Mesophyll conductance in leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) seedlings grown under elevated CO_2 concentration and low N availability

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Abstract

To test the hypothesis that mesophyll conductance (g_m) would be reduced by leaf starch accumulation in plants grown under elevated CO_2 concentration [CO_2], we investigated g_m in seedlings of Japanese white birch grown under ambient and elevated [CO_2] with an adequate and limited nitrogen supply by using simultaneous gas exchange and chlorophyll fluorescence measurements. Both elevated [CO_2] and limited nitrogen supply decreased area-based leaf N accompanied with a decrease in the maximum rate of Rubisco carboxylation ($V_{c,max}$) on a CO_2 concentration at chloroplast stroma (C_c) basis. Conversely, only seedlings grown at elevated [CO_2] under limited nitrogen supply had significantly higher leaf starch content with significantly lower g_m among the treatment combinations. Based on a leaf anatomical analysis using microscopic photographs, however, there were no significant difference in the area of chloroplast surfaces facing intercellular space per unit leaf area among treatment combinations. Thicker cell walls were suggested in plants grown under limited N by increases in leaf mass per area subtracting non-structural carbohydrates. These results suggest that starch accumulation and/or thicker cell walls in the leaves grown at elevated [CO_2] under limited N supply might hinder CO_2 diffusion in chloroplasts and cell walls, which would be an additional cause of photosynthetic down-regulation as well as a reduction in Rubisco activity related to the reduced leaf N under elevated [CO_2].

Abbreviations: A, net CO_2 assimilation rate; A_c, net photosynthetic rate limited by Rubisco activity; A_j, net photosynthetic rate limited by RuBP regeneration; C_c, CO_2 concentration in the chloroplast stroma; C_i, intercellular CO_2 concentration; F_s, chlorophyll fluorescence under actinic light; F_m', the maximum chlorophyll fluorescence under actinic light; g_m, mesophyll conductance to CO_2 supply from substomatal cavities to sites of carboxylation; J_{max}, the maximum rate of electron transport driving RuBP regeneration; J_T, total rate of electron transport though PSII to photosynthesis and photorespiration; K_c, K_o, Rubisco Michaelis constant for CO_2 and O_2, respectively; LMA, leaf mass per area; N_{area}, area-based leaf nitrogen content; NSC, non-structural carbohydrates; PFD, photon flux density; R_d, mitochondrial respiration in the light; R_n, mitochondrial respiration in the dark; S_c, the areas of chloroplast surfaces facing intercellular space per unit leaf area; V_{c,max_Cc}, V_{c,max_Ci}, C_c- and C_i-based maximum carboxylation capacity of Rubisco, respectively; Φ_{CO2}, quantum yield of CO_2 assimilation; Φ_e, quantum yield of electron transport though PSII to photosynthesis and photorespiration; Φ_{PSII}, quantum yield of PSII electron transport; Γ^*, CO_2 compensation point in the absence of dark respiration

Introduction

Elevated atmospheric CO_2 concentration [CO_2] temporally stimulates the photosynthetic rate, but eventually results in photosynthetic down-regulation with carbohydrate accumulation after long-term exposure, even when root growth is not limited during FACE (free-air CO_2 enrichment) experiments (Nie et al. 1995, Rogers and Ellsworth 2002, Bernacchi et al. 2003, 2005, Ainsworth and Long 2005, Rogers et al. 2006, Eguchi et al. 2008). Some mechanisms underlying photosynthetic down-regulation in leaves grown under elevated [CO_2] have been proposed, such as a reduced Rubisco content accompanied with reduced leaf N content (Seneweera et al. 2011), and a reduced ratio of Rubisco to total N via a reduction in RNA expression induced by sugar accumulation (e.g. Sage 1994, Drake et al. 1997, Sims et al. 1998, Stitt and Krapp 1999). Aside from the amount and activity of Rubisco, a decrease in mesophyll conductance from the stomatal cavity to chloroplast stroma due to starch accumulation has been proposed as a mechanism of photosynthetic down-regulation in crop plants with large carbon sinks based on a negative correlation between the photosynthetic rate and starch accumulation (Nakano et al. 2000, Sawada et al. 2001).

The mesophyll conductance to CO_2 supply from substomatal cavities to sites of carboxylation (g_m) may pose a significant limitation to photosynthesis (Harley et al. 1992, Loreto et al. 1992, Terashima et al. 2006, Warren 2008). Simultaneous measurements of gas exchange and chlorophyll fluorescence make it possible to estimate g_m, combined with measurements under non-photorespiratory conditions (Harley et al. 1992, Epron et al. 1995, Warren and Dreyer 2006). Recent studies have reported that g_m can respond to environmental conditions, such as growth irradiance (Hanba et al. 2002), nutrient supply (Warren 2004), and short-term variations in temperature (Bernacchi et al. 2002, Warren and Dreyer 2006), and ambient CO_2 concentration (Centritto et al. 2003, Düring 2003, Flexas et al. 2007, Vrábl et al. 2009). g_m is considered to be determined not only by intrinsic anatomical features (Oguchi et al. 2003, Eguchi et al. 2004, Terashima et al. 2006) but possibly by physiological reactions involving a carbonic anhydrase or aquaporins (Bernacchi et al. 2002, Long and Bernacchi 2003, Hanba et al. 2004, Terashima et al. 2006, Flexas et al. 2007). g_m is also known to be smaller in tree species than in herbaceous ones, which generally results in lower photosynthetic rates in tree species (Epron et al. 1995, Flexas et al. 2008, 2012).

The effects of long-term elevated [CO_2] on g_m have been reported as species- and experiment dependent (Singsaas et al. 2003, Bernacchi et al. 2005). Bernacchi et al. (2005) reported

that g_m is unaffected in soybean grown at elevated [CO_2] though the effect of starch accumulation on g_m was not assessed. It is proposed that starch accumulation in the chloroplast hinders CO_2 diffusion through an increase in the length of the diffusion pathway around large starch grains (Krieg 1986). To our knowledge, there has been little study on g_m in plants grown under elevated CO_2 in relation to starch accumulation. In the previous studies, limited N supply was found to be capable of inducing starch accumulation accompanied by the photosynthetic down-regulation in Japanese white birch (*Betula platyphylla* var. *japonica*) grown under elevated [CO_2] (Kitao et al. 2005, 2007).

