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Effects of Internal Conductance on the Temperature Dependence of the Photosynthetic Rate in Spinach Leaves from Contrasting Growth Temperatures

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The photosynthetic rate may be strongly limited by internal conductance from the intercellular airspace to the chloroplast stroma (g_i). However, the effects of growth and leaf temperature on g_i are still unclarified. In this work, we determined the temperature dependence of g_i in spinach leaves grown at 30/25°C (high temperature; HT) and 15/10°C (low temperature; LT), using the concurrent measurements of the gas exchange rate and stable carbon isotope ratio. Moreover, we quantified the effects of g_i on the temperature dependence of the photosynthetic rate. We measured g_i and the photosynthetic rate at a CO_2 concentration of 360 μl l^{-1} under saturating light (A_{360}) at different leaf temperatures. The optimum temperature for A_{360} was 28.5°C in HT leaves and 22.9°C in LT leaves. The optimum temperatures for g_i were almost similar to those of A_{360} in both HT and LT leaves. There was a strong linear relationship between A_{360} and g_i. The photosynthetic rates predicted from the C_3 photosynthesis model taking account of g_i agreed well with A_{360} in both HT and LT leaves. The temperature coefficients (Q_{10}) of g_i between 10 and 20°C were 2.0 and 1.8 in HT and LT leaves, respectively. This suggests that g_i was determined not only by physical diffusion but by processes facilitated by protein(s). The limitation of the photosynthetic rate imposed by g_i increased with leaf temperature and was greater than the limitation of the stomatal conductance at any temperature, in both HT and LT leaves. This study suggests that g_i substantially limits the photosynthetic rate, especially at higher temperatures.

Keywords: CO_2 concentration in the chloroplast (C_c) — Internal conductance — Photosynthesis — Stomatal conductance — Temperature acclimation — Temperature dependence.

Abbreviations: C_a, ambient CO_2 concentration; C_c, chloroplast CO_2 concentration; C_i, intercellular CO_2 concentration; g_i, internal conductance; g_s, stomatal conductance; HT, high temperature (30/25°C); Lg_i, limitation of the photosynthetic rate by gi; Lg_s, limitation of the photosynthetic rate by gs; LT, low temperature (15/10°C); RuBP, ribulose-1,5-bisphosphate; S_c, surface area of chloroplasts exposed to intercellular airspace.

Introduction

Alteration of plant growth temperature results in the change in the temperature dependence of the leaf photosynthetic rate (Berry and Björkman 1980, Yamori et al. 2005, Yamori et al. 2006). The temperature dependence of the photosynthetic rate is affected by the intercellular CO_2 concentration (C_i, Berry and Björkman 1980, Farquhar et al. 1980, Kirschbaum and Farquhar 1984, Hikosaka et al. 2006). The effects of growth temperature on C_i are different among species. In some studies, C_i was found to decrease when plants were grown at low temperatures (Ferrar et al. 1989, Williams and Black 1993, Hikosaka et al. 1999, Hikosaka et al. 2005), but in others, C_i did not change (Ferrar et al. 1989, Hendrickson et al. 2004). In *Quercus myrsinaefolia*, C_i was markedly lower in leaves grown at a low temperature due to low stomatal conductance, and the average C_i were 230 and 300 μl l^{-1} for leaves grown at the low and high temperatures, respectively (Hikosaka et al. 1999). The authors claimed that such differences in C_i might cause a shift of the optimum temperature of the photosynthetic rate by approximately 3°C.

It was assumed that the CO_2 concentration in the chloroplast stroma (C_c) was equal to the C_i (Farquhar et al. 1980). However, it is now clear that this assumption is incorrect. Recent studies showed that C_c is significantly lower than C_i due to the finite internal conductance from the intercellular airspace to the chloroplast stroma (g_i) and that this drawdown in CO_2 concentration from C_i to C_c significantly limits photosynthesis (Loreto et al. 2003, Warren et al. 2003, Hanba et al. 2004, Warren et al. 2004, Niinemets et al. 2005, Terashima et al. 2006). Both stomatal conductance (g_s) and g_i correlate with the photosynthetic rate and are of the similar magnitude (for a review, see Evans and Loreto 2000). Therefore, the effect of leaf and growth temperatures on g_i may partly explain the temperature dependence of the photosynthetic rate.

Some authors reported that the magnitude of g_i is determined by leaf anatomical features such as cell wall

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thickness (Nobel 1991, Kogami et al. 2001, Miyazawa and Terashima 2001) and the surface areas of mesophyll cells (S_{mes}) and chloroplasts (S_c) exposed to intercellular air spaces (Nobel 1991, von Caemmerer and Evans 1991, Evans et al. 1994, Syvertsen et al. 1995, Evans 1998, Evans and Loreto 2000, Evans 2004). On the other hand, those leaf anatomical features failed to explain the rapid response of g_i to environmental conditions (Delfine et al. 1998, Delfine et al. 1999, Flexas et al. 2002, Bernacchi et al. 2002, Centritto et al. 2003). Therefore, it has been suggested that g_i is closely associated with proteins, such as aquaporins (Terashima and Ono 2002, Uehlein et al. 2003, Flexas et al. 2004, Hanba et al. 2004) and/or carbonic anhydrase (Makino et al. 1992, Sasaki et al. 1996, Gillon and Yakir 2000).

Makino et al. (1994) suggested that g_i is affected by the growth temperature in rice leaves (*Oryza sativa*). They found a decreased photosynthetic rate per unit Rubisco content at low C_i in leaves grown at low temperature, and argued that this effect was the result of a decrease in C_c because Rubisco was not inactivated at these temperatures. On the other hand, in leaves of *Nerium oleander*, simultaneous measurements of gas exchange and Chl fluorescence suggested that C_c was not different between the growth temperatures (Hikosaka and Hirose 2001). There is only one report on the temperature dependence of g_i. Bernacchi et al. (2002) determined the temperature dependence of g_i by simultaneous measurements of gas exchange and Chl fluorescence in tobacco leaves (*Nicotiana tabacum*) grown at 25/18°C (day/night). They showed that g_i strongly depended on the leaf temperature, with an optimum temperature at 35–37.5°C, and progressively limited the photosynthetic rate at higher temperatures. However, g_i obtained by the simultaneous measurements of gas exchange and Chl fluorescence may not be reliable, because the method is based on a simple assumption that the difference between the electron transport rates estimated from gas exchange and that from Chl fluorescence is fully explained by g_i. There are many reports that alternative pathways, such as the water–water cycle and cyclic electron flow, are stimulated under conditions under which photosynthetic rates are limited, such as low and high temperatures (for reviews, see Arnon 1995, Asada 2000, Heber 2002, Öquist and Huner 2003, Sharkey 2005). Therefore, the method employing the concurrent measurements of the gas exchange rate and stable carbon isotope ratio has been widely regarded to be more reliable (for a review, see Evans and Loreto 2000). Thus, the dependence of g_i on measurement and growth temperatures should be evaluated by the latter method.

