Plant, Cell and Environment (2007) **30**, 53–66 doi: 10.1111/j.1365-3040.2006.01605.x

High temperature acclimation of C_4 photosynthesis is linked to changes in photosynthetic biochemistry

SIMON A. DWYER[1,2], OULA GHANNOUM[1,3], ADRIENNE NICOTRA[2] & SUSANNE VON CAEMMERER[1]

[1]Molecular Plant Physiology Group, Research School of Biological Sciences and [2]School of Botany and Zoology, Australian National University, GPO Box 475, Canberra, ACT 2601, and [3]Centre for Plant and Food Science, University of Western Sydney, Locked Bag 1797, South Penrith DC, NSW 1797, Australia

ABSTRACT

With average global temperatures predicted to increase over the next century, it is important to understand the extent and mechanisms of C_4 photosynthetic acclimation to modest increases in growth temperature. To this end, we compared the photosynthetic responses of two C_4 grasses (*Panicum coloratum* and *Cenchrus ciliaris*) and one C_4 dicot (*Flaveria bidentis*) to growth at moderate (25/20 °C, day/night) or high (35/30 °C, day/night) temperatures. In all three C_4 species, CO_2 assimilation rates (A) underwent significant thermal acclimation, such that when compared at growth temperatures, A increased less than what would be expected given the strong response of A to short-term changes in leaf temperature. Thermal photosynthetic acclimation was further manifested by an increase in the temperature optima of A, and a decrease in leaf nitrogen content and leaf mass per area in the high- relative to the moderate-temperature-grown plants. Reduced photosynthetic capacity at the higher growth temperature was underpinned by selective changes in photosynthetic components. Plants grown at the higher temperature had lower amounts of ribulose-1,5-bisphosphate carboxylase/oxygenase and cytochrome *f* and activity of carbonic anhydrase. The activities of photosystem II (PSII) and phosphenolpyruvate carboxylase were not affected by growth temperature. Chlorophyll fluorescence measurements of *F. bidentis* showed a corresponding decrease in the quantum yield of PSII (Φ_{PSII}) and an increase in non-photochemical quenching (Φ_{NPQ}). It is concluded that through these biochemical changes, C_4 plants maintain the balance between the various photosynthetic components at each growth temperature, despite the differing temperature dependence of each process. As such, at higher temperatures photosynthetic nitrogen use efficiency increases more than A. Our results suggest C_4 plants will show only modest changes in photosynthetic rates in response to changes in growth temperature, such as those expected within or between seasons, or the warming anticipated as a result of global climate change.

Correspondence: Simon A. Dwyer. Fax: +61 2 6125 5075; e-mail: simon.dwyer@anu.edu.au

Key-words: carbonic anhydrase; chlorophyll fluorescence; nitrogen; Rubisco; stomatal conductance; temperature.

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the first and rate-limiting enzyme of the CO_2 fixation cycle of photosynthesis, reacts with both CO_2 (carboxylation) and O_2 (oxygenation). The oxygenation reaction of Rubisco (photorespiration) is often wasteful, consuming chemical energy, taking up catalytic sites, contributing to inhibitory compounds and resulting in net carbon loss (Osmond 1981; Jordan & Ogren 1984). The ratio of oxygenation to carboxylation increases with temperature as the CO_2/O_2 specificity of Rubisco decreases, offsetting carbon gains from increased enzyme activity (Jordan & Ogren 1984). C_4 photosynthesis overcomes the problem of photorespiration at high temperature by way of a biochemical 'pump' which concentrates CO_2 into a specialized compartment (normally bundle sheath cells) where Rubisco is exclusively located (Hatch 1987). Through this combination of biochemical and anatomical modifications in the leaf, Rubisco can fix CO_2 at close to its saturated rate (V_{cmax}), which increases exponentially with temperature (Hatch 1987; von Caemmerer & Quick 2000; Kubien *et al*. 2003). Hence, C_4 plants have higher CO_2 assimilation rates (A) at high temperatures and higher photosynthetic temperature optima (T_{opt}) than their C_3 counterparts (Berry & Björkman 1980).

These physiological properties are reflected in the geographic distribution of the C_4 pathway, which is positively correlated with growing season temperature (Hattersley 1983; Ehleringer, Cerling & Helliker 1997). C_4 grasses dominate many warm and high-light environments such as the Australian rangelands and the North American tallgrass prairies (Hattersley 1983; Ehleringer *et al*. 1997; Knapp & Medina 1999). The high productivity achieved by C_4 plants under warm conditions leads to an agricultural and ecological importance that is disproportionately high relative to their small taxonomic representation (Holn *et al*. 1977; Ehleringer *et al*. 1997; Brown 1999). While the adaptation of C_4 plants to warm environments is well established and understood, it is not clear how, and to what extent, C_4 photosynthesis acclimates to changes in growth temperature.

In contrast, the acclimation of C_3 photosynthesis to growth temperature has been well studied (Berry & Björkman 1980; Quinn & Williams 1985), and the field is undergoing a resurgence of interest as our awareness of global warming increases. C_3 plants grown at high temperature tend to have higher T_{opt} and less photosynthetic inhibition at very high temperatures compared to counterparts grown at low temperature (Yamasaki *et al*. 2002; Haldimann & Feller 2005; Yamori, Noguchi & Terashima 2005). This is related to changes in several factors, such as the temperature dependence of chloroplast electron transport (Quinn & Williams 1985; Yamasaki *et al*. 2002), the activation state of Rubisco which is mediated by Rubisco activase (Crafts-Brandner & Salvucci 2000; Law, Crafts-Brandner & Salvucci 2001; Salvucci & Crafts-Brandner 2004) and possibly in the properties of Rubisco itself (Yamori *et al*. 2005).

The situation is different for C_4 photosynthesis, where Rubisco operates at a higher rate, and where photosynthesis is complicated by the presence of two photosynthetic cycles (C_3 and C_4) and two photosynthetic cell types (bundle sheath and mesophyll; Furbank, Hatch & Jenkins 2000). The matter is further complicated by the three main biochemical subtypes that are recognized (although even these do not fully encompass the biochemical diversity of C_4 photosynthesis). These subtypes are grouped according to the main C_4 acid decarboxylation pathway in the bundle sheath: NAD-ME (NAD malic enzyme), NADP-ME (NADP malic enzyme) and PCK (phosphoenol pyruvate carboxykinase; Hatch, Kagawa & Craig 1975; Kanai & Edwards 1999). The C_4 subtypes also possess subtly different anatomy and physiology, which may influence temperature acclimation.

Very few studies have examined the temperature acclimation of C_4 photosynthesis, and most of these have compared plants grown at very low and very high temperatures to assess the extremes of physiological tolerance (Björkman *et al*. 1972; Pearcy 1977; Björkman, Badger & Armond 1980). Others were mostly interested in the performance of C_4 photosynthesis at low temperature (Pietrini & Massacci 1998; Kubien & Sage 2004a,b; Naidu & Long 2004). Little or no work has been done comparing the acclimation of C_4 photosynthesis to growth temperatures which are more reflective of the variations that plants are most likely to experience within or between growing seasons. An understanding of such responses is essential for predictions of how agricultural and wild C_4 populations will respond to climate variations such as those predicted to occur with global climate change (Intergovernmental Panel on Climate Change 2001).

This study was carried out to investigate the response of C_4 photosynthesis in one NAD-ME grass (*Panicum coloratum*), one NADP-ME grass (*Cenchrus ciliaris*) and one NADP-ME dicot (*Flaveria bidentis*) species to growth at moderate and high temperatures. The two chosen grass species represent the taxonomically and ecologically most common C_4 subtypes. *F. bidentis* is used as a model organism for molecular and genetic engineering studies. The main aims of this study were to investigate the extent to which C_4 photosynthesis may acclimate in response to moderate changes in growth temperatures, and to gauge the diversity of the response between subtypes and between functional groups (monocots and dicots). The study also aimed at elucidating the underlying mechanisms of the response of C_4 photosynthesis to growth temperature. To these ends, the three species were grown at either moderate- or high-temperature regimes. Subsequently, leaf gas exchange, chlorophyll fluorescence and several biochemical parameters were measured. Through this combination of biochemical and physiological techniques, we show that C_4 plants tend to adjust photosynthetic capacity in such a way that A differs to a much smaller extent than what would be predicted by the strong temperature dependence of C_4 photosynthesis. This is achieved through a reallocation of leaf nitrogen between the photosynthetic components involved in light capture, electron transport, and the C_3 and C_4 cycles.