We hypothesized that leaf starch accumulation would decrease g_m, which could be a cause of apparent photosynthetic down-regulation under elevated [CO_2] as well as a reduction in Rubisco activity in Japanese white birch. To test this hypothesis, we investigated the effects of elevated CO_2 on g_m in its leaves, which are feasible to induce starch accumulation grown at elevated CO_2 while limiting N supply, via simultaneous measurements of gas exchange and chlorophyll fluorescence (Harley et al. 1992).

Materials and methods

Plants and growing conditions

Two-year-old seedlings of Japanese white birch (*Betula platyphylla* Sukatchev var. *japonica* Hara) obtained from a commercial nursery (Oji Forestry & Landscaping Co., Ltd., Sapporo, Japan) were transplanted in free-draining plastic pots (diameter: 18 cm, depth: 15 cm, volume: approx. 3 l) filled with clay loam soil mixed with Kanuma pumice soil (1:1 in volume) to simulate typical of immature volcanic ash characterized by Japanese land. Pots were placed on the trays to prevent nutrient drainage. The seedling height was 15 to 20 cm. The seedlings were placed in natural daylight (approx. 90% of full sunlight) phytotron (26/16 °C, day/night) for about 45 days from the end of May to the beginning of July. One chamber was used for each CO_2 treatment: 360 μmol mol^{-1} (ambient CO_2 treatment, Ambient); and 720 μmol mol^{-1} (elevated CO_2 treatment, Elevated). To reduce the chamber effects, the treatments and seedlings were switched between the chambers about every 10 days. Details of the CO_2 regulation are as described previously (Koike 1995, Koike et al. 1996). Two nitrogen levels were applied: 700 mg per plant (high nitrogen, +N), or 100 mg per plant (low nitrogen, −N).

Measurements of CO_2 assimilation rate and chlorophyll fluorescence

Plants grown in the growth chambers were transferred to the laboratory and supplied with adequate water in the evening prior to measurement. The CO_2 assimilation rate and chlorophyll fluorescence were measured simultaneously on fully expanded mature leaves, which were developed after treatments began (approx. 30 days old) with a portable fluorometer (PAM-2000, Walz, Effeltrich, Germany) combined with a portable photosynthesis system (LI-6400, Li-Cor, Nebraska, USA) using a PAM-2000 adapter chamber (Li 6400-06, Li-Cor). As we observed small differences in the number of newly-developed leaves in the main shoots of seedlings (from 8 to 11) 45 days after treatment among combinations of CO_2 and N, the 30-day-old leaves used in the present study could be considered to be at virtually the same developmental stage. Firstly, we measured the CO_2 assimilation rate and chlorophyll fluorescence under various light intensities and non-photorespiratory conditions (at 1 % O_2) in order to derive the relationship between the quantum yield of CO_2 assimilation (Φ_{CO2}) and that of electron transport (Φ_{PSII}). The leaf temperature was maintained at 30 °C and relative humidity at the inlet of the chamber between 70 and 80%. Φ_{CO2} is calculated as: $\Phi_{CO2} = (A + R_d)/PFD$) (Epron et al. 1995, Valentini et al. 1995), where A is the net CO_2 assimilation rate (μmol m^{-2} s^{-1}), R_d the mitochondrial respiration in the light (μmol m^{-2} s^{-1}), and PFD the photon flux density (μmol m^{-2} s^{-2}). The ratio of R_d to mitochondrial respiration in the dark (R_n) is species-specific, but dependent on leaf age and leaf temperature (Villar et al. 1995). In a preliminary study, we derived the R_d:R_n ratio at a leaf temperature of 30°C for approximately 30-day-old leaves of *B. platyphylla* seedlings grown under ambient and elevated CO_2 using the Laisk method (1977). There was no significant difference in the R_d:R_n ratio between the CO_2 treatments (n = 4 for each treatment), and the mean value of the pooled data (n = 8) was 0.59 ± 0.03 (mean ± SE). In the present study, R_d was estimated from R_n assuming that the R_d:R_n ratio was constant across the treatment combinations as follows:

$$R_d = 0.59\ R_n\ (r^2 = 0.998,\ p < 0.001,\ n = 8) \qquad (1).$$

The fluorescence under actinic light, F_s, and the maximum fluorescence, F_m' (determined under saturating light of 8000 μmol m^{-2} s^{-1} PFD for 1 s), were used to calculate the quantum yield of PSII electron transport, Φ_{PSII} (= $(F_m' - F_s)/F_m'$) (Genty et al. 1989). Subsequently, we measured the CO_2 assimilation rate and chlorophyll fluorescence under various ambient CO_2 concentrations (C_a) (2000, 720, 360, 200, and 100 μmol mol^{-1}), at 21% O_2 and saturating light (1200 μmol m^{-2} s^{-1} PFD). We

chose a light intensity (1200 μmol m^{-2} s^{-1}), which was sufficient to saturate the net photosynthetic rate but not strong enough to induce a light-dependent decrease in the photosynthetic rate based on a preliminary study. The C_i-based maximum carboxylation capacity of Rubisco (V_{c,max_Ci}) was estimated from the following equation (Farquhar et al. 1980, Long and Bernacchi 2003):

$$A_c = (C_i - \Gamma^*)/(C_i + K_c(1 + (O/K_o))V_{c,max\_Ci} - R_d \quad (2),$$

where A_c is the net photosynthetic rate limited by Rubisco activity, and the CO_2 photo-compensation point (Γ^*), the Michaelis-Menten constants for CO_2 (K_c) and O_2 (K_o) on the C_i basis were estimated after Bernacchi et al. (2001) and Long and Bernacchi (2003) assuming g_m is infinite.

