C_4 Photosynthesis at Low Temperature. A Study Using Transgenic Plants with Reduced Amounts of Rubisco[1]

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C_4 plants are rare in the cool climates characteristic of high latitudes and elevations, but the reasons for this are unclear. We tested the hypothesis that CO_2 fixation by Rubisco is the rate-limiting step during C_4 photosynthesis at cool temperatures. We measured photosynthesis and chlorophyll fluorescence from 6°C to 40°C, and in vitro Rubisco and phospho*enol*pyruvate carboxylase activity from 0°C to 42°C, in *Flaveria bidentis* modified by an antisense construct (targeted to the nuclear-encoded small subunit of Rubisco, anti-*RbcS*) to have 49% and 32% of the wild-type Rubisco content. Photosynthesis was reduced at all temperatures in the anti-*Rbcs* plants, but the thermal optimum for photosynthesis (35°C) did not differ. The in vitro turnover rate (*k*cat) of fully carbamylated Rubisco was 3.8 mol mol^{-1} s^{-1} at 24°C, regardless of genotype. The in vitro *k*cat (Rubisco Vcmax per catalytic site) and in vivo *k*cat (gross photosynthesis per Rubisco catalytic site) were the same below 20°C, but at warmer temperatures, the in vitro capacity of the enzyme exceeded the realized rate of photosynthesis. The quantum requirement of CO_2 assimilation increased below 25°C in all genotypes, suggesting greater leakage of CO_2 from the bundle sheath. The Rubisco flux control coefficient was 0.68 at the thermal optimum and increased to 0.99 at 6°C. Our results thus demonstrate that Rubisco capacity is a principle control over the rate of C_4 photosynthesis at low temperatures. On the basis of these results, we propose that the lack of C_4 success in cool climates reflects a constraint imposed by having less Rubisco than their C_3 competitors.

C_4 plants often dominate the warm climate regions of the earth when they have access to at least moderate light intensities (Sage et al., 1999). Conversely, C_4 species are relatively rare in the cool climates characteristic of high latitudes or high elevations (Teeri and Stowe, 1976; Tieszen et al., 1979; Rundel, 1980; Long, 1983; Sage et al., 1999). In North America, temperature is the best predictor of the success of C_4 grasses, which rarely occur when the minimal temperature of the warmest month of the growing season is below 8°C (Teeri and Stowe, 1976). Globally, C_4 plants are rare at latitudes and elevations where the average growing season temperatures are less than approximately 16°C (Sage et al., 1999). The transition from C_4- to C_3-dominated landscapes generally occurs between 30°N and 40°N and between 1,500 and 3,000 m elevation (Sage et al., 1999).

The reason for the relative lack of C_4 plants in cool regions remains unclear. The lower quantum yield of photosynthesis (ϕ_{CO2}, the initial slope of the light-response curve) in C_4 versus C_3 species at low temperatures has been proposed to account for the differences in the biogeography of the two pathways (Ehleringer and Björkman, 1977; Ehleringer, 1978). However, differences in quantum yield only effect carbon uptake when light is limiting, and in plant canopies CO_2 assimilation is a function of both light-saturated and light-limited photosynthetic rates (Ehleringer, 1978; Long, 1999). Alternatively, one or more of the carbon-concentrating reactions of the mesophyll might be inherently chilling sensitive. Pyruvate orthophosphate dikinase (PPDK, EC 2.7.9.1) and phospho*enol*pyruvate (PEP) carboxylase (PEPCase, EC 4.1.1.31) can both dissociate below 8°C to 12°C in vitro (Sugiyama and Boku, 1976; Edwards et al., 1985; Krall and Edwards, 1993). However, considerable species and ecotypic variability has been noted, and these steps are not fundamentally prone to failure at low temperatures in vivo (Leegood and Edwards, 1996; Matsuba et al., 1997).

The possibility that the bundle sheath reactions may limit C_4 photosynthesis at suboptimal temperatures has received less attention. Björkman and Pearcy (1971) found that the activation energy (Ea) of photosynthesis was similar to that of Rubisco (EC 4.1.1.39) in several warm-climate C_3 and C_4 species. Variation in Rubisco activity was correlated with differences in the photosynthetic capacity of *Atriplex lentiformis* (Torr.) Wats. grown at different temperatures (Pearcy, 1977). In *Bouteloua gracilis* Lag. and *Muhlenbergia montanum* (Nutt.) A.S. Hitch., the light-saturated net pho-

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tosynthetic rate and the maximal in vitro Rubisco activity are equivalent below 17°C and 22°C, respectively (Pittermann and Sage, 2000, 2001). These findings indicate that Rubisco capacity can limit the rate of C_4 photosynthesis below approximately 20°C.

A Rubisco limitation can be quantified if the amount of the enzyme is changed without affecting the activities of other enzymes (Stitt and Schulze, 1994; Stitt, 1995). This enables determination of a flux control coefficient for Rubisco (C_{ra}) within the context of the carbon assimilation pathway (Kacser and Burns, 1973; Stitt, 1995). A C_{ra} of 1 indicates complete control of photosynthetic flux by Rubisco; a 10% change in the amount of enzyme will result in a 10% change in the flux through the pathway. A control coefficient between zero and one would indicate that the control of photosynthesis is shared between Rubisco and other processes. To determine C_{ra}, a series of plants with differences in the content of Rubisco is needed (Stitt and Schulze, 1994). Antisense molecular techniques provide a means of meeting this requirement.

In the C_4 dicot *Flaveria bidentis* L. Kuntze, Rubisco content has been reduced with antisense-RNA constructs targeting the nuclear-encoded small-subunit of Rubisco (*Rbc*S; Chitty et al., 1994; Furbank et al., 1996, 1997). The amounts of other photosynthetic enzymes are not effected by the transformation (Furbank et al., 1996). Reducing Rubisco by antisense in *F. bidentis* results in plants that have reduced steady-state photosynthetic rates under a range of conditions (Furbank et al., 1996; Siebke et al., 1997; von Caemmerer et al., 1997). Anti-*Rbc*S *F. bidentis* has been used to show that Rubisco is an important control over C_4 photosynthesis at temperatures near the thermal optimum, where C_{ra} was as high as 0.7 (Furbank et al., 1997). The effect of temperature variation on this control is unknown.

In the present study, we tested the hypothesis that Rubisco is the primary rate-limiting step during C_4 photosynthesis at low temperatures. We used three *F. bidentis* genotypes with a 3-fold difference in Rubisco content. To examine the nature of the rate limitation during the temperature response of C_4 photosynthesis, we measured gas exchange and chlorophyll *a* fluorescence across a range of temperatures from 6°C to 40°C and the in vitro activities of Rubisco and PEPCase from 0°C to 42°C. To quantify the control of CO_2 assimilation by Rubisco and whether this control is affected by temperature, we determined the C_{ra} at temperatures ranging from 6°C to 40°C.

RESULTS

The antisense constructs led to significant reductions in the amount of Rubisco present in *F. bidentis* leaves (Table I). The 136-13 and 141-1 anti-*Rbc*S lines had 49% and 32% of the wild-type Rubisco content, respectively. The Rubisco turnover rate (*k*cat) did not differ between any of the lines, indicating that the antisense constructs had no effect on the kinetics of the enzyme. There was no difference in the activity of PEPCase between the genotypes (Table I). In each genotype, the Arrhenius plots for Rubisco and PEPCase indicated enzyme dissociation at low temperatures in vitro (Table I). The Ea of Rubisco increased from approximately 57 to 100 kJ mol^{-1} between 12°C and 18°C, whereas the Ea of PEPCase increased from 71 to 180 kJ mol^{-1} at similar temperatures. The wild-type plants had higher chlorophyll content than the anti-*Rbc*S lines, but there were no changes in the ratio of chlorophyll a/b (Table I).