MATERIALS AND METHODS

Plant culture

The three C_4 species, *P. coloratum*, *C. ciliaris* (both members of the Paniceae, widely introduced as pasture species to Australia) and *F. bidentis* (Asteraceae, commonly used as a model organism for molecular studies) were grown in a controlled environment, walk-in growth cabinets (Phoenix Research, Edwardstown, SA, Australia) under either a high temperature (35/30 °C day/night) or moderate temperature (25/20 °C day/night) regimes. All other growth conditions were matched. Photoperiod was 10 h, relative humidity 70%, and light intensity at the leaf level was 550 μmol quanta m^{-2} s^{-1}. Plants were grown from seed in a sterilized, general-purpose potting mix in 5 L pots, and supplemented with a slow-release fertilizer (Osmocote Plus; Scotts, Baulkham Hills, NSW, Australia). All plants were initially germinated under high temperature before being moved to the appropriate cabinet shortly after germination. Throughout growth, the plants were supplied with adequate water and fertilizer. Plants were used for measurements between 8 and 10 weeks after sowing. For the two grasses, physiological and biochemical measurements were made in the middle part of the most recently fully expanded leaf of the main tiller. For *F. bidentis*, measurements were made on one of the most recently expanded leaf pair, on either side of the main vein.

Leaf gas exchange and chlorophyll *a* fluorescence measurements

The temperature response of leaf gas exchange was measured concurrently with chlorophyll *a* fluorescence using a Li-Cor 6400 open gas-exchange system with an attached pulse amplitude modulated fluorometer (LI-6400-40; Li-Cor, Lincoln, NE, USA) at leaf temperatures between 25 and 42 °C. Measurements were taken at an inlet CO_2 partial pressure (p_a) of 665 μbar and an actinic irradiance of 2000 μmol quanta m^{-2} s^{-1} (10% from blue LEDs). Plants were dark adapted overnight and the maximum quantum

yield of photosystem II (PSII) (F_v/F_m) measured at 25 °C (Genty, Briantais & Baker 1989). Plants were then light adapted for 45 min to obtain steady-state *A*. The temperature response of *A* and chlorophyll fluorescence was measured by beginning at 25 °C and raising the leaf temperature by 3 °C steps to a maximum of between 40 and 42 °C. Each step increase in leaf temperature was achieved in 5–10 min, and then leaves were allowed another 5 min to reach steady state. Measurements between 25 and 31 °C were made with the gas exchange system at room temperature. To achieve higher leaf temperatures, the system was moved inside the 35/30 °C growth cabinet. This move resulted in a slight but consistent increase in *A* but had no effect on statistical analyses of the data. Incoming air was humidified by adding water to the CO_2 scrub canister of the gas exchange system; however, relative humidity could not be kept constant as temperatures were increased. At each leaf temperature, the standard gas exchange parameters were logged along with the steady-state fluorescence (F_s) and maximum fluorescence (F'_m) signals for the calculation of the quantum yield of PSII (Φ_{PSII}; Genty *et al*. 1989) and the quantum yield of non-photochemical quenching (Φ_{NPQ}; Hendrickson, Furbank & Chow 2004):

$$\Phi_{NPQ} = \frac{F_s}{F'_m} - \frac{F_s}{F_m}. \quad (1)$$

Φ_{NPQ} is a parameter related to the more commonly used NPQ, representing xanthophyll and ΔpH-regulated heat dissipation, the major variable components of heat dissipation (Hendrickson *et al*. 2004; Kramer *et al*. 2004). Φ_{NPQ} accounts for the proportion of quanta absorbed by PSII that is used in these energy-dissipating processes, and thus represents quantum efficiency in an analogous way to Φ_{PSII}. The T_{opt} for each plant was calculated by fitting a cubic function through the temperature response data (r^2 values were all between 0.97 and 0.99) and determining the turning point.

The LI-6400 detects the fluorescence signal at 710 nm to avoid conflict with the red-measuring LEDs. However, this wavelength may permit some interference from fluorescence emanating from PSI (Pfündel 1998; Kramer *et al*. 2004). We used a Walz fluorometer (PAM-101; Walz, Effeltrich, Germany) with a blue-measuring beam and two detection wavelengths (660–710 nm and greater than 710 nm) to estimate PSI interference according to an equation adapted from Pfündel (1998: 189):

$$\begin{aligned}\frac{F_v}{F_m} &= \frac{F_m - F_0}{F_m} \\ &= \frac{(F_m^{PSII} + F_{PSI}) - (F_0^{PSII} + F_{PSI})}{F_m^{PSII} + F_{PSI}} \\ &= \frac{F_m^{PSII} - F_0^{PSII}}{F_m^{PSII} + F_{PSI}}.\end{aligned} \quad (2)$$

This interference was assumed stable between light and dark measurements, and was accounted for in all fluorescence calculations.

The saturating flash delivered by the red LEDs of the LI-6400-40 system was not truly saturating for the F'_m signal in the C_4 plants, despite its intensity (~ 8000 μmol quanta m^{-2} s^{-1}). Therefore, F'_m was extrapolated by using the multiple-flash option included in the latest edition of the LI-6400 software (Open v5.3) to calculate Φ_{PSII} and Φ_{NPQ} for *F. bidentis* (similar to the method described in Earl & Ennahli 2004). Fluorescence parameters calculated from the multiple-flash method will be referred to, unless otherwise noted.

To estimate dark (mitochondrial) respiration (R_d), gas exchange measurements were repeated on different plants in the dark to determine the temperature response of R_d. This measured response was fitted to an Arrhenius function relating R_d to leaf temperature, activation energy (E_a) and R_d at 25 °C [R_d(25 °C); von Caemmerer 2000]. To estimate R_d during gas exchange measurements in the light, R_d(25 °C) was measured for each plant at the beginning of gas exchange measurements and the E_a fitted to dark measurements then used to estimate R_d as a function of temperature. Estimated R_d was used for calculation of the quantum requirement for CO_2 fixation, Φ_{CO_2}:

$$\Phi_{CO_2} = \frac{A + R_d}{I\alpha_{leaf}}, \quad (3)$$

where *I* is the incident irradiance and α_{leaf} the absorptance of the leaf to irradiance of a given quality. Leaf absorptance to the red and blue LED light source of the LI-6400 was determined on several plants from each condition using a Li-Cor 1800 spectroradiometer with integrating sphere. All plants had α_{leaf} of approximately 0.9, and this was chosen as the generic value used in all calculations of Φ_{CO_2}.

Biochemical analysis

Carbonic anhydrase (CA) activity was determined by the exchange of ^{18}O from $^{13}C^{18}O_2$ to $H_2{}^{16}O$ measured by mass spectrometry (Badger & Price 1989; Jenkins, Furbank & Hatch 1989; von Caemmerer *et al*. 2004). For phosphoenolpyruvate carboxylase (PEP-C) activity, leaf discs (~ 1 cm^2) were ground on ice in 600 μL extraction buffer (100 mM Hepes-KOH, pH 7.4, 5 mM DTT, 0.1% BSA, 0.05% Triton ×100, 2 mM EDTA, 5 mM $MgCl_2$, 0.1% BSA, 1% PVPP) with 24 μL protease inhibitor cocktail (P-9599; Sigma, St. Louis, MO, USA) and the homogenate centrifuged at 16.1 *g* for 30 s. Twenty microliters leaf extract was added to a cuvette containing an assay buffer (50 mM EPPS-OH, pH 8.0, 2 mM EDTA, 18 mM $MgCl_2$, 0.2 mM NADH, 5 mM glucose-6-phosphate, 1 mM $NaHCO_3$ and 12 units malate dehydrogenase), and the carboxylase reaction initiated with 4 mM PEP. The rate of consumption of NADH was determined by the absorptance change at 340 nm (assuming an optical density change of 0.00622 nmol $NADH^{-1}$ mL^{-1}). Leaf Rubisco content was determined similarly to the method described in Ruuska *et al*. (1998), which utilizes a tight-binding radioactively labelled inhibitor of Rubisco catalytic sites ([^{14}C]CABP). The amount of functional PSII centres in fresh leaf sections

was quantified by measuring O_2 evolution in response to very short flashes of light according to the method of Chow, Hope & Anderson (1989, 1991). Following the measurements, leaf samples were frozen in liquid N_2 and stored at –80 °C for later chlorophyll determination according to Porra, Thompson & Kriedemann (1989).