In this study, we determined the temperature dependences of g_i and the photosynthetic rate at a CO_2 concentration of $360\,\mu l\,l^{-1}$ under saturating light conditions in spinach leaves grown at 30/25 (high temperature; HT) and 15/10°C (low temperature; LT). The value of g_i was estimated for intact leaves by the concurrent measurements of the gas exchange rate and stable carbon isotope ratio. Based on the results of these measurements, we addressed several key questions. (i) Does g_i strongly depend on the leaf temperature? This could indicate whether the processes concerned with g_i are controlled by proteins which facilitate CO_2 diffusion. (ii) Does the temperature dependence of g_i change depending on the growth temperature? (iii) To what extent do such changes in g_i contribute to the changes in the temperature dependence of the photosynthetic rate depending on the growth temperature?

Results

Temperature dependences of photosynthetic rate, g_s and g_i

The temperature dependences of the net photosynthetic rate at an ambient CO_2 concentration (C_a) of $360\,\mu l\,l^{-1}$ under saturating light were different depending on the growth temperature (Fig. 1a), as has been previously reported (Yamori et al. 2005, Yamori et al. 2006).

The optimum temperatures for the photosynthetic rate were 28.5°C in HT leaves and 22.9°C in LT leaves, when the temperature dependences of the photosynthetic rates were fitted by cubic curves. g_s in HT leaves was greater than that in LT leaves at any temperature, and the optimum temperatures for g_s were 20–25°C in both HT and LT leaves (Fig. 1b). Temperature dependences of the actual carbon isotope discrimination (Δ) and the differences between the expected carbon isotope discrimination (Δ_i) and Δ were almost constant in both HT and LT leaves, except for somewhat greater Δ in HT leaves and $\Delta_i - \Delta$ in LT leaves at low temperatures (Fig. 1c). Δ in HT leaves was greater than that in LT leaves at any temperature, while, $\Delta_i - \Delta$ values were almost similar between HT and LT leaves except for at the lowest temperatures. Using $\Delta_i - \Delta$, g_i was calculated by Equation 1 (see Materials and Methods). The g_i were different depending on the growth temperature (Fig. 1d). The optimum temperatures for g_i were almost similar to those of the photosynthetic rate in both HT and LT leaves. The values for g_i at the respective optimum temperatures were $0.22\,mol\,m^{-2}\,s^{-1}$ in HT leaves and $0.21\,mol\,m^{-2}\,s^{-1}$ in LT leaves. At higher temperatures, we found a decrease in g_i in the LT leaves, but this effect was not clear in HT leaves. At lower temperatures, the effect of temperature on g_i was very similar in HT and in LT leaves. The temperature coefficients (Q_{10}) for g_i between 10 and 20°C were 1.99 in HT leaves and 1.84 in LT leaves, respectively.

When the data of HT and LT leaves were pooled separately, the relationships between the photosynthetic rate and g_s were barely observed (Fig. 2a; HT, $R^2 = 0.51$;

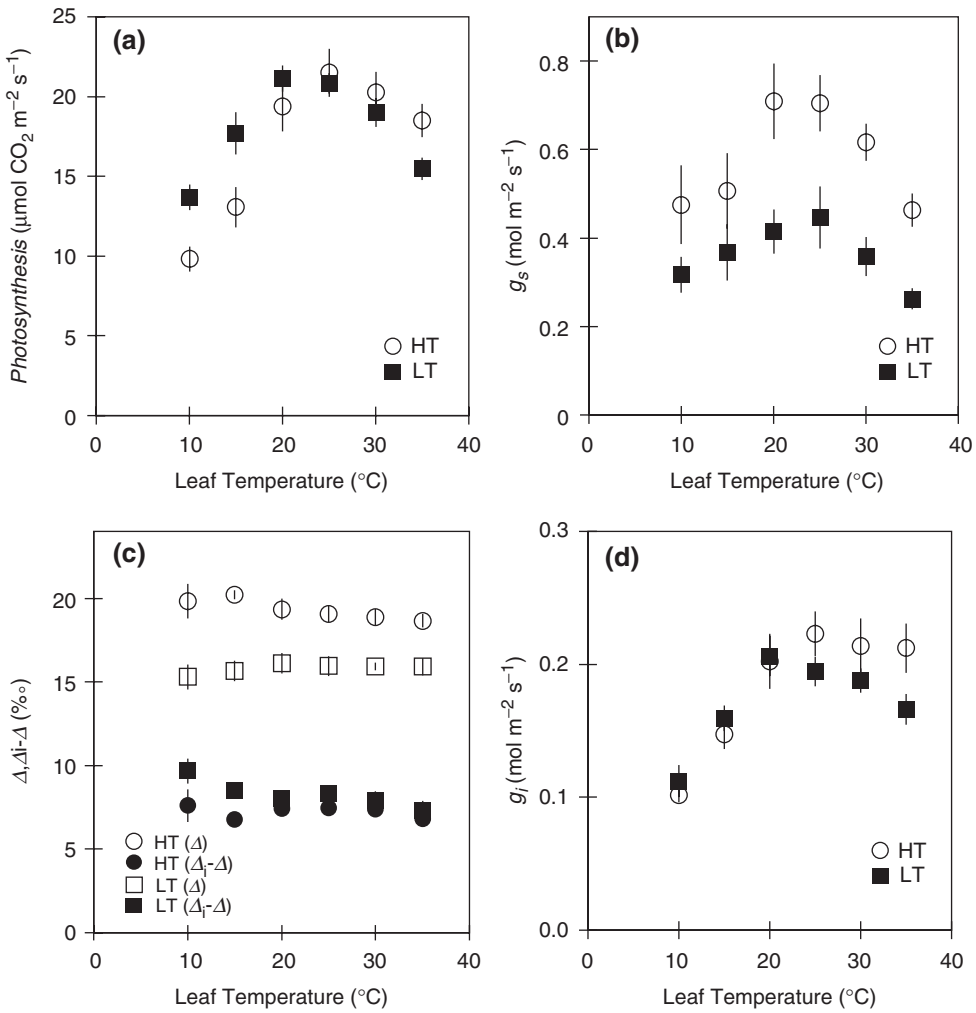

Fig. 1 Temperature dependences of net photosynthetic rate (a), stomatal conductance (b; g_s), the actual carbon isotope discrimination (Δ) and the differences between the expected carbon isotope discrimination (Δ_i) and Δ (c), and internal conductance (d; g_i). HT (circles) and LT (squares) denote the leaves grown at day/night air temperature of 30/25 and 15/10°C, respectively. Gas exchange was measured at an ambient CO_2 concentration of 360 μl l^{-1} under a saturating light intensity of 1,500 μmol m^{-2} s^{-1}. Internal conductance was estimated by the concurrent measurements of the gas exchange rate and stable carbon isotope ratio. Data represent means ± SE, n = 5.

LT, $R^2 = 0.74$). On the other hand, there were strong linear regressions between the photosynthetic rate and g_i, in both HT and LT leaves (Fig. 2b; HT, $R^2 = 0.97$; LT, $R^2 = 0.86$). The temperature dependences of g_i/g_s were different depending on the growth temperature (Fig. 2c). The values of g_i/g_s showed similar values at the respective growth temperatures in HT and LT leaves.