We also estimated the C_i-based maximum rate of electron transport driving RuBP regeneration (J_{max_Ci}) from the following equation (Farquhar et al. 1980, Ethier and Livingston 2004):

$$A_j = (C_i - \Gamma^*)/(C_i + 2\Gamma^*)\ J_{max\_Ci}/4 - R_d \quad (3)$$

where A_j is the net photosynthetic rate limited by RuBP regeneration.

Estimation of mesophyll conductance by the variable J method

We used 'the variable J method' to estimate g_m based on gas exchange and chlorophyll fluorescence measurements (Harley et al. 1992, Valentini et al. 1995, Long and Bernacchi 2003). The relationship between the quantum yield of CO_2 assimilation and PSII electron transport under non-photorespiratory conditions was used to estimate the total electron transport rate (J_T).

Φ_{PSII} is linearly related to the apparent quantum efficiency of the photosynthetic linear electron flow, which can be estimated under non-photorespiratory conditions by Φ_{CO2} as:

$$\Phi_{PSII} = k\ \Phi_{CO2} + b \quad (4),$$

where k is the slope and b is the y-axis intercept.

The relationships between the quantum yield of CO_2 assimilation under the non-photorespiratory condition (Φ_{CO2}) and that of electron transport (Φ_{PSII}) were almost identical among the combinations

of CO_2 and N supply, suggesting little effects of CO_2 and N supply on alternative electron pathways other than photosynthesis and photorespiration (Biehler and Fock 1996, Kitao et al. 2003), allowing the use of 'the variable J method'.

Quantum yield of electron transport through PSII to photosynthesis and photorespiration (Φ_e) is estimated as

$$\Phi_e = 4\,\Phi_{CO2} = 4\,(\Phi_{PSII} - b) / k \qquad (5),$$

where 4 is the number of electrons needed to fix one CO_2.

The total rate of electron transport though photosystem II to photosynthesis and photorespiration is calculated as

$$J_T = PFD\,\Phi_e \qquad (6),$$

g_m is estimated as

$$g_m = A/(C_i - \Gamma^*(J_T + 8(A + R_d))/(J_T - 4(A + R_d))) \qquad (7),$$

where A and C_i are taken from the gas exchange measurements described above, and C_c-based Γ^* at leaf temperature of 30 °C was estimated after Bernacchi et al. (2002).

The calculated values of g_m were used to convert A-C_i curves into A-C_c curves using the following equation:

$$C_c = C_i - (A/g_m) \qquad (8),$$

where C_c is the CO_2 concentration in the chloroplast stroma. Because the sensitivity to errors in estimating g_m was relatively low between 100 and 300 μmol mol^{-1} C_i (Harley et al. 1992), we used g_m estimated at ambient CO_2 (C_a) of 360 μmol mol^{-1}, corresponding to C_i 267 ± 5 μmol mol^{-1} (mean ± SE), as a representative for each leaf.

From A-C_c curves, the C_c-based maximum carboxylation capacity of Rubisco (V_{c,max_Cc}) was calculated using the temperature dependence of the kinetic parameters of Rubisco on a C_c basis (Bernacchi et al. 2002) with the following equation:

$$A_c = (C_c - \Gamma^*)/(C_c + K_c(1 + (O/K_o))V_{c,max\_Cc} - R_d \quad (9).$$

We also estimated the C_c-based maximum rate of electron transport (J_{max_Cc}) from the following equation (Farquhar et al. 1980, Ethier and Livingston 2004):

$$A_j = (C_c - \Gamma^*)/(C_c + 2\Gamma^*)\ J_{max\_Cc}/4 - R_d \quad (10).$$

Estimation of mesophyll conductance by the curve-fitting method

In addition, we estimated g_m using the curve-fitting method proposed by Ethier and Livingston (2004), for a validation of g_m estimated by the variable J method. Substituting Eq. 8 in Eq. 9 gives a quadratic equation (von Caemmerer 2000) whose solution is the positive root:

$A_c = (-b + (b^2 - 4ac)^{\wedge 0.5})/2a$,

$a = -1/g_m$,

$b = (V_{c,max\_Cc} - R_d)/g_m + C_i + K_c(1 + O/K_o)$,

$c = R_d(C_i + K_c(1 + O/K_o)) - V_{c,mac\_Cc}\ (C_i - \Gamma^*)$ (11).

C_c-based kinetic constants of Rubisco (Γ^*,K_c, and K_o) were estimated after Bernacchi et al. (2002). The value of R_d was estimated as described above. We set these constants as a known priori and then estimated g_m and V_{c,max_Cc} from a non-linear least-squares fit.

Light microscopy

Interveinal sections of leaf samples were fixed in an aqueous 4% solution of glutaraldehyde and post-fixed in 1% osmium tetroxide, before being dehydrated through a graded ethanol series and embedded in epoxy resin. The embedded samples were then cut into transverse, thin sections (1 μm) and stained with 1% toluidine blue. Following Evans et al. (1994), the areas of chloroplast surfaces facing intercellular space per unit leaf area (S_c) were calculated from the microscopic photographs. A curvature factor (F) was determined assuming that the shape of the cells was a cylinder with hemispherical ends (Thain, 1983; Miyazawa and Terashima, 2001). The mean value

of F was 1.48 for the palisade and 1.39 for the spongy cells.