Total leaf nitrogen and nitrogen budget

Leaf sections were measured, dried at 80 °C then weighed to determine the leaf mass per area (LMA). Sections were then ground to a fine powder and % nitrogen (N) per dry mass was determined using a CHN analyser (Model EA 1110; Carlo Erba Instruments, Milan, Italy). Leaf N was converted to a leaf area basis using the mean values for LMA. Nitrogen bound in photosynthetic components was calculated by assuming that 16% per mass of protein complexes is N, and that the molecular mass of Rubisco is 550 000 (eight catalytic sites per molecule), PSII reaction centres is 417 000 and cytochrome b_6f complexes is 194 000 (assuming a 1:1 ratio of cytochrome *f*: b_6f complex) (Terashima & Evans 1988; Hikosaka & Terashima 1995; Ghannoum *et al*. 2005). For PEP-C, it was assumed that an activity of 1 μmol m^{-2} s^{-1} equates to 2.4 mg m^{-2} of the protein (Leegood & von Caemmerer 1988). Nitrogen in chlorophyll was calculated assuming four N atoms per chlorophyll (Tanaka & Tanaka 2006).

Cytochrome *f* measurement and thylakoid nitrogen

A concentrated (2–4 mM chlorophyll) crude thylakoid extract from 30 to 45 leaves was prepared as described in Ghannoum *et al*. (2005). Thylakoid extracts were diluted to a chlorophyll concentration of approximately 170 nmol mL^{-1} in a solubilizing buffer [50 mM $NaPO_4$ (pH 6.5), 5 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$ and 0.33 M sorbitol and 1% (v/v) Triton]. The spectra of the hydroquinol-reduced solution, referenced to ferricyanide-oxidized solution, was measured using a dual-beam spectrophotometer (model 557; Perkin Elmer, Foster City, CA, USA). Cytochrome *f* (cyt *f*) concentration was calculated according to Bendall, Davenport & Hill (1971).

Thylakoid N was determined as described in Ghannoum *et al*. (2005), where pure thylakoids were isolated by centrifugation in 30% Percol in buffer [50 mM $NaPO_4$ (pH 6.5), 5 mM $MgCl_2$, 2 mM EDTA and 0.33 M sorbitol]. Subsamples of known chlorophyll content were dried in tin cups at 80 °C and analysed for %N.

Statistical analyses

Two-way analyses of variance (ANOVA) were conducted with species (three levels) and growth temperature (two levels) as the factors. Interactions and species main effects were investigated using Fisher's least significant difference test. The acceptable probability of a type I error (*P*) was set at 5% for all tests.

RESULTS

Temperature response of gas exchange parameters

The response of *A* to leaf temperature followed the same trend for the three C_4 species at both growth temperatures. Between leaf temperatures of 25 °C and T_{opt}, *A* increased by 28–32% and 51–71% for 25 °C- and 35 °C-grown plants, respectively (Fig. 1). Above the T_{opt}, *A* declined to a greater extent in the 25 °C-grown relative to the 35 °C-grown grasses, *P. coloratum* and *C. ciliaris*. In contrast, *A* declined little above the T_{opt} in the dicot, *F. bidentis*, irrespective of growth temperature (Fig. 1). The T_{opt} for *A* increased with growth temperature in all three species, though this increase was minor in *F. bidentis* (Table 1). At both 25 °C and T_{opt}, *A* was lower in 35 °C- compared to 25 °C-grown plants for all three C_4 species (Fig. 1 & Table 1). Despite this reduction in photosynthetic capacity, when measured at growth temperature, *A* was higher in 35 °C-compared to 25 °C-grown plants (Fig. 1 circles & Table 1). This difference was small in *C. ciliaris* (< 1%), greater in *P. coloratum* (13%) and relatively large in *F. bidentis* (21%). All species showed increasing measured R_d with leaf temperature. In *P. coloratum* and *F. bidentis*, growth temperature had no effect on R_d at any leaf temperature. In *C. ciliaris*, R_d was higher in 25 °C-grown plants compared to their 35 °C-grown counterparts (Fig. 1 & Table 1).

Stomatal conductance (g_s) increased with leaf temperature for all three C_4 species, with *F. bidentis* showing the strongest response of g_s to leaf temperature (Fig. 2a). In *F. bidentis*, g_s tended to be lower (when compared at 25 °C) in 35 °C-grown than 25 °C-grown plants, but no difference was apparent for either of the grasses. Leaf-air vapour pressure difference (VPD_L) increased with leaf temperature despite efforts to control it by increasing humidity inside the gas exchange chamber (Fig. 2b). The combined increase in g_s and VPD_L led to a large increase in transpiration rates (not shown). The resultant cooling effect limited the maximal leaf temperature achievable during gas exchange measurements, particularly in *F. bidentis*. Intercellular CO_2 partial pressure (p_i, Fig. 2c) fluctuated with leaf temperature but, despite the large increases in VPD_L, remained above levels that were CO_2-saturating for the three C_4 species (i.e. where *A* is limited by Rubisco or electron transport), as determined by the CO_2 response curves (not shown). This was the result of the increasing g_s and the relatively high measurement p_a.

Chlorophyll *a* fluorescence

After an overnight dark adaptation period, no difference was apparent in F_v/F_m between growth temperatures for the two grasses. For *F. bidentis*, F_v/F_m was higher in 25 °C-grown relative to 35 °C-grown plants (Table 1).

Detailed analysis of the temperature response of chlorophyll fluorescence characteristics was undertaken for *F. bidentis*. Figure 3 shows fluorescence parameters calculated from both the F'_m extrapolated from the multiple flash

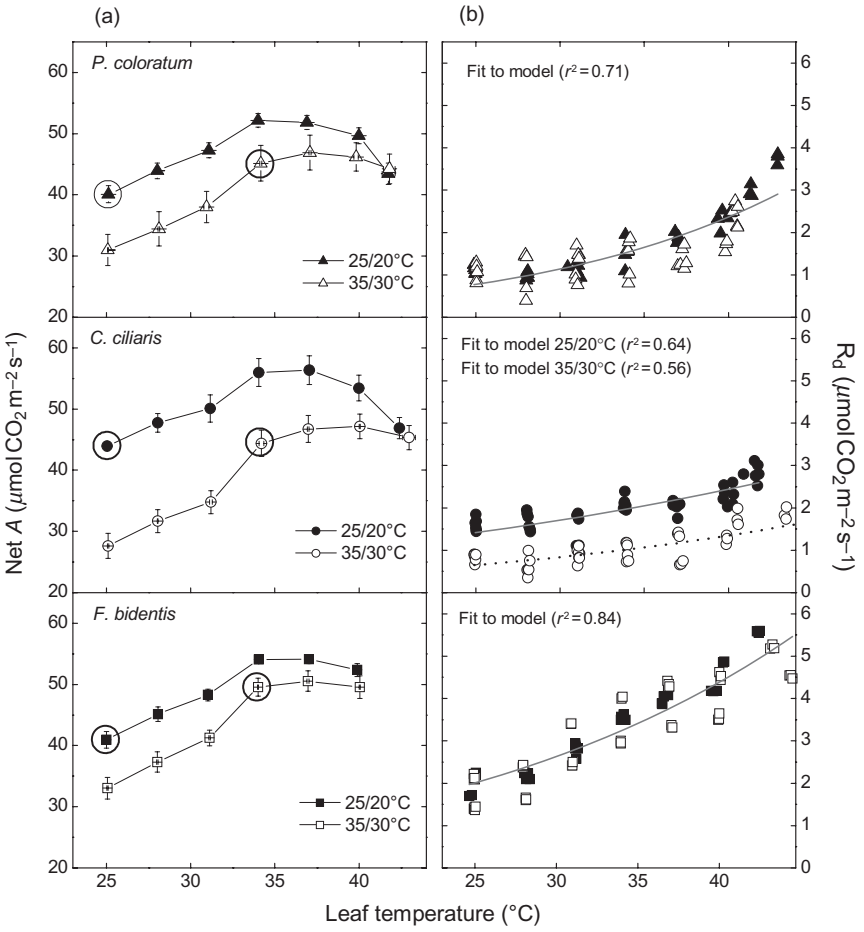

Figure 1. (a) Net CO_2 assimilation rate (A) and (b) measured dark respiration rate (R_d), for *Panicum coloratum*, *Cenchrus ciliaris* and *Flaveria bidentis* for plants grown at 25/20 °C (solid symbols) and 35/30 °C (hollow symbols). For A, values are the means ± SE of four to five different plants. Circles indicate A at growth temperatures. Note the Y-axes in (a) do not start from zero. In (b), two plants from each condition were measured. Lines are a fit for the activation energy (E_a) of the process and R_d (25 °C) to a modified Arrhenius function (von Caemmerer 2000; Eq. 2.32). Fitted values are given in Table 1. Gas exchange measurements were taken at a p_a of 665 μbar and an irradiance of 2000 μmol m^{-2} s^{-1}. p_i remained saturating throughout measurements despite the increase in leaf-air vapour pressure difference (VPD_L).