The C_i tended to decrease with increasing temperature, in both HT and LT leaves (Fig. 3a). C_i in HT leaves was approximately 30 μl l^{-1} greater than that in LT leaves (Fig. 3a). The C_c were almost constant at any temperature in both HT and LT leaves (Fig. 3b). C_c in HT leaves was approximately 50 μl l^{-1} higher than that in LT leaves (Fig. 3b). The C_i/C_a ratio decreased with increasing temperature (Fig. 3c), whereas, C_c/C_i and C_c/C_a were almost constant at any temperature in both HT and LT leaves (Fig. 3d, e). C_c/C_i was considerably lower than C_i/C_a in both HT and LT leaves. The CO_2 drawdowns caused by low g_s (the difference between the ambient and intercellular CO_2 concentrations, $C_a - C_i$) at 25°C were 43.2 and 75.1 μl l^{-1} in HT and LT leaves, respectively. On the other hand, the CO_2 drawdowns caused by low g_i (the difference between intercellular and chloroplast CO_2 concentrations, $C_i - C_c$) at 25°C were 96.8 and 108 μl l^{-1} in HT and LT leaves, respectively. The reduction in both g_s and g_i caused the CO_2 drawdowns from ambient air to the chloroplast stroma in both HT and LT leaves.

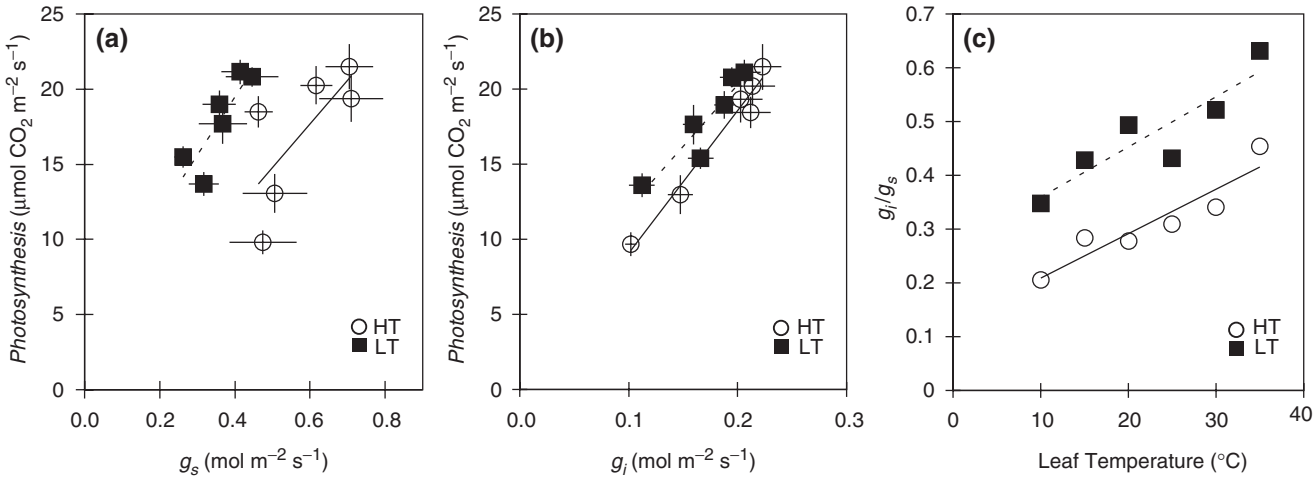

Fig. 2 Relationships between photosynthetic rate and stomatal conductance (a; g_s) or internal conductance (b; g_i). The regression lines shown are: (a) HT (solid line), $y = 29.14x + 0.214$ ($R^2 = 0.51$); LT (dotted line), $y = 38.64x + 4.016$ ($R^2 = 0.74$), (b) HT (solid line), $y = 93.96x - 0.144$ ($R^2 = 0.97$); LT (dotted line), $y = 81.28x + 4.065$ ($R^2 = 0.86$). Temperature dependence of the ratio between internal conductance and stomatal conductance (c). The regression lines shown are: HT (solid line), $y = 0.0081x + 0.136$ ($R^2 = 0.87$); LT (dotted line), $y = 0.0092x + 0.273$ ($R^2 = 0.81$). Abbreviations and symbols are the same as those in Fig. 1. Data represent means ± SE, $n = 5$.

Effects of g_s and g_i on the temperature dependence of the photosynthetic rate

We evaluated the effects of g_s and g_i on the temperature dependence of the photosynthetic rate (Fig. 4). As described in model A in Materials and Methods, $P_c(C_a)$ was calculated assuming infinite values for g_s and g_i, $P_c(C_i)$ was calculated assuming a finite value for g_s and an infinite value for g_i, and $P_c(C_c)$ was calculated using finite values for g_s and g_i. The values for the measured photosynthetic rates were taken from the data set presented in Fig. 1a. At low temperatures, $P_c(C_a)$, $P_c(C_i)$ and $P_c(C_c)$ nearly matched the measured photosynthetic rate, both in HT and in LT leaves (Fig. 4a, b). The extents of the decrease in all of the calculated photosynthetic rates at low temperatures in the HT leaf were greater than those in the LT leaf. At moderately high temperatures, $P_c(C_a)$ and $P_c(C_i)$ were much higher than the measured photosynthetic rates in both HT and LT leaves. However, $P_c(C_c)$ agreed well with the measured photosynthetic rates in both HT and LT leaves at high temperatures.

When the temperature dependences of the photosynthetic rates were fitted by cubic curves, the optimum temperatures for $P_c(C_a)$, $P_c(C_i)$, $P_c(C_c)$ and the measured photosynthetic rate were 29.3, 28.8, 28.4 and 28.5°C in HT leaves (Fig. 4a). In contrast, in LT leaves, the optimum temperatures of $P_c(C_a)$, $P_c(C_i)$, $P_c(C_c)$ and the measured photosynthetic rate were 25.7, 24.7, 22.9 and 22.9°C (Fig. 4b). For both leaf types, we found that the optimum temperatures for the calculated photosynthetic rate $P_c(C_c)$ were very similar to those of the measured photosynthetic rate in both HT and LT leaves (Fig. 4a, b). The temperature dependences of $P_c(C_c)$ were closely related to those of the measured photosynthetic rates over the entire temperature range. We analyzed the relationship between the measured photosynthetic rate and $P_c(C_c)$ in both HT and LT leaves. The measured photosynthetic rate (y) agreed with $P_c(C_c)$ (x), when the data of HT and LT leaves were pooled ($y = 0.95\ x$, $R^2 = 0.70$).

