1, and the value of the photosynthetic parameters of *R. pseudoacacia* grown at ambient and elevated  $[\text{CO}_2]$  are set out in Table 1. Elevated



**Fig. 1** Response of the net photosynthetic rate to intercellular  $\text{CO}_2$  concentration for *R. pseudoacacia* grown at ambient ( $370 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and elevated ( $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ )  $[\text{CO}_2]$ . Values are mean  $\pm$  SD for individual trees in three rings in each  $\text{CO}_2$  treatment

$[\text{CO}_2]$  affected the net photosynthetic rate,  $A_{\text{growth}}$ ,  $A_{\max}$ ,  $V_{\text{cmax}}$ , and  $J_{\max}$ . The  $A_{\text{growth}}$  was significantly higher (8%) for plant materials grown at elevated  $[\text{CO}_2]$  relative to controls ( $P < 0.01$ ; see Fig. 1; Table 1). In contrast, under enhanced  $[\text{CO}_2]$  and light saturation ( $A_{\max}$ ), the photosynthetic rate of *R. pseudoacacia* grown at ambient  $[\text{CO}_2]$  was about  $32 \mu\text{mol m}^{-2} \text{s}^{-1}$ , and at elevated  $[\text{CO}_2]$  was  $24 \mu\text{mol m}^{-2} \text{s}^{-1}$  (27% less;  $P < 0.001$ ). Moreover,  $V_{\text{cmax}}$  (−15%) and  $J_{\max}$  (−19%) were significantly lower for materials grown at elevated  $[\text{CO}_2]$  ( $P < 0.001$ ).

### RuBisCo content in leaves

Figure 2 shows the relation between the RuBisCo content and the  $V_{\text{cmax}}$  in leaves of *R. pseudoacacia* grown at ambient and elevated  $[\text{CO}_2]$ . The RuBisCo content and  $V_{\text{cmax}}$  in leaves grown at elevated  $[\text{CO}_2]$  was significantly less than ambient  $[\text{CO}_2]$  ( $P < 0.001$ ). Strong correlation between  $V_{\text{cmax}}$  and RuBisCo content was observed across  $\text{CO}_2$  treatments, moreover ( $R^2 = 0.79$ ).

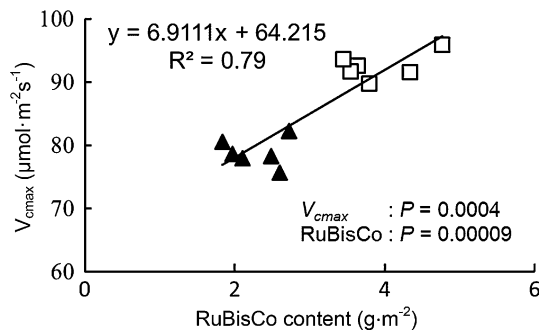
### Leaf physiological variables and leaf structural variables

Table 2 shows the leaf N content, C/N ratio, Chl *a* + *b*, Chl *a/b*, PNUE, WUE and LMA in current year leaves of *R. pseudoacacia* grown at ambient and elevated  $[\text{CO}_2]$ . The leaf N content of *R. pseudoacacia* grown at ambient  $[\text{CO}_2]$  was  $2.87 \text{ g m}^{-2}$ , and at elevated  $[\text{CO}_2]$  it was  $2.24 \text{ g m}^{-2}$ . The leaf N content at elevated  $[\text{CO}_2]$  was significantly less (by 23%) than at ambient  $[\text{CO}_2]$  ( $P < 0.05$ ). The C/N ratio and PNUE were sharply lower at elevated  $[\text{CO}_2]$ , but the difference was not significant. The Chl *a* + *b* in leaves grown at ambient and elevated  $[\text{CO}_2]$  were, respectively, 0.26 and  $0.24 \text{ g m}^{-2}$ ; the difference was not significant. The Chl *a/b* was slightly higher in leaves grown at elevated  $[\text{CO}_2]$  than at ambient  $[\text{CO}_2]$ , but the difference was not

**Table 1**  $A_{\text{growth}}$ ,  $A_{\text{max}}$ ,  $V_{\text{cmax}}$  and  $J_{\text{max}}$  of *R. pseudoacacia* grown at ambient ( $370 \mu\text{mol mol}^{-1}$ ) and elevated ( $500 \mu\text{mol mol}^{-1}$ )  $[\text{CO}_2]$ , and relative changes between the treatments

