Elevated CO₂ Induces Biochemical and Ultrastructural Changes in Leaves of the C₄ Cereal Sorghum

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We analyzed the impact of growth at either 350 (ambient) or 700 (elevated) μL L⁻¹ CO₂ on key elements of the C₄ pathway (photosynthesis, carbon isotope discrimination, and leaf anatomy) using the C₄ cereal sorghum (Sorghum bicolor L. Moench.). Gas-exchange analysis of the CO₂ response of photosynthesis indicated that both carboxylation efficiency and the CO₂ saturated rate of photosynthesis were lower in plants grown at elevated relative to ambient CO₂. This was accompanied by a 49% reduction in the phosphoenolpyruvate carboxylase content of leaves (area basis) in the elevated CO₂-grown plants, but no change in Rubisco content. Despite the lower phosphoenolpyruvate carboxylase content, there was a 3-fold increase in CO₂ isotope discrimination in leaves of plants grown at elevated CO₂ and bundle sheath leakiness was estimated to be 24% and 33%, respectively, for the ambient and elevated CO₂-grown plants. However, we could detect no difference in quantum yield. The ratio of quantum yield of CO₂ fixation to PSII efficiency was lower in plants grown at elevated CO₂, but only when leaf internal was below 50 μL L⁻¹. This suggests a reduction in the efficiency of the C₄ cycle when [CO₂] is low, and also implies increased electron transport to acceptors other than CO₂. Analysis of leaf sections using a transmission electron microscope indicated a 2-fold decrease in the thickness of the bundle sheath cell walls in plants grown at elevated relative to ambient CO₂. These results suggest that significant acclimation to increased CO₂ concentrations occurs in sorghum.

The C₄ photosynthetic pathway differs from the C₃ pathway in that it involves two carboxylation steps rather than one. In the first step, CO₂ is fixed into C₄ acids by phosphoenolpyruvate carboxylase (PEPC) in mesophyll cells. In the second step, these C₄ acids are transported into bundle sheath cells, where they are decarboxylated and the CO₂ is refixed by Rubisco. Efficient functioning of the C₄ pathway is facilitated by the distinctive Kranz anatomy of C₄ leaves that allows separation of the two carboxylation steps while at the same time maintaining short diffusion pathways for the transfer of metabolites (Leegood, 1997). Another important structural feature is the very low permeability of bundle sheath cell walls, which minimizes leakage of accumulated CO₂ back to the mesophyll (Hatch et al., 1995). This distinctive combination of biochemistry and anatomy has been estimated to result in a 3- to 20-fold increase in the CO₂ concentration in bundle sheath cells, relative to that in the surrounding air (Jenkins, 1997; Laisk and Edwards, 1998). The main advantages of possessing the C₄ pathway arise both directly and indirectly, from the improved carboxylation efficiency (CE) with which Rubisco operates in bundle sheath cells relative to that in the mesophyll of C₃ plants. This improved efficiency is the result of both the higher CO₂ concentration (CO₂) around Rubisco and the suppression of photorespiration (oxygenation reaction of Rubisco). The improved operating efficiency of Rubisco produces secondary advantages for C₄ plants with respect to both water- and nitrogen-use efficiencies (Sage and Pearcy, 1987; Long, 1999). Based on an estimated bundle sheath CO₂ concentration of 10 to 100 times that in air, it has been calculated that C₄ photosynthesis needs only 13% to 20% of the Rubisco required by C₃ plants to sustain the same carbon fixation rate (Long, 1999). However, others have suggested that the bundle sheath CO₂ concentration may be lower than this (e.g. Laisk and Edwards, 1998) and thus the amount of Rubisco required may be as much as 42% of that found in C₃ plants. C₄ plants also allocate significant amounts of N to PEPC and the ratio of PEPC to Rubisco activity has been shown to decline as N becomes more limiting (Sage et al., 1987). The preferential allocation of N to Rubisco, rather than PEPC, probably helps to prevent a build up of CO₂ in the bundle sheath above carboxylation capacity, thus reducing the potential for increased leakiness. When grown at very low N, the advantage of C₄ photosynthesis over C₃ tends to decline and photosynthetic nitrogen use efficiency of C₃ plants may be higher (Sage and Pearcy, 1987). Furthermore, under limiting N, C₄ plants become more responsive to elevated CO₂ concentrations and there is some evidence, based on δ¹³C values of plant tissue, of an impairment of the CO₂-concentrating mechanism under these conditions (Wong and Osmond, 1991). Growth at elevated CO₂ concentrations was also found to result in an increase in carbon isotope discrimination (Δ) for the C₄ crop, maize (Vogel, 1980) and the C₄ savannah grass, Eragrostis pilosa (Watling and Press, 1998). Measurements of Δ in C₄ plants have also been shown to vary in response to other environmental variables such as water availability.
(Buchmann et al., 1996; Saliendra et al., 1996) and light (Buchmann et al., 1996). Models relating C₄ photosynthesis to Δ suggest that changes in Δ are largely the result of increases in bundle sheath leakiness (Farquhar et al., 1989). However, measurements of on-line isotope discrimination during gas-exchange found little or no short-term response to environmental variables in C₄ plants (Henderson et al., 1992), suggesting that the observed long-term variations in Δ may represent acclimatory responses.