We evaluated the limitation of the photosynthetic rate by g_s and g_i in HT and LT leaves (Fig. 4c, d). The limitations of the photosynthetic rate by g_s (Lg_s) and g_i (Lg_i) are expressed as the proportionate decreases in the photosynthetic rate, compared with the rates calculated by assuming infinite g_s and g_i, respectively (Equations 3 and 4). Lg_s and Lg_i increased with leaf temperature in both HT and LT leaves. Lg_s in HT leaves increased from 0.013 at 10°C to 0.136 at 35°C (Fig. 4c), and from 0.040 to 0.254 in LT leaves (Fig. 4d). On the other hand, Lg_i in HT leaves increased from 0.063 at 10°C to 0.287 at 35°C (Fig. 4c), and from 0.151 to 0.421 in LT leaves (Fig. 4d). At all temperatures examined, Lg_i was greater than Lg_s, in both HT and LT leaves. In addition, Lg_s and Lg_i in LT leaves were always greater than those in HT leaves at any temperature.

Effects of differences in g_s and g_i on the temperature dependence of the photosynthetic rate

Figure 5a, b show the temperature dependences of the predicted photosynthetic rates at various C_c using Equation A-3 in HT and LT leaves, respectively (see Appendix). In these predictions, it is assumed that C_c were constant for the overall temperature range. Temperature dependences of the predicted photosynthetic rates differed depending

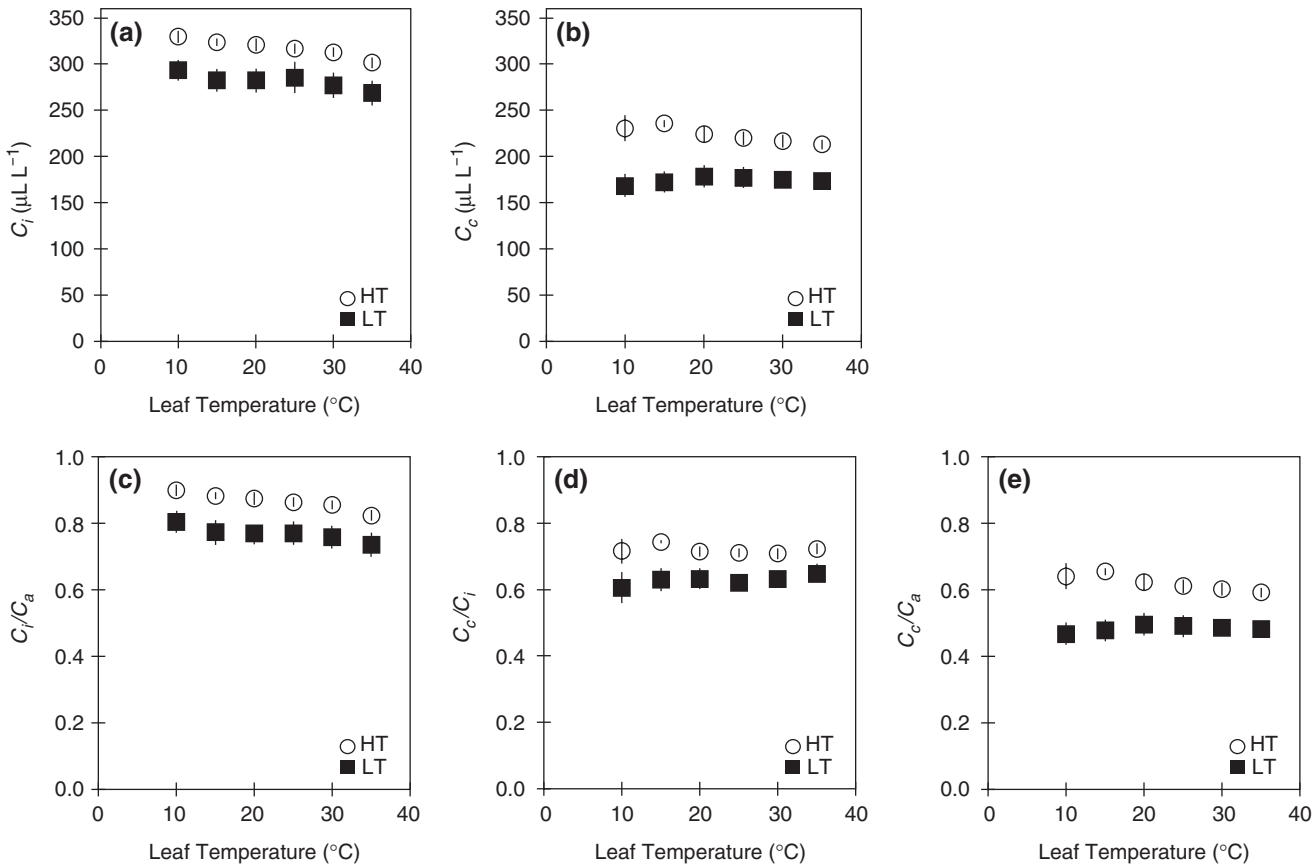

Fig. 3 The temperature dependence of the CO_2 concentration in the intercellular air space (a; C_i) and in the chloroplast (b; C_c), and the temperature dependence of the ratio between intercellular and ambient CO_2 concentration (c; C_i/C_a), the ratio of chloroplast to intercellular CO_2 concentration (d; C_c/C_i) and the ratio of chloroplast to ambient CO_2 concentration (e; C_c/C_a). Abbreviations and symbols are the same as those in Fig. 1. Data represent means ± SE, n = 5.

on C_c in both HT and LT leaves. The optimum temperature of the photosynthetic rate shifted towards lower temperatures with the decrease in C_c. These findings indicate that the optimum temperature in LT leaves was lower than that in HT leaves if C_c in LT leaves was lower than that in HT leaves. It is obvious that the optimum temperatures of the photosynthetic rate do not change very much if the differences in C_c between HT and LT leaves are small. On the other hand, the optimum temperatures of the photosynthetic rate change greatly if the differences in C_c between HT and LT leaves are large.

C_i and C_c were different between different growth temperatures by approximately 30 and 50 μl l^{-1}, respectively (Fig. 3a, b). We examined the effects of the differences in g_s and g_i on the temperature dependence of the photosynthetic rate, since the temperature dependence of the photosynthetic rate is thought to be affected by C_c (Hikosaka et al. 2006). Fig. 5c, d showed the effects of differences in C_i on the temperature dependence of the photosynthetic rate in HT and LT leaves, respectively. We estimated the temperature dependence of the photosynthetic rate in HT leaves at values for C_i obtained for LT leaves (Fig. 5c) and, similarly, we estimated the temperature dependence in LT leaves at values for C_i obtained for HT leaves (Fig. 5d). A change in C_i hardly affected the temperature dependence of the photosynthetic rate (Fig. 5c, d). The effect of C_i on the optimum temperature was small, and produced a difference of only 0.7–1.0°C.

Figure 5e, f showed the effects of differences in C_c on the temperature dependence of the photosynthetic rate in HT and LT leaves, respectively. We estimated the temperature dependence of the photosynthetic rate in HT leaves at values for C_c obtained for LT leaves (Fig. 5e) and, similarly, we estimated the temperature dependence in LT leaves at values for C_c obtained for HT leaves (Fig. 5f). A change in C_c affected the temperature dependence of the photosynthetic rate (Fig. 5e, f). The effect of a different C_c on the optimum temperature of the photosynthetic rate was larger than that of C_i, and produced shifts of 1.5–1.8°C.