Parameter	CO <sub>2</sub> concentration		Elevated/ambient	P value
	Ambient	Elevated		
$A_{\text{growth}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	18.1 (0.1)	19.7 (0.3)	1.08	0.0024
$A_{\text{max}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	32.7 (1.3)	24.1 (1.2)	0.73	0.0002
$V_{\text{cmax}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	92.5 (2.1)	78.8 (0.2)	0.85	0.0004
$J_{\text{max}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	89.8 (4.9)	72.9 (6.3)	0.81	0.0005

Values are mean  $\pm$  SD for individual trees in three rings in each CO<sub>2</sub> treatment. The significance of the CO<sub>2</sub> treatment effect is indicated by the P value. The difference was tested by ANOVA



**Fig. 2** Relation between the RuBisCo content and the  $V_{\text{cmax}}$  in leaves for *R. pseudoacacia* grown at ambient  $[\text{CO}_2]$  ( $370 \mu\text{mol mol}^{-1}$ , open squares) and elevated  $[\text{CO}_2]$  ( $500 \mu\text{mol mol}^{-1}$ , closed triangles). The significance of the CO<sub>2</sub> treatment effect is indicated by the P value. CO<sub>2</sub> treatment had significant effect on  $V_{\text{cmax}}$  ( $P = 0.0004$ ) and RuBisCo content ( $P = 0.00009$ ). The difference was tested by ANOVA

significant. The WUE in leaves was almost identical at elevated and ambient  $[\text{CO}_2]$ . We observed large differences in LMA. The LMA in leaves grown at ambient  $[\text{CO}_2]$  was  $145.5 \text{ g m}^{-2}$ , whereas at elevated  $[\text{CO}_2]$  it was  $107.0 \text{ g m}^{-2}$ . The LMA at elevated  $[\text{CO}_2]$  was significantly less (by 27%) than at ambient  $[\text{CO}_2]$  ( $P < 0.01$ ).

Table 3 shows the RuBisCo/N, Chl/N,  $V_{\text{cmax}}/\text{N}$ ,  $J_{\text{max}}/\text{N}$  and  $J_{\text{max}}/V_{\text{cmax}}$  of *R. pseudoacacia* grown at ambient or elevated  $[\text{CO}_2]$ , and relative changes between the treatments. The RuBisCo/N of *R. pseudoacacia* grown at

ambient  $[\text{CO}_2]$  was  $1.37/\text{g m}^{-2}$ , and at elevated  $[\text{CO}_2]$  it was  $1.02/\text{g m}^{-2}$ . The RuBisCo/N at elevated  $[\text{CO}_2]$  was significantly less (by 26%) than at ambient  $[\text{CO}_2]$  ( $P < 0.05$ ). The value of  $V_{\text{cmax}}/\text{N}$  and  $J_{\text{max}}/\text{N}$  were sharply higher at elevated  $[\text{CO}_2]$ , but the difference was not significant. However, Chl/N was significantly greater (by 22%) at elevated  $[\text{CO}_2]$  than at ambient  $[\text{CO}_2]$  ( $P < 0.05$ ). The ratio of the capacities of RuBP regeneration to RuBP carboxylation ( $J_{\text{max}}/V_{\text{cmax}}$ ) was almost identical at elevated and ambient  $[\text{CO}_2]$ .

### Acetylene reduction activity

Figure 3 shows the acetylene reduction activity of a N-fixer that is symbiotic with *R. pseudoacacia*, under ambient and elevated  $[\text{CO}_2]$ . The acetylene reduction activity of *Frankia* sp. grown at elevated  $[\text{CO}_2]$  was significantly greater than for that grown at ambient  $[\text{CO}_2]$  ( $P < 0.01$ ). The acetylene reduction activity of *Frankia* sp. grown at ambient  $[\text{CO}_2]$  was  $12.2 \mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ nodule h}^{-1}$ , and the acetylene reduction activity of *Frankia* sp. grown at elevated  $[\text{CO}_2]$  was  $15.0 \mu\text{mol C}_2\text{H}_4 \text{ g}^{-1} \text{ nodule h}^{-1}$ .