It has been known for some time that environmental variables, such as water availability and salinity, can trigger switches between C₃ and crassulacean acid metabolism photosynthesis in some plants (Winter, 1985). A small number of species have also been reported to exhibit shifts between C₃ and C₄ characteristics in response to environmental variables. These species include sedges from the genus Eleocharis (Ueno, 1996a, 1996b) and grasses from the tribe Orycteae (Keeley, 1998), both of which develop C₃-like traits when they are in aquatic environments, but become more C₄-like when in the terrestrial phase. Another example is the aquatic plant Hydrilla verticillata that switches from C₃ to C₄ photosynthesis when CO₂ availability declines (Reiskind et al., 1997). Despite such examples, and the impacts of both N and CO₂ reported above, the extent to which C₃ photosynthesis may be regulated by environmental variables remains relatively unexplored, especially in comparison with the C₃ pathway.

Under circumstances where CO₂ concentrations are high, as may be the case, at least internally, for the aquatic sedges and grasses, there is no particular advantage in operating a CO₂-concentrating mechanism such as the C₄ pathway. This is because as [CO₂] in the environment increases, the efficiency of C₃ photosynthesis will improve, relative to C₄ photosynthesis, because of the extra cost of operating a CO₂-concentrating mechanism that is incurred by the C₄ pathway (two extra ATP are required for regeneration of phosphoenolpyruvate [PEP]; Kanai and Edwards, 1999). Thus, under high [CO₂], C₃ photosynthesis becomes energetically more favorable than C₄. Furthermore, when [CO₂] is high, C₄ efficiency may be further compromised because the supply of C₄ acids may exceed Rubisco carboxylation capacity, resulting in increased leakiness of CO₂ from the bundle sheath. In an analogous situation, increased leakiness has been demonstrated for transgenic Flaveria bidentis, in which levels of Rubisco in bundle sheath cells were reduced (von Caemmerer et al., 1997).

Although there have been a number of papers in which the impact of elevated CO₂ concentration on growth of C₄ plants has been examined (for review, see Wand et al., 1999), few have explored the possibility that the C₄ pathway itself may be sensitive to changes in CO₂ concentration. In this paper we report the results of an experiment designed to explore the extent to which key features of the C₄ syndrome, specifically leaf anatomy, photosynthetic light and CO₂ utilization, Δ, and enzyme contents may be affected by increased CO₂ concentrations. We grew the C₄ crop, sorghum (Sorghum bicolor L. Moench.), at both 350 and 700 µL L⁻¹ CO₂ and found evidence suggesting modification of the C₄ pathway, at both anatomical and metabolic levels, in the plants grown at elevated CO₂.