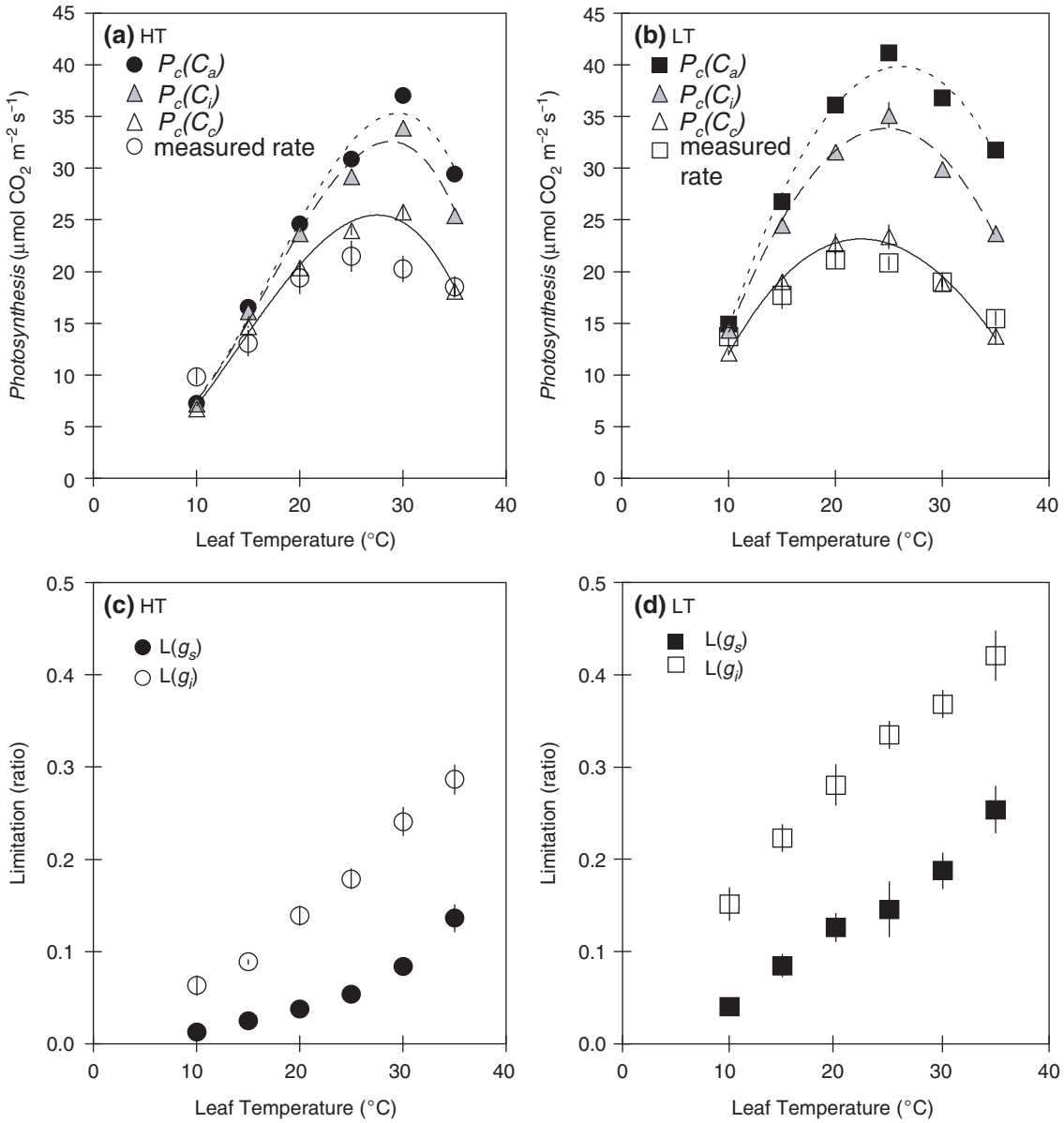

Fig. 4 Effects of stomatal conductance (g_s) and internal conductance (g_i) on the temperature dependence of the photosynthetic rate in HT (a) and LT leaves (b). $P_c(C_a)$ was calculated from the photosynthetic rate at $C_a = C_i = C_c = 360\,\mu l\,l^{-1}$ assuming that both g_s and g_i were infinite (HT; filled circle, LT; filled square). $P_c(C_i)$ was calculated from the photosynthetic rate at $C_c = C_i$ assuming values for g_s obtained in this study, while g_i was assumed to be infinite (HT, LT; gray triangle). $P_c(C_c)$ was calculated from the photosynthetic rate assuming values for g_s and g_i obtained from measurements in this study (HT, LT; open triangle). The data were fitted by cubic curves. The values of the measured photosynthetic rates were equal to those presented in Fig. 1 (HT, open circle; LT, open square). The limitation of the photosynthetic rate by g_s (Lg_s, HT; filled circle, LT; filled square) and g_i (Lg_i, HT; open circle, LT; open square) in HT (c) and LT leaves (d). Data represent means ± SE, n = 5.

Discussion

Temperature dependences of the photosynthetic rate and g_i

We clearly showed that the temperature dependences of g_i were different depending on the growth temperature, particularly at high temperatures (Fig. 1d). The optimum temperatures of g_i were very similar to those of the photosynthetic rate, in both HT and LT leaves (Fig. 1a, d). We also showed that the photosynthetic rate positively depended on g_i, in both HT and LT leaves (Fig. 2b; HT, $R^2 = 0.97$; LT, $R^2 = 0.86$). Using the method based on the simultaneous measurements of gas exchange and