### N allocation patterns in the leaves

Figure 4 shows the N allocation patterns in the leaves of *R. pseudoacacia* grown at ambient and elevated  $[\text{CO}_2]$ . The N

**Table 2** Leaf N content, C/N ratio, Chl *a* + *b*, Chl *a/b*, PNUE, WUE and LMA of *R. pseudoacacia* grown at ambient ( $370 \mu\text{mol mol}^{-1}$ ) and elevated ( $500 \mu\text{mol mol}^{-1}$ )  $[\text{CO}_2]$ , and relative changes between the treatments

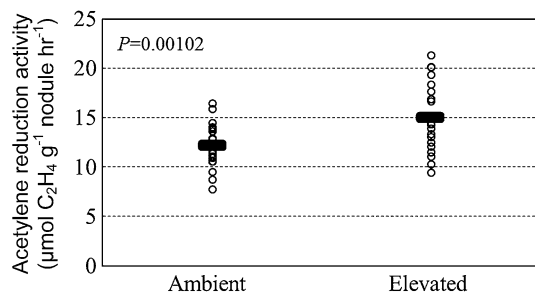
Parameter	CO <sub>2</sub> concentration		Elevated/ambient	P value
	Ambient	Elevated		
Leaf N content ( $\text{g m}^{-2}$ )	2.87 (0.24)	2.24 (0.21)	0.77	0.0164
C/N ratio ( $\text{g m}^{-2}/\text{g m}^{-2}$ )	11.5 (1.0)	11.1 (0.9)	0.97	ns
Chl <i>a</i> + <i>b</i> ( $\text{g m}^{-2}$ )	0.26 (0.02)	0.24 (0.02)	0.92	ns
Chl <i>a/b</i> ratio	9.33 (1.51)	9.78 (0.57)	1.04	ns
PNUE ( $\mu\text{mol g}^{-1} \text{s}^{-1}$ )	12.53 (0.51)	11.81 (1.83)	0.94	ns
WUE ( $\mu\text{mol mmol}^{-1}$ )	16.57 (1.96)	17.01 (0.98)	1.02	ns
LMA ( $\text{g m}^{-2}$ )	145.5 (8.5)	107.0 (15.0)	0.73	0.0043

Values are mean  $\pm$  SD for individual trees in three rings in each CO<sub>2</sub> treatment. The significance of the CO<sub>2</sub> treatment effect is indicated by the P value (ns = nonsignificant). The difference was tested by ANOVA

**Table 3** RuBisCo/N, Chl/N,  $V_{\text{cmax}}/N$ ,  $J_{\text{max}}/N$  and  $J_{\text{max}}/V_{\text{cmax}}$  of *R. pseudoacacia* grown at ambient ( $370 \mu\text{mol mol}^{-1}$ ) and elevated ( $500 \mu\text{mol mol}^{-1}$ ) [ $\text{CO}_2$ ], and relative changes between the treatments

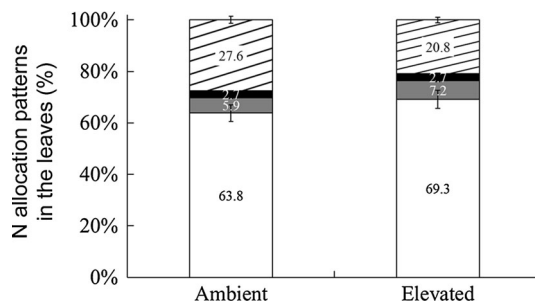
Parameter	$\text{CO}_2$ concentration		Elevated/ambient	<i>P</i> value
	Ambient	Elevated		
RuBisCo/N ( $\text{g m}^{-2}/\text{g m}^{-2}$ )	1.37 (0.23)	1.02 (0.19)	0.74	0.0217
Chl/N ( $\text{g m}^{-2}/\text{g m}^{-2}$ )	0.09 (0.008)	0.11 (0.01)	1.22	0.0361
$V_{\text{cmax}}/N$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}/\text{g m}^{-2}$ )	32.25 (2.48)	35.42 (3.22)	1.09	ns
$J_{\text{max}}/N$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}/\text{g m}^{-2}$ )	29.27 (4.14)	33.82 (5.31)	1.15	ns
$J_{\text{max}}/V_{\text{cmax}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}/\mu\text{mol m}^{-2} \text{s}^{-1}$ )	0.97 (0.03)	0.93 (0.07)	0.95	ns

Values are mean  $\pm$  SD for individual trees in three rings in each  $\text{CO}_2$  treatment. The significance of the  $\text{CO}_2$  treatment effect is indicated by the *P* value (ns = nonsignificant). The difference was tested by ANOVA