Leaf N, sugar and starch concentration

Leaf N, soluble sugar and starch contents were determined for the leaves used for the gas exchange and chlorophyll fluorescence measurements. Dried leaf samples, kept at 70°C, were weighted for the calculation of leaf mass per area (LMA), then ground using a mortar. The leaf N content was determined by an NC analysis system composed of a nitrogen/carbon determination unit (Sumigraph, NC-800, Sumica Chem. Anal. Service, Osaka, Japan), a gas chromatograph (GC-8A, Shimadzu, Kyoto, Japan) and a data processor (Chromatopac, C-R6A, Shimadzu). Sugars were extracted with 80% ethanol and determined by the phenol-sulfuric acid method (Dubois et al. 1956). Starch in the residue was solubilized by potassium hydroxide and digested to glucose with amyloglucosidase (A9228, Sigma, St. Louis, MO) solution (Kabeya et al. 2003). The digested glucose was determined with Wako Autokit Glucose (439-90901, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Statistical analysis

Two-factorial ANOVA ($N \times CO_2$) was used to test the differences in the treatment means of net CO_2 assimilation rate (A), stomatal conductance (g_s), total rate of electron transport (J_T), mitochondrial respiration rate in the dark (R_n), C_i-based $V_{c,max}$, C_c-based $V_{c,max}$, C_i-based J_{max}, C_c-based J_{max}, the ratio of C_c-based J_{max} to $V_{c,max}$ (J_{max_Cc}:V_{c,max_Cc}), area-based leaf N, leaf starch and sugar content, g_m estimated at a C_a of 360 µmol mol^{-1} (g_{m_360}), S_c, leaf mass per area subtracting total non-structural carbohydrates (LMA-NSC) by the aov function in R (R Development Core Team, 2014). Furthermore, as the interaction term between N and CO_2 was significant for J_{max_Cc}:V_{c,max_Cc}, leaf starch content and g_{m_360}, significant differences in their means among the combinations of $N \times CO_2$ were re-tested using one-factorial ANOVA and the Holm pairwise comparisons. One-factorial analysis of covariance (ANCOVA) was used to test the effect of N supply on C_i and C_c-based $V_{c,max}$ taking area-based leaf N (N_{area}) as a covariate (glm function in R; R Development Core Team 2014).

Results

Photosynthetic traits, including the net CO_2 assimilation rate (A), stomatal conductance (g_s), total rate of electron transport (J_T), dark respiration rate (R_n), C_i- and C_c-based maximum carboxylation capacity of Rubisco (V_{c,max_Ci} and V_{c,max_Cc}, respectively), C_i- and C_c-based maximum rate of electron transport (J_{max_Ci} and J_{max_Cc}, respectively), and the ratio of C_c-based J_{max} to $V_{c,max}$ (J_{max_Cc}:V_{c,max_Cc}), in the seedlings of *B. platyphylla* grown under ambient and elevated [CO_2], with adequate and limited nitrogen supply are shown in Table 1. Both elevated [CO_2] and limited N supply significantly decreased A and J_T measured at the same C_a (360 μmol mol^{-1}), whereas g_s was only affected by the limited N supply. The limited N supply also decreased R_n, but no significant effect of elevated [CO_2] was observed. The C_c-based $V_{c,max}$ was generally higher than the C_i-based $V_{c,max}$. Both C_i- and C_c-based $V_{c,max}$ were significantly decreased by elevated [CO_2] and limited N supply. The C_i- and C_c-based J_{max} were almost identical and decreased by elevated [CO_2] and limited N supply as well as A, J_T and $V_{c,max}$. A significant ($p < 0.05$) effect of the interaction between CO_2 and N supply on J_{max_Cc}:V_{c,max_Cc} was observed (Table 1). Significant differences among the combinations were re-tested by one-way ANOVA and the Holm pairwise comparisons. The ranking of J_{max_Cc}:V_{c,max_Cc} was Elevated − N:1.41^a > Elevated + N:1.12^b > Ambient − N:0.98^b > Ambient + N:0.94^b (different letters indicate significant differences at $p < 0.05$). The significantly higher J_{max_Cc}:V_{c,max_Cc} observed in the plants grown at elevated [CO_2] under limited N supply was attributed to the severer decrease in V_{c,max_Cc} than that in J_{max_Cc} (by 70% and 50% relative to the plants grown at ambient [CO_2] under adequate N supply, respectively).

We estimated g_m using two different methods: the variable J method (Harley et al. 1992, Valentini et al. 1995, Long and Bernacchi 2003) and the curve-fitting method (Ethier and Livingston 2004). As shown in Figure 1, a positive and significant correlation ($r^2 = 0.49$, $p < 0.001$) was observed in g_m estimated using the two different methods, which can support the validity of the variable J method used in the present study.

Based on the response of g_m to sub-stomatal CO_2 concentration (C_i), g_m showed generally decreasing trends with higher C_i above 470 and below 100 μmol mol^{-1} in all treatment combinations (Fig. 2). Because the sensitivity to errors in estimating g_m was relatively low between 100 and 300 μmol mol^{-1} C_i (Harley et al. 1992), we used g_m estimated at ambient CO_2 (C_a) of 360 μmol mol^{-1}, corresponding to C_i 267 ± 5 μmol mol^{-1} (mean ± SE), as a representative for each leaf. As a significant ($p < 0.05$) effect of the interaction of CO_2 and N supply on g_m at 360 μmol mol^{-1} C_a (g_{m_360}) was observed (Table 2), significant differences among the combinations were re-tested by one-way ANOVA and the Holm pairwise comparisons. The ranking of g_{m_360} was

Ambient + N: $0.16^{a} \pm 0.01$ > Elevated + N: $0.13^{a} \pm 0.008$ > Ambient − N: $0.12^{a} \pm 0.01$ > Elevated − N: $0.06^{b} \pm 0.003$ mol m^{-2} s^{-1} (means ± SE, different letters indicating statistically significant differences at $p < 0.05$, Fig. 3). The leaf starch content also exhibited the significant effect of the interaction of CO_2 and N supply (Table 2). Based on the Holm pairwise comparisons, the ranking of starch was Elevated − N: $4.38^{a} \pm 0.80$ > Ambient − N: $0.61^{b} \pm 0.12$ > Elevated + N: $0.27^{b} \pm 0.07$ > Ambient + N: $0.24^{b} \pm 0.09$ g m^{-2} (Fig. 3A). The leaf sugar concentration significantly increased under limited N supply (Table 2, Fig. 3B). A stronger negative correlation was observed between leaf starch and g_{m_360} than between leaf sugar and g_{m_360} (Fig. 3A,B).