RESULTS

In interpreting the CO₂ response of photosynthesis in sorghum, we have used the model of C₄ photosynthesis developed by von Caemmerer and Furbank (1999) in which the initial slope of the A/ci response is an indicator of PEPC activity (CE), whereas the CO₂ saturated rate (A_sat), is determined by either Rubisco activity, the rate of PEP regeneration, the electron transport rate, or PEPC activity if it is very low. This model has been supported by data obtained both from mutants deficient in PEPC (Dever et al., 1997), and transgenic plants with reduced amounts of Rubisco (von Caemmerer et al., 1997). There was a significant [CO₂] effect on the A/ci response of sorghum in our experiment (Fig. 1a). In the plants grown at the higher CO₂ concentration CE was 28% lower and A_sat was 16% lower, although this latter value was not statistically significant (Table I). These results suggest that growth at elevated CO₂ had a significant impact on PEPC activity and possibly on some or all of the components that determine A_sat. Despite these changes, rates of assimilation were similar when plants were measured at growth [CO₂] (indicated by arrows in Fig. 1a). In addition, there was no difference in the CO₂ compensation point 1.42 and 1.51 µL L⁻¹, respectively, for plants grown at either ambient or elevated CO₂, implying that rates of photorespiration were equally low in both.

Chlorophyll (Chl) fluorescence measurements indicated that PSII efficiency (ΦPSII) varied with ci in a similar way to A in both the ambient- and elevated-CO₂ grown plants (Fig. 1b). However, when ci was below 50 µL L⁻¹, the ratio of CO₂ fixation (ΦCO₂) to ΦPSII, which is a measure of the energy efficiency of CO₂ fixation, was lower in the elevated CO₂-grown plants (Fig. 1c). Thus, at low values of ci, less CO₂ was fixed per electron transported in the elevated CO₂-grown plants than in their ambient CO₂-grown counterparts. In conjunction with the gas-exchange data, this provides further evidence of a reduction in the efficiency of the C₄ cycle in sorghum grown at elevated CO₂. However, it also suggests an increase in electron transport to processes other than CO₂ fixation, such as photorespiration, O₂ reduction (Mehler reaction), or nitrogen assimilation.

PEPC and Rubisco contents of the same leaves used for gas-exchange measurements were determined from western blots. The PEPC content (area basis) of sorghum grown at elevated CO₂ was 51% of that
found in the ambient CO₂-grown plants, but there was no change in Rubisco content with growth CO₂ (Table II and Fig. 2). The lower PEPC content of the elevated CO₂-grown sorghum is consistent with the lower CE observed in these plants; however, the lower $A_{sat}$ does not appear to have been the result of any change in PEPC content and instead, may have been due to the decline in PEPC and/or the changes in PEP regeneration and electron transport. Despite the difference in PEPC content, there was no significant difference in either leaf N or chl content (area basis) between the two CO₂ treatments (Table II).

Two previous studies with sorghum have also found that leaf N did not vary significantly with [CO₂] (Reeves et al., 1994; Henning et al., 1996).

Measurements of $\Delta$ made on dried leaf material indicated a significant increase in discrimination against $^{13}$C when plants were grown at elevated relative to ambient CO₂ (Table III). Bundle sheath leakiness (φ), calculated on the basis of the ratio of internal [CO₂] to external [CO₂] ($c_i/c_a$) observed during gas-exchange measurements, was also higher in the elevated CO₂-grown plants than in those grown at ambient CO₂ (Table III). The magnitude of φ is determined by both the physical conductance of bundle sheath cell walls and also the extent of PEPC over-cycling, which occurs if the delivery of CO₂ to the bundle sheath is in excess of its utilization by the C₃ cycle (Farquhar et al., 1989; von Caemmerer and Furbank, 1999). In the current experiment it is unlikely that PEPC over-cycling was significantly higher in the plants grown at elevated CO₂ because of their lower PEPC to Rubisco ratio, relative to ambient CO₂-grown plants. Thus the higher φ may have been due to changes in bundle sheath conductance and/or the higher $c_i$ in the plants grown at elevated CO₂. Increased φ should also result in a decline in the light-use efficiency of C₄ plants, because CO₂ that leaks from the bundle sheath is either lost or refixed by PEPC in the mesophyll, thus increasing the energy expended per CO₂ fixed. However, when we measured the photon flux density (PFD) response of photosynthesis in our experiment, there was no difference in quantum yield between the ambient and elevated CO₂ grown sorghum (Fig. 3).