**Fig. 3** Acetylene reduction activity of *Frankia* sp., symbiotic with *R. pseudoacacia*, grown at ambient [ $\text{CO}_2$ ] ( $370 \mu\text{mol mol}^{-1}$ ) and elevated [ $\text{CO}_2$ ] ( $500 \mu\text{mol mol}^{-1}$ ). The black bars are means of 24 measurements (open circles) for each [ $\text{CO}_2$ ] treatment. The significance of the  $\text{CO}_2$  treatment effect is indicated by the *P* value. The difference was tested by ANOVA

allocation patterns in leaves of *R. pseudoacacia* grown at elevated [ $\text{CO}_2$ ] differed from that at ambient [ $\text{CO}_2$ ], especially to RuBisCo and remaining N. For  $N_R$  there was a slight decrease in *R. pseudoacacia* grown at elevated [ $\text{CO}_2$ ] relative to ambient [ $\text{CO}_2$ ], from an allocation of approximately 27.6% N to 20.8%. Remaining N was slightly greater in *R. pseudoacacia* grown at elevated [ $\text{CO}_2$ ]



**Fig. 4** N allocation patterns in the leaves (%) of *Robinia pseudoacacia* grown at ambient [ $\text{CO}_2$ ] ( $370 \mu\text{mol mol}^{-1}$ ) and elevated [ $\text{CO}_2$ ] ( $500 \mu\text{mol mol}^{-1}$ ). The diagonally hatched box denotes N allocated to RuBisCo ( $N_R$ ); the black box denotes N allocated to electron transport proteins ( $N_E$ ); the dark-gray box denotes N allocated to LHC, PSI and PSII ( $N_L$ ); the white interior box denotes others. Values are mean  $\pm$  SD for individual six trees in three rings in each  $\text{CO}_2$  treatment

relative to ambient [ $\text{CO}_2$ ], from an allocation of approximately 63.8–69.3%. *R. pseudoacacia* grown at ambient [ $\text{CO}_2$ ] allocated 2.7% N to  $N_E$  and 5.9% N to  $N_L$ ; at elevated [ $\text{CO}_2$ ] these figures were 2.7 and 7.2%.

## Discussion

During the fourth year of exposure of *R. pseudoacacia* to elevated [ $\text{CO}_2$ ] in the FACE system, we observed changes in the photosynthesis parameters and in N allocation patterns in the leaves, as well as changes to leaf structural and biochemical characteristics and acetylene reduction activity of *Frankia* sp. symbiotic with *R. pseudoacacia*. Previous studies determined that long-term exposure to elevated [ $\text{CO}_2$ ] in controlled environments results in a decline in photosynthetic capacity, associated with reduced levels of RuBisCo and organic N per unit leaf area (Choi et al. 2005a, b; Koike et al. 2007; Lambers et al. 2008; Choi et al. 2009; Robinson et al. 2012). We also observed a photosynthetic down-regulation of *R. pseudoacacia* in response to elevated [ $\text{CO}_2$ ]. This may be associated with reduction of N and RuBisCo content, and also changed N allocation patterns in the leaves, corresponding to conditions giving rise to down-regulation (Tables 1, 2, 3; Figs. 1, 2, 3, 4).

Photosynthetic characteristics are determined mainly by leaf structure (such as LMA), leaf N content, and the N allocation patterns in the leaves. The LMA underwent various changes with light, atmospheric [ $\text{CO}_2$ ], nutrient, temperature and other factors (Poorter et al. 2009). The LMA, which represents the light-intercepting capability of a leaf per unit area, is an index related to many important physiological characteristics (Reich et al. 1999). Changes in leaf morphology and anatomy may occur due to the effects of elevated [ $\text{CO}_2$ ] on the leaf carboxylation rate per unit leaf area (Luo et al. 1994; Peterson et al. 1999). In the present study there was a significantly lower LMA and leaf N content of *R. Pseudoacacia* grown at elevated [ $\text{CO}_2$ ] than at ambient [ $\text{CO}_2$ ]. There have been similar findings in