Based on the anatomical analysis, there were no effects of CO_2 and N on the cumulated chloroplast surface area that faces the intercellular spaces on a leaf area basis (S_c) (Table 2), and a low correlation between S_c and g_{m_360} was observed (Fig. 4).

The area-based leaf N content (N_{area}) decreased significantly both by elevated [CO_2] and limited N supply (Fig. 5, Table 2). Concomitantly, C_i- and C_c-based $V_{c,max}$ decreased both by elevated [CO_2] and limited N (Figs. 5A and B, Table 1). One-factorial ANCOVA with N_{area} as a covariate provides that the slope of C_i-based $V_{c,max}$ to N_{area} in plants grown under adequate N supply at both ambient and elevated [CO_2] was significantly lower than that under limited N supply ($F_{1,15} = 4.6$, $p = 0.049$) (Fig. 5A). This result also indicates a steeper decline in C_i-based $V_{c,max}$ with decreasing N_{area} caused by elevated [CO_2] in plants grown under limited N supply. Conversely, the slope of C_c-based $V_{c,max}$ to N_{area} in plants grown under adequate N supply with different CO_2 concentrations was not significantly different from that in plants grown under limited N supply ($F_{1,15} = 1.5$, $p = 0.24$); the intercepts were not significantly different ($F_{1,15} = 0.46$, $p = 0.51$) (Fig. 5B).

To assess leaf structural change, we used corrected values of LMA, which is subtracting total mass of nonstructural carbon (NSC = soluble sugar + starch) from the measured LMA. Limited N significantly increased LMA-NSC, whereas elevated [CO_2] had no significant effect on LMA-NSC (Table2, Fig. 6). Conversely, no difference was observed in leaf thickness among treatment combinations (data not shown).

Discussion

In the present study, elevated [CO_2] and limited N supply were found to be capable of inducing leaf starch accumulation in the Japanese white birch (Fig. 3A), accompanied with photosynthetic down-regulation indicated by a decrease in $V_{c,max}$ both on C_i and C_c basis (Table 1, Figs. 5) (Kitao et al.

2005, 2007). Conversely, photosynthetic down-regulation, i.e., a decrease in $V_{c,max}$, was also observed in plants with adequate N supply under elevated [CO_2] (Table 1, Fig 5). Decreases in A and J_T measured at a C_a of 360 μmol mol^{-1} by elevated [CO_2] also suggests photosynthetic down-regulation (Table 1, Ainsworth and Long 2005, Rogers et al. 2006). Limited N supply reduced R_n, whereas elevated [CO_2] had no significant effect on R_n in Japanese white birch, as has been reported (Gonzalez-Meler et al. 2004). J_{max} on the basis of both C_i and C_c was decreased by elevated [CO_2] and limited N supply (Table 1). Although both C_c-based $V_{c,max}$ and C_c-based J_{max} decreased with elevated CO_2 and limited N supply, the extent of decrease in $V_{c,max}$ was greater than that in J_{max} (Table 1). Consequently, an increase in J_{max}:$V_{c,max}$ was observed in plants grown under the combination of elevated [CO_2] and limited N supply on a C_c basis, even after considering mesophyll conductance (von Caemmerer 2000, Leakey et al. 2009). This suggests that the limited N supply had a severe effect on $V_{c,max}$ relative to J_{max} in plants grown under elevated [CO_2] (Ainsworth and Long 2005, Rogers et al. 2006, Leakey et al. 2009).

g_m is considered to respond in terms of matching the availability of CO_2 and photosynthetic capacity (Long and Bernacchi 2003), where g_m declined at limiting PFD or high C_i (Düring 2003, Flexas et al. 2007, Vrábl et al. 2009). In the present study, we also observed declines in g_m at lower and higher C_i than ambient levels in all treatment combinations (Fig. 2). Although responses of g_m to C_i are reportedly species-dependent such that some species have constant g_m at a certain range of C_i (von Caemmerer and Evans 1991, Loreto et al. 1992, Tazoe et al. 2009), Japanese white birch is suggested to be a species changing g_m to match the availability of CO_2 (cf. Düring, 2003, Flexas et al. 2007, Vrábl et al. 2009). Recently, Tholen et al. (2012) reported that an increase in photorespiration would result in an apparent decrease in g_m at C_i below ambient levels even when no intrinsic change in diffusion properties of the mesophyll occurs. Furthermore, a possibility has also been proposed that the C_i-dependent g_m response is a methodological artifact at both low and high C_i regions (Gu and Sun 2014). However, as we compared g_m at the same C_i and the same irradiance, g_{m_360} used in the present study estimated by the variable J method could reflect the difference in g_m among the treatment combinations, which is also validated by the correlation with the curve-fitting method (Fig. 1, Ethier and Livingston 2004).

g_{m_360} is unaffected in plants grown under elevated CO_2 with adequate N supply (Fig. 3, Table 2), as has been reported in a N-fixing plant, soybean at FACE experiments (Bernacchi et al. 2005). However, seedlings grown at elevated [CO_2] under limited N supply had significantly lower g_{m_360} accompanied with higher leaf starch content among the treatment combinations (Fig. 3).

Although g_m is generally coordinated with g_s across diverse species (Flexas et al. 2013), a significant decline in g_m was observed in plants grown under elevated [CO_2] with limited N supply (Fig. 3) despite of no significant effect of elevated [CO_2] on g_s (Table 1). This suggests some factor existing to interfere the coordination between g_m and g_s in these plants (see discussion below). The lower g_{m_360} might lead to an apparently greater decrease in the C_i-based $V_{c,max}$ relative to the C_c-based $V_{c,max}$ in the seedlings grown under elevated [CO_2] with limited N supply (Fig. 5).

As the cumulated chloroplast surface area that faces the intercellular spaces on a leaf area basis (S_c) represents the area for CO_2 dissolution, an increase in S_c may contribute to increase g_m (Oguchi et al. 2003, Terashima et al. 2006). In the present study, there were no significant effects of both CO_2 and N on S_c, suggesting that leaf anatomical traits related to g_m might not be affected by elevated [CO_2].