method and the F'_m actually achieved (i.e. by the standard method). Compared to the standard method, the multiple-flash method gave higher Φ_{PSII} and Φ_{PSII}/Φ_{CO_2} and lower Φ_{NPQ}. The single-flash method led to a flatter shape for Φ_{PSII} and Φ_{NPQ} as a function of leaf temperature (Fig. 3). That is, the difference between the two methods increased with leaf temperature, suggesting that fluorescence saturation was becoming harder to achieve. While the multiple-flash method gave sensible values, the calculated ratio of Φ_{PSII}/Φ_{CO2} for the single flash-method fell below the theoretical minimum (Fig. 3).

According to the multiple-flash data, with increasing leaf temperature Φ_{PSII} increased while Φ_{NPQ} decreased in *F. bidentis*. Φ_{PSII} was higher and Φ_{NPQ} lower in 25 °C-grown compared to 35 °C-grown plants. Neither parameters was different when compared at growth temperature (Fig. 3 & Table 1). The ratio Φ_{PSII}/Φ_{CO_2} was relatively stable with increasing leaf temperature and the small differences between growth temperatures were not significant (Fig. 3 & Table 1).

Biochemical analysis

The amount of Rubisco catalytic sites per unit leaf area was significantly lower at the higher growth temperature for all species by 23–27% (Table 2). The activity of CA was also lower in the 35 °C- compared to the 25 °C-grown plants by an average of 23% in *P. coloratum*, 54% in *C. ciliaris* and 18% in *F. bidentis* (Table 2). In contrast, the activity of PEP-C was not affected by growth temperature in any of the three C_4 species (Table 2).

Total chlorophyll content on an area basis did not change significantly with growth temperature (Table 2). For all species, the proportion of chl *a* to total chlorophyll was lower, while the proportion of chl *b* to total chl was higher at 35 °C relative to 25 °C growth (Table 2). This led to a decrease in the chl *a*/*b* ratio at the higher growth temperature. On a leaf area or chlorophyll basis, the amount of functional PSII centres was not affected by growth temperature in any of the species. Cytochrome *f* measurements were not replicated due to the large number of leaves (35–40) used to prepare a highly concentrated thylakoid sample for the assay. Cytochrome *f* was reduced at 35 °C relative to 25 °C growth temperature by 47% in *P. coloratum* and 21% in *C. ciliaris*, while it was reduced by a small extent (7%) in *F. bidentis* (Table 2).

Leaf nitrogen and mass per area

The leaves of all three C_4 species had 9–10% lower LMA at high relative to moderate growth temperature (Table 2).

Table 1. Leaf gas exchange and fluorescence parameters for three C_4 species grown at two different temperature regimes. Values are the means ± SE for four to five replicates, except where noted. Different letters indicate significant differences *within* species, according to a two-way analysis of variance and Fisher's least significant difference tests ($P < 0.05$)

Parameter/Growth temperature	*Panicum coloratum*		*Cenchrus ciliaris*		*Flaveria bidentis*	
	25/20 °C	35/30 °C	25/20 °C	35/30 °C	25/20 °C	35/30 °C
T_{opt} for A (°C)	36.1 ± 1.0a	38.1 ± 1.0b	36.5 ± 1.0a	39.7 ± 1.0b	36.2 ± 1.0a	37.2 ± 1.0b
A at growth temperature (μmol CO_2 m^{-2} s^{-1})	40.1 ± 1.4a	45.2 ± 2.9b	44.0 ± 0.8a	44.4 ± 2.1b	41.0 ± 1.8a	49.6 ± 1.5b
A at 25 °C (μmol CO_2 m^{-2} s^{-1})	40.1 ± 1.4a	31.0 ± 2.6b	44.0 ± 0.8a	27.6 ± 2.0b	41.0 ± 1.8a	33.1 ± 1.8b
Maximum A (μmol CO_2 m^{-2} s^{-1})	52.3 ± 1.1a	46.9 ± 2.9b	56.5 ± 2.3a	47.3 ± 2.0b	54.4 ± 0.7a	50.6 ± 1.7b
Inhibition of A at max. temperature (% of max)	16.9 ± 2.5a	6.1 ± 0.4b	17.0 ± 0.3a	4.0 ± 1.2b	3.9 ± 1.42a	2.1 ± 0.6a
g_s at 25 °C (mol H_2O m^{-2} s^{-1})	0.18 ± 0.02a	0.13 ± 0.01a	0.16 ± 0.01a	0.12 ± 0.01a	0.31 ± 0.02a	0.17 ± 0.04b
A at 25 °C/g_s at 25 °C (μmol CO_2 mol^{-1} H_2O)	225 ± 12a	237 ± 16a	278 ± 12a	230 ± 17a	135 ± 6a	225 ± 37b
p_i/p_a at growth temperature	0.35 ± 0.02a	0.30 ± 0.09a	0.33 ± 0.03a	0.27 ± 0.02a	0.65 ± 0.01a	0.61 ± 0.03a
F_v/F_m	0.779 ± 0.004a	0.768 ± 0.005a	0.790 ± 0.002a	0.796 ± 0.002a	0.790 ± 0.002a	0.767 ± 0.002b
Φ_{PSII} at growth temperature[a]	–	–	–	–	0.37 ± 0.01a	0.40 ± 0.01a
Φ_{NPQ} at growth temperature[a]	–	–	–	–	0.39 ± 0.01a	0.36 ± 0.01a
Φ_{PSII}/Φ_{CO_2} at 25 °C[a]	–	–	–	–	13.4 ± 0.2a	12.5 ± 0.5a
E_a for dark respiration (kJ mol^{-1})[b]	57.8 ± 4.4		28.2 ± 3.4	37.4 ± 5.4	40.3 ± 2.1	
Measured R_d (25 °C) (μmol CO_2 m^{-2} s^{-1})[b]	0.77 ± 0.06		1.42 ± 0.08	0.65 ± 0.06	2.01 ± 0.07	

[a]*F. bidentis* only.
[b]Based on two plants from each condition.
T_{opt}, temperature optima; A, CO_2 assimilation rate; g_s, stomatal conductance; p_i, intercellular CO_2 partial pressure; p_a, inlet CO_2 partial pressure; F_v/F_m, maximum quantum yield of photosystem II (PSII); Φ_{PSII}, quantum yield of PSII; Φ_{NPQ}, quantum yield of non-photochemical quenching; E_a, activation energy; R_d, dark respiration rate.

On an area basis, leaf N content decreased substantially (16–27%) at the higher growth temperature in all three species (Table 2). On a dry mass basis, leaf N content was significantly lower in all species at the higher growth temperature, although this reduction was only marginal in *P. coloratum* (2%) compared to the other two species (15 and 9% in *C. ciliaris* and *F. bidentis*, respectively). This indicates that the reduction in leaf N content was mainly due to the reduction in LMA in *P. coloratum*, but not in *C. ciliaris* and *F. bidentis* (Table 2). When compared at growth temperature, the ratio of A/N was between 38 and 46% higher in 35 °C- compared to 25 °C-grown plants for all three C_4 species. When compared at a common (25 °C) leaf temperature, the A/N ratio was between 2 and 14% higher for 25 °C-grown plants (Table 3).