N-fixing trees and other tree species, in which the LMA decreases in specimens grown at elevated  $[\text{CO}_2]$ , and in some herbaceous plants grown in a FACE system (Norby 1996; Peterson et al. 1999; von Caemmerer et al. 2001; Gutiérrez et al. 2009; Liu et al. 2012; Novriyanti et al. 2012; Koike et al. 2015). Other studies found that the LMA increases in specimens grown at elevated  $[\text{CO}_2]$ , however, (Staudt et al. 2001; Poorter et al. 2009). The LMA of *R. pseudoacacia* grown at elevated  $[\text{CO}_2]$ , given that there is no restricting root zone in the FACE system and that the plant is undergoing symbiosis with  $\text{N}_2$  fixative micro-organisms, was significantly lower than at ambient  $[\text{CO}_2]$ . In a review of the FACE system, Koike et al. (2015) stated that N-fixing Alder species, in symbiosis with the N-fixing micro-organism *Frankia* sp., usually had lower LMA (high SLA; reciprocal of LMA) when grown at elevated than at ambient  $[\text{CO}_2]$ , because these N-fixing species are able to act as a sink of photosynthates (Koike 1993; Koike et al. 1997; Tobita et al. 2011; Liu et al. 2012). Fast-growing species mostly have low LMA and lower leaf tissue densities, moreover. This is an important finding, because it was determined in the FACE experiment that this is due mainly to reduced foliage thickness in both leaves and phyllodes of *R. pseudoacacia*. Also, PNUE and WUE are almost identical at elevated  $[\text{CO}_2]$  and ambient  $[\text{CO}_2]$ . It is likely that the combination of decreasing leaf N content and of LMA with photosynthetic rate led to almost identical in PNUE and WUE at elevated  $[\text{CO}_2]$ . Moreover, we found that the acetylene reduction activity was increased in specimens of *R. pseudoacacia* grown at elevated  $[\text{CO}_2]$ , relative to those grown at ambient  $[\text{CO}_2]$  (Table 2; Fig. 3). Several studies have found that  $\text{N}_2$ -fixing trees (e.g. *Alnus glutinosa*, *A. hirsute*, *Elaeagnus angustifolia*, *R. pseudoacacia*) grown at elevated  $[\text{CO}_2]$  increase the nitrogenase activity of  $\text{N}_2$ -fixing micro-organisms, or fixed more N, and made their leaf N concentration smaller (Norby 1987; Luo et al. 1994; Koike et al. 1997; Vogel et al. 1997; Olesniewicz and Thomas 1999; Tobita et al. 2005; Millett et al. 2012). Plants grown at elevated  $[\text{CO}_2]$  commonly accumulate carbohydrate in leaves and other tissues as starch, soluble sugars and structural compounds. Decreased leaf N may be due to a combination of dilution from increased carbohydrate and decreased investment in RuBisCo or other enzymes, or the effect of watering (Coleman et al. 1993; Ainsworth and Long 2005).

Also important is that in *R. pseudoacacia* grown at elevated  $[\text{CO}_2]$ , the decreases of  $J_{\text{max}}$ ,  $V_{\text{cmax}}$ , Chl and RuBisCo content in the leaf corresponded to conditions giving photosynthetic down-regulation found with LMA, and leaf N content decreased (Tables 1, 2; Figs. 1, 2). Values of  $J_{\text{max}}$  and  $V_{\text{cmax}}$  were reduced in elevated  $[\text{CO}_2]$ , by 19 and 15%, respectively ( $P < 0.001$ ). Also,  $A_{\text{max}}$  was

27% less at elevated  $[\text{CO}_2]$  ( $P < 0.001$ ). Ainsworth and Long (2005) reported in FACE experiments that  $V_{\text{cmax}}$  was at least 20% less at elevated  $[\text{CO}_2]$ , and that leaf N was reallocated from RuBisCo to other functions. These results indicate that the photosynthetic down-regulation, inclusive of  $V_{\text{cmax}}$  and  $J_{\text{max}}$ , Chl and RuBisCo content at elevated  $[\text{CO}_2]$ , are due to a decrease in leaf N content. RuBisCo is the rate-limiting enzyme in photosynthetic carbon assimilation and photorespiratory oxygen reduction. Photosynthesis at elevated  $[\text{CO}_2]$  is limited by the electron transport capacity (Farquhar et al. 1980) or RuBP regeneration (Onoda et al. 2005). The parameters  $V_{\text{cmax}}$  and  $J_{\text{max}}$  are strongly correlated with RuBisCo content, leaf structure (LMA), cytochrome and especially leaf N content (von Caemmerer and Farquhar 1981; Evans 1987; Onoda et al. 2005; Yamori et al. 2010). In the present study, we found a decrease in RuBisCo/N ( $-26\%$ ,  $P = 0.02$ ) at elevated  $[\text{CO}_2]$  (Table 3). This implies that the reason for the reduction in RuBisCo content is that leaf N was not distributed sufficiently to RuBisCo at elevated  $[\text{CO}_2]$  (Table 3; Figs. 2, 4). Also,  $V_{\text{cmax}}$  and  $J_{\text{max}}$  were significantly less at elevated  $[\text{CO}_2]$ , with corresponding RuBisCo content. This fact also indicates that the decrease in the  $J_{\text{max}}/V_{\text{cmax}}$  reduces the  $\text{CO}_2$  stimulation of photosynthesis, and the photosynthetic rate was limited by RuBP regeneration rather than RuBP carboxylation in *R. pseudoacacia* specimens exposed to elevated  $[\text{CO}_2]$ .