Thicker cell walls accompanied with higher cellulose contents have been reported in *Arabidopsis thaliana* grown under elevated CO_2 (Teng et al. 2006). In the present study, thicker cell walls, indicated by the higher LMA-NSC (Osborne and Beerling 2003), might be a cause of the decreased g_m in plants grown under elevated [CO_2] and limited N supply (Terashima et al. 2006). However, because plants grown under ambient [CO_2] and limited N, which also had higher LMA-NSC, showed no significant difference in g_m from Ambient + N and Elevated + N plants, there is a high probability that starch accumulation in Elevated − N plants hindered CO_2 diffusion in chloroplasts, leading to the decrease in g_m (Nakano et al. 2000, Sawada et al. 2001).

Sawada et al. (2000) suggested that the suppression of photosynthesis in single-rooted soybean leaves during the acclimation to elevated CO_2 was largely caused by the hindrance of CO_2 diffusion due to starch accumulation in the chloroplast. In the present study, however, C_c-based $V_{c,max}$, which represents photosynthetic activity taking differences in g_m into account, still decreased under elevated [CO_2] accompanied with reduced N_{area}, maybe due to N dilution by the enhanced growth as a whole plant (Kitao et al. 2005, 2007, Seneweera et al. 2011).

Consequently, leaf starch accumulation and/or thicker cell wall at elevated [CO_2] under limited N supply declined g_m, which could be an additional cause of photosynthetic down-regulation as well as a reduction in Rubisco activity due to the reduced area-based leaf N content.

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Table 1. Photosynthetic traits, including the net CO_2 assimilation rate (A), stomatal conductance (g_s), total rate of electron transport (J_T), mitochondrial respiration rate in the dark (R_n), C_i-based maximum carboxylation capacity of Rubisco (V_{c,max_Ci}), C_c-based maximum carboxylation capacity of Rubisco (V_{c,max_Cc}), C_i-based maximum rate of electron transport (J_{max_Ci}), C_c-based maximum rate of electron transport (J_{max_Cc}), and ratio of C_c-based J_{max} to $V_{c,max}$ (J_{max_Cc}:V_{c,max_Cc}), in the seedlings of Japanese white birch grown under ambient and elevated CO_2 with adequate and limited N supply. A, g_s, and J_T were measured at saturating light intensity (1200 μmol m^{-2} s^{-1}), a leaf temperature of 30°C and 360 μmol mol^{-1} CO_2. R_n was measured in the darkness at a leaf temperature of 30°C and 360 μmol mol^{-1} CO_2. Values are mean ± SE (n = 4–6). The summary of the two-way ANOVA to test the effects of the CO_2 treatment ($F_{1,15}$), N supply ($F_{1,15}$), and CO_2 treatment × N supply ($F_{1,15}$) is shown. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

	Ambient CO_2		Elevated CO_2		F statistics		
	+N	−N	+N	−N	CO_2	N	CO_2 × N
A (μmol m^{-2} s^{-1})	13.7 ± 0.4	9.4 ± 0.3	11.4 ± 0.2	5.7 ± 0.4	69 ***	154 ***	2.8
g_s (mol m^{-2} s^{-1})	0.30 ± 0.02	0.24 ± 0.02	0.30 ± 0.02	0.22 ± 0.04	0.18	4.7 *	0.03
J_T (μmol m^{-2} s^{-1})	126.9 ± 1.4	77.4 ± 3.2	98.4 ± 2.9	47.0 ± 3.2	92 ***	226 ***	0.08
R_n (μmol m^{-2} s^{-1})	1.35 ± 0.04	0.97 ± 0.03	1.23 ± 0.10	0.86 ± 0.04	4.5	38 ***	0.01
V_{c,max_Ci} (μmol m^{-2} s^{-1})	102.9 ± 2.3	65.1 ± 3.1	81.5 ± 4.0	36.3 ± 2.2	70 ***	157 ***	1.3
V_{c,max_Cc} (μmol m^{-2} s^{-1})	142.5 ± 1.9	79.4 ± 4.1	104.5 ± 5.1	46.5 ± 3.3	77 ***	195 ***	0.35
J_{max_Ci} (μmol m^{-2} s^{-1})	131.7 ± 3.9	77.9 ± 4.6	115.5 ± 2.0	63.6 ± 2.0	22 ***	199 ***	0.65
J_{max_Cc} (μmol m^{-2} s^{-1})	133.8 ± 4.8	78.1 ± 4.7	116.6 ± 1.9	64.4 ± 2.0	20 ***	180 ***	0.19
J_{max_Cc}:V_{c,max_Cc}	0.94 ± 0.03	0.98 ± 0.02	1.12 ± 0.04	1.41 ± 0.07	39 ***	10 **	5.1 *

Table 2. Summary of the two-factorial ANOVA to test for the effects of growth [CO_2] ($F_{1,15}$), N supply ($F_{1,15}$) and their interaction ($F_{1,15}$) on area-based leaf N (N_{area}), leaf starch and sugar content, g_m estimated at a C_a of 360 µmol mol^{-1} (g_{m_360}), the areas of chloroplast surfaces facing intercellular space per unit leaf area (S_c), and leaf mass per area subtracting total non-structural carbohydrates (LMA-NSC). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

	F-statistics		
	CO_2	N	Interaction
N_{area}	18 ***	120 ***	0.89
Leaf starch content	16 **	16 **	10 **
Leaf sugar content	0.17	23 ***	0.88
g_{m_360}	30 ***	32 *	5.6 **
S_c	1.7	2.7	1.0
LMA-NSC	2.1	10 *	1.9