Reducing the amount of Rubisco by antisense led to a large reduction in the net CO_2 assimilation rate (*A*) relative to the wild type at all measurement temperatures (Fig. 1a). Furthermore, the ratio of wild type to antisense photosynthesis increased as leaf temperature was reduced. *F. bidentis* had a photosynthetic thermal optimum of about 35°C when grown under these conditions, regardless of genotype (Fig. 1a). Dark respiration (Rd) did not vary with genotype ($P = 0.67$, ANOVA; Fig. 1a). The Ea of net photosyn-

Table I. *Biochemical characteristics of Flaveria bidentis wild type and anti-RbcS plants*

The concentration of Rubisco active sites was determined by the ^{14}CABP-labeling assay described in "Materials and Methods." The *k*cat and enzyme activity data are values at 24°C. The Rubisco *k*cat and PEP carboxylase activity values were determined by the incorporation of ^{14}C into acid-stable products. Activation energies were calculated as described by Berry and Raison (1981) across the temperature ranges indicated by superscript letters. Chlorophyll determinations follow Porra et al. (1989). Each value represents the mean (± SE) of four or five measurements. Values with different capital letters are statistically different from each other ($P < 0.05$, Tukey).

Plant	Rubisco			PEP Carboxylase		Gross CO_2 Assimilation	Chlorophyll	
	Catalytic Sites	*k*cat	Ea	Activity	Ea	Ea[a]	Content	a/b
	μmol m^{-2}	*mol mol^{-1} s^{-1}*	*kJ mol^{-1}*	*μmol m^{-2} s^{-1}*	*kJ mol^{-1}*	*kJ mol^{-1}*	*μmol m^{-2}*	
Wild type	13.2 ± 0.9A	3.9 ± 0.3	100.6 ± 2.0[b] 56.1 ± 1.4[c]	159.9 ± 6.8	175.2 ± 3.8[b] 71.6 ± 1.0[c]	72.7 ± 1.4A	592 ± 59A	3.7 ± 0.1
anti-*Rbc*S 136–13	6.5 ± 0.9B	3.8 ± 0.2	97.4 ± 6.1[b] 57.0 ± 2.1[c]	186.3 ± 13.5	193.6 ± 3.8[b] 69.4 ± 3.4[c]	87.6 ± 1.6B	453 ± 37B	3.7 ± 0.2
anti-*Rbc*S 141–1	4.2 ± 0.5B	3.7 ± 0.1	100.9 ± 2.4[b] 59.3 ± 1.1[c]	146.9 ± 13.3	183.4 ± 6.6[b] 74.0 ± 1.9[c]	96.2 ± 2.6C	469 ± 41AB	3.8 ± 0.2

[a] Measured between 5°C and 30°C. [b] Measured between 0°C and 12°C. [c] Measured between 18°C and 42°C.

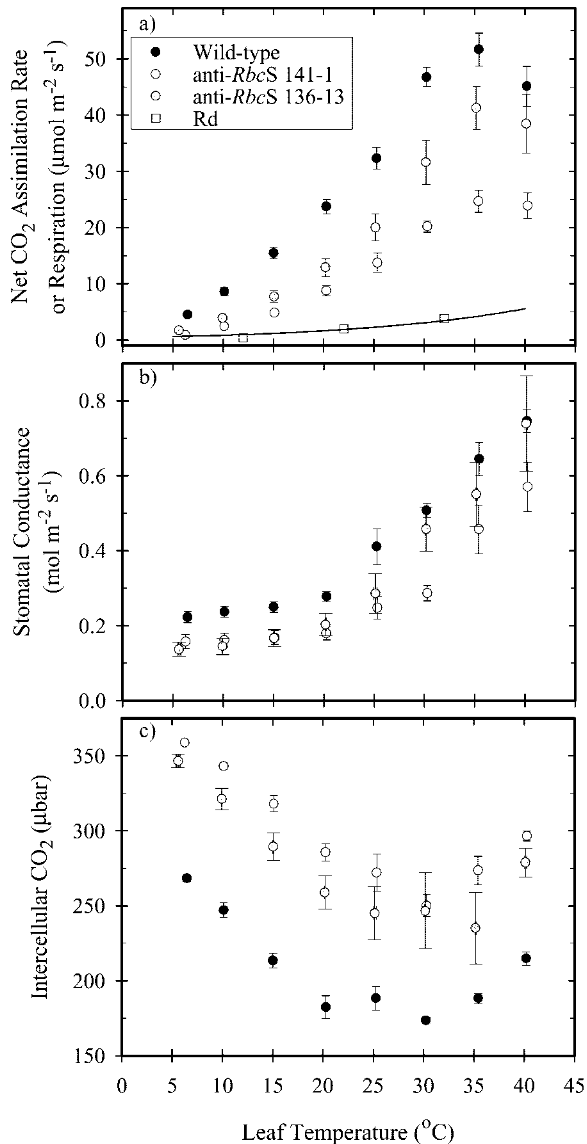

Figure 1. The temperature responses of the rates of net CO_2 assimilation and Rd (a), stomatal conductance (b), and the partial pressure of Ci (c) in *F. bidentis* wild type (●) and anti-*Rbc*S (○ and ◍). Photosynthesis was measured at a temperature- and genotype-dependent PPFD that was just sufficient to saturate photosynthesis at 370 μbar CO_2 and 200 mbar O_2. Each point represents the mean (± SE) of measurements on five different leaves. Rd was determined as the *y* intercept of the light response of photosynthesis in the wild-type and 141-1 lines. Respiration did not differ between genotypes, and pooled values are shown here. The relationship between Rd and temperature is described by $Rd = 3.76e^{-3}\ T^2 + 5.74e^{-4}\ T + 8.56e^{-9}$ (where *T* is leaf temperature [°C], $R^2 = 0.927$). This was used to correct the net assimilation for respiration in all ensuing calculations.

thesis was about 20% higher in the anti-*Rbc*S lines (Table I). The antisense lines had lower stomatal conductance than the wild type below 20°C (Fig. 1b) but maintained a higher intercellular CO_2 (Ci) across the range of temperatures measured, due to their greatly reduced photosynthetic rates (Fig. 1c). The Ci corresponding to an ambient CO_2 of 370 μbar was sufficient to saturate photosynthesis at each measurement temperature in all genotypes (data not shown). Stomatal conductance was relatively stable in each genotype below 20°C, and Ci rose markedly as temperature declined below this point.

At 30°C, the ratios of variable to maximal fluroescence (F_v/F_m) were 0.80 ± 0.01 and 0.82 ± 0.01 (+ SE; $n = 8$) for the wild type and 141-1 lines, respectively. At a light intensity of 1,500 μmol m^{-2} s^{-1}, the wild-type leaves had a higher quantum yield of photosystem II (PSII; Φ_{PSII}) than the anti-*Rbc*S leaves at temperatures below the thermal optimum (Fig. 2a). The same pattern was detected when the temperature curves were measured under illumination that was just saturating, although in that case Φ_{PSII} at low temperatures (<15°C) was about 15% higher than the data obtained at a constant PPFD of 1,500 μmol m^{-2} s^{-1} (data not shown). The wild-type leaves maintained a greater proportion of open PSII than the antisense plants below 25°C, as indicated by the higher photochemical quenching (qP) values (Fig. 2b); at higher temperatures there was no difference between the two groups. There were no statistical differences in non-photochemical quenching between the two genotypes (Fig. 2c).