Leaf sections taken from the youngest fully expanded leaves of the sorghum plants were analyzed using a transmission electron microscope. Examination of the micrographs indicated that plants grown at ambient CO₂ had significantly thicker bundle sheath cell walls than elevated CO₂-grown plants (Fig. 4). Sections from three plants at each CO₂ concentration were analyzed and on average, bundle sheath cell walls of the ambient CO₂-grown plants were twice as thick as those of the elevated CO₂-grown plants, (3.6 ± 0.3 and 1.6 ± 0.1 μm, respectively). This anatomical data provides further evidence that the decline in C₄ pathway efficiency observed in the sorghum plants grown at elevated CO₂ may be, at least partly, the result of changes in the conductance of bundle sheath cell walls to CO₂.

### Table I. CE and the $A_{sat}$ (μmol CO₂ m⁻² s⁻¹) for sorghum grown at either 350 or 700 μL L⁻¹ CO₂

<table>
<thead>
<tr>
<th>Growth [CO₂] μL L⁻¹</th>
<th>CE</th>
<th>$A_{sat}$ (μmol CO₂ m⁻² s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>350</td>
<td>1.16 (0.04)</td>
<td>31.7 (1.3)</td>
</tr>
<tr>
<td>700</td>
<td>0.83 (0.02)</td>
<td>26.7 (0.3)</td>
</tr>
</tbody>
</table>

Parameters were determined using the data shown in Figure 1. Values are means ± se, n = 3. Means superscripted with the same letter are not significantly different at α = 0.05.
PEPC activity and a paper (Watling and Press, 1997) we reported that of the plants grown at ambient CO₂. In contrast, there also for reduced amounts of PEPC (Dever et al., 1997), and activity is very low, because CO₂ levels in the bundle sheath will not be saturating for Rubisco. Similar changes in the A/cᵢ response have been reported both for mutants of the C₄ dicot Amaranthus edulis, with reduced amounts of PEPC (Dever et al., 1997), and also for Amaranthus retroflexus in which PEPC content varied with N availability (Sage et al., 1987). In agreement with the predictions of the model and with these earlier reports, we found that PEPC content of the plants grown at elevated CO₂ was only 51% that of the plants grown at ambient CO₂. In contrast, there was no difference in the Rubisco content of leaves from the two CO₂ treatments. Maroco et al. (1998) also found no change in Rubisco content for heterozygous PEPC mutants of A. edulis with a similar reduction in PEPC content to that which we observed for the plants grown at elevated CO₂. In an earlier paper (Watling and Press, 1997) we reported that [CO₂] had no impact on photosynthesis of sorghum grown at elevated and ambient CO₂. At present we are unable to account entirely for this difference. However, the level of N supplied to plants was higher in the former study than the present one, and nitrogen supply can affect PEPC:Rubisco ratios (Sage et al., 1987) and the response of C₄ plants to [CO₂] (Wong and Osmond, 1991; Ghannoum and Conroy, 1998).

Although the changes in the A/cᵢ response that we observed for elevated CO₂-grown sorghum are entirely consistent with the concurrent decline in PEPC content, they could also be explained by changes in bundle sheath conductance. As modeled by von Caemmerer and Furbank (1999), increases in the permeability of the bundle sheath to CO₂ can cause a decline in both CE and Aₛat because of increased leakage of CO₂ from the bundle sheath. These predictions are supported by work with transgenic F. bidentis, in which expression of carbonic anhydrase in the bundle sheath was increased, resulting in increased leakage of bicarbonate from the bundle sheath and a decline in both CE and Aₛat (Ludwig et al., 1998). Our data also suggest that there was an increase in φ in the plants grown at elevated CO₂, and this was accompanied by significant changes in the physical characteristics of the bundle sheath cell walls as indicated by electron microscopy. Increases in φ can be the result of an increased PEPC to Rubisco ratio (over-cycling of PEPC), and/or changes in the physical conductance of the bundle sheath to CO₂ (Farquhar et al., 1989). However, as we observed a decline in the PEPC to Rubisco ratio, it is most likely that the increased φ was due to changes in bundle sheath conductance, perhaps exacerbated by the in-

Table II. PEPC and Rubisco content (area basis) and N and Chl concentrations for sorghum grown at either 350 or 700 μL L⁻¹ CO₂

<table>
<thead>
<tr>
<th>Growth [CO₂]</th>
<th>PEPC % 350 CO₂</th>
<th>Rubisco % 350 CO₂</th>
<th>N g m⁻²</th>
<th>Total Chl μmol m⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>350</td>
<td>100 (15.0)ᵃ</td>
<td>100 (7.6)ᵃ</td>
<td>0.59 (0.01)ᵃ</td>
<td>371.0 (18.0)ᵃ</td>
</tr>
<tr>
<td>700</td>
<td>51.0 (8.1)b</td>
<td>95.0 (6.8)ᵃ</td>
<td>0.56 (0.02)ᵇ</td>
<td>392.0 (10.0)ᵃ</td>
</tr>
</tbody>
</table>

Values are means ± se, n = 5. Means superscripted with the same letter are not significantly different at α = 0.05.