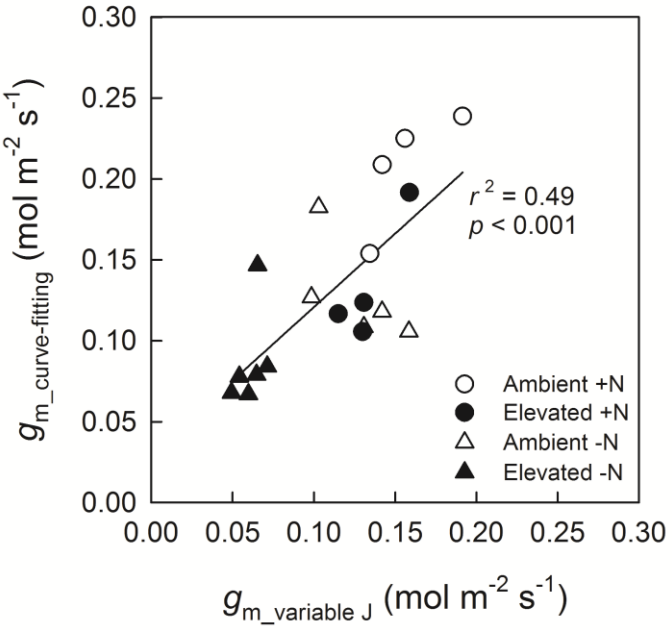

Figure 1. The relationship between mesophyll conductance (g_m) estimated by the variable J method and the curve-fitting method in leaves of Japanese white birch grown under ambient (open) and elevated (closed) [CO_2] with high (circles) and low (triangles) nitrogen supplies. The data were fitted by linear regression.

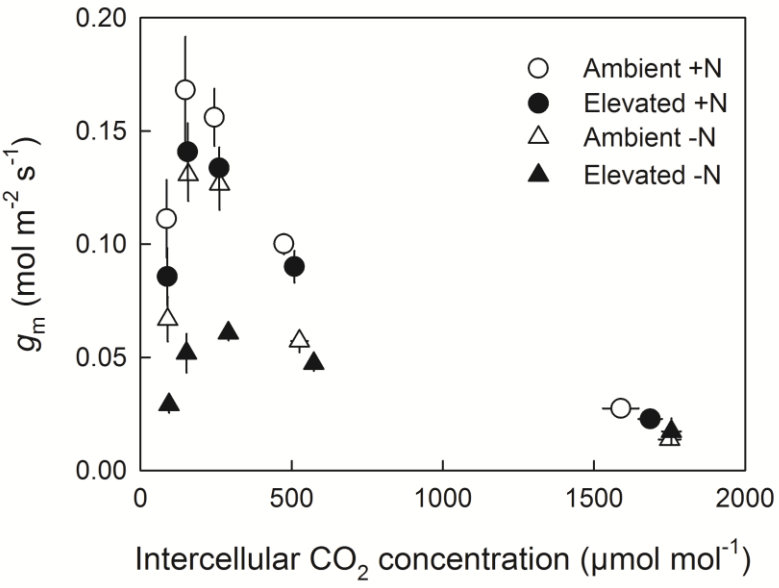

Figure 2. Mesophyll conductance (g_m) as a function of intercellular CO_2 concentration (C_i) in leaves of Japanese white birch grown under ambient (open) and elevated (closed) [CO_2] with high (circles) and low (triangles) nitrogen supplies. Values are mean (n = 4–6) ± SE.

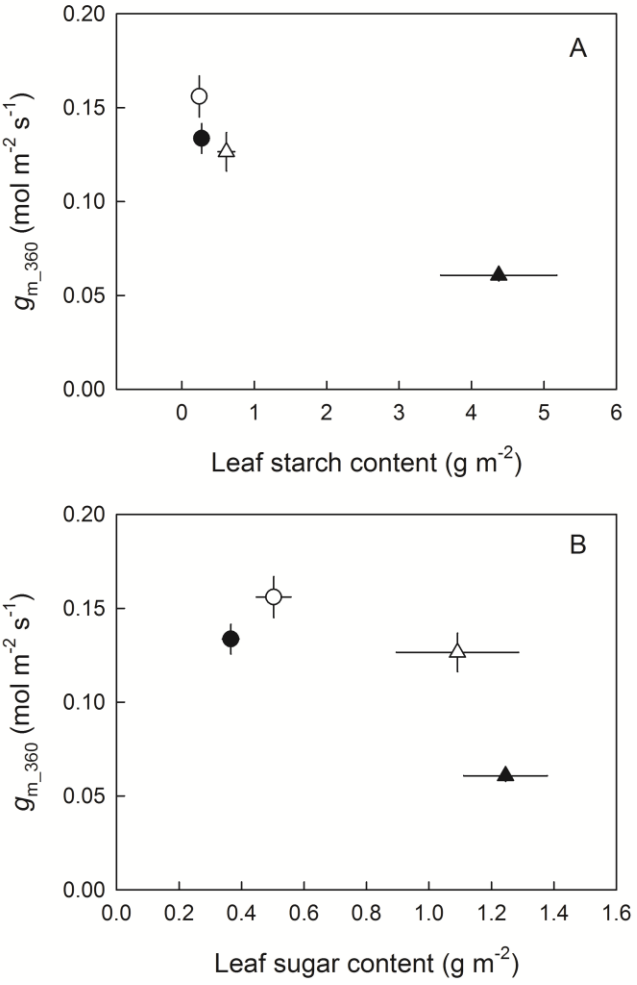

Figure 3. The relationship between mesophyll conductance (g_{m_360}) at ambient CO_2 of 360 µmol mol^{-1} and leaf starch content (A) and leaf sugar concentration (B) in leaves of Japanese white birch grown under ambient (open) and elevated (closed) [CO_2] with high (circles) and low (triangles) nitrogen supplies. Values are mean (n = 4–6) ± SE.