The instantaneous quantum requirement of PSII per CO_2 ($\Phi_{PSII}/\Phi_{CO2}{}^*$) was constant in the wild-type leaves above 25°C and increased at lower temperatures (Fig. 3a). The anti-*Rbc*S leaves had a higher $\Phi_{PSII}/\Phi_{CO2}{}^*$ than the wild type at all temperatures. The quantum requirement of photosynthesis in *F. bidentis* was sensitive to temperature, increasing more than 3-fold as temperature was reduced from 40°C to 10°C (Fig. 3a). The increase in the quantum requirement for CO_2 fixation with declining leaf temperature is consistent with increased leakage (ϕ) of CO_2 from the bundle sheath (Fig. 3b).

The in vitro *k*cat increased with increasing assay temperature, but there were no differences between the genotypes (Fig. 4a). Dividing the gross CO_2 assimilation rate by the concentration of Rubisco catalytic sites yielded the in vivo *k*cat (Fig. 4b). At 15°C or lower, in vitro and in vivo *k*cat were the same in each genotype. Above 20°C, in vitro *k*cat exceeded the in vivo value in each genotype. The in vivo *k*cat in the anti-*Rbc*S lines was greater than the wild-type value above 30°C (Fig. 4b).

The Rubisco control coefficient (C_{ra}) was determined from the relationship between gross photosynthesis and the concentration of Rubisco catalytic sites (Fig. 5a). At low-measurement temperatures, this relationship is linear, and a higher amount of Rubisco increased photosynthesis. At warmer temperatures, the relationship between photosynthesis and Rubisco content reached a plateau as other limitations became important. The control coefficient was inversely related to temperature, being about 0.68 at the thermal optimum and rising to 0.99 at the

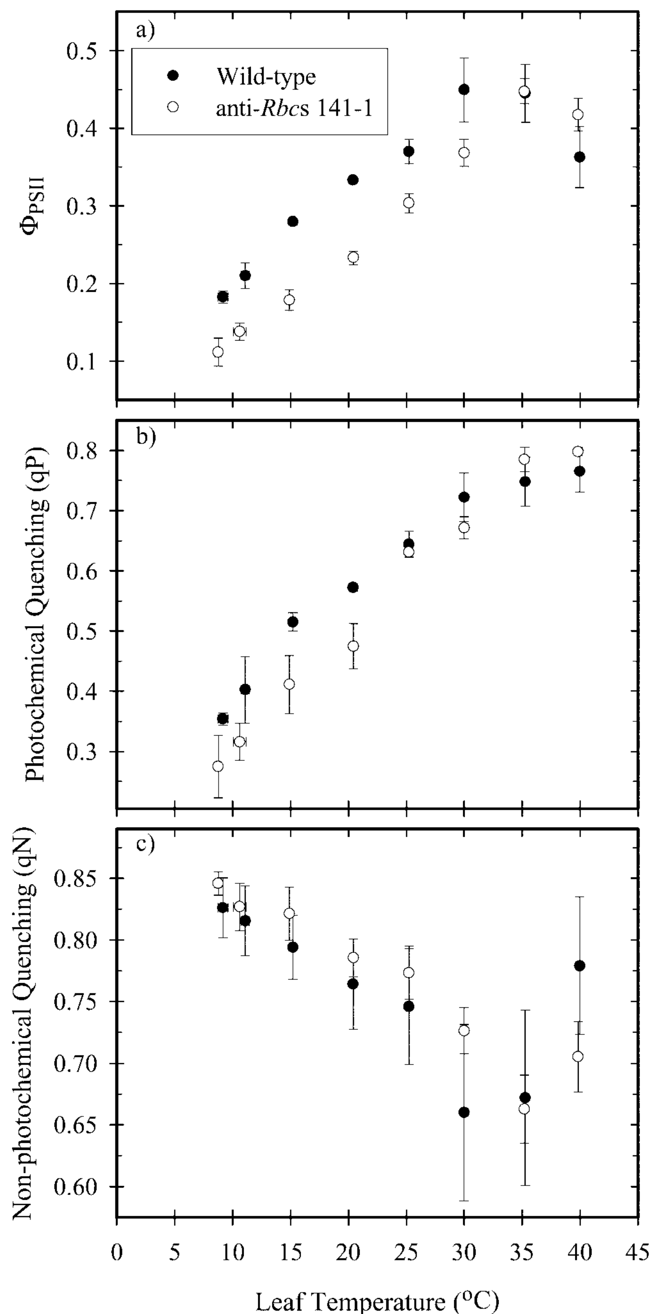

Figure 2. The temperature responses of the quantum yield of PSII (a; Φ_{PSII}), photochemical quenching (b; qP), and non-photochemical quenching (c; qN) in *F. bidentis* wild type (●) and anti-*Rbc*S (141-1, ○). Measurements were made at a constant PPFD of 1,500 μmol m^{-2} s^{-1}, 370 μbar CO_2, and 200 mbar O_2. Each point represents the mean (± SE) of measurements on three different leaves.

lowest measurement temperature; at 6°C, C_{ra} was statistically equivalent to one (Fig. 5b).

DISCUSSION

C_4 plants are largely excluded from cool climates, probably because of poor photosynthetic performance at low temperatures relative to C_3 species (Osmond et al., 1982). This poor performance may reflect an inherent biochemical limitation, and different steps of C_4 photosynthesis have been suggested to be the rate-limiting factor at low temperatures. Using *F. bidentis* modified with antisense constructs to reduce Rubisco content, we determined the pattern of control exerted by Rubisco on C_4 photosynthesis over the 6°C to 40°C temperature range. Although control is shared near the thermal optimum, Rubisco becomes a principle control over C_4 photo-