DISCUSSION

Responses of C₄ Photosynthesis to Elevated CO₂

We observed significant [CO₂] effects on photosynthetic characteristics of the C₄ crop sorghum, with plants grown at elevated CO₂ having lower CE than their ambient CO₂-grown counterparts. According to the model of C₄ photosynthesis developed by von Caemmerer and Furbank (1999), this is consistent with a decline in the PEPC content of leaves, as the initial slope of the A/cᵢ response is proportional to PEPC activity and Aₛat may also decline if PEPC activity is very low, because CO₂ levels in the bundle sheath will not be saturating for Rubisco. Similar changes in the A/cᵢ response have been reported both for mutants of the C₄ dicot Amaranthus edulis, with reduced amounts of PEPC (Dever et al., 1997), and also for Amaranthus retroflexus in which PEPC content varied with N availability (Sage et al., 1987). In agreement with the predictions of the model and with these earlier reports, we found that PEPC content of the plants grown at elevated CO₂ was only 51% that of the plants grown at ambient CO₂. In contrast, there was no difference in the Rubisco content of leaves from the two CO₂ treatments. Maroco et al. (1998) also found no change in Rubisco content for heterozygous PEPC mutants of A. edulis with a similar reduction in PEPC content to that which we observed for the plants grown at elevated CO₂. In an earlier paper (Watling and Press, 1997) we reported that

[ambient lanes 1-4] [elevated lanes 5-8]

**Figure 2.** Western blots of Rubisco and PEPC for leaf samples taken from S. bicolor grown at ambient (350 μL L⁻¹) or elevated (700 μL L⁻¹) CO₂.

Table III. Δ obtained from leaf dry matter and estimated bundle-sheath leakiness (φ) for sorghum grown at either 350 or 700 μL L⁻¹ CO₂

<table>
<thead>
<tr>
<th>Growth [CO₂]</th>
<th>Δ</th>
<th>φ</th>
</tr>
</thead>
<tbody>
<tr>
<td>μL L⁻¹</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>350</td>
<td>1.05 (0.18)ᵃ</td>
<td>24.0 (0.6)ᵃ</td>
</tr>
<tr>
<td>700</td>
<td>3.51 (0.09)b</td>
<td>33.0 (0.3)b</td>
</tr>
</tbody>
</table>

The cᵢ/cₛ values used to estimate φ were obtained during gas-exchange measurements and were 0.19 and 0.26, respectively, for 350 or 700 μL L⁻¹ CO₂-grown plants, measured at growth CO₂. Values are means ± se, n = 5. Means superscripted with the same letter are not significantly different at α = 0.05.
crease in $c_i$. If this is the case, it is possible that the decline in PEPC content was a response to the increase in leakiness, brought about by the change in bundle sheath conductance, rather than a direct response to increased [CO$_2$]. If there had been no decline in the PEPC to Rubisco ratio, the magnitude of $\phi$ would have been even higher and $C_4$ efficiency further compromised. Maroco et al. (1998) also observed a decline in PEPC content in transgenic *F. bidentis* with reduced amounts of Rubisco, although von Caemmerer et al. (1997) did not.