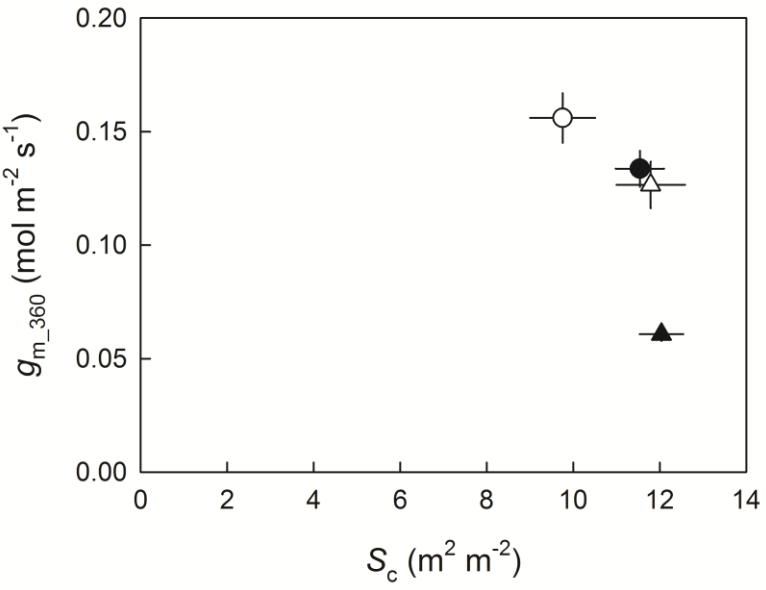

Figure 4. The relationship between mesophyll conductance (g_{m_360}) at ambient CO_2 of 360 µmol mol^{-1} and the areas of chloroplast surfaces facing intercellular space per unit leaf area (S_c) in leaves of Japanese white birch grown under ambient (open) and elevated (closed) [CO_2] with high (circles) and low (triangles) nitrogen supplies. Values are mean (n = 4–6) ± SE.

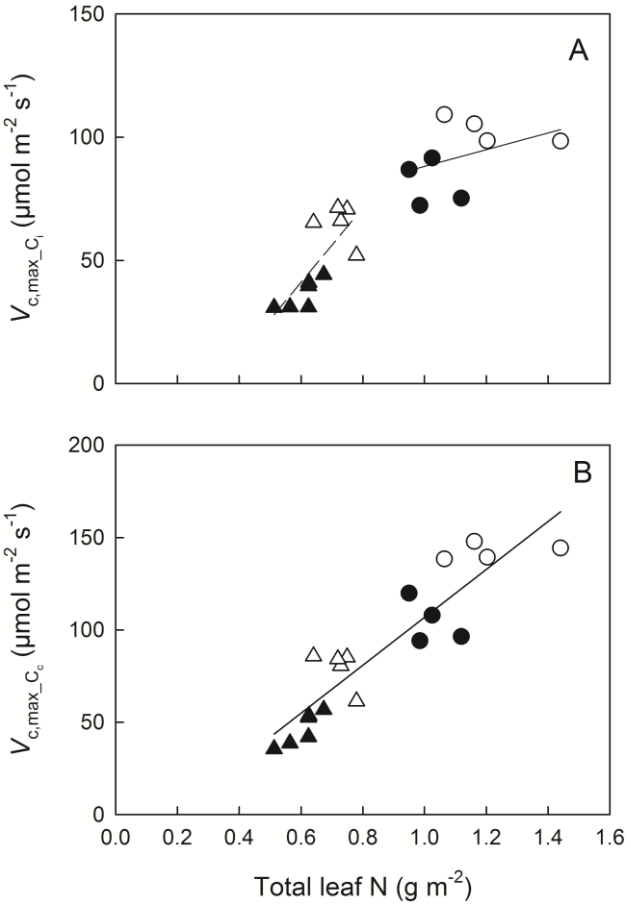

Figure 5. C_i- (V_{c,max_Ci}, A) and C_c-based maximum carboxylation capacity of Rubisco (V_{c,max_Cc}, B) as a function of total leaf nitrogen in leaves of Japanese white birch grown under ambient (open) and elevated (closed) [CO_2] with high (circles) and low (triangles) nitrogen supply. In the upper panel (A), the linear regressions for the adequate and limited N supplies are as follows: $y = 33.9x + 54.3$ ($r^2 = 0.16$, $p = 0.33$) (solid line) and $y = 153.4x - 51.5$ ($r^2 = 0.57$ and $p < 0.01$) (dashed line), respectively. A single regression line is presented in the lower panel (B) because the regressions generated for each N supply were not statistically different. The linear regression for the combined data is $y = 129.8x - 23.0$ ($r^2 = 0.81$ and $p < 0.001$).

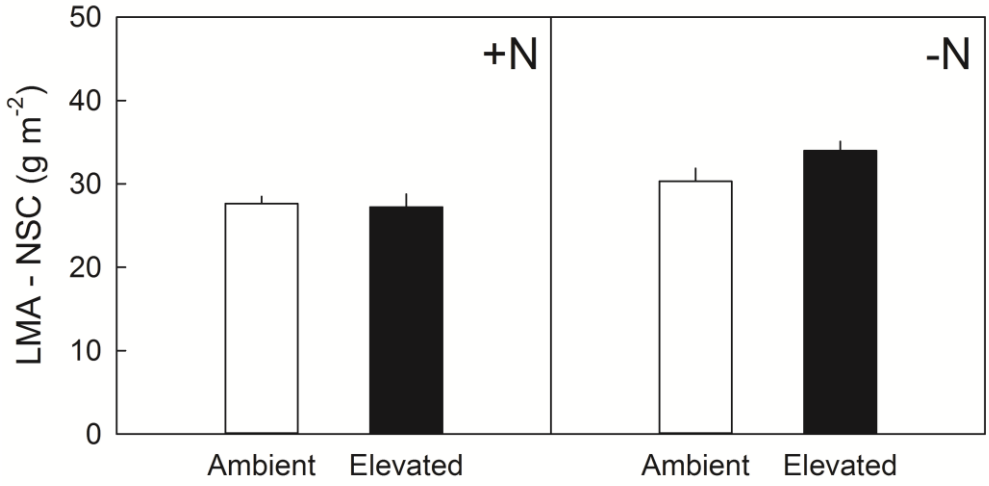

Figure 6. Leaf mass per area subtracting total non-structural carbohydrates (LMA-NSC) in white birch seedlings grown under ambient (open) and elevated (closed bars) [CO_2] with high (left) and low (right panel) nitrogen supplies. Values are mean (n = 4–6) + SE.