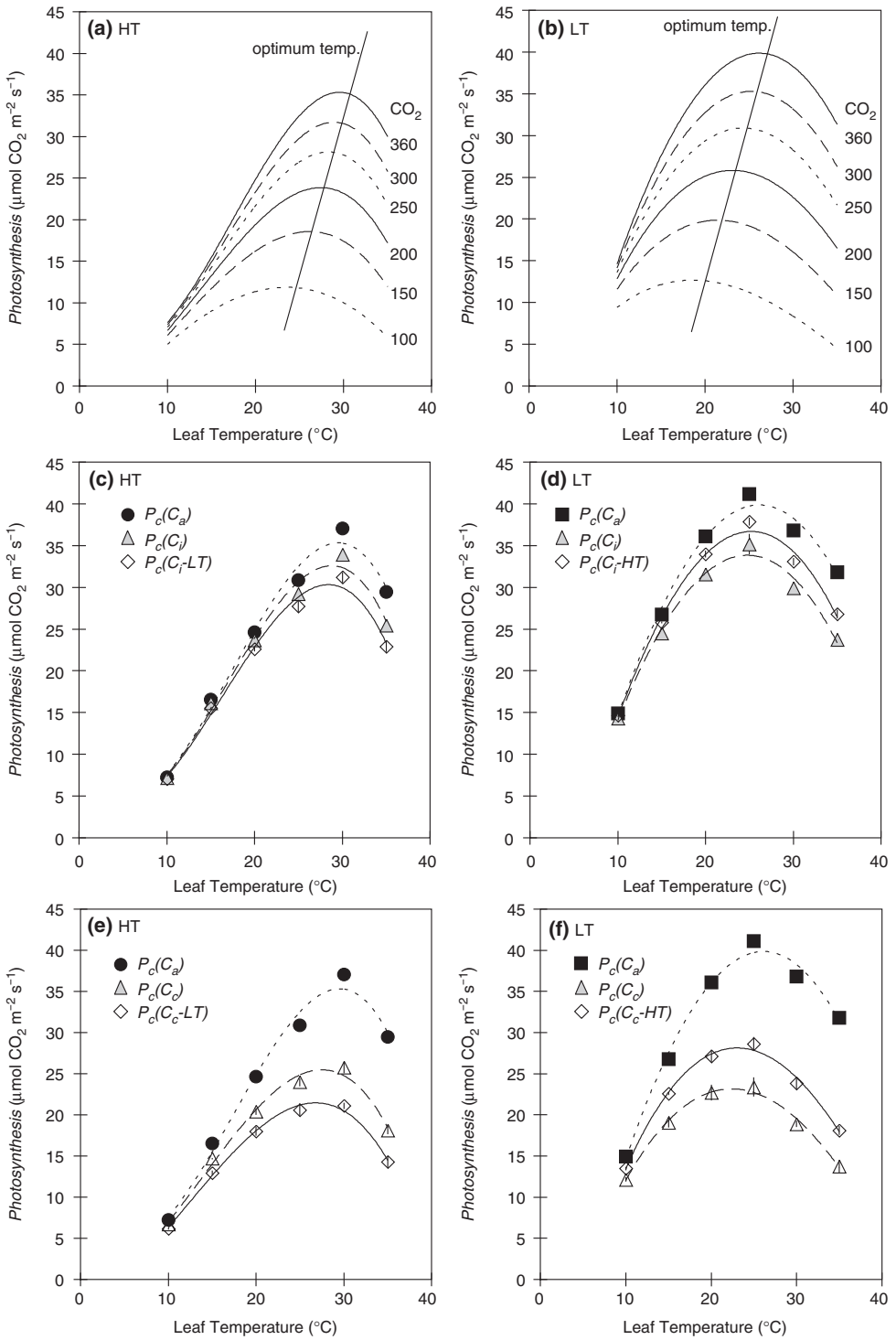

Fig. 5 Temperature dependence of the predicted photosynthetic rate at different C_c in HT (a) and LT leaves (b). It is assumed that C_c was constant for the overall temperature range. The effects of differences in stomatal conductance (g_s) and internal conductance (g_i) on the temperature dependence of the photosynthetic rate in HT (c, e) and LT leaves (d, f). Abbreviations and symbols for $P_c(C_a)$, $P_c(C_i)$ and $P_c(C_c)$ in HT and LT leaves are the same as those in Fig. 4. $P_c(C_i - LT)$ in (c) HT was calculated from the photosynthetic rate in HT leaves at C_i obtained for LT leaves. $P_c(C_i - HT)$ in (d) LT was calculated from the photosynthetic rate in LT leaves at C_i obtained for HT leaves. $P_c(C_c - LT)$ in (e) HT was calculated from the photosynthetic rate in HT leaves at C_c obtained for LT leaves. $P_c(C_c - HT)$ in (f) LT was calculated from the photosynthetic rate in LT leaves at C_c obtained for HT leaves. The data were fitted by cubic curves. Data represent means ± SE, $n = 5$.

Chl fluorescence, Bernacchi et al. (2002) showed that g_i was strongly dependent on the leaf temperature, with a sharp peak at 35–37.5°C in tobacco leaves grown at 25/18°C (day/night). The temperature dependence of g_i, as presented in this work, differed from the data shown by Bernacchi et al. (2002), especially at higher leaf temperatures. It is possible that the discrepancy between our results and the previously published data for the temperature dependence of g_i resulted from differences in plant species and/or in growth conditions. Alternatively, it is also probable that the discrepancy is due to the difference in the method. The measurement of g_i by the combination of gas exchange and Chl fluorescence is based on the assumption that the difference between the electron transport rates estimated from gas exchange and estimated from Chl fluorescence is fully explained by g_i. However, it has been reported that alternative pathways, such as the water–water cycle and cyclic electron flow, are stimulated under conditions in which photosynthetic rates are limited, such as low and high temperature (for a review, see Arnon 1995, Asada 2000, Heber 2002, Öquist and Huner 2003, Sharkey 2005). If such alternative pathways are actually enhanced in tobacco leaves at temperatures higher and lower than the optimum temperature, the sharp peak in g_i reported by Bernacchi et al. (2002) may be due to the underestimation of g_i at such temperatures.

The Q_{10} value between 10 and 20°C for the diffusivity of CO_2 in pure water was determined to be around 1.4 (Hesketh et al. 1983). If g_i is partly determined by proteins, it is thought that the Q_{10} for g_i should be approximately 2 or higher (Nobel 1999). The observed Q_{10} between 10 and 20°C was approximately 2 for g_i both in HT and in LT leaves (Fig. 1d), suggesting that the determinants of g_i were not only physical diffusion, but processes facilitated by proteins, such as aquaporin (Terashima and Ono 2002, Uehlein et al. 2003, Flexas et al. 2004, Hanba et al. 2004) and carbonic anhydrase (Makino et al. 1992, Sasaki et al. 1996, Gillon and Yakir 2000). Moreover, the temperature dependence of g_i was affected by the growth temperature (Fig. 1d). This also strongly suggests the involvement of protein(s) in CO_2 diffusion. If this is the case, the difference in the decrease in g_i at higher temperatures can be attributed to the differences in the thermal stability and/or the suppressed enzyme activities involved in CO_2 diffusion (Fig. 1d).

Recently, the role of aquaporins in CO_2 diffusion has received much interest. Aquaporins increase the CO_2 permeability of plant cell membranes (Terashima and Ono 2002, Uehlein et al. 2003, Flexas et al. 2004, Hanba et al. 2004). Hanba et al. (2004) overexpressed an aquaporin 2 of *Hordeum vulgare* L. (HvPIP2;1; an aquaporin in the plasma membrane-type subfamily) in rice (*Oryza sativa* L.). They demonstrated that the overexpression of HvPIP2;1, which increased the level of aquaporins detected by an antibody raised against HvPIP2;1 by 135%, enhanced g_i in intact rice leaves by 40%. Terashima et al. (2006) roughly estimated that more than two-thirds of CO_2 transported across the plasma membrane is via aquaporins. Carbonic anhydrase is also thought to be involved in CO_2 diffusion (Makino et al. 1992, Sasaki et al. 1996, Gillon and Yakir 2000). However, Price et al. (1994) showed that the antisense reduction of carbonic anhydrase activity to 2% of wild-type levels did not reduce the light-saturated photosynthetic rate at an ambient CO_2 concentration of 360 $\mu l\,l^{-1}$ at 25°C. Therefore, the role of carbonic anhydrase in internal conductance is still unclear.