Growth temperature may have selectively affected photosynthetic components independently of the whole leaf changes in leaf N. Accordingly, we calculated the percentage contribution of measured photosynthetic components to total leaf N (Table 3). The N allocation to Chl, PSII and PEP-C was increased at the higher growth temperature. The N investment in Rubisco was maintained between the two growth temperatures while the allocation to the cytochrome b_6f complex decreased with high-temperature growth in *P. coloratum*, but did not change in the other two species (Table 3). This variation indicates that the decline of leaf N with increased growth temperature was not confined to the important photosynthetic components measured, and is more likely generalized across the many biochemical leaf processes.

DISCUSSION

Acclimation of C_4 photosynthesis to growth temperature

C_4 photosynthesis is generally considered less plastic than C_3 photosynthesis due to the constraints of regulating an additional biochemical cycle, two cell types and the rigid positioning of chloroplasts within bundle sheath cells (von Caemmerer & Furbank 2003; Kubien *et al*. 2003; Sage & McKown 2006). However, this study has demonstrated considerable potential for acclimation in a dicot and two monocots, representing both NAD- and NADP-ME subtype C_4 plants. C_4 plants grown at moderate and high temperatures underwent significant photosynthetic thermal acclimation such that, despite a considerable reduction in photosynthetic capacity, high-temperature-grown plants had higher A when compared at growth temperatures. The magnitude of the increase in A at growth temperature differed considerably between the three species, being relatively slight in the two grasses. This was accompanied by an increase in T_{opt} at the higher growth temperature. The thermal acclimation of A is particularly striking considering

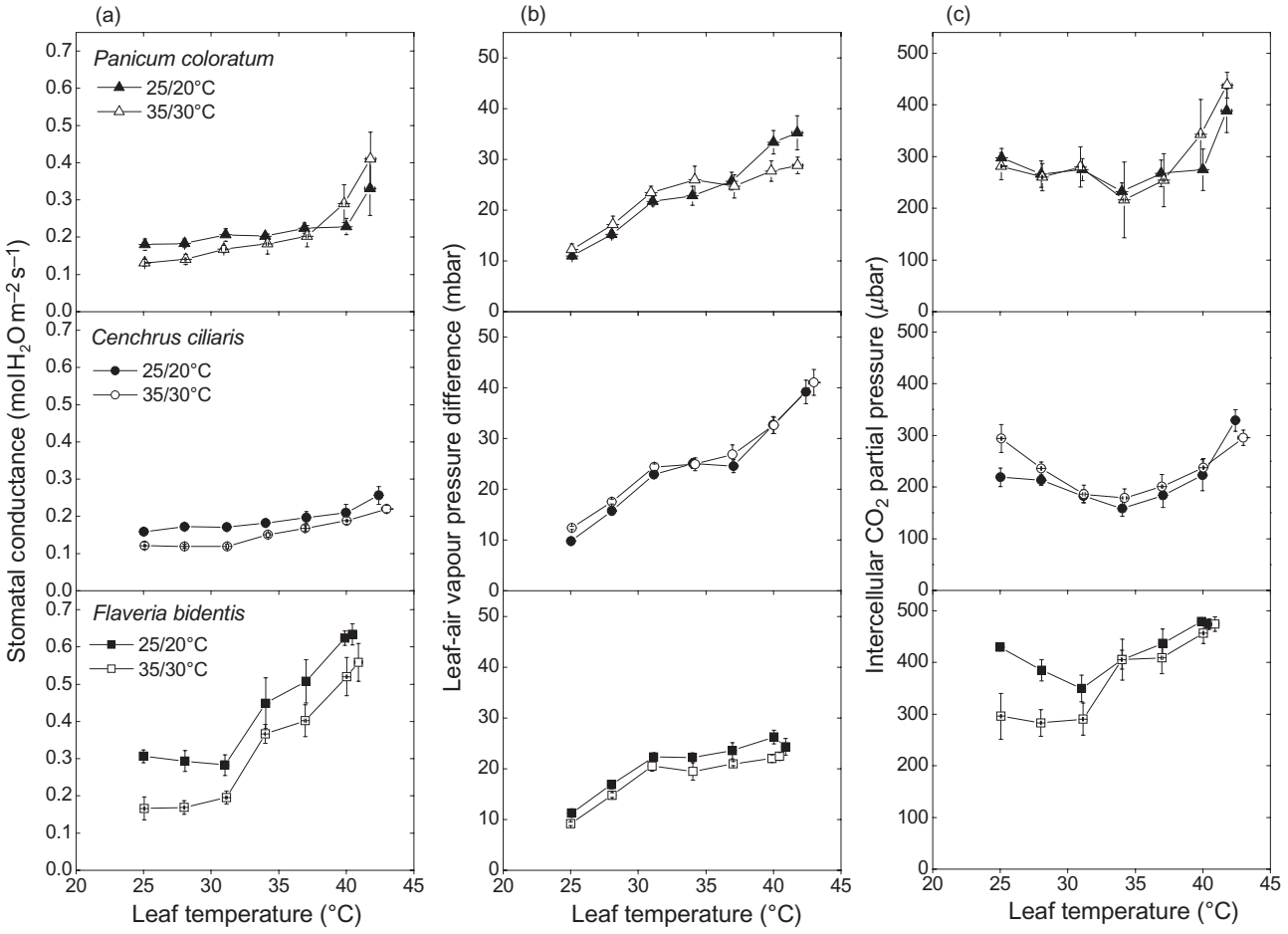

Figure 2. (a) Stomatal conductance to H_2O, (b) leaf-air vapour pressure difference and (c) calculated intercellular CO_2 partial pressure measured as a function of leaf temperature for all species and growth temperatures. Gas exchange measurements were taken at p_a of 665 μbar and an irradiance of 2000 μmol m^{-2} s^{-1}.

the relatively moderate difference between the two temperature regimes imposed on the plants. The results suggest that thermal acclimation of *A* occurs in response to the usual temperature fluctuations encountered by C_4 plants during the course of a growing season (Pearcy *et al*. 1974), between growing seasons, or longer term variation due to climate change.

Over the coming century, we expect atmospheric CO_2 concentrations and average air temperatures to increase, alongside many other environmental consequences of climate change (such as in seasonality, rainfall timing and distribution, humidity and light; Karl & Trenberth 2003). In the short term, when the CO_2 concentration around a C_3 plant is increased, faster rates of photosynthesis result, due to greater rates of carboxylation and decreased rates of oxygenation by Rubisco (Farquhar, von Caemmerer & Berry 1980). This diminishes the relative photosynthetic advantage of the CO_2 concentrating mechanism of C_4 plants (Collatz, Berry & Clark 1998). However, when C_3 plants are grown at raised CO_2, they commonly do not exhibit increases in photosynthetic rates of the magnitude that short-term measurements would predict. Instead, the tendency for a C_3 leaf is to reduce Rubisco content and electron transport capacity so that photosynthetic rates are relatively similar between plants grown at ambient and raised CO_2 (a response often referred to as down-regulation; Drake, Gonzalez-Meler & Long 1997; Ainsworth & Long 2005). A large part of the advantage of increased CO_2 to a C_3 plant instead comes through increased nitrogen use efficiency (because less photosynthetic protein is required for a similar photosynthetic rate) and increased water use efficiency (due to the lower g_s that may be required; Nowak, Ellsworth & Smith 2004).