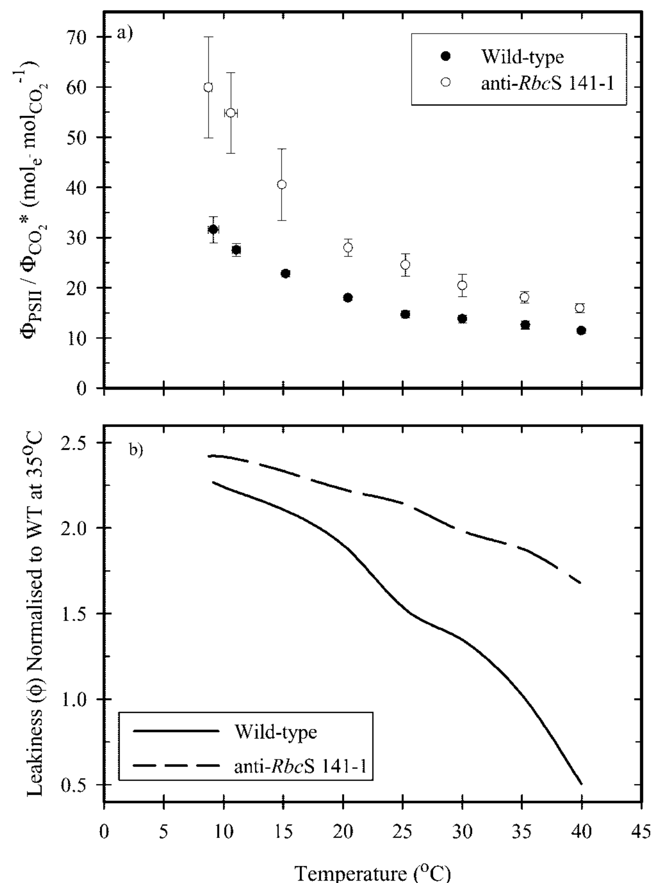

Figure 3. a, The ratio of the quantum yields of PSII (Φ_{PSII}) and gross CO_2 assimilation (Φ_{CO2}*) in wild-type (●) and anti-*Rbc*S (141-1, ○) *F. bidentis* as a function of temperature. b, Modeled leakiness (ϕ) of CO_2 from the bundle sheath, relativized to the estimated value for the wild type at the thermal optimum (35°C). Chlorophyll *a* fluorescence was measured in the 660- to 710-nm waveband, thereby reducing the contribution of PSI; Φ_{PSII} was assessed using the technique of Genty et al. (1989). Gross assimilation was determined using the temperature correction for respiration described in Figure 1. Each value is the mean (± SE) of measurements on three different leaves. Leakiness (ϕ) was determined from the relationship $\Phi_{PSII}/\Phi_{CO2}^* = 4 + 2.66m/(1 - \phi)$, assuming equal contributions of linear and cyclic electron transport (e.g. $m = 0.5$) and that 43% of quanta are absorbed by PSII (Siebke et al., 1997). Leakiness values are shown relative to the wild-type value at the thermal optimum (35°C) because of the potential uncertainty surrounding the calculation of ϕ from fluorescence data. At 35°C, ϕ in the wild type was determined to be 0.39 ± 0.09 (n = 3).

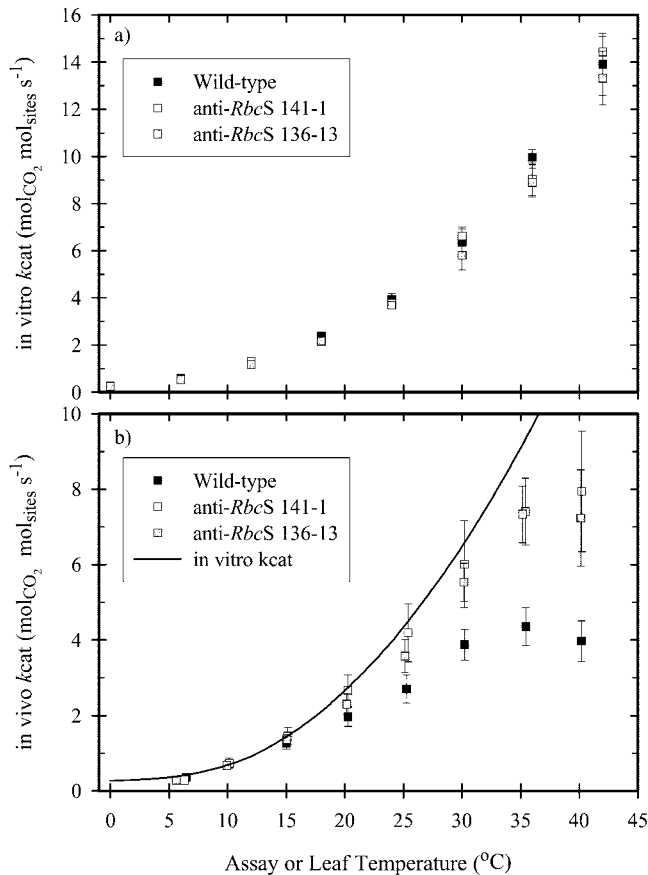

Figure 4. The temperature dependence of the in vitro (a), and in vivo (b) *k*cat for Rubisco in wild-type (■) and anti-*Rbc*S (□, ▩) *F. bidentis*. Note the different scales on the two panels. The in vitro data reflect the activity of the fully carbamylated enzyme; in vivo *k*cat is estimated as gross photosynthesis divided by the number of Rubisco catalytic sites. Each value represents the mean (± SE) of four measurements.

synthesis at low temperatures. Several lines of evidence support this assertion. The in vitro activity of Rubisco matches the in vivo rate of gross photosynthesis at low temperatures, which indicates that the enzyme is the rate-limiting step. The apparent leakage of CO_2 from the bundle sheath increases at low temperature in the wild type to an extent similar to the increase accompanying the reduction of Rubisco by antisense. Finally, the Rubisco C_{ra} increases to near unity at low temperatures, which indicates almost complete control of C_4 photosynthesis by Rubisco.

Rubisco Kinetics in Vitro and in Vivo

Between 6°C and 15°C, the *k*cat of Rubisco in vitro and the rate of gross photosynthesis in vivo are equivalent in wild-type *F. bidentis*. This indicates a strong Rubisco limitation of C_4 photosynthesis at low temperatures. Reducing Rubisco content extends its control of C_4 photosynthesis to higher temperatures, as shown by the wider thermal range across which the in vivo and the in vitro *k*cat values are equivalent in the anti-*Rbc*S lines. The in vivo *k*cat was less than the in vitro *k*cat of Rubisco above 15°C in wild-type plants, and above 25°C in the anti-*Rbc*S lines. A similar finding has been previously reported in the C_4 grass *B. gracilis* (Pittermann and Sage, 2000). In *B. gracilis* populations from high elevation, the maximal in vitro Rubisco activity and net photosynthesis were equivalent below 22°C, whereas in plants from lower elevations, they were equivalent below 17°C. The high-elevation *B. gracilis* plants had 13% less Rubisco than low-elevation plants, which increased the temperature at which Rubisco activity became non-limiting.

At temperatures where Rubisco exerts high control, the activation energy (Ea) of gross assimilation (A^*) should reflect the Ea of the enzyme. In *F. bidentis*, the situation is complicated by the increase in the Ea of Rubisco observed below 15°C. The Ea of A^* between 5°C and 30°C was between the values for Rubisco above and below the break in the thermal response of

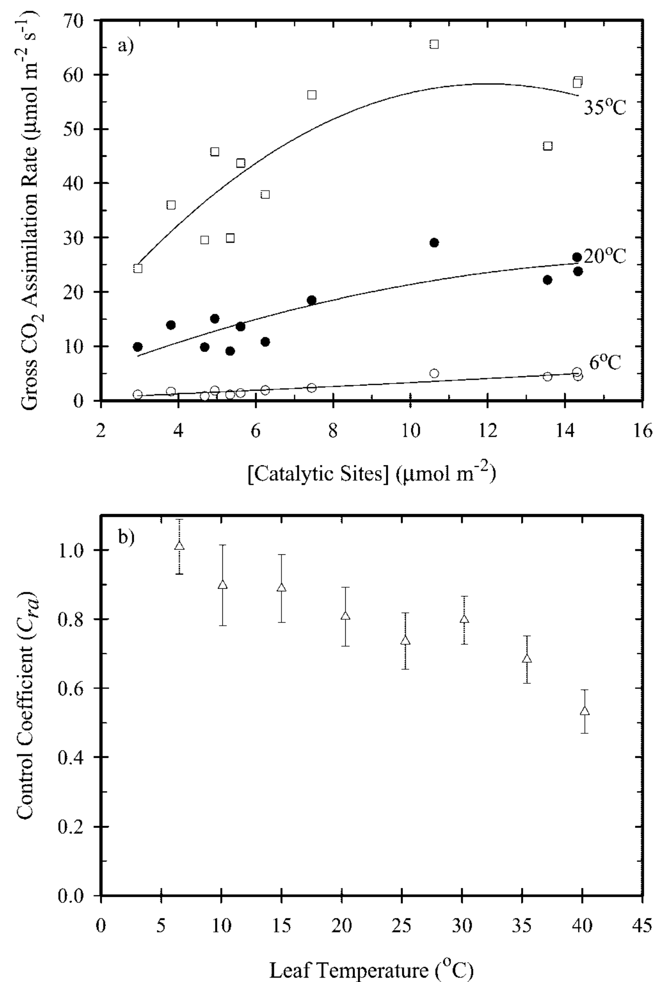

Figure 5. a, The relationship between gross photosynthesis and Rubisco catalytic site concentration at three temperatures; b, Rubisco C_{ra} in *F. bidentis* as a function of leaf temperature. The error bars in b were determined from the SE of the regression of photosynthesis on catalytic sites.