Several authors have argued that g_i is largely constitutive and determined by leaf structural traits such as the surface area of chloroplasts exposed to intercellular air spaces (S_c) and cell wall thickness (for a recent review, see Terashima et al. 2006). There is no doubt that g_i is proportional to S_c and inversely related to cell wall thickness, since S_c represents the effective area for CO_2 diffusion and cell wall thickness affects the path length for CO_2 diffusion in the liquid phase. LT leaves in spinach had thick palisade tissue comprising approximately three cell layers, while HT leaves had thin palisade tissue comprising approximately two cell layers (unpublished data). Therefore, it is possible that LT leaves have a greater S_c than HT leaves. However, Park and Tsunoda (1979) observed that rice plants grown at low temperature had swollen chloroplasts filled with excess starch. Large starch grains might significantly reduce S_c and disturb CO_2 diffusion in the chloroplast. Starch accumulation was also observed in spinach leaves grown at low temperature (Guy et al. 1992). In addition, changes in cell wall thickness and lipid properties of the membranes depending on the growth temperature might affect the CO_2 permeability. If this is the case, it may counteract the effect of the increased number of palisade cell layers on S_c in LT leaves. This may be the explanation for the observation that we found little difference in g_i between HT and LT leaves, compared with maximum values at the respective optimum temperature for g_i (Fig. 1d).

Effects of g_s and g_i on the temperature dependence of the photosynthetic rate

We evaluated the effects of g_s and g_i on the temperature dependence of the photosynthetic rate (Fig. 4a, b). The photosynthetic rate calculated assuming an infinite value for g_i [$P_c(C_a)$ and $P_c(C_i)$] deviated from the measured photosynthetic rate at high temperatures, in both HT and LT leaves. However, the photosynthetic rate calculated using finite values for g_s and g_i [$P_c(C_c)$] agreed well with the measured photosynthetic rates at high temperature, and properly reproduced the shift in the optimum temperature

for the photosynthetic rate in both HT and LT leaves (Fig. 4a, b).

The limitation of the photosynthetic rate by g_s (Lg_s) and g_i (Lg_i) was estimated by Equations 3 and 4. Both g_s and g_i became increasingly limiting to the photosynthetic rate as the temperature increased (Fig. 4c, d). The CO_2 drawdowns caused by finite g_s and g_i (the difference between ambient and chloroplast CO_2 concentration, $C_a - C_c$) were mainly the result of the limitation by g_i rather than that by g_s, because Lg_i was greater than Lg_s at all temperatures in both HT and LT leaves (Fig. 4c, d). This clearly showed that g_i imposed a very substantial limitation to the photosynthetic rate, irrespective of the growth temperatures, especially at high temperatures. In our previous study, we showed that the photosynthetic performance is largely determined by Rubisco kinetics at low temperatures, but by the Rubisco kinetics and Rubisco activation state at high temperatures (Yamori et al. 2006). In this study, we showed that, at high temperature, the photosynthetic performance is determined not only by the Rubisco kinetics and Rubisco activation state but also by g_i.

The temperature dependence of the photosynthetic rate is thought to be affected by C_i and C_c (Hikosaka et al. 2006). The values for C_i and C_c in LT leaves were less than those in HT leaves by approximately 30 and 50 $\mu l\,l^{-1}$, respectively (Fig. 3a, b). As a sensitivity analysis, we estimated the temperature dependence of the photosynthetic rate in HT leaves at the C_c obtained for LT leaves (Fig. 5e) and, similarly, that in LT leaves at the C_c obtained for HT leaves (Fig. 5f). The difference in C_c affected the temperature dependence and the optimum temperature of the photosynthetic rate by 1.5–1.8°C. Therefore, we conclude that the differences in C_c contribute to the change in the temperature dependence of the photosynthetic rate and may partly explain the shift in the optimum temperature.

In many plants, g_i is relatively low (for a review, see Evans and Loreto 2000, Ethier and Livingstone 2004, Terashima et al. 2006). These impose a significant limitation to photosynthesis. Furthermore, g_i was strongly dependent on the leaf temperature and became increasingly limiting to the photosynthetic rate as the temperature increased. Under such conditions, it is impossible to evaluate the photosynthetic rate accurately, if g_i is not taken into account for the C_3 photosynthesis model (Farquhar et al. 1980). When the temperature dependences of Rubisco kinetic parameters and the Rubisco activation state provided in the previous study (Yamori et al. 2006), and g_i provided in this study were all taken into account, the C_3 photosynthesis model will be much improved for estimation of the photosynthetic rate over a wide range of temperatures.

We clearly showed that g_i was strongly dependent on the leaf temperature and had an optimum temperature. Moreover, the temperature dependences of g_i changed depending on the growth temperature. The temperature dependence of g_i provided evidence for the view that CO_2 transfer from the leaf intercellular airspace into the chloroplast would be controlled by processes facilitated by proteins, such as aquaporins and carbonic anhydrase. We also found that g_i became more limiting to the photosynthetic rate as the temperature increased, in both HT and LT leaves. The limitation by g_i was larger than that by g_s at any temperature, in both HT and LT leaves. These results showed that the photosynthetic rate is significantly limited by g_i at any temperature, but especially at higher temperatures. Together with our previous study (Yamori et al. 2006), we can be fairly certain that, at low temperatures, the photosynthetic performance is largely determined by Rubisco kinetics, while at high temperatures, it is determined by the Rubisco kinetics, Rubisco activation state and g_i.

Materials and Methods

Plant growth conditions

Spinach (*Spinacia oleracea* L. cv. Torai) plants were grown in vermiculite, as described in Yamori et al. (2005). The day/night lengths were 8 and 16 h, respectively. Photosynthetically active photon flux density (PPFD) in the daytime was 230 $\mu mol\,photons\,m^{-2}\,s^{-1}$. The day/night air temperatures were either 30/25 or 15/10°C. These are referred to as high temperature (HT) and low temperature (LT) conditions, respectively. The leaves grown at HT and LT are called HT and LT leaves, respectively. The plants were watered once a week and fertilized with 200 ml of a nutrient solution containing 2 mM KNO_3, 2 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.665 mM NaH_2PO_4, 25 μM Fe-EDTA, 5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 25 μM H_3BO_4, 0.25 μM Na_2MoO_4, 50 μM NaCl and 0.1 μM $CoSO_4$ once a week.