The high concentrations of CO$_2$ in bundle sheath cells of $C_4$ plants act to suppress the oxygenase reaction of Rubisco, but do not remove it altogether, as has been demonstrated through measurements of Gly metabolism in maize (Marek and Stewart, 1983), $^{18}$O$_2$ labeling also in maize (de Veau and Burris, 1989), NH$_4^+$ production in *A. edulis* (Lacuesta et al., 1997), and increased O$_2$-sensitivity, relative to wild-type plants, in PEPC-deficient mutants of *A. edulis* (Maroco et al., 1998). If bundle sheath conductance was greater in sorghum grown at elevated CO$_2$, as is implied by our data, then it might be expected that the plants would show an increased sensitivity to O$_2$. Although we did not make direct measurements of the O$_2$ sensitivity of photosynthesis in our experiment, we did find a decrease in the $\phi$CO$_2$ to $\phi$PSII ratio, at low $c_i$ for the plants grown at elevated as compared with ambient CO$_2$. This implies both a decline in the energy efficiency of CO$_2$ fixation and also an increase in electron transport to acceptors other than CO$_2$, and is consistent with increased rates of photorespiration in the elevated CO$_2$-grown plants when exposed to low [CO$_2$]. At higher CO$_2$ concen-

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**Figure 3.** The relationship between absorbed PFD and CO$_2$ assimilation rate for *S. bicolor* grown at ambient (350 μL L$^{-1}$) or elevated (700 μL L$^{-1}$) CO$_2$.

**Figure 4.** Transmission electron micrographs of leaf sections showing bundle sheaths from *S. bicolor* grown at either 350 (a) or 700 μL L$^{-1}$ CO$_2$ (b). bsc, Bundle sheath cell; m, mesophyll; vb, vascular bundle. Scale bar = 15 μm (both micrographs). Bundle sheath cell walls (indicated by arrows) were approximately twice as thick in ambient relative to elevated CO$_2$ grown plants.
trations, the $\Phi CO_2$ to $\Phi PSII$ ratio was similar in both ambient- and elevated CO$_2$-grown plants. Presumably, this was because the ratio of CO$_2$ to O$_2$ in the bundle sheath cells increased as both PEPC activity and $c_i$ increased. Despite the decline in the $\Phi CO_2$ to $\Phi PSII$ ratio observed at low $c_i$, we did not observe any significant increase in CO$_2$ compensation point for elevated CO$_2$-grown sorghum, as might be expected if photorespiration rates had increased. However, it is possible that the differences in photorespiration were too small to be detected by the gas-exchange system we used, whereas small changes in energy-use efficiency of CO$_2$ fixation were detected by the Chl fluorescence measurements.

Theory predicts that increases in $\phi$ in C$_4$ plants should be accompanied by a decline in the quantum yield of CO$_2$ fixation, because CO$_2$ diffusing from the bundle sheath is either lost or fixed by PEPC in the mesophyll, increasing the energy expended per CO$_2$ fixed (Farquhar, 1983; Hatch et al., 1995). In this context, quantum yields have been reported to vary between both the different C$_4$ subtypes and C$_4$ monocots and dicots; this has been attributed to variation in $\phi$ postulated to be the result of differences in bundle sheath conductance associated with the presence or absence of a suberin lamella in cell walls (Hattersley, 1982; Ehleringer and Pearcy, 1983; Ohsugi et al., 1988). However, concurrent measurements of quantum yield and $\phi$ have rarely been made in the same plants. Furthermore, von Caemmerer et al. (1997) were able to demonstrate a significant increase in $\phi$ for transgenic F. bidentis with reduced Rubisco content, but found no difference in quantum yield between the transgenic and wild-type plants. In our experiment, although the isotope data indicated that there had been a significant increase in $\phi$ for sorghum grown at elevated CO$_2$, we also could not detect any difference in quantum yield. Von Caemmerer et al. (1997) suggested that the inability to find a correlation between $\phi$ and quantum yield may be due to two factors. First, the extent to which the Q-cycle contributes to proton translocation is unknown, but may be significant in C$_4$ plants (Furbank et al., 1990). And second, the relationship between $\phi$ and the quantum requirement of CO$_2$ fixation is non-linear, so that a relatively large increase in $\phi$ actually has a rather small impact on quantum yield, which may be undetectable. However, if the latter is true, it is then difficult to argue that increases in $\phi$ are significantly disadvantageous to C$_4$ plants.