N absorbed in roots enters photosynthetic proteins in the leaves, specifically LHC proteins, RuBisCo for  $\text{CO}_2$  fixation, electron transport systems, and other remaining soluble proteins (Kitaoka and Koike 2004). Elevated  $[\text{CO}_2]$  led to increased leaf carbohydrates and less leaf protein when photosynthesis was down-regulated. Photosynthetic down-regulation can occur through sugar-mediated repression of RuBisCo, and is related to reallocation of N away from the photosynthetic apparatus or an effect on photosynthetic enzymes (Dewar et al. 1998; Stitt and Krapp 1999; Makino et al. 2000). Changes in the N allocation patterns within the photosynthetic apparatus are an important mode of acclimation to changes in light, temperature and atmospheric  $[\text{CO}_2]$  (Katahata et al. 2007; Lambers et al. 2008). Models of optimal photosynthesis suggest that N allocation patterns within leaves reflect a trade-off between  $\text{CO}_2$  fixation and LHC proteins (Evans 1989; Hikosaka and Terashima 1995). Consistent with this hypothesis, allocation of N to RuBisCo was negatively correlated with elevated  $[\text{CO}_2]$ . The  $\text{N}_R$  at elevated  $[\text{CO}_2]$  was between 27.6 and 20.8% in our study. This is because *R. pseudoacacia* leaves grown at elevated  $[\text{CO}_2]$  allocated N less rapidly to RuBisCo than to other photosynthetic proteins, such as  $\text{N}_E$  and  $\text{N}_L$ , than did specimens grown at ambient  $[\text{CO}_2]$ . Remaining N increased more rapidly than



N in other photosynthetic proteins (such as  $N_E$  and  $N_L$ ) in leaves grown at elevated  $[CO_2]$  than in leaves grown at ambient  $[CO_2]$ . Although PSI and PSII were found as one unit of megacomplex, we empirically use the terms PSI and PSII (Yokono et al. 2015). Relatively less N was allocated to RuBisCo in leaves of *R. pseudoacacia* grown at elevated  $[CO_2]$ , consistent with  $J_{max}/V_{cmax}$  ratios generated from  $P_N/C_i$  curves.

These results suggest that leaf N is reallocated from RuBisCo to remaining N at elevated  $[CO_2]$ . RuBisCo is a soluble protein, whereas the reaction center and LHC proteins are thylakoid membrane proteins. Soluble proteins are resolved more easily than membrane protein (Makino et al. 1988; Katahata et al. 2007). Remobilizing the N from RuBisCo may, therefore, be more efficient than remobilizing it from light-harvesting proteins. These responses in the *R. pseudoacacia* leaves are related to decreased RuBisCo and leaf N content, resulting in a low carbon gain at elevated atmospheric  $[CO_2]$ , because the same photosynthetic carbon assimilation rate can be maintained with lower RuBisCo content (Hilbert et al. 1991). The  $A_{growth}$  was significantly higher in *R. pseudoacacia* grown at elevated  $[CO_2]$  than at ambient  $[CO_2]$ , however. We have suggested that *R. pseudoacacia* allocates more  $N_L$  (LHC, PSI and PSII) in leaves to improve light harvesting but less  $N_R$  in leaves, because sufficient  $CO_2$  for carbon fixing is available at elevated  $[CO_2]$ . Moreover, *R. pseudoacacia* allocated same  $N_E$  and more N to remaining N at elevated  $[CO_2]$ . According to these observations, *R. pseudoacacia* grown at elevated  $[CO_2]$  is well prepared for sudden changes in environmental conditions, such as light, temperature or atmospheric  $[CO_2]$ , with N stored as soluble remaining N storage in soluble proteins.

**Author contribution statement** DSC, HT and YW conducted experiments, and measured the photosynthetic rates and acetylene reduction activities. TK directed the experiment and managed the experimental facilities. DSC and TS analyzed leaf nitrogen allocation and also the various nutrients. DSC, TK and RDG discussed the results and co-wrote this paper.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interests.

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