Warmer temperatures increase the catalytic rate at which Rubisco works. In C_3 plants, the increase in catalytic rate is offset by a decrease in the ratio of carboxylation to oxygenation (Jordan & Ogren 1984). Rubisco in C_4 plants is almost CO_2 saturated, so the increase in catalytic rate at higher temperatures is reflected in the rate at which Rubisco assimilates CO_2 (von Caemmerer & Quick 2000; Kubien *et al*. 2003). This leads to the very strong temperature dependence of photosynthesis in C_4 plants, which is demonstrated by the 30–60% increase in net carbon assimilation as leaf temperatures were raised from 25 to 35 °C

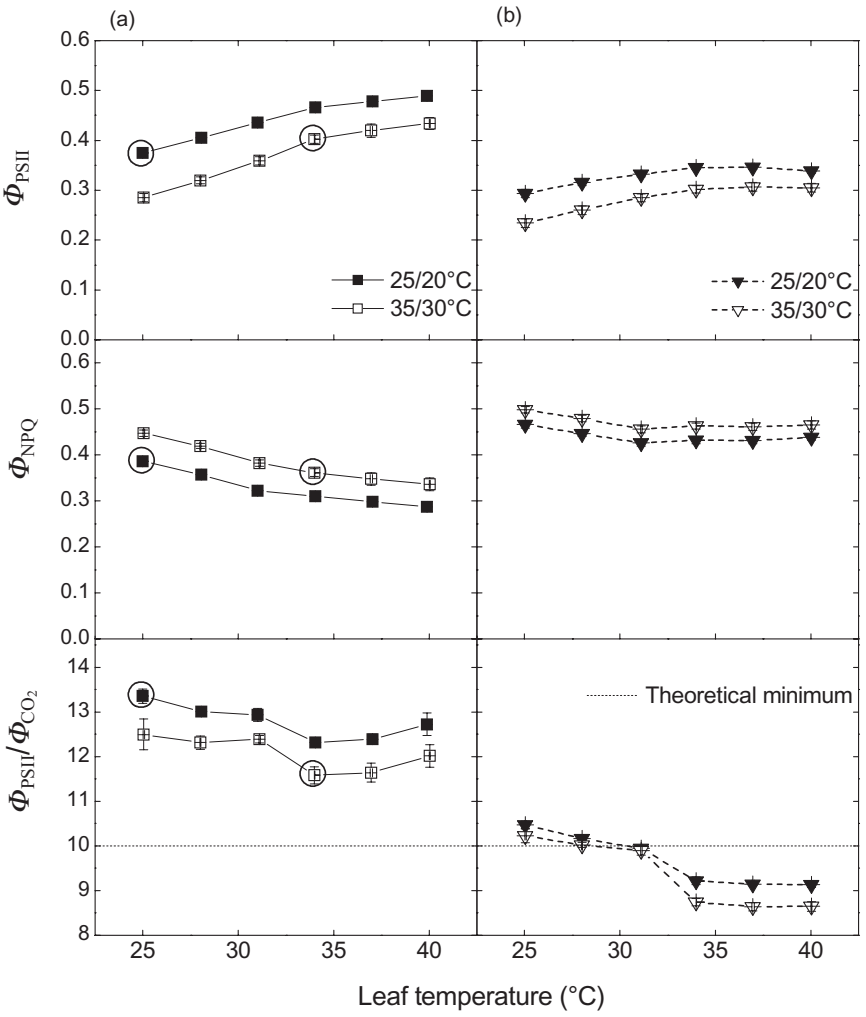

Figure 3. Fluorescence parameters: quantum yield of photosystem II (Φ_{PSII}), quantum yield of non-photochemical quenching (Φ_{NPQ}) and the ratio of Φ_{PSII} and quantum requirement for CO_2 fixation (Φ_{PSII}/Φ_{CO_2}) for *Flaveria bidentis*, determined by the (a) multiple-flash method or (b) single-flash method. Values are the means ± SE of four replicates for each temperature regime. Note the Φ_{PSII}/Φ_{CO_2} scale does not start from zero. F_m (for calculation of Φ_{NPQ}) was determined from plants dark adapted overnight. If 50% of absorbed irradiance is being used by each of PSI and PSII, then Φ_{PSII}/Φ_{CO_2} gives the quanta used in photochemistry per CO_2 fixed. Units are not given on the figure to avoid making assumptions about the proportions of irradiance absorbed by each photosystem. Encircled symbols indicate growth temperatures.

Table 2. Leaf biochemical parameters for three C_4 species grown at two different temperature regimes. Values are the means ± SE of between three and five replicates, unless otherwise stated. Different letters indicate significant differences *within* species, according to a two-way analysis of variance and Fisher's least significant difference tests ($P < 0.05$)

Parameter/Growth temperature	*Panicum coloratum*		*Cenchrus ciliaris*		*Flaveria bidentis*	
	25/20 °C	35/30 °C	25/20 °C	35/30 °C	25/20 °C	35/30 °C
Rubisco (μmol m^{-2})	12.2 ± 1.1a	8.89 ± 0.82b	6.80 ± 0.22a	5.17 ±0.18b	14.7 ± 0.4a	11.3 ± 0.4b
CA (μmol CO_2 m^{-2} s^{-1})	1156 ± 62a	885 ± 15b	843 ± 21a	387 ± 72b	1492 ± 17a	1219 ± 43b
PEP-C (μmol CO_2 m^{-2} s^{-1})	71.3 ± 9.5a	82.3 ± 8.3a	168 ± 21a	132 ± 4a	231 ± 5a ($n = 2$)	210a ($n = 1$)
Chl $a + b$ (μmol m^{-2})	372 ± 6a	347 ± 28a	319 ± 7a	300 ± 26a	499 ± 38a	533 ± 51a
Chl a / $a + b$	0.832 ± 0.003a	0.818 ± 0.003b	0.850 ± 0.000a	0.834 ± 0.003b	0.834 ± 0.003a	0.830 ± 0.006b
Chl b / $a + b$	0.168 ± 0.004a	0.182 ± 0.004b	0.150 ± 0.005a	0.166 ± 0.004b	0.166 ± 0.004a	0.166 ± 0.004b
PSII (μmol m^{-2})	1.22 ± 0.02	1.17 ± 0.06	1.15 ± 0.05	1.20 ± 0.03	1.24 ± 0.02	1.28 ± 0.02
Cyt f (μmol m^{-2})	[b]1.13 ± 0.12	0.597 ± 0.039	0.639 ± 0.040	0.505 ± 0.018	0.869 ± 0.060	0.808 ± 0.039
Cyt f / Chl (mmol mol^{-1})	[b]3.04 ± 0.33	1.72 ± 0.11	2.00 ± 0.13	1.68 ± 0.06	1.74 ± 0.12	1.52 ± 0.07
LMA (g m^{-2})	45.6 ± 1.4a	37.1 ± 1.0b	32.4 ± 1.4a	27.7 ± 0.8b	40.2 ± 1.0a	37.0 ± 0.4b
Leaf N (mmol m^{-2})	163 ± 10a	130 ± 5b	111 ± 4a	81.1 ± 3.7b	183 ± 8a	153 ± 7b
Leaf N/mass (mol g^{-1})	3.58 ± 0.23a	3.50 ± 0.13b	3.43 ± 0.13a	2.93 ± 0.13b	4.54 ± 0.20a	4.13 ± 0.20b
Thylakoid N (mmol m^{-2})[a]	17.4	25.3	21.2	15.6	30.4	32.1

[a]Mean from one or two replicates only, hence no SE or statistical analysis.
[b]SE refers to assay error. No statistics were conducted because no measure of within treatment variation was available.
Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; CA, carbonic anhydrase; PEP-C, phosphoenolpyrurate carboxylase; PSII, photosystem II; LMA, leaf mass per area.

Table 3. The ratio of CO_2 assimilation rates (A) to leaf N and the fraction of total leaf N invested in measured photosynthetic components for three C_4 species grown at two temperatures. Values are means ± SE. Different letters indicate significant differences *within* species, according to a two-way analysis of variance and Fisher's least significant difference tests ($P < 0.05$)

Parameter/Growth temperature	*Panicum coloratum*		*Cenchrus ciliaris*		*Flaveria bidentis*	
	25/20 °C	35/30 °C	25/20 °C	35/30 °C	25/20 °C	35/30 °C
$A_{(\text{growth temperature})}$/N [mmol CO_2 (mol N)$^{-1}$ s^{-1}]	0.252 ± 0.019a	0.362 ± 0.019b	0.409 ± 0.016a	0.563 ± 0.016b	0.237 ± 0.016a	0.347 ± 0.016b
$A_{(25\ °C)}$/N [mmol CO_2 (mol N)$^{-1}$ s^{-1}]	0.252 ± 0.015a	0.246 ± 0.015b	0.409 ± 0.013a	0.351 ± 0.013b	0.237 ± 0.013a	0.231 ± 0.013b
Rubisco (%)	5.93 ± 0.37a	5.40 ± 0.20a	4.82 ± 0.18a	5.03 ± 0.23a	6.36 ± 0.27a	5.85 ± 0.26a
PEP-C (%)	1.21 ± 0.17a	1.75 ± 0.17b	4.16 ± 0.15a	4.49 ± 0.15b	3.49 ± 0.15a	3.79 ± 0.15b
Chl (%)	0.919 ± 0.057a	1.08 ± 0.04b	1.15 ± 0.04a	1.49 ± 0.07b	1.10 ± 0.05a	1.40 ± 0.06b
PSII (%)	3.58 ± 0.22a	4.31 ± 0.16b	4.94 ± 0.18a	7.09 ± 0.32b	3.25 ± 0.14a	4.01 ± 0.18b
Cyt b_6f complex (%)	0.777 ± 0.048a	0.515 ± 0.019b	0.642 ± 0.024a	0.698 ± 0.032a	0.533 ± 0.022a	0.593 ± 0.026a

Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; PSII, photosystem II.