The model developed by Farquhar (1983), describing the relationship between C$_4$ photosynthesis and $^{13}$C discrimination, indicates that the magnitude of $\Delta$ in C$_4$ plants is largely determined by the extent of $\phi$. As described above, $\phi$ itself is a function of the PEPC to Rubisco ratio and the physical conductance of the bundle sheath to CO$_2$. When C$_4$ plants are grown at elevated CO$_2$ concentrations, however, a third factor may influence the magnitude $\Delta$. This is the proportion of CO$_2$ fixed directly by Rubisco in the bundle sheath that has diffused in from the mesophyll, rather than being delivered via PEPC. If this proportion increases, as may occur when bundle sheath conductance increases in combination with an increase in $c_i$ and a decline in PEPC activity, as appears to occur in the elevated CO$_2$ grown sorghum, then the opportunity for Rubisco to discriminate against $^{13}$CO$_2$ increases and $\Delta$ will also increase. That is, under elevated CO$_2$, there may be an increased exchange of CO$_2$ between the atmosphere and the bundle sheath and this is reflected in the increase in $\Delta$. This type of change in $\Delta$ may result either from an increase in the rate of diffusion of CO$_2$ into the bundle sheath (indicating an increase in direct fixation of CO$_2$ by Rubisco) or an increase in the rate of CO$_2$ leakage from the bundle sheath into the atmosphere (i.e. CO$_2$ that is lost from the bundle sheath, but not recycled by PEPC; Hatch et al., 1995). The former may be analogous to similar changes in $\Delta$ observed during transitions between the various phases of crassulacean acid metabolism photosynthesis (Roberts et al., 1997).

Environmental Regulation of C$_4$

The benefits of operating the C$_4$ pathway, relative to the C$_3$ pathway, are greatest under conditions of high light and temperature and a low CO$_2$ to O$_2$ ratio. Thus, if the C$_4$ syndrome is subject to environmental regulation, it might be expected to occur under those conditions that least favor C$_4$ photosynthesis. In the current experiment sorghum was exposed to elevated CO$_2$ concentrations under conditions of limiting N, and PFDs that were approximately one-half of those generally experienced in the regions where sorghum, and C$_4$ grasses in general, predominate (Doggett, 1988). We observed changes in both photosynthetic and anatomical characteristics that suggested modifications of the C$_4$ syndrome had occurred in response to the increased CO$_2$ concentration. Similar modifications have been reported for grasses from the tribe Orcuttieae, which contains a number of species that have both aquatic and terrestrial phases in their life cycle (Keelley, 1998). One genus, Neostapfia, exhibits C$_4$ characteristics in the terrestrial form, but in aquatic leaves there is a reduction in the thickness of bundle sheath cell walls, an increase in $\Delta$, and a decline in the PEPC to Rubisco ratio, characteristics that are identical to those we observed for the elevated CO$_2$ grown sorghum. In a second genus, Orcuttia, C$_4$ activity is maintained in the aquatic plants, but in the absence of Kranz anatomy (Keelley, 1998). Similar changes have also been reported for the sedge Eleocharis vivipara on switching from a terrestrial to an aquatic habitat (Ueno, 1996a, 1996b). A further example of environmental regulation is given by the aquatic plant Hydrilla verticillata, which switches from C$_3$ to C$_4$ metabolism when CO$_2$ concentrations decline (Reis-
kind et al., 1997). Expression of the C₄ syndrome can also be affected by light availability. Maize seedlings that developed in low light or darkness were shown to have Rubisco mRNA present in both bundle sheath and mesophyll cells, whereas high-light grown seedlings showed localization of Rubisco to the bundle sheath cells only (Langdale et al., 1988). These observations indicate that expression of the C₄ phenotype is flexible with respect to environmental factors, in at least some species.

There are a number of consequences that arise from the knowledge that the C₄ phenotype may be subject to some level of environmental regulation. First, it may mean that C₄ plants are more flexible in the face of environmental change than has previously been thought. In particular, this could have consequences for the persistence of C₄-dominated communities in response to climate change and rising atmospheric CO₂ concentrations, both in the future and the past (Cerling et al., 1997; Collatz et al., 1998). Second, the fact that C₄ can be expressed in a variety of forms, including without the presence of the distinctive (and often diagnostic) Kranz anatomy (Keeley, 1998), means that it may be invisible in the fossil record. Of particular interest is the fact that the carbon isotope signatures of C₄ plants can vary with environmental factors such as light, water availability and, as shown in our paper, [CO₂]. This has obvious consequences for interpretation of paleoecological data that is based on carbon isotope signatures of