Gas exchange measurements and estimation of internal conductance

Gas exchange measurements were performed at an ambient CO_2 concentration of 360 $\mu l\,l^{-1}$ under saturating light of 1,500 $\mu mol\,m^{-2}\,s^{-1}$, using a laboratory-constructed system with an infrared CO_2/H_2O gas analyzer (LI-7000; Li-Cor Inc., Lincoln, NE, USA) as described by Hanba et al. (1999, 2002) with slight modifications. The photosynthetic rates were measured at least 30 min after the attainment of the temperature. Gas exchange parameters were calculated according to the method of von Caemmerer and Farquhar (1981). Internal conductance (g_i) was estimated for intact leaves by the concurrent measurements of the gas exchange rate and stable carbon isotope ratio (Hanba et al. 1999, Hanba et al. 2002). In the present study, g_i was calculated using the equation reported by Scartazza et al. (1998):

$$g_i = \frac{(b - a_i)A/C_a}{(\Delta_i - \Delta) - f\Gamma^*/C_a} \quad (1)$$

where Δ is the actual carbon isotope discrimination, Δ_i is the expected carbon isotope discrimination assuming infinite g_i, A is the photosynthetic rate, C_a is the ambient CO_2 concentration, a_i is the carbon isotope discrimination during CO_2 diffusion/hydration into water (1.8‰), and b is the carbon isotope discrimination caused by carboxylation by Rubisco and phosphoenolpyruvate carboxylase (30‰). The symbols f and Γ^* represent the discrimination caused by photorespiration and the CO_2 compensation point when day respiration is zero, respectively. Here, we assumed that f was too small to have a significant effect on g_i (von Caemmerer and Evans 1991). Δ was calculated from carbon isotope ratios of CO_2 in air leaving and entering the gas exchange chamber. CO_2 samples were collected using dry ice–ethanol and liquid nitrogen traps. The carbon isotope ratio was determined with an isotope mass spectrometer (MAT 252, Theremo Finnigan, Bremen, Germany).

The CO_2 concentration in the chloroplast stroma (C_c) was calculated using the equation:

$$C_c = C_i - A/g_i \tag{2}$$

where C_i is the concentration of CO_2 in the intercellular air spaces.

Models

A. Effects of g_s and g_i on the temperature dependence of the photosynthetic rate. We compared the measured photosynthetic rates and the predicted photosynthetic rates in HT and LT leaves, respectively (Fig. 4). The predicted photosynthetic rates in HT and LT leaves were calculated for three conditions. For condition (A), we calculated the temperature dependence of $P_c(C_a)$ at $C_a = C_i = C_c = 360\ \mu l\,l^{-1}$ assuming that both g_s and g_i were infinite, using Equation A-2 (see Appendix). For condition (B), we calculated the temperature dependence of $P_c(C_i)$ at $C_c = C_i$ assuming that g_s was the value obtained in this study, while g_i was infinite, using Equation A-2. For condition (C), we calculated the temperature dependence of $P_c(C_c)$ assuming that both g_s and g_i were the values obtained in this study, using Equation A-3 (see Appendix). To obtain these predicted photosynthetic rates, we assumed that day respiration rates (R_d) were half the respective dark respiration rates that were reported for HT and LT leaves in our previous studies (Yamori et al. 2005, Yamori et al. 2006). We first calculated the temperature dependence of $K_c(1 + O/K_o)$ values, using Equation A-4 (see Appendix). The temperature dependences of the Rubisco activation state (R^*), the maximum rate of ribulose-1,5-bisphosphate (RuBP) carboxylation (V_{cmax}) and the CO_2 compensation point in the absence of day respiration (Γ^*) were obtained from spinach leaves grown under the conditions identical to the present experiment (Yamori et al. 2006). $IS(C_c)$ in Equation A-4 was determined from g_i obtained in this study and $IS(C_i)$ obtained for spinach leaves (Yamori et al. 2005), using Equation A-5 (see Appendix). The data for these parameters at intervals of 5°C from 10 to 35°C were obtained by fitting cubic curves. The resultant temperature dependences of $K_c(1 + O/K_o)$ were generally similar to those reported by Jordan and Ogren (1984) who determined temperature dependences of K_c and K_o values in Rubisco purified from spinach leaves grown at 23/18°C (day/night).

Next, we calculated absolute values of V_{cmax} ($\mu mol\,m^{-2}\,s^{-1}$) to match the Rubisco contents between the measured photosynthetic rates in this study and the estimated photosynthetic rates. V_{cmax} on a leaf area basis was estimated from Equation A-3, at each temperature, using photosynthetic rates and C_c obtained in this study. Then, V_{cmax} values estimated for each temperature were averaged for the HT and LT leaves, respectively. V_{cmax} values thus estimated at 25°C were 54.1 and 73.1 $\mu mol\,m^{-2}\,s^{-1}$ for HT and LT leaves, respectively.

B. Limitation of photosynthetic rate by g_s and g_i. The limitations of the photosynthetic rate imposed by g_s and g_i were calculated from the predicted photosynthetic rates assuming that these conductances were either infinite or finite, according to Farquhar and Sharkey (1982). The limitations of the photosynthetic rate by g_s (Lg_s) and g_i (Lg_i) were estimated as:

$$Lg_s = \frac{P_c(C_a) - P_c(C_i)}{P_c(C_a)} \tag{3}$$

$$Lg_i = \frac{P_c(C_i) - P_c(C_c)}{P_c(C_i)} \tag{4}$$

Appendix

C_3 photosynthesis model

Photosynthetic rate (P) under RuBP carboxylation-limited conditions is expressed as a function of the CO_2 concentration at the intercellular spaces (C_i, $\mu l\,l^{-1}$):

$$P_c(C_i) = \frac{V_{cmax}(C_i - \Gamma^*)}{C_i + K_c(1 + O/K_o)} - R_d \tag{A-1}$$

where V_{cmax} ($\mu mol\,m^{-2}\,s^{-1}$) is the maximum rate of RuBP carboxylation on the leaf area basis, K_c ($\mu l\,l^{-1}$) and K_o ($ml\,l^{-1}$) are the Michaelis constants for CO_2 and O_2, respectively, O ($ml\,l^{-1}$) is the O_2 concentration, R_d ($\mu mol\,m^{-2}\,s^{-1}$) is the day respiration rate, and Γ^* ($\mu l\,l^{-1}$) is the CO_2 compensation point in the absence of day respiration (Farquhar et al. 1980). When the Rubisco activation state (R^*) is taken into account (see Yamori et al. 2006), $P_c(C_i)$ is expressed as:

$$P_c(C_i) = \frac{V_{cmax}(C_i - \Gamma^*)}{C_i + K_c(1 + O/K_o)} \times R^* - R_d \tag{A-2}$$

$P_c(C_c)$ is expressed as a function of the chloroplastic CO_2 concentration (C_c, $\mu l\,l^{-1}$):

$$P_c(C_c) = \frac{V_{cmax}(C_c - \Gamma^*)}{C_c + K_c(1 + O/K_o)} \times R^* - R_d \tag{A-3}$$

Because the initial slope [$IS(C_c)$] of A vs. the C_c curve is identical to $dP_c(C_c)/dC_c$ at $C_c = \Gamma^*$, V_{cmax} is expressed as:

$$V_{cmax} = \frac{IS(C_c) \times \{\Gamma^* + K_c(1 + O/K_o)\}}{R^*} \tag{A-4}$$

Using the initial slope of A vs. C_i, $IS(C_i)$, obtained for spinach leaves grown under conditions identical to the present experiment (Yamori et al. 2005), $IS(C_c)$ was calculated as:

$$IS(C_c) = \frac{g_i \times IS(C_i)}{g_i - IS(C_i)} \quad \text{(A-5)}$$

where g_i is the internal conductance, the conductance for CO_2 diffusion from the intercellular airspace to the chloroplast stroma.

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