the enzyme, as would be expected if the thermal response of the enzyme controls the thermal response of CO_2 assimilation. The Ea of Rubisco from *F. bidentis* determined between 18°C to 42°C is similar to the 50 to 60 kJ mol^{-1} reported for a range of C_4 species (Sage, 2002). A break in the thermal response of Rubisco has been previously noted in rice (*Oryza sativa*; Sage, 2002), and in both C_3 and C_4 species of *Atriplex* (Björkman and Pearcy, 1971). In wild-type *F. bidentis*, the Ea of PEPCase in vitro above 18°C is equivalent to that of photosynthesis. However, during the temperature response measurements, photosynthesis was operating on the plateau of the *A*/Ci curve (data not shown), above the low-CO_2 region where PEPCase is postulated to control C_4 photosynthesis (von Caemmerer and Furbank, 1999).

Leakage of CO_2 from the Bundle Sheath

Reducing Rubisco capacity by antisense results in greater CO_2 leakage (ϕ) than in wild-type *F. bidentis* across the 6°C to 40°C range, as indicated by the increase in Φ_{PSII}/Φ_{CO2}*. In C_4 plants, the degree of overcycling, and hence CO_2 ϕ, should increase if the ratio of the mesophyll to bundle sheath reaction rates increase (Henderson et al., 1992). As indicated by the similarity of the PEPCase activities in the three genotypes, the mesophyll reactions in the anti-*Rbc*S leaves proceed at rates similar to those of the wild type. In this case, the CO_2 concentration in the bundle sheath will increase, and ϕ will increase. Consistent with this, anti-*Rbc*S *F. bidentis* shows increased carbon isotope discrimination at 25°C, relative to the wild type (von Caemmerer et al., 1997); in C_4 plants, increased leakage of CO_2 from the bundle sheath increases the discrimination against ^{13}C (Farquhar, 1983). It is thus evident that the antisense plants are unable to reduce the rates of the mesophyll reactions to compensate for the reduced flow of carbon through Rubisco. Other explanations for the increase in Φ_{PSII}/Φ_{CO2}* at low temperatures cannot be completely excluded, but appear unlikely. Increases in the strength of alternative electron sinks, such as the Mehler reaction, should lead to increased Φ_{PSII}/Φ_{CO2}* (Krall and Edwards, 1991). However, the direct reduction of O_2 likely accounts for less than 10% of total electron flux in C_3 species, and there is no evidence of substantial rates in C_4 plants (Badger et al., 2000). A mesophyll limitation of C_4 photosynthesis, such as by PPDK, would reduce bundle sheath CO_2 levels and hence reduce both ϕ and Φ_{PSII}/Φ_{CO2}*, at least until photorespiration begins to increase. Thus, although alternative explanations cannot be completely excluded, they are not consistent with the results presented here and in previous studies.

The quantum requirement of CO_2 assimilation is constant above 15°C in a range of C_4 dicots and monocots, indicating that the stoichiometry of the C_4 and C_3 cycles is unaltered at intermediate and warm temperatures (Oberhuber and Edwards, 1993). Similar results are reported here; Φ_{PSII}/Φ_{CO2}* is relatively constant in wild-type *F. bidentis* at temperatures where the control of photosynthesis is shared between Rubisco and other processes. In wild-type plants, the values of Φ_{PSII}/Φ_{CO2}* at warm temperatures (>25°C) shown here are similar to those reported by Siebke et al. (1997). As Rubisco becomes the principle control over the rate of photosynthesis at cooler temperatures (<20°C), bundle sheath leakiness increases, indicating overcycling of the CO_2-concentrating reactions. In plants with reduced Rubisco, ϕ begins to increase at warmer temperatures than in wild-type leaves, as would be predicted from a higher Rubisco control of photosynthesis.

The Control of C_4 Photosynthesis

The C_{ra} defines the control exerted by Rubisco over the flow of carbon through the entire C_4 photosynthetic pathway (Stitt, 1995). The high C_{ra} below 20°C determined here shows that Rubisco dominates control at suboptimal temperatures, whereas the intermediate value at the thermal optimum shows that the control of photosynthetic carbon flux is shared between Rubisco and other processes. If several enzymes are colimiting, then reducing any one of them by antisense should result in a control coefficient close to one for that enzyme. This cannot be excluded here, although the agreement between the in vitro and in vivo *k*cat values of Rubisco would support the strong control exerted by this enzyme at lower temperatures. Theoretical models of C_4 photosynthesis at the leaf level indicate conditions under which other biochemical processes may dominate the control of *A*, such as at low light or low CO_2 (von Caemmerer and Furbank, 1999). At low PPFD, Rubisco control is not indicated, because the net CO_2 assimilation rate of anti-*Rbc*S *F. bidentis* is the same as that of a null-transformant control (Furbank et al., 1996; Siebke et al., 1997). Using *Amaranthus edulis* mutants with reduced PEPCase, Dever et al. (1997) have shown that PEPCase has a high C_{ra} for C_4 photosynthesis at low CO_2, but this control is reduced when CO_2 is saturating (Dever et al., 1997). For PPDK and NADP malate dehydrogenase, the control coefficients are 0.3 and 0, respectively, at 25°C (Furbank et al., 1997). Model predictions indicate that at or above the thermal optimum in air, C_4 photosynthesis is limited by the regeneration of PEP or ribulose 1,5-bisphosphate (von Caemmerer and Furbank, 1999), but our results indicate that Rubisco continues to exert significant control at or above the thermal optimum in *F. bidentis*.

Consequences for C_4 Photosynthesis in Cool Climates

A limitation by Rubisco capacity on C_4 photosynthesis at low temperature represents a mechanism to explain the relative rarity of the C_4 syndrome in cool

climate habitats. The carboxylation efficiency of Rubisco improves at low temperatures, because both the relative availability of CO_2 versus O_2 and the specificity of Rubisco for CO_2 increase as temperature declines (Badger and Collatz, 1977; Berry and Raison, 1981). This improved efficiency is more advantageous to C_3 species, because they typically have three to four times as much Rubisco as C_4 plants (Ku et al., 1979; Long, 1999). Furthermore, the high partial pressure of CO_2 in the bundle sheath ensures that little oxygenation occurs in C_4 plants, so the improved efficiency of Rubisco at low temperatures will not directly affect C_4 photosynthesis.

The minimal amount of Rubisco theoretically required for C_4 plants to match C_3 photosynthetic rates increases at lower temperatures (Long, 1999). Consistent with this, Rubisco accounts for only 4% to 8% of soluble leaf protein in the summer-active Death Valley native *Tidestromia* sp. but about 20% in cool-coastal *Atriplex* sp. (Osmond et al., 1982). Conversely, high-elevation ecotypes of *B. gracilis* contain less Rubisco than plants from low-elevation populations (Pittermann and Sage, 2000). Whereas C_3 species frequently acclimate to low temperatures by increasing the amount of Rubisco (Treharne and Eagles, 1970; Holaday et al., 1992; Hurry et al., 1995), in C_4 plants, the responses are more variable. In *A. lentiformis*, the Rubisco activity of plants grown at 23°C/18°C (day/night) is 60% greater than that of plants grown at 43°C/30°C (Pearcy, 1977), whereas Rubisco activity is insensitive to growth temperatures between 19°C and 31°C in maize (*Zea mays*; Ward, 1987) and between 14°C and 26°C in *Muhlenbergia glomerata*, a C_4 grass native to boreal Canada (Kubien, 2003).

The Rubisco content of C_4 species may be limited by the compartmentalization of the enzyme, because it is restricted to a reduced fraction of the leaf volume relative to C_3 species (Dengler and Nelson, 1999). Increasing the content of Rubisco would likely require changes in the proportion and arrangement of mesophyll and bundle sheath tissues within the leaves of C_4 plants or in the positioning of chloroplasts within the bundle sheath tissue. As a response to low temperatures this seems unlikely, because the spatial arrangement of these compartments influences the intercellular communication required for the efficient operation of the CO_2-concentrating mechanism (Dengler and Nelson, 1999). In addition, the Rubisco to chlorophyll ratio is the same in C_3 and C_4 species if the amount of the enzyme in the C_4 species is expressed on the basis of chlorophyll extracted from isolated bundle sheath cells (Ku et al., 1979; O. Ghannoum, personal communication). If this ratio is fixed, then C_4 species could not match the Rubisco content of C_3 plants, and the potential to increase the enzyme may be limited. Even if the bundle sheath cells could accommodate additional Rubisco, increasing the amount of the enzyme would have a negative effect on the nitrogen economy of C_4 species, thus mitigating one of the ecological advantages maintained over C_3 vegetation (Long, 1999; Pittermann and Sage, 2000). If this constraint exists, a potential solution would be to increase the *k*cat of the enzyme. Both *k*cat and K_m for CO_2 are higher in Rubisco from C_4 species than from C_3 plants (Seemann et al., 1984; Sage and Seemann, 1993; Sage, 2002). This increased turnover has not enabled C_4 species to become common in cool climates.

In summary, we propose that C_4 plants cannot contain sufficient Rubisco to match the photosynthetic rates of ecologically similar C_3 species at low temperatures. A reduction in the amount of Rubisco by C_4 species is possible because of the high CO_2 concentration in the bundle sheath and is one of the fundamental advantages C_4 plants have over their C_3 competitors (Osmond et al., 1982). High control of C_4 photosynthesis by Rubisco is a disadvantage at low temperatures and may be an inherent feature of the C_4 pathway that precludes such species from becoming common in cool climates.

MATERIALS AND METHODS

Plant Growth

Wild-type and anti-*Rbc*S transgenic *Flaveria bidentis* were germinated in sand in a naturally lit greenhouse. The transgenic plants were T_2 progeny of the 141-1 (one insert) and 136-13 (four inserts) primary transformants (Furbank et al., 1996). Four (wild type) or 7 (transgenic) weeks after germination, the seedlings were transplanted to 12-L pots containing 69% (v/v) Promix (Plant Products, Brampton, Canada), 17% (v/v) sand, and 17% (v/v) plant-compost. Plants were subsequently moved to a controlled environment chamber (GC-20, Enconair, Winnipeg, Canada) and maintained under a 16-h photoperiod with a maximal PPFD of 750 μmol m^{-2} s^{-1}. The day/night temperature and relative humidity were 28°/20°C and 50%/75%, respectively. Plants were watered daily and fertilized weekly with 0.5× Hoagland solution supplemented with 3 mM NH_4NO_3.

Gas-Exchange Measurements

The photosynthetic responses to temperature and CO_2 were measured with an open type leaf gas-exchange system using an infrared gas analyzer (Li-6262, Li-Cor, Lincoln, NE) to detect both CO_2 and water vapor. In this system, mass flow controllers (model 840, Sierra Instruments, Monterey, CA) were used to supply N_2, O_2, and CO_2 at the desired levels. All temperature and CO_2 responses were measured at 200 ± 5 mbar O_2. The air stream was humidified by passing the mixture through a water-filled flask which was set to a specific temperature in a water bath. For measurements at lower temperatures, the flask was placed on ice and filled with either water or a 70% (w/v) Suc solution. After humidification, CO_2 was injected, and the flow of air was measured by a mass-flow transducer (831, Edwards, Wilmington, MA) before being passed through the temperature-controlled leaf cuvette and an infrared gas analyzer. Leaf temperature was measured by placing three fine wire (36-gauge) thermocouples in contact with the abaxial surface of the leaf. Illumination was provided by a cool-light source (KL-2500, Schott, Mainz, Germany). All gas exchange measurements were made on the youngest fully expanded leaf and were calculated according to von Caemmerer and Farquhar (1981).

Photosynthetic temperature responses were measured either at a constant PPFD of 1,500 μmol m^{-2} s^{-1} or at a temperature-dependent PPFD that was sufficient to saturate photosynthesis. These points were determined by evaluating the photosynthetic responses to light at 12°C, 22°C, and 32°C, using a portable photosynthesis system (Li-6400, Li-Cor). The *y* intercept of light response curve was taken as an estimate of Rd at each temperature. This approach was used to mitigate the potential for photoinhibition, particularly at the lower temperatures. Light intensity in the cuvette was

measured using a photodiode (G1738, Hamamatsu, Bridgewater, NJ) calibrated against a quantum sensor (Li-190s, Li-Cor). The temperature responses were measured at an ambient CO_2 of 370 ± 2 μbar. The leaf to air vapor pressure deficit was maintained at 12 ± 2 mbar at temperatures greater than 10°C; at cooler temperatures, vapor pressure deficit was reduced. All temperature response measurements were initiated at 30°C; leaf temperature was subsequently increased in 5°C intervals to 40°C and then decreased to the lower temperatures. At each temperature, the leaf was allowed to equilibrate for a minimum of 15 min before measurement. After the last measurement was completed, the leaf was warmed to about 15°C, and two leaf discs (1.55 cm^2 each) were rapidly removed and frozen in liquid N_2. Leaf samples were stored at −80°C until enzymes were assayed.

Chlorophyll *a* Fluorescence Measurements

Chlorophyll *a* fluorescence was determined simultaneously with gas exchange during the temperature response measurements of the wild-type and 141-1 lines. We used a PAM-101 (Walz, Effeltrich, Germany) equipped with an emitter-detector unit (ED-101BL, Walz) that provides excitation light at 470 nm and detection in the 660- to 710-nm waveband. This enabled us to isolate the fluorescence signal originating from PSII (Pfündel, 1998). Each leaf was allowed to dark-adapt at 30°C for 30 min before the ratio of variable to maximal fluorescence (F_v/F_m) was assessed. Reaction center closure was achieved by applying a 0.8-s pulse of saturating light (approximately 4,000 μmol m^{-2} s^{-1}). Once a leaf had reached steady state at a given temperature, the quantum yield of PSII (Φ_{PSII}) was measured (Genty et al., 1989). Saturating pulses were applied at 90-s intervals; at each temperature, the average of three measurements was taken. There was no reduction in F_m' with successive pulses. Thirty seconds after the last Φ_{PSII} estimate was obtained, F_o' was assessed by rapidly darkening the leaf in the presence of far-red light. Leaf absorbance was determined from the chlorophyll concentration (Siebke et al., 1997). Fluorescence nomenclature and calculations follow van Kooten and Snel (1990).

Enzyme and Chlorophyll Assays

The in vitro activities of Rubisco and PEPCase were assayed from 0°C to 42°C using leaf discs harvested from the leaves used for gas exchange analysis. Leaf samples (3.1 cm^2) were rapidly ground (<90 s) at 0°C using a ten-broek glass-in-glass homogenizer containing 7 mL of extraction buffer (100 mM HEPES, pH 7.6, 2 mM Na-EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 9 mg mL^{-1} polyvinyl polypyrrolidone, 2 mg mL^{-1} bovine serum albumin, 2 mg mL^{-1} polyethylene glycol, 2.8% (v/v) Tween-80, 2 mM NaH_2PO_4, 11 mM amino-*n*-caproic acid, and 2.2 mM benzamide). Chlorophyll content was determined spectrophotometrically in *N*,*N*-dimethylformamide, using two aliquots of the crude extract (Porra et al., 1989).

Rubisco was quantified in aliquots of the crude extract, using a [^{14}C]carboxy-arabinitol bisphosphate (CABP)-binding assay and assuming 6.5 binding sites per Rubisco (Butz and Sharkey, 1989). The CABP assay buffer consisted of 100 mM Bicine, 20 mM $MgCl_2$, and 10 mM $NaHCO_3$ at pH 8.2. The leaf extract (40 μL) was incubated in 40 μM ^{14}CABP (specific activity, 27 Bq $nmol^{-1}$) at room temperature for 15 min, followed by a 2.5-h incubation at 37°C in the presence of rabbit anti-Rubisco serum. The Rubisco-^{14}CABP complexes were filtered with 0.45-μm Supor filters (Gelman, Ann Arbor, MI) and thoroughly washed with a 10 mM sodium phosphate buffer (pH 7.6, containing 10 mM $MgCl_2$ and 150 mM NaCl). The radioactivity bound to the filters was measured by liquid scintillation spectroscopy.

A 3.33-mL aliquot of the crude leaf extract was added to 370 μL of a Rubisco activating solution (100 mM Bicine, pH 8.2) containing 280 mM $MgCl_2$ and 200 mM $NaHCO_3$, giving final concentrations of 28 mM $MgCl_2$ and 20 mM $NaHCO_3$, respectively (Sage and Seemann, 1993). This mixture was incubated at room temperature for 20 to 25 min to fully carbamylate Rubisco. The carbamylated extract was then kept on ice until being assayed. The remaining crude extract was kept on ice for subsequent determination of PEPCase activity.

Rubisco activity was assayed in a buffer containing 100 mM Bicine (pH 8.2), 1 mM Na-EDTA, 20 mM $MgCl_2$, 5 mM DTT, 1 unit mL^{-1} ribulose-5-P kinase, 1.7 unit mL^{-1} phospho-ribulo-isomerase, 2 mM ATP, 2 mM Rib-5-P, and 12 mM $NaH^{14}CO_3$ (specific activity, 27 Bq $nmol^{-1}$, ICN Pharmaceuticals, Costa Mesa, CA; Pittermann and Sage, 2000). The assay buffer (400 μL) was allowed to equilibrate at a given temperature for 90 s, after which the assay commenced with the addition of 100 μL of the carbamylated extract. Assays ran for 30 to 60 s and were terminated by the addition of 500 μL of 2 N HCl. The radioactivity of acid-stable products was measured by liquid scintillation spectroscopy. Rubisco *k*cat (mol CO_2 fixed [mol active sites]$^{-1}$ s^{-1}) was determined from the in vitro activity and the concentration of catalytic sites.

PEPCase activity was assayed in a buffer containing 50 mM Bicine (pH 8.2), 1 mM Na-EDTA, 5 mM $MgCl_2$, 5 mM DTT, 4.6 mM PEP, and 4.6 mM G-6-P, 0.2 mM NADH, 0.3 unit mL^{-1} MDH, and 5.4 mM $NaH^{14}CO_3$ (Pittermann and Sage, 2000). Assays were initiated by the addition of 20 μL of the crude extract to 480 μL of the assay buffer, and terminated by the addition of 500 μL of 2 N HCl. Acid stable radioactivity was determined by liquid scintillation spectroscopy.

C_{ra}

A flux control coefficient (C_{ra}) was calculated to determine the extent to which Rubisco controls C_4 photosynthesis across the range of measurement temperatures (Kacser and Burns, 1973; Stitt, 1995). The coefficient was defined as:

$$C_{ra} = \frac{\delta A^*}{\delta [E]} \left(\frac{[E]}{A^*} \right) \quad (1)$$

where A^* is the gross CO_2 assimilation rate (A + Rd, where Rd is respiration) and $[E]$ is the concentration of Rubisco catalytic sites. To obtain the relationship between photosynthesis and Rubisco content, we regressed gross CO_2 assimilation against catalytic site concentration, using the data from the temperature response measurements. We made no underlying assumptions regarding the shape of this relationship and simply used the curve that gave the best fit at each temperature. The derivatives of each curve were then taken at each wild-type catalytic site concentration to determine $\delta A^*/\delta[E]$ between 6°C and 40°C. A second-order polynomial was fit to the respiration rates, determined during the light response measurements, to provide an estimate of respiration at each temperature.

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LITERATURE CITED

Badger MR, Collatz GJ (1977) Studies on the kinetic mechanism of ribulose-1,5-bisphosphate carboxylase and oxygenase reactions, with particular reference to the effect of temperature on kinetic parameters. Carnegie Inst Wash Year Book **76:** 355–361

Badger MR, von Caemmerer S, Ruuska S, Nakano H (2000) Electron flow to oxygen in higher plants and algae: rates and control of direct photoreduction (Mehler reaction) and Rubisco oxygenase. Philos Trans R Soc Lond B **355:** 1433–1446

Berry JA, Raison JK (1981) Responses of macrophytes to temperature. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology, Vol 12A. Springer-Verlag, Berlin, pp 277–338

Björkman O, Pearcy RW (1971) Effect of growth temperature on the temperature dependence of photosynthesis in vivo and on the CO_2 fixation by carboxydismutase in vitro in C_3 and C_4 species. Carnegie Inst Wash Year Book **70:** 511–520

Butz ND, Sharkey TD (1989) Activity ratios of ribulose-1,5-bisphosphate carboxylase accurately reflect carbamylation ratios. Plant Physiol **89:** 735–739

Chitty JA, Furbank RT, Marshall JS, Chen Z, Taylor WC (1994) Genetic transformation of the C_4 plant *Flaveria bidentis*. Plant J **6:** 949–956

Dengler NC, Nelson T (1999) Leaf structure and development in C_4 plants. *In* RF Sage, RK Monson, eds, C_4 Plant Biology. Academic Press, Toronto, pp 133–172

Dever LV, Bailey KJ, Leegood LC, Lea PJ (1997) Control of photosynthesis in *Amaranthus edulis* mutants with reduced amounts of PEP carboxylase. Aust J Plant Physiol **24:** 469–476

Edwards GE, Nakamoto H, Burnell JN, Hatch MD (1985) Pyruvate, Pi-dikinase and NADP-malate dehydrogenase in C_4 photosynthesis: properties and mechanism of light/dark regulation. Annu Rev Plant Physiol **36:** 255–286

