Effects of low atmospheric CO_2 and elevated temperature during growth on the gas exchange responses of C_3, C_3–C_4 intermediate, and C_4 species from three evolutionary lineages of C_4 photosynthesis

Patrick J. Vogan · Rowan F. Sage

Abstract This study evaluates acclimation of photosynthesis and stomatal conductance in three evolutionary lineages of C_3, C_3–C_4 intermediate, and C_4 species grown in the low CO_2 and hot conditions proposed to favo r the evolution of C_4 photosynthesis. Closely related C_3, C_3–C_4, and C_4 species in the genera *Flaveria*, *Heliotropium*, and *Alternanthera* were grown near 380 and 180 µmol CO_2 mol^{-1} air and day/night temperatures of 37/29°C. Growth CO_2 had no effect on photosynthetic capacity or nitrogen allocation to Rubisco and electron transport in any of the species. There was also no effect of growth CO_2 on photosynthetic and stomatal responses to intercellular CO_2 concentration. These results demonstrate little ability to acclimate to low CO_2 growth conditions in closely related C_3 and C_3–C_4 species, indicating that, during past episodes of low CO_2, individual C_3 plants had little ability to adjust their photosynthetic physiology to compensate for carbon starvation. This deficiency could have favored selection for more efficient modes of carbon assimilation, such as C_3–C_4 intermediacy. The C_3–C_4 species had approximately 50% greater rates of net CO_2 assimilation than the C_3 species when measured at the growth conditions of 180 µmol mol^{-1} and 37°C, demonstrating the superiority of the C_3–C_4 pathway in low atmospheric CO_2 and hot climates of recent geological time.

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P. J. Vogan
Department of Integrative Biology, University of Guelph,
50 Stone Road East, Guelph, ON N1G 2W1, Canada

R. F. Sage (✉)
Department of Ecology and Evolutionary Biology,
University of Toronto, 25 Willcocks St.,
Toronto, ON M5S 3B2, Canada
e-mail: r.sage@utoronto.ca

Introduction

The C_4 photosynthetic pathway has independently evolved more than 60 times, making it one of the most convergent of complex traits in the biosphere (Christin et al. 2011; Sage et al. 2011a). Phylogenetic analyses of known C_4 clades demonstrate that the C_4 pathway is relatively young in geological time, having repeatedly arisen in the past 30 million years (Christin et al. 2008, 2011; Vincentini et al. 2008). A leading hypothesis for the rise of C_4 photosynthesis is the carbon starvation hypothesis, which proposes that, in warm environments, low atmospheric CO_2 conditions promoted high rates of photorespiration and reduced carboxylation capacity of Rubisco in C_3 plants; in response, C_4 photosynthesis evolved to compensate for the inhibitory effects of photorespiration (Ehleringer et al. 1991, 1997; Monson 2003; Sage 2004). Over the past 200 million years, atmospheric CO_2 levels were well above current levels until the late-Oligocene epoch (30–25 million years ago, MYA). In the late Oligocene, atmospheric CO_2 declined to between 200 and 400 µmol CO_2 mol^{-1} air (Pagani et al. 1999; Pearson and Palmer 2000; Royer et al. 2001). During the last 0.8 MYA, they oscillated between 180 and 300 µmol mol^{-1} (Petit et al. 1999; Gerhart and Ward 2010). Consistent with the CO_2 starvation hypothesis, the decline in CO_2 in the late-Oligocene corresponds to the first wave of C_4 origins,

while a possible late-Miocene CO_2 decline to below 300 µmol mol^{-1} (Kurschner et al. 2008; Tripati et al. 2009) corresponds to an additional wave of C_4 evolution and the expansion of C_4 grasslands in low- to mid-latitudes (Christin et al. 2008, 2011; Vincentini et al. 2008; Arakaki et al. 2011). Climates were as warm or warmer during the Miocene and Pliocene than today (Zachos et al. 2008), such that leaf temperatures >35°C would have regularly occurred at low latitudes, as indicated by measurements in modern ecosystems (Monson and Jaeger 1991; Vogan 2010). The synchrony between low CO_2 in the Miocene and C_4 origins is also consistent with ecophysiological studies that show superior growth, carbon gain, quantum yield, and resource-use efficiency in C_4 relative to C_3 plants at low to current CO_2 levels and temperatures over 30°C (Ehleringer and Pearcy 1983; Dippery et al. 1995; Tissue et al. 1995; Sage 1995; Ehleringer et al. 1997). For example, C_4 biomass dominates C_3 biomass in prairie sod grown at 200 µmol mol^{-1}; at 360 µmol mol^{-1}, the C_4 to C_3 biomass ratio is similar (Johnson et al. 1993; Polley et al. 2002).

While the timing of C_4 origins and the ecophysiological data are consistent with the CO_2 starvation hypothesis, these results do not address how low CO_2 would have actually led to the evolution of the C_4 pathway. For the CO_2 starvation hypothesis to be valid, the intervening stages between the C_3 and C_4 conditions would have to enhance fitness in a low CO_2, high temperature environment. The intermediate stage during C_4 evolution involves the formation of a photorespiratory CO_2 concentration mechanism in plants known as C_3–C_4 intermediates (Monson and Rawsthorne 2000). In C_3–C_4 intermediates, the photorespiratory cycle is distributed between two tissue compartments, the bundle sheath and the mesophyll, rather than occurring in a single photosynthetic cell as in C_3 plants. This two-tissue metabolic cycle restricts photorespiratory CO_2 production to the bundle-sheath cells, thus facilitating its recapture by bundle-sheath chloroplasts, and boosting the efficiency of bundle-sheath Rubisco (von Caemmerer 1989). In combination, the changes leading to C_3–C_4 intermediacy compensate for high rates of photorespiration as evidenced by reduced CO_2 compensation points of photosynthesis (Γ) and elevated rates of net CO_2 assimilation in C_3–C_4 relative to C_3 species at low atmospheric CO_2 concentration (Morgan and Brown 1979; Ku et al. 1983, 1991; Rajendrudu et al. 1986; Monson and Rawsthorne 2000; Vogan et al. 2007).

To best evaluate the CO_2 starvation hypothesis, closely related C_3, C_3–C_4 intermediate, and C_4 species should be grown and measured in the hot, low CO_2 conditions proposed to favor the evolution of the C_4 pathway. Because plants can acclimate to variation in growth CO_2 and temperature, inferences based on plants grown away from the conditions where C_4 photosynthesis evolved could be misleading. The large majority of C_4 lineages have arisen in warm, sub-tropical to tropical locations, and most C_3–C_4 species grow in hot environments (Monson and Rawsthorne 2000; Edwards and Smith 2010; Sage et al. 2011a, b), hence, it is best to evaluate their performance at the elevated temperatures common in their habitat. Also, multiple evolutionary lineages should be simultaneously studied. To address evolutionary hypotheses where a genetic model is lacking, comparisons of numerous independent lineages are preferred, as each lineage is but one replicated observation (Felsenstein 1985). Previous studies addressing the carbon starvation hypothesis rarely met these criteria. No study has compared responses of multiple evolutionary lines of C_3, C_3–C_4, and C_4 species grown and measured at subambient CO_2. C_3–C_4 intermediates have elevated photosynthetic capacities relative to C_3 species at sub-ambient CO_2; however, this observation is based on measurements of plants grown at the atmospheric CO_2 levels at the time of the experiment (330–375 µmol mol^{-1}; Ku et al. 1983, 1991; Rajendrudu et al. 1986; Vogan et al. 2007). High temperatures aggravate photorespiration in C_3 plants and thus the selection pressures for C_4 evolution would be greatest in hot environments (Sage 2004), but few studies have examined performance of C_3–C_4 intermediates relative to C_3 species growing in hot environments. Schuster and Monson (1990) and Monson and Jaeger (1991) did evaluate C_3–C_4 *Flaveria* species grown in warm to hot conditions, but the plants grew at atmospheric CO_2 levels of the recent past (350–360 µmol mol^{-1}).

One potential outcome of growth in low CO_2 and elevated temperature is leaf-level biochemical acclimation which might enhance carbon acquisition in C_3 species and reduce the relative benefit of the C_3–C_4 mechanism. In C_3 species, this can occur by increased investment in Rubisco content (Sage and Coleman 2001). C_3–C_4 species may also acclimate and thus retain their advantage; for example, they may enhance the efficiency of photorespiratory CO_2 re-fixation. A third acclimation response, increased stomatal conductance leading to higher intercellular CO_2 levels, has been observed in low CO_2-grown plants of the C_3 forb *Solanum dimidiatum* (Maherali et al. 2002). Increased stomatal conductance, however, would greatly enhance water loss in hot environments and could thus be of limited value. In hot environments with high vapor pressure differences between leaf and air, it is possible that C_3–C_4 intermediate plants have reduced stomatal conductance relative to C_3 species, since they may rely on the photorespiratory CO_2 pump to offset reduced intercellular CO_2 levels. This could lead to greater water-use efficiency in C_3–C_4 relative to C_3 species growing in warm, low CO_2 conditions.

In this study, we evaluated photosynthetic and stomatal acclimation to low CO_2 in closely related C_3, C_4 and C_3–C_4 plants from three separate evolutionary lineages of C_4

photosynthesis: *Flaveria* (Asteraceae), *Heliotropium* (Boraginaceae), and *Alternanthera* (Amaranthaceae). Each species studied occurs in hot environments where daytime temperatures during the growing season exceed 35°C (Supplemental Fig. S1; Monson and Jaeger 1991). The C_3–C_4 species *Heliotropium convolvulaceum* grows on sand dunes in the Mojave and Chihuahuan deserts of southwestern North America. Episodic monsoon rains support summer activity of *H. convolvulaceum*, when leaf temperatures exceed 35°C for much of the day (Fig. S1). *Heliotropium calcicola* (C_3), *Flaveria robusta* (C_3), and *F. ramosissima* (C_3–C_4) are common in semi-arid regions of central Mexico where they also exploit monsoon rains to remain active on hot summer days (Frohlich 1978; Powell 1978). *Alternanthera sessilis* (C_3) and *A. tenella* (C_3–C_4) are herbaceous species native to moist (sub)tropical climates of the Caribbean basin, Central America, and South America (http://www.tropicos.org). Plants were grown at high temperatures (37/29°C) in low and current atmospheric CO_2 concentrations of 180 and 380 µmol mol^{-1}. The response of net CO_2 assimilation rate (A) to intercellular CO_2 concentration (C_i) was measured at 30 and 40°C and the response of A to variation in leaf temperature (T) was assessed at the growth CO_2 concentrations. Stomatal acclimation to low CO_2 was evaluated using responses of stomatal conductance (g) to C_i (Santrucek and Sage 1996). Associated with these measurements, we measured leaf nitrogen (N), Rubisco, and chlorophyll contents to determine if N allocation to photosynthetic processes is altered by growth CO_2.

Materials and methods

Source material and growth conditions

Flaveria bidentis (C_4) seeds were obtained from offspring of seeds originally collected by Powell (1978). E. Sudderth provided collected seeds of *F. ramosissima* (C_3–C_4) from Tehuacan, Mexico (Sudderth et al. 2009). R.F. Sage collected seeds of *F. robusta* (C_3; 20 km W of Colima, Mexico), *Heliotropium convolvulaceum* (C_3–C_4; 10 km NE of Overton, NV, USA), and *H. texanum* (C_4; 6 km E of Zapata, TX, USA; Vogan et al. 2007). *Heliotropium calcicola* (C_3) seeds were collected by Michael Frohlich in northern Mexico (Vogan et al. 2007). Seeds of *Alternanthera sessilis* (C_3) and *A. tenella* (C_3–C_4) were from offspring of plants collected by A.S. Ragavendra (Hyderabad, India), while *A. caracasana* (C_4) seeds were collected by A.M. Powell in Alpine, TX, USA. Within their respective genera, these species are closely related (Frohlich 1978; McKown et al. 2005; Sanchez-del Pino et al. 2009). Vouchers specimens are stored at the Royal Ontario Museum, Toronto, ON.

Plants were grown from rooted cuttings in growth chambers (GC-20; BioChambers, Winnipeg, MB, Canada) set to deliver day/night temperatures of 37/29°C, a 14-h photoperiod, and a mean irradiance at plant height (±SE) of 561 ± 11 µmol photons m^{-2} s^{-1}. Plants grew in 4-L pots containing equal parts sand, Pro-Mix (Premier Horticulture, Quakertown, PA, USA) and sterilized topsoil. Plants were watered daily, fertilized three times weekly with a full-strength Hoagland's solution, and trimmed monthly. Growth CO_2 levels (±SE) were 184 ± 5 and 381 ± 8 µmol mol^{-1}, and were maintained with a soda-lime scrubber controlled by a PP Systems WMA-2 gas analyzer (PP Systems International, Amesbury, MA, USA). To minimize growth chamber effects, plants were rotated weekly within a chamber, and the treatments were switched between chambers for the three replicated sets of measurements.

### Photosynthetic gas exchange and leaf nitrogen analysis

Gas exchange of a youngest fully expanded leaf was measured using an LI-6400 photosynthesis system (Li-Cor, Lincoln, NE, USA) at a saturating light intensity of 1,500 µmol photons m^{-2} s^{-1}. The response of A and g to leaf temperature (T) was measured at 380 and 180 µmol CO_2 mol^{-1} air by first lowering leaf T from 25 to 15°C and then raising it in 5°C increments to 45°C. Photosynthetic responses to intercellular CO_2 (A/C_i responses) were logged at 30 and 40°C during the course of an A/T response measurement. Leaf-to-air vapor pressure difference during the A/T responses rose from 1.5 kPa at low temperature to 3.5 kPa at the warmer temperatures, but was maintained at 2–2.7 kPa for the A/C_i measurements. The photosynthetic thermal optimum was calculated by determining maximum A from a cubic regression of the A/T responses. CO_2 compensation points (Γ) of A were estimated by extrapolating the initial slope of the A/C_i plots to the x-intercept.

After each gas exchange response, two 2.6 cm^2 leaf discs were sampled from measured leaves. One was assayed for N content using a C:N analyzer (Costech Analytical Technologies, Valencia, CA, USA); the second was assayed for Rubisco content using the ^{14}C-CABP binding method (Sage et al. 1993). Chlorophyll was spectrophotometrically assayed following extraction in acetone (Evans 1983). Nitrogen allocation to electron transport components was calculated from leaf chlorophyll content based on a value of 55 mol N allocated to electron transport for every mol of chlorophyll (Evans 1983). Rubisco N was estimated assuming a protein N content of 16% (Sage et al. 1987).

Results

Growth CO_2 concentration did not affect leaf N, relative N allocation to Rubisco and electron transport, leaf C:N

Fig. 1 The response of net CO_2 assimilation rate to intercellular CO_2 concentration for current ambient CO_2-grown (380 µmol CO_2 mol^{-1}; **a**, **b** low CO_2-grown (180 µmol mol^{-1}; **c**, **d** *Alternanthera* species. Measurements were conducted at a photon flux density of 1,500 µmol m^{-2} s^{-1}, leaf-to-air vapor pressure difference of 2–2.7 kPa and leaf temperatures of 30°C (**a**, **c**) and 40°C (**b**, **d**). Each *symbol* is the mean ± SE of three measurements from independent plants
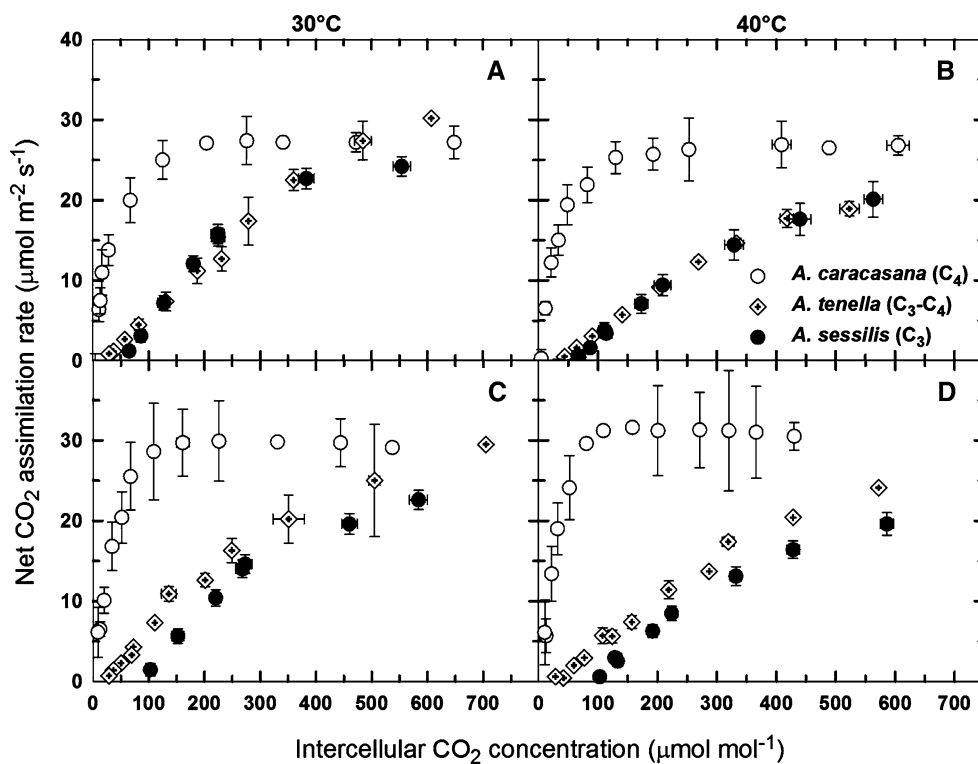
ratios, or specific leaf mass (Supplemental Table S1). Leaf Rubisco content was 60% lower and C:N was roughly 25% higher in C_4 species than in the other functional types, but there was no apparent biochemical acclimation to low CO_2 in any species or photosynthetic type (Table S1).

Growth CO_2 had no effect on the A/C_i responses of a given species. The CO_2 compensation point and net CO_2 assimilation rate at the highest measurement CO_2 level (A_{800}) were the same between plants grown at 180 versus 380 µmol mol^{-1} (Figs. 1, 2 and 3; Table 1). Increasing the measurement temperature from 30 to 40°C increased Γ in C_3 species by an average of 25 µmol mol^{-1}, while in C_3–C_4 intermediates, the increase was 16 µmol mol^{-1}. *Flaveria ramosissima*, a C_3–C_4 species with some C_4-cycle activity, exhibited lower Γ than *Alternanthera tenella* and *Heliotropium convolvulaceum* which lack significant C_4-cycle activity (Table 1; Monson et al. 1986; Devi et al. 1995; Vogan et al. 2007). These species' gas exchange results did not otherwise differ. Net CO_2 assimilation rates at the respective growth CO_2 levels were lower in both C_3 and C_3–C_4 species at 40°C compared to 30°C; the decline in A with increasing temperature was approximately 30% greater in C_3 than in C_3–C_4 species (Table 1). At 380 µmol mol^{-1} and 40°C, A was slightly higher in the C_3–C_4 species than C_3 plants, while at 180 µmol mol^{-1}, A was 67% greater in the C_3–C_4 species. C_4 species exhibited the highest rates of A at all measurement temperatures and CO_2 levels.

The thermal optimum of A (T_{OPT}) at 380 µmol mol^{-1} ranged from 30 to 32°C for the C_3 species, from 31 to 32°C

for the C_3–C_4 intermediates and from 34 to 42°C for the C_4 species (Table 2). When the measurement CO_2 concentration was lowered to 180 µmol mol^{-1}, T_{OPT} of the C_4 species was unaffected, while it decreased to 29°C in the C_3–C_4 species and 25°C in the C_3 species (Table 2; Fig. 4; Supplemental Table S2). At low measurement CO_2, A at T_{OPT} was highest in the C_4 species of each genus, and was 34% greater in the C_3–C_4 intermediates than the C_3 species. At the daytime growth temperature (37°C), A in the C_3–C_4 species was not different from C_3 species at the current ambient CO_2, but at low CO_2, A in C_3–C_4 species was 1.6–2 times higher.

The g versus C_i responses were not affected by growth CO_2 in any species, and were similar in all three photosynthetic types of *Alternanthera* and *Flaveria* (Figs. 5, 6 and 7). The C_i/C_a ratio increased with reduction in C_a below 300 µmol mol^{-1} in both the C_3 and C_3–C_4 species. There was no consistent evidence for stomatal acclimation within any species or photosynthetic type as the C_i/C_a *versus* C_i responses were similar between CO_2 treatments. At 180 µmol mol^{-1}, the larger A values in the C_3–C_4 intermediates relative to the respective C_3 species of *Flaveria* and *Heliotropium* resulted in significantly lower C_i/C_a in the C_3–C_4 species than in C_3 plants; this difference was not apparent at current ambient CO_2 levels. Given the similarity in g between the two photosynthetic types at low CO_2, the difference in C_i/C_a between C_3 and C_3–C_4 species at sub-ambient CO_2 was driven entirely by higher A in the C_3–C_4 species. Stomatal conductance also increased at low

Fig. 2 The response of net CO_2 assimilation rate to intercellular CO_2 concentration for current ambient CO_2-grown (380 μmol CO_2 mol^{-1}; (**a**, **b**) low CO_2-grown (180 μmol mol^{-1}; **c**, **d**) *Flaveria* species measured at 30°C **a** and **c** and 40°C (**b**, **d**). Measurement conditions were as described for Fig. 1. Each *symbol* is the mean ± SE of three measurements from independent plants
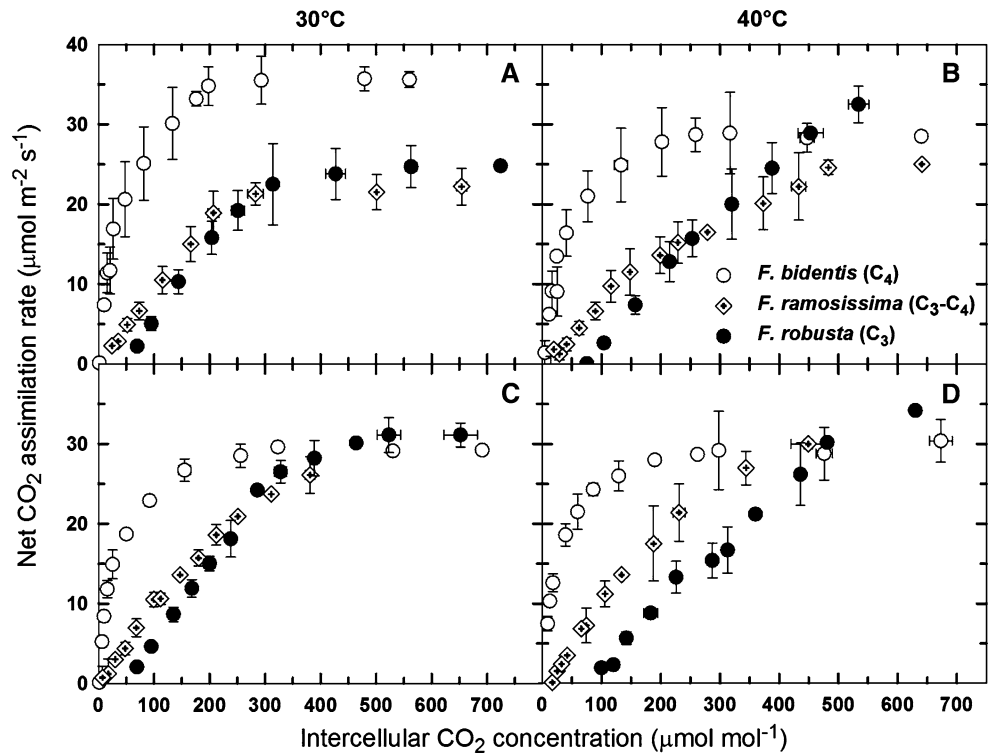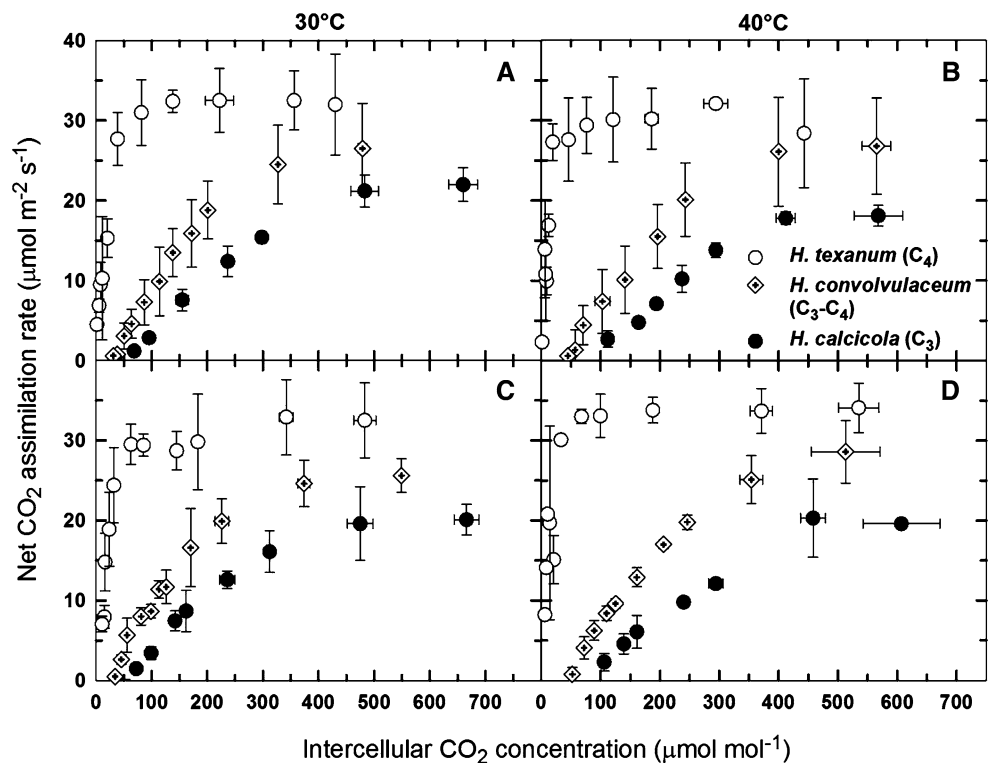

Fig. 3 The response of net CO_2 assimilation rate to intercellular CO_2 concentration for current ambient CO_2-grown (380 μmol CO_2 mol^{-1}; **a**, **b**) low CO_2-grown (180 μmol mol^{-1}) *Heliotropium* species measured at 30°C (**a**, **c**) and 40°C (**b**, **d**). Measurement conditions were as described for Fig. 1. Each *symbol* is the mean ± SE of three measurements from independent plants

CO_2 in all three C_4 species, but to a lesser extent than in the C_3 and C_3–C_4 species, such that the C_i/C_a values declined or showed little change at the lowest measurement C_i.

Photosynthetic water-use efficiencies (PWUE) in C_3 and C_3–C_4 species of each genus at 40°C were similar at

380 μmol mol^{-1}. There was a trend for PWUE to be 85 and 186% greater in the intermediates *F. ramosissima* and *H. convolvulaceum*, respectively, than in the respective C_3 species *F. robusta* and *H. calcicola* at 180 μmol mol^{-1} and 40°C ($P = 0.07$ and 0.08, respectively; Table 3). In

Table 1 CO_2 compensation points (Γ) and net CO_2 assimilation rates at growth CO_2 concentrations and near CO_2 saturation for *Flaveria*, *Alternanthera*, and *Heliotropium*

Species	Type	Leaf T (°C)	Γ (μmol mol⁻¹)	A_{180} (μmol CO_2 m⁻² s⁻¹)	A_{380} (μmol CO_2 m⁻² s⁻¹)	A_{800} (μmol CO_2 m⁻² s⁻¹)
F. robusta	C_3	30	50.9 ± 1.1[B]	9.8 ± 1.1[B]	19.1 ± 1.8[C]	28.3 ± 1.8[A]
		40	77.1 ± 4.0[A]	6.7 ± 0.4[A]	15.0 ± 1.0[B]	30.1 ± 2.7[A]
A. sessilis	C_3	30	54.2 ± 1.8[B]	6.9 ± 2.1[A]	14.2 ± 1.4[B]	24.7 ± 1.6[AB]
		40	76.4 ± 2.9[A]	4.9 ± 3.1[A]	9.1 ± 1.2[A]	22.9 ± 1.8[AB]
H. calcicola	C_3	30	52.2 ± 2.0[B]	7.9 ± 0.9[AB]	13.1 ± 1.2[B]	21.7 ± 1.5[B]
		40	77.4 ± 0.8[A]	4.9 ± 1.0[A]	10.0 ± 0.9[A]	20.1 ± 1.1[B]
All C_3		30	52.4 ± 1.4[B]	8.2 ± 1.6[AB]	15.5 ± 1.5[BC]	24.9 ± 1.6[AB]
		40	77.0 ± 2.6[A]	5.5 ± 1.8[A]	11.4 ± 1.1[AB]	24.4 ± 2.0[AB]
F. ramosissima	C_3–C_4	30	11.6 ± 1.6[E]	13.7 ± 2.2[C]	19.4 ± 1.6[C]	25.9 ± 2.2[AB]
		40	21.4 ± 2.5[C]	11.4 ± 1.6[BC]	15.8 ± 2.7[B]	24.7 ± 2.1[AB]
A. tenella	C_3–C_4	30	18.1 ± 1.2[D]	9.8 ± 2.2[B]	16.9 ± 2.3[BC]	29.0 ± 0.3[A]
		40	32.5 ± 2.8[C]	5.2 ± 0.9[A]	12.8 ± 1.6[AB]	24.3 ± 1.4[AB]
H. convolvulaceum	C_3–C_4	30	22.4 ± 2.1[D]	10.9 ± 1.7[B]	22.0 ± 1.8[C]	33.7 ± 4.1[A]
		40	45.8 ± 6.8[BC]	11.1 ± 1.6[BC]	24.1 ± 2.7[CD]	32.8 ± 3.8[A]
All C_3–C_4		30	17.4 ± 1.5[D]	11.5 ± 2.0[BC]	19.4 ± 1.9[C]	29.5 ± 2.1[A]
		40	33.2 ± 3.4[C]	9.2 ± 1.2[B]	17.6 ± 2.4[BC]	27.3 ± 2.6[AB]
F. bidentis	C_4	30	2.8 ± 1.2[F]	24.0 ± 3.6[D]	27.8 ± 2.5[D]	32.5 ± 4.1[A]
		40	3.0 ± 1.1[F]	22.8 ± 2.1[D]	25.2 ± 2.6[D]	29.1 ± 3.6[A]
A. caracasana	C_4	30	2.8 ± 0.6[F]	22.3 ± 1.8[D]	28.7 ± 3.9[D]	28.8 ± 3.4[AB]
		40	3.3 ± 0.9[F]	25.0 ± 4.4[D]	29.2 ± 4.1[D]	30.1 ± 3.3[A]
H. texanum	C_4	30	3.4 ± 1.3[F]	29.7 ± 3.0[D]	30.7 ± 3.2[D]	31.0 ± 4.4[A]
		40	5.3 ± 1.7[F]	31.6 ± 3.1[D]	32.1 ± 4.6[D]	32.5 ± 5.1[A]
All C_4		30	3.0 ± 0.9[F]	25.3 ± 2.7[D]	29.1 ± 3.3[D]	30.8 ± 4.2[AB]
		40	3.9 ± 1.0[F]	26.5 ± 3.6[D]	28.8 ± 3.6[D]	30.6 ± 3.7[AB]

Low-CO_2 and current ambient-CO_2 grown plants were pooled for each value because A/C_i responses were not different between the two treatments

All species values are mean ± SE of three individuals

Different letters indicate significant differences at $P < 0.05$ between measurements within a column

Table 2 The photosynthetic thermal optimum (T_{OPT}) of net CO_2 assimilation rate (A) and net CO_2 assimilation rate at T_{OPT} and at 37°C for C_3, C_4 and C_3–C_4 intermediate photosynthetic types

Species	Measurement CO_2 concentration (μmol mol⁻¹)	T_{OPT} (°C)	A at T_{OPT} (μmol CO_2 m⁻² s⁻¹)	A at 37°C (μmol CO_2 m⁻² s⁻¹)
All C_3	180	24.8 ± 0.7[A]	9.4 ± 0.8[A]	6.9 ± 1.2[A]
	380	31.3 ± 0.4[B]	16.6 ± 1.4[C]	15.8 ± 1.1[BC]
All C_3–C_4	180	29.1 ± 0.5[B]	12.6 ± 0.6[B]	10.5 ± 1.0[B]
	380	31.7 ± 0.3[B]	20.3 ± 2.2[CD]	18.9 ± 1.6[C]
All C_4	180	36.2 ± 0.5[C]	25.7 ± 2.8[D]	24.9 ± 2.1[D]
	380	38.7 ± 1.2[C]	32.6 ± 3.0[D]	31.4 ± 2.8[D]

Plants were measured near their growth CO_2 concentration (180 or 380 μmol mol⁻¹)

Species values are means ± SE of three individuals

Different letters indicate significant differences at $P < 0.05$ among measurements within a column

See Supplemental Table S2 for the complete species dataset

Alternanthera, there was no significant difference in PWUE between the C_3–C_4 species and the C_3 species. C_4 species exhibited significantly greater PNUE than C_3 and C_3–C_4 species at low and current CO_2 concentrations. There were no significant PNUE differences between C_3 and C_3–C_4 species at the current atmospheric CO_2 level. At low

Fig. 4 The response of net CO_2 assimilation rate to leaf temperature for C_3, C_3–C_4 and C_4 species of *Alternanthera*, *Flaveria*, and *Heliotropium*. Plants were measured near their growth CO_2 concentration: 180 μmol mol^{-1} (**a**, **c**, **e**) and 380 μmol mol^{-1} (**b**, **d**, **f**). Results from *Alternanthera* species are presented in (**a**, **b**), *Flaveria* species in (**c**, **d**), and *Heliotropium* species (**e**, **f**)
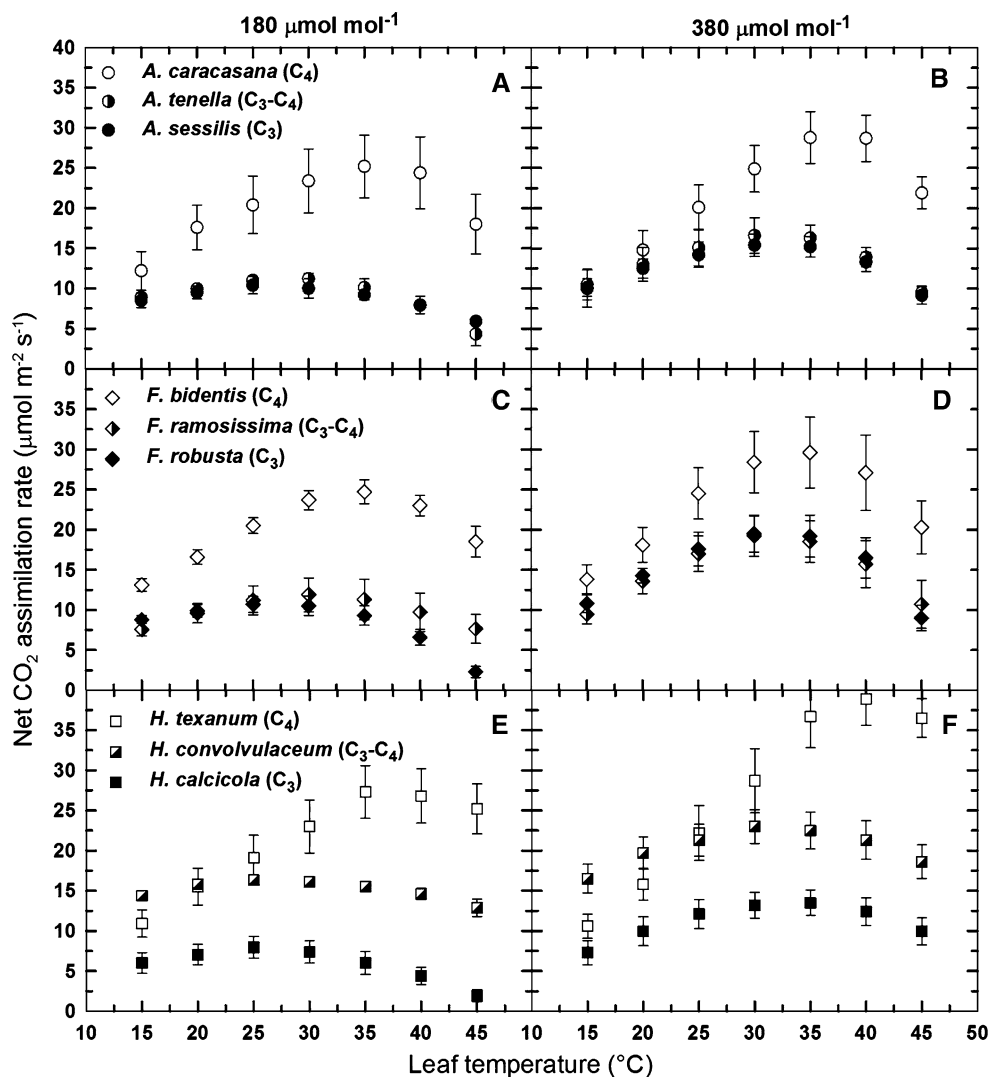
growth CO_2, C_3–C_4 PNUE was 63% greater than C_3 PNUE. Because leaf N, chlorophyll and Rubisco N content were not different between these two functional types, the difference in PNUE resulted from greater *A* in the C_3–C_4 than C_3 species at 180 μmol mol^{-1}.

Discussion

Acclimation responses to low CO_2

Hypothesized acclimation responses to low CO_2 in C_3 plants include increased Rubisco content and reduced investment in electron transport capacity (Sage and Reid 1992; Sage and Coleman 2001). Such responses have been observed sporadically in C_3 plants; for example, *Abutilon theophrasti* and *Solanum dimidiatum* increase Rubisco content relative to electron transport capacity in low growth CO_2 (Tissue et al. 1995; Anderson et al. 2001). None of the

species in this study, however, showed significant photosynthetic acclimation to low CO_2. There was no increase in Rubisco content or apparent change in electron transport investment in any species grown at 180 μmol mol^{-1} CO_2 relative to 380 μmol mol^{-1}. We hypothesized that, if changes in leaf *N* allocation did occur, they might be attenuated in C_3–C_4 relative to C_3 species. This hypothesis was not supported as there was no significant change in *N* allocation in any species of any functional type when grown at low CO_2. This lack of plasticity could have constrained the ability of C_3 plants to adjust to periods of low atmospheric CO_2 in order to ameliorate carbon starvation. As a result, evolutionary selection for CO_2 scavenging mechanisms may have been enhanced. For example, re-fixation of photorespired CO_2 within the bundle-sheath may have improved carbon balance of some C_3 species sufficiently to initiate the evolution of C_3–C_4 intermediacy and, subsequently, C_4 photosynthesis (Monson and Rawsthorne 2000; Muhaidat et al. 2011).

Fig. 5 The responses of stomatal conductance (*open symbols*) and C_i/C_a ratio (*filled symbols*) to variation in intercellular CO_2 concentration at 30°C (**a**, **c**) or 40°C (**b**, **d**) for three *Alternanthera* species grown near current CO_2 conditions (380 μmol mol^{-1} (**a**, **b**) or low CO_2 conditions (180 μmol mol^{-1} (**c**, **d**). Measurement conditions were the same as indicated in Fig. 1. Each *symbol* represents the mean ± SE of three measurements from separate plants
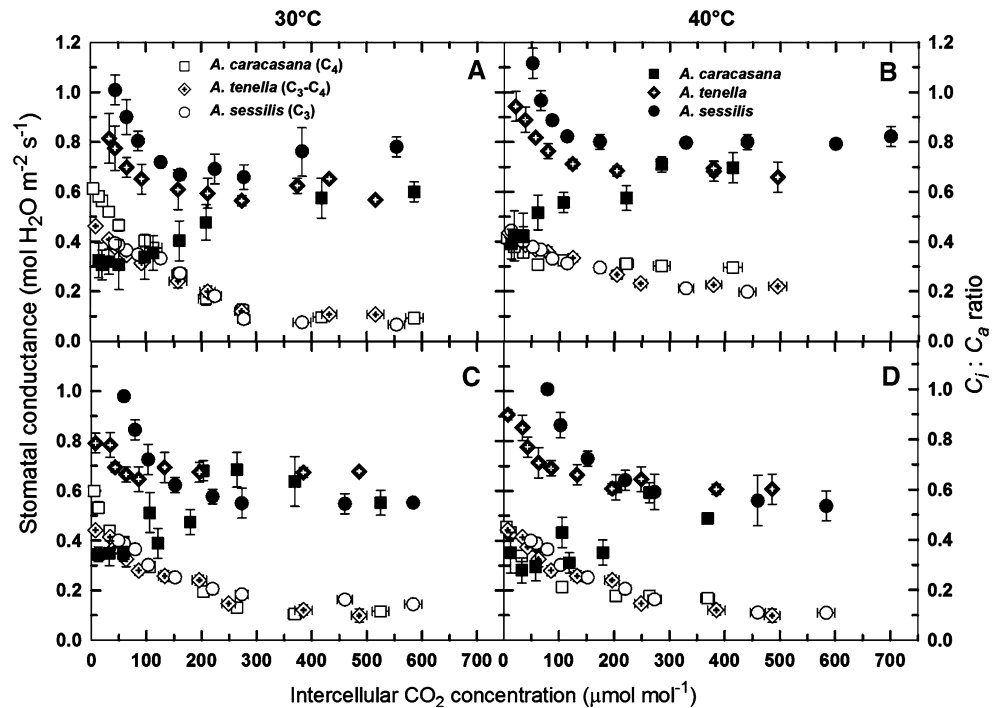

Fig. 6 The responses of stomatal conductance (*open symbols*) and C_i/C_a ratio (*filled symbols*) to variation in intercellular CO_2 concentration at 30°C (**a**, **c**) or 40°C (**b**, **d**) for three *Flaveria* species grown near current CO_2 conditions (380 μmol mol^{-1} (**a**, **b**) or low CO_2 conditions (180 μmol mol^{-1} (**c**, **d**). Measurement conditions were the same as indicated in Fig. 1. Each *symbol* represents the mean ± SE of three measurements from separate plants
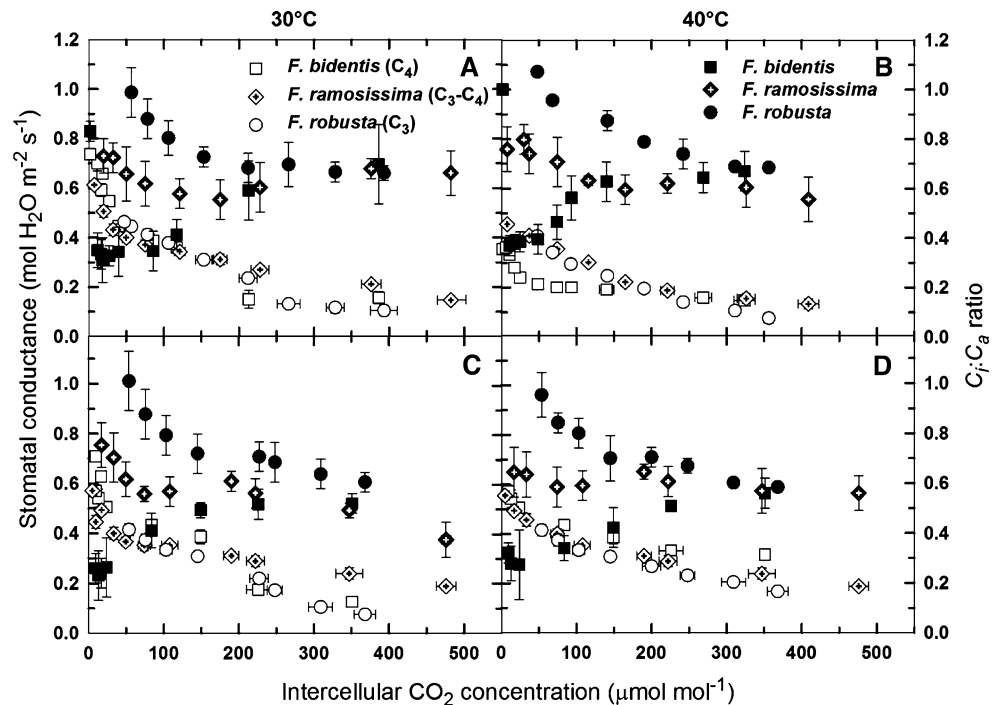
Our results also demonstrate a low ability of stomata to compensate for CO_2 deficiency. The responses of g versus C_i and C_i/C_a versus C_i were measured as an index of stomatal acclimation to low CO_2. A common acclimation response to high growth CO_2 is a reduction in g at high measurement CO_2 and reduced sensitivity of stomata to variation in C_i (Santrucek and Sage 1996; Tognetti et al. 2000; Lodge et al. 2001; Herrick et al.

2004). Stomata can acclimate to low CO_2 by increasing g and the sensitivity to CO_2 variation, as has been shown for *Solanum dimidiatum* (Maherali et al. 2002). This increases C_i and thus partially compensates for low CO_2. Such a response was not observed in any species in this experiment, as the g versus C_i responses generally overlapped between the plants grown at low- and present-day levels of CO_2. Correspondingly, the response of C_i/C_a

Fig. 7 The responses of stomatal conductance (*open symbols*) and C_i/C_a ratio (*filled symbols*) to variation in intercellular CO_2 concentration at 30°C **a**, **c** or 40°C (**b**, **d**) for three *Heliotropium* species grown near current CO_2 conditions (380 μmol mol^{-1} (**a**, **b**) or low CO_2 conditions (180 μmol mol^{-1} (**c**, **d**). Measurement conditions were the same as indicated in Fig. 1. Each *symbol* represents the mean ± SE of three measurements from separate plants
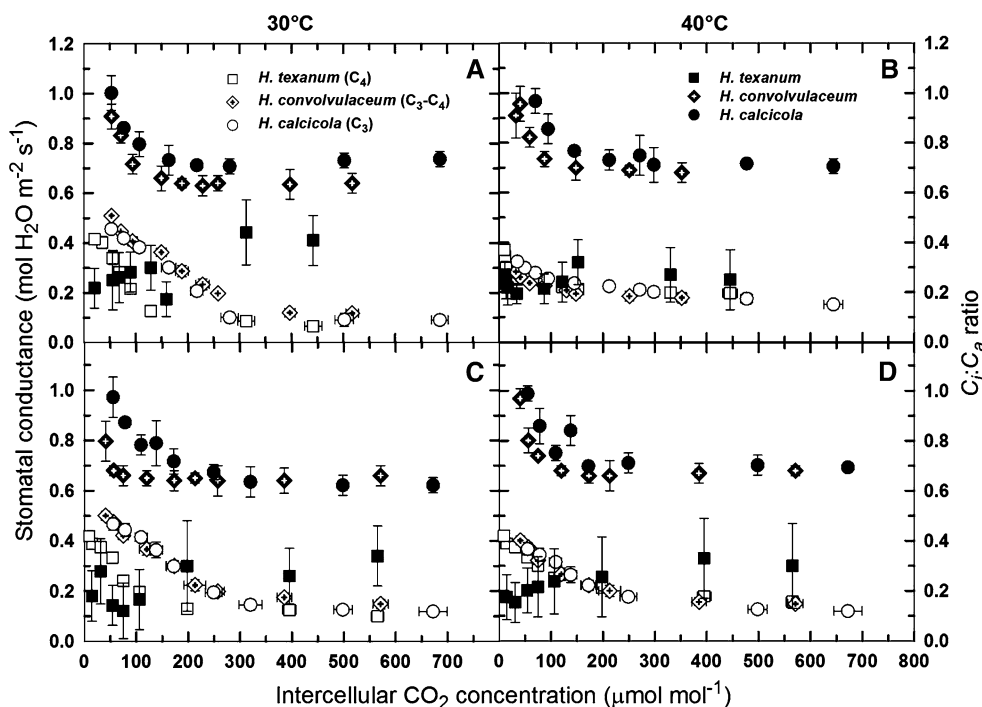

Table 3 Photosynthetic water-use and nitrogen-use efficiencies (PWUE and PNUE, respectively) of *Flaveria*, *Alternanthera* and *Heliotropium* species at cuvette CO_2 concentrations ([CO_2]) of 180 and 380 μmol mol^{-1}

Species	Type	[CO_2]	PWUE (μmol CO_2 mol^{-1} H_2O)	PNUE (μmol CO_2 mmol^{-1} N s^{-1})
F. robusta	C$_3$	180	102 ± 20[A]	0.030 ± 0.007[A]
		380	264 ± 87[AB]	0.088 ± 0.010[C]
A. sessilis	C$_3$	180	101 ± 37[A]	0.038 ± 0.006[A]
		380	199 ± 42[B]	0.091 ± 0.014[C]
H. calcicola	C$_3$	180	70 ± 34[A]	0.036 ± 0.008[A]
		380	142 ± 36[AB]	0.102 ± 0.003[C]
All C$_3$		180	**91 ± 28[A]**	**0.035 ± 0.007[A]**
		380	**202 ± 51[B]**	**0.094 ± 0.009[C]**
F. ramosissima	C$_3$–C$_4$	180	189 ± 73[A]	0.065 ± 0.009[B]
		380	244 ± 46[AB]	0.105 ± 0.019[C]
A. tenella	C$_3$–C$_4$	180	116 ± 23[A]	0.055 ± 0.012[B]
		380	235 ± 12[B]	0.081 ± 0.009[C]
H. convolvulaceum	C$_3$–C$_4$	180	200 ± 50[AB]	0.050 ± 0.009[B]
		380	253 ± 33[B]	0.157 ± 0.034[CD]
All C$_3$–C$_4$		180	**168 ± 48[AB]**	**0.057 ± 0.010[B]**
		380	**244 ± 32[B]**	**0.114 ± 0.021[C]**
F. bidentis	C$_4$	180	470 ± 145[C]	0.182 ± 0.018[D]
		380	517 ± 110[C]	0.174 ± 0.016[D]
A. caracasana	C$_4$	180	393 ± 54[C]	0.167 ± 0.018[D]
		380	451 ± 66[C]	0.198 ± 0.007[D]
H. texanum	C$_4$	180	418 ± 50[C]	0.234 ± 0.073[E]
		380	552 ± 115[C]	0.240 ± 0.022[E]
All C$_4$		180	**427 ± 78[C]**	**0.194 ± 0.039[DE]**
		380	**507 ± 98[C]**	**0.204 ± 0.015[DE]**

All values are taken at a leaf temperature of 40°C and are means ± SE of three individuals per species

PWUE was calculated as the ratio of (A/g_s) · VPD at a VPD of 2–2.7 kPa

PNUE was calculated as the ratio of A to leaf N content

Different letters indicate significant differences at $P < 0.05$ among measurements within a column

ratio to C_i did not differ between plants grown at 180 or 380 μmol mol^{-1}. The responses of C_i/C_a to intercellular CO_2 concentrations were similar between C_3 and C_3–C_4 species above 300 μmol mol^{-1}, but at sub-ambient CO_2 concentrations, C_i/C_a was significantly lower in the C_3–C_4 species. The absence of differences between the two photosynthetic types in the g versus C_i responses indicate that the lower C_i/C_a values of the C_3–C_4 species are driven by inherently greater A at low CO_2 rather than differences in stomatal acclimation. In C_4 species, the greater A at low CO_2 enabled by the C_4 cycle prevented any rise in C_i/C_a at low measurement CO_2. This difference in the C_i/C_a response between C_4, C_3 and C_3–C_4 plants demonstrates the assimilatory strength of the C_4 metabolic cycle in CO_2-depleted atmospheres (Huxman and Monson 2003; Vogan and Sage 2011).

Carbon balance of photosynthetic types under low CO_2 and high temperature

While the A/C_i responses of all nine species were unaffected by growth at low relative to current CO_2 levels, there are substantial differences between the A/C_i responses of the three photosynthetic types that impact carbon balance. The primary differences were lower Γ and 40% (at 30°C) and 67% (at 40°C) greater A at 180 μmol mol^{-1} in the C_3–C_4 species compared to the C_3 species. There was also a trend towards greater PWUE in the C_3–C_4 species *F. ramosissima* and *H. convolvulaceum,* and greater PNUE in the C_3–C_4 intermediates, relative to their respective C_3 relatives at low but not current levels of atmospheric CO_2. These differences are likely due to the enhanced capacity of the C_3–C_4 species to recapture photorespired CO_2 (Ku et al. 1983, 1991; Rajendrudu et al. 1986; Vogan et al. 2007). The A/T responses of the different photosynthetic types further illustrate the benefits of the C_3–C_4 pathway to photosynthetic performance at low CO_2 and high temperature. While T_{OPT} and A at T_{OPT} are not significantly different between C_3 and C_3–C_4 species at ambient CO_2, the reduction of CO_2 to 180 μmol mol^{-1} resulted in a significantly greater decline in T_{OPT} in C_3 species (6.5°C) than in C_3–C_4 species (2.6°C). Greater ability to recapture photorespired CO_2 moderates the effects of lowering CO_2 on T_{OPT}, A at T_{OPT}, and A at the growth temperature of 37°C in the C_3–C_4 species.

The enhanced photosynthetic performance of C_3–C_4 intermediates under conditions of low CO_2 and high temperature are important given the contribution of A to growth and fitness. Reductions in A at sub-ambient CO_2 resulted in equivalent reductions in biomass yield in *Abutilon theophrasti*, *Avena sativa*, *Brassica kaber*, *Nicotiana tabacum,* *Phaseolus vulgaris*, and *Prosopis glandulosa* (Polley et al. 1992; Johnson et al. 1993; Dippery et al. 1995; Cowling

and Sage 1998; Ward et al. 1999; Campbell et al. 2005). The amount of vegetative biomass has a large influence on fruit number and seed set, and reductions in plant growth often result in substantially lower plant fitness (Solbrig 1981; Farris and Lechowicz 1990). Consistently, reproductive output is substantially reduced by low CO_2 (Sage 1995; Campbell et al. 2005). Several studies have also evaluated fitness of plants with mutations that reduce photosynthetic rate. For example, a 25–30% reduction in A in single-gene mutants of *Amaranthus hybridus* lowers seed number, seed size and seedling survival at light levels above 400 μmol photons m^{-2} s^{-1} (Arntz et al. 2000a, b). *Arabidopsis thaliana* mutants with reduced chlorophyll content and 15% lower A have a 15% lower growth rate and 25% lower seed weight than wild-type plants (Janacek et al. 2009).

Reduced fitness in C_3 species during the low CO_2 conditions of the past 25 million years likely created opportunities for novel mechanisms of carbon scavenging. At the atmospheric CO_2 concentrations of the late-Pleistocene (180–280 μmol mol^{-1}), for example, photorespiration in hot climates would have reduced A by over 50% (Ehleringer et al. 1991). While initially inhibitory, high rates of photorespiration create an opportunity to improve Rubisco efficiency and A if the release of photorespired CO_2 occurs within an inner compartment where Rubisco is present (Monson and Rawsthorne 2000). In C_3–C_4 species, photorespired CO_2 is produced in the inner region of the bundle sheath cells, resulting in observed 67% enhancements of A relative to C_3 species. In hot environments during low CO_2 intervals, as would have been present on subtropical sand dunes where *H. convolvulaceum* grows, the ability to recapture photorespired CO_2 using the C_3–C_4 mechanism could have maintained fitness in habitats too extreme for C_3 species. Such extreme settings may have thus enabled natural experiments in CO_2 scavenging, such as C_3–C_4 intermediacy, to proceed without interference from C_3 competitors (Sage 2004).

Conclusion

The results of this study show that improved carbon economy is the primary benefit of C_3–C_4 intermediacy under low CO_2 and high temperature. There was no acclimation to low CO_2 via shifts in leaf N allocation or stomatal behavior in any photosynthetic type. The inherently greater A at high temperature and low CO_2 of the C_3–C_4 intermediates compared to their C_3 relatives stands out as the major difference between these two photosynthetic types. The greater carbon assimilation capacity of C_3–C_4 intermediates under these conditions and their somewhat greater PWUE and PNUE likely provided advantages in growth and

reproduction, and sustained these species in the hot, low latitude environments of recent geological time. In contrast, C_3 species appear to be severely limited in their ability to alleviate CO_2 starvation through photosynthetic or stomatal acclimation. In the past century, CO_2 levels have risen to values not seen for millions of years (Pagani et al. 2005), and, given the similarity in photosynthetic performance between C_3 and C_3–C_4 species at current atmospheric CO_2 concentrations, the critical environmental stress selecting for C_3–C_4 photosynthesis appears to have already disappeared. As a consequence, the potential for C_4 evolution in the future may have been eliminated.

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