(Fig. 1). This physiological response has led to predictions that C_4 plants will be advantaged by increased temperatures through increases in A (Long 1991; Sage & Kubien 2003). Our results cast doubt over these predictions, and instead show that C_4 plants grown at increased temperatures reduce photosynthetic capacity in such a way that photosynthetic rates did not increase nearly as much as might be expected, despite changes in enzymatic activity. This acclimation was clearly demonstrated in two typical C_4 grasses with differing biochemical subtypes. The dicot *F. bidentis*, showed the same response, but to a far lesser extent. This intimates that this type of response may be a generalizable C_4 phenomenon, the degree of which is determined by a plant's ecology or functional type. Our results suggest that (similarly to the C_3 response to increased CO_2) C_4 plants will be advantaged by warmer temperatures through slightly higher assimilation rates, but also through more efficient enzyme use, and hence nitrogen use.

Chlorophyll fluorescence measurements

The maximum quantum yield of chlorophyll fluorescence (F_v/F_m) is commonly used as an indicator of photoinhibition or stress in plants (Maxwell & Johnson 2000). No difference was found in F_v/F_m between growth temperatures in the grasses, suggesting that 35 °C plants were no more stressed than 25 °C plants. In contrast, F_v/F_m was lower for *F. bidentis* grown at the higher temperature which may be indicative of some stress from growth at high temperatures. Nevertheless, these F_v/F_m values were not particularly low compared to *F. bidentis* plants measured previously, suggesting that any photoinhibition was minor (Pfündel 1998). The difference in photosynthetic capacity between growth temperatures was much smaller in *F. bidentis* compared to the two grasses, which suggests F_v/F_m is disconnected from the reduced photosynthetic capacity observed at high temperatures.

Fluorescence parameters calculated from single flashes using the LI-6400-40 system have previously been shown to be non-saturating for PSII fluorescence yield in C_4 plants (Earl & Ennahli 2004), and this was verified during this study. The fluorescence parameters calculated from single flashes in this study were low relative to A compared to previous findings using different fluorescence set-ups (Oberhuber & Edwards 1993; Kubien *et al*. 2003). From a modelling perspective, too, the values obtained from single flashes are unreasonably low. If we assume that half of the irradiance absorbed by the leaf is being used in PSII and that Φ_{PSII} is directly proportional to chloroplast electron transport, then the ratio Φ_{PSII}/Φ_{CO_2} can be interpreted quantitatively as the quanta required per CO_2 fixed. This quantitative interpretation needs to be viewed with considerable caution due to the ambiguities of fluorescence; however, we will take it into account in order to relate the parameter to the predicted energetic requirements of C_4 photosynthesis (von Caemmerer & Furbank 1999). The theoretical minimum quantum requirement for CO_2 fixation in C_4 photosynthesis (without any leakiness of CO_2 from the bundle sheath), assuming 5 ATP and 2 quanta per ATP, is 10 quanta per CO_2 fixed (Edwards & Baker 1993; von Caemmerer & Furbank 1999). The single-flash method yields Φ_{PSII}/Φ_{CO2} values that are at or below this theoretical minimum (Fig. 3). When leakiness of CO_2 from the bundle sheath is taken into account, this theoretical minimum is increased, making the single-flash values even more improbable (Farquhar 1983; Henderson *et al*. 1994; Hatch, Agostino & Jenkins 1995). Multiple-flash Φ_{PSII}/Φ_{CO_2} values were higher, which could account for inefficiencies from leakiness and reflected values obtained elsewhere for C_4 plants (Oberhuber & Edwards 1993; Kubien *et al*. 2003). The difficulty in achieving fluorescence saturation for measurements with the LI-6400 system appears not to be a problem in C_3 plants, but rather a phenomenon confined to C_4 plants. C_4 plants tend to have lower quantum yields and typically higher light saturation points than C_3 plants, which could make saturation more difficult to achieve, but even so, it is surprising that a flash of the intensity generated by the LI-6400-40 (~ 8000 μmol quanta m^{-2} s^{-1}) is not saturating. Given the availability of the multiple-flash option in the latest LI-6400-40 software, and its ease of use, we recommend that this option be used in fluorescence

measurements of C_4 plants, and other plants where fluorescence saturation is difficult to achieve.

Reallocation of photosynthetic nitrogen in C_4 plants grown at different temperatures

The thermal acclimation of *A* observed in this study was coupled with reductions in LMA, leaf N content and specific photosynthetic proteins at the higher compared to the moderate growth temperatures. In *P. coloratum*, the reduction in leaf N content per area was predominantly accounted for by the reduction in LMA at the higher growth temperature. In the other two C_4 species, *C. ciliaris* and *F. bidentis*, the reduction in leaf N was only partially accounted for by the changes in LMA. However, in all three C_4 species, the extent of reductions in photosynthetic proteins at the higher growth temperature differed between processes, indicating that thermal acclimation of *A* is not simply driven by changes in leaf thickness or density (i.e. LMA), but also by the response of the photosynthetic apparatus to changes in growth temperature. For example, the amount of functional PSII centres and the activity of PEP-C were not affected by growth temperature, while the amount of Rubisco and cyt *f* and the activity of CA were greatly reduced at the higher growth temperature. Consequently, the proportion of total leaf N allocated to measured photosynthetic components was altered at 35 °C- compared to 25 °C-grown plants, with some components accounting for a greater proportion of leaf N (chlorophyll, PSII, PEP-C), the electron transport component cyt b_6f accounting for less, and Rubisco the same (Table 3). These selective changes could reflect that the activities of Rubisco, CA and Cyt *f* found in 25 °C-grown leaves may be in excess of those needed at 35 °C; the same may not apply for the activities of PSII and PEP-C. The reallocation of N could refect the different temperature dependence of photosynthetic processes. Rubisco, for example, is close to CO_2 saturated in C_4 photosynthesis and therefore its potential rate of carboxylation (V_{cmax}, modelled in Fig. 4) will have a temperature dependence that reflects the increase in maximum catalytic rate (k_{cat}; Furbank & Hatch 1987; von Caemmerer 2000; Kubien *et al*. 2003). In contrast, PEP-C operates at subsaturating HCO_3^- concentrations, so increases in its k_{cat} are not fully reflected in the activity of this enzyme. Thus, the relative abundance of the two enzymes must be altered in order to maintain the balance between movement of CO_2 into the bundle sheath (via PEP-C) and its fixation there by Rubisco. Further evidence for this 'balancing' of different photosynthetic processes according to their differing temperature sensitivity comes from chlorophyll fluorescence measured in *F. bidentis* (Fig. 3). At a common leaf temperature (25 °C), high-temperature-grown plants had higher Φ_{NPQ} and lower Φ_{PSII} indicating an excess of light energy absorbed by PSII. This is consistent with similar light capture capacity (chlorophyll and PSII centres) but reduced downstream electron transport capacity (cyt *f*) and energy consumption (Rubisco) in high-temperature-grown plants. When plants are compared at growth temperatures, however, there is no difference in either Φ_{NPQ} or Φ_{PSII}, suggesting the balance between light capture and downstream processes is similar between growth temperatures and that plants had acclimated in such a way as to maintain this balance.