Ehleringer JR (1978) Implications of quantum yield differences on the distributions of C_3 and C_4 grasses. Oecologia **31:** 255–267

Ehleringer JR, Björkman O (1977) Quantum yields for CO_2 uptake in C_3 and C_4 plants. Plant Physiol **59:** 86–90

Farquhar GD (1983) On the nature of carbon isotope discrimination in C_4 species. Aust J Plant Physiol **10:** 205–226

Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanion SJ, von Caemmerer S, Ashton AR (1997) Genetic manipulation of key photosynthetic enzymes in the C_4 plant *Flaveria bidentis*. Aust J Plant Physiol **24:** 477–485

Furbank RT, Chitty JA, von Caemmerer S, Jenkins CLD (1996) Antisense RNA inhibition of RbcS gene expression reduces Rubisco level and photosynthesis in the C_4 plant *Flaveria bidentis*. Plant Physiol **111:** 725–734

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta **990:** 87–92

Henderson SA, von Caemmerer S, Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C_4 species. Aust J Plant Physiol **19:** 263–285

Holaday AS, Martindale W, Alred R, Brooks AL, Leegood RC (1992) Changes in activities of enzymes of carbon metabolism in leaves during exposure of plants to low temperature. Plant Physiol **98:** 1105–1114

Hurry V, Keerberg O, Pärnik T, Gardeström P, Öquist G (1995) Cold-hardening results in increased activity of enzymes involved in carbon metabolism in leaves of winter rye (*Secale cereale* L.). Planta **195:** 554–562

Kacser H, Burns JA (1973) The control of flux. Symp Soc Exp Biol **27:** 65–104

Krall JP, Edwards GE (1991) Environmental effects on the relationship between the quantum yields of carbon assimilation and *in vivo* PSII electron transport in maize. Aust J Plant Physiol **18:** 267–278

Krall JP, Edwards GE (1993) PEP carboxylases from two C_4 species of *Panicum* with markedly different susceptibilities to cold inactivation. Plant Cell Physiol **34:** 1–11

Ku MSB, Schmitt MR, Edwards GE (1979) Quantitative determination of RuBP carboxylase-oxygenase protein in leaves of several C_3 and C_4 plants. J Exp Bot **30:** 89–98

Kubien DS (2003) On the performance of C_4 photosynthesis at low temperatures and its relationship to the ecology of C_4 plants in cool climates. PhD thesis. University of Toronto

Leegood RC, Edwards GE (1996) Carbon metabolism and photorespiration: temperature dependence in relation to other environmental factors. *In* NR Baker, ed, Photosynthesis and the Environment. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 191–221

Long SP (1983) C_4 Photosynthesis at low temperatures. Plant Cell Environ **6:** 345–363

Long SP (1999) Environmental responses. *In* RF Sage, RK Monson, eds, C_4 Plant Biology. Academic Press, Toronto, pp 215–249

Matsuba K, Imaizumi N, Kaneko S, Samejima M, Ohsugi R (1997) Photosynthetic responses to temperature of phosphoenolpyruvate carboxykinase type C_4 species differing in cold sensitivity. Plant Cell Environ **20:** 268–274

Oberhuber W, Edwards GE (1993) Temperature dependence of the linkage of quantum yield of photosystem II to CO_2 fixation C_4 and C_3 plants. Plant Physiol **101:** 507–512

Osmond CB, Winter K, Ziegler H (1982) Functional significance of different pathways of CO_2 fixation in photosynthesis. *In* OL Lange, PS Nobel, CB Osmond, H Ziegler, eds, Physiological Plant Ecology II. Encyclopedia of Plant Physiology, Vol 12B. Springer-Verlag, Berlin, pp 480–547

Pearcy RW (1977) Acclimation of photosynthetic and respiratory carbon dioxide exchange to growth temperature in *Atriplex lentiformis* (Torr.) Wats. Plant Physiol **59:** 795–799

Pfündel E (1998) Estimating the contribution of photosystem I to total leaf chlorophyll fluorescence. Photosynth Res **56:** 185–195

Pittermann J, Sage RF (2000) Photosynthetic performance at low temperature of *Bouteloua gracilis* Lag., a high-altitude C_4 grass from the Rocky Mountains, USA. Plant Cell Environ **23:** 811–823

Pittermann J, Sage RF (2001) The response of the high altitude C_4 grass *Muhlenbergia montana* (Nutt.) A.S. Hitchc. to long- and short-term chilling. J Exp Bot **52:** 829–838

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents, verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta **975:** 384–394

Rundel PW (1980) The ecological distribution of C_4 and C_3 grasses in the Hawaiian Islands. Oecologia **45:** 354–359

Sage RF (2002) Variation in the k_{cat} of Rubisco in C_3 and C_4 plants and some implications for photosynthetic performance at high and low temperature. J Exp Bot **53:** 609–620

Sage RF, Seemann JR (1993) Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in response to reduced light intensity in C_4 plants. Plant Physiol **102:** 21–28

Sage RF, Wedin DA, Li M (1999) The biogeography of C_4 photosynthesis, patterns and controlling factors. *In* RF Sage, RK Monson, eds, C_4 Plant Biology. Academic Press, Toronto, pp 313–373

Seemann JR, Badger MR, Berry JA (1984) Variations in the specific activity of ribulose-1,5-bisphosphate carboxylase between species utilizing different photosynthetic pathways. Plant Physiol **74:** 791–794

Siebke K, von Caemmerer S, Badger M, Furbank RT (1997) Expressing an RbcS antisense gene in transgenic *Flaveria bidentis* leads to an increased quantum requirement for CO_2 fixed in photosystems I and II. Plant Physiol **115:** 1163–1174

Stitt M (1995) The use of transgenic plants to study the regulation of plant carbohydrate metabolism. Aust J Plant Physiol **22:** 635–646

Stitt M, Schulze D (1994) Does Rubisco control the rate of photosynthesis and plant growth? an exercise in molecular ecophysiology. Plant Cell Environ **17:** 465–487

Sugiyama T, Boku K (1976) Differing sensitivity of pyruvate orthophosphate dikinase to low temperature in maize cultivars. Plant Cell Physiol **17:** 851–854

Teeri JA, Stowe LG (1976) Climatic patterns and the distribution of C_4 grasses in North America. Oecologia **23:** 1–12

Tieszen LL, Senyimba MM, Imbamba SK, Troughton JH (1979) The distribution of C_3 and C_4 grasses and carbon isotope discrimination along an altitudinal and moisture gradient in Kenya. Oecologia **37:** 337–350

Treharne KJ, Eagles CF (1970) Effect of temperature on photosynthetic activity of climatic races of *Dactylis glomerata* L. Photosynthetica **4:** 107–117

van Kooten O, Snel JFH (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynth Res **25:** 147–150

von Caemmerer S, Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas-exchange of leaves. Planta **153:** 376–387

von Caemmerer S, Furbank RT (1999) Modelling C_4 photosynthesis. *In* RF Sage, RK Monson, eds, C_4 Plant Biology. Academic Press, Toronto, pp 173–211

von Caemmerer S, Millgate A, Farquhar GD, Furbank RT (1997) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase by antisense RNA in the C_4 plant *Flaveria bidentis* leads to reduced assimilation rates and increased carbon isotope discrimination. Plant Physiol **113:** 469–477

Ward DA (1987) The temperature acclimation of photosynthetic responses to CO_2 in *Zea mays* and its relationship to the activities of photosynthetic enzymes and the CO_2-concentrating mechanism of CO_2 photosynthesis. Plant Cell Environ **10:** 407–411