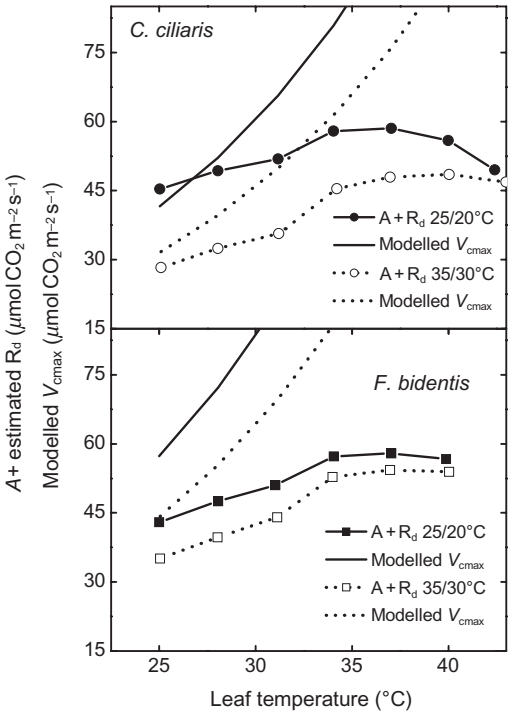

Figure 4. Comparison of the *gross* CO_2 assimilation rate [*A* + estimated dark respiration rate (R_d); lines with symbols] with the modelled maximum ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate (V_{cmax}; lines without symbols). Modelling is shown for *Cenchrus ciliaris* (top) and *Flaveria bidentis* (bottom) for both 25/20 °C (solid lines) and 35/30 °C (dotted lines). Modelled V_{cmax} was calculated by multiplying the measured Rubisco site concentration for each species and condition (Table 2) by the k_{cat} at a given temperature. The temperature dependence of k_{cat} was modelled by a modified Arrhenius function (von Caemmerer 2000; Eq. 2.32), from the k_{cat} at 25 °C for each species (6.1 s^{-1} for *C. ciliaris*, Ghannoum *et al*. 2005; and 3.1 s^{-1} for *F. bidentis*, Kubien *et al*. 2003), assuming an activation energy of 56.1 kJ mol^{-1} (Kubien *et al*. 2003).

Selective changes in photosynthetic proteins may serve to economize the N allocation within the photosynthetic apparatus at each growth temperature. A good indicator of this economization is the change in the ratio of *A*/N (Table 3). When compared at growth temperatures, the *A*/N ratio is consistently higher for the high-temperature-grown plants (Table 3). This occurs in spite of the significant reductions in leaf N and photosynthetic capacity. Therefore, when environmental conditions allow for it, C_4 plants acclimate in such a way as to forsake the opportunity of fixing more CO_2 (at the expense of using more N) for the more conservative option of fixing CO_2 at a slightly higher rate while using less N. In either case, *A*/N improves, however, the latter acclimation strategy places a greater premium on saving N. This

is reminiscent of the well-documented acclimation of C_3 photosynthesis in response to growth at elevated CO_2 concentration (Drake *et al*. 1997; Ainsworth & Long 2005).

The biochemical limits to C_4 photosynthesis at high temperature

At low leaf temperatures, C_4 photosynthesis is most likely limited by Rubisco k_{cat} (Kubien *et al*. 2003; Kubien & Sage 2004b). At warmer leaf temperatures, V_{cmax} predicted from k_{cat} and the Rubisco catalytic site concentration becomes in excess of the realized A (Fig. 4), suggesting other limitations have been imposed. Traditional understanding suggests that above approximately 25 °C, A is limited by either chloroplast electron transport (because V_{cmax} exceeds the maximum rate of electron transport) or by the rate of enzymatic PEP or RuBP regeneration (von Caemmerer & Furbank 1999). More recently, it has been argued that Rubisco becomes deactivated at high temperatures due to the inability of Rubisco's chaperone enzyme, Rubisco activase, to keep pace with the increased rate of inhibitory binding to catalytic sites (Crafts-Brandner & Salvucci 2000, 2002; Salvucci & Crafts-Brandner 2004).

In this study, measurements of A were taken at saturating p_i (i.e. not limited by carboxylation by PEP-C). Changes in NPQ pathways have been used to differentiate between Rubisco and other limitations (e.g. Salvucci & Crafts-Brandner 2004). Our fluorescence measurements show Φ_{NPQ} actually tended to decrease as temperature increased, suggesting that downstream limitations (i.e. by Rubisco activation) decreased as temperature increased (Fig. 3).

For modelled V_{cmax} to mirror estimated *in vivo* V_{cmax} ($A + R_d$), Rubisco activation state would need to decrease from 100% in *C. ciliaris* and about 75% in *F. bidentis* at 25 °C to 40% or less at the maximum leaf temperatures, in a roughly linear fashion (Fig. 4). This is a similar level of deactivation to that reported for several C_3 plants and for the C_4 plant *Zea mays* (Law & Crafts-Brandner 1999; Crafts-Brandner & Salvucci 2002). As Rubisco activation state may regulate downwards to match any electron transport limitation (Ruuska *et al*. 2000), measurements of activation state such as what have been performed on *Z. mays* (Crafts-Brandner & Salvucci 2002), would not disentangle whether electron transport or Rubisco activation is the rate-limiting factor (von Caemmerer 2000; Sharkey 2005). The evidence from this study is not parsimonious with a simple limitation via Rubisco activation state, but nor does it suggest that the traditional view is necessarily true.

Temperature response of stomata in C_4 plants

Stomatal conductance (g_s) increased with leaf temperature for all three species (Fig. 2). Documented responses of g_s to temperature are variable; some species increase g_s with temperature, while others decrease or even show an optimum curve (Hall, Schulze & Lange 1976; Ball, Woodrow & Berry 1987; Sage & Pearcy 1987; Šantrůček & Sage 1996; Lu, Quinones & Zeiger 2000; Kubien *et al*. 2003). Arguably, some of this variation can be explained by variation in VPD_L (Hall *et al*. 1976). Increases in VPD_L are conventionally expected to reduce g_s at a constant temperature (Hall *et al*. 1976), but here we found an increase in g_s coinciding with a very large increase in VPD_L (as temperature increased). It was posited that this was an artefact due to slow stomatal induction, as all temperature responses were measured in the same direction, but by measuring a temperature response curve backwards (i.e. from 42 °C, decreasing to 25 °C), we found g_s decreased again, rejecting this hypothesis. Additional measurements on *F. bidentis* (not shown), where leaf temperature was increased and decreased while maintaining relative humidity, further suggested a direct temperature response. We attempted to relate our data to a well-established model predicting g_s from A, p_i and relative humidity (Ball *et al*. 1987; Collatz, Ribas-Carbo & Berry 1992), however, the model predicted a decline in g_s because the increase in A was insufficient to overcome the decrease in relative humidity. Elsewhere, g_s in *F. bidentis* has been shown to increase between leaf temperatures of 5 and 40 °C, while VPD_L was maintained at a very moderate 12 mbar (Kubien *et al*. 2003). This suggests a temperature effect on g_s independent of VPD_L: a phenomenon that has not been clearly expounded in the literature.

Few studies have focused on long-term stomatal acclimation to increased temperature. *F. bidentis*, which showed the strongest increase in g_s to immediate leaf temperature, showed lower g_s when grown at the higher temperature. Šantrůček & Sage (1996) noted a similar response in a C_3 dicot, *Chenopodium album*, grown at elevated temperature. In the present study, relative humidity was kept constant between growth conditions, so the response cannot be considered a direct adaptation to reduce excessive water loss through transpiration. Variation in g_s has been linked with photosynthetic rates (Wong, Cowan & Farquhar 1979), and possibly the reduction in photosynthetic capacity in *F. bidentis* has led to a reduced capacity for g_s. For both grasses, g_s appeared lower in high-temperature plants, the expected response according to this theory, but in contrast to *F. bidentis* this difference was not significant.

CONCLUSIONS

The three C_4 plants studied here exhibited marked acclimation when grown at 35 °C compared to 25 °C, showing that C_4 plants do exhibit a degree of phenotypic plasticity to certain environmental changes. Growth at the higher temperature was characterized by a reduction in some photosynthetic components, but not others. In this way, the balance between the functioning of the various photosynthetic components was maintained, despite differences in temperature response between them. Photosynthetic rate was thus increased less than what might be predicted at high temperatures, and was effected with a lower nitrogen cost. Our results indicate that in response to increased temperatures, C_4 plants will not simply increase their photosynthetic

rates as has been predicted, but will acclimate by adjusting capacity and reallocating nitrogen resources between photosynthetic components.

ACKNOWLEDGMENTS

During this work S.D. was supported by an ANU Honours Scholarship. The assistance of the following people was indispensable: Asaph Cousins, Wah Soon Chow, John Evans, Vanda Quinn (all from the Research School of Biological Sciences, Australian National University) and Robert Furbank (Commonwealth Scientific and Industrial Research Organisation).

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Received 16 June 2006; received in revised form 21 September 2006; accepted for publication 25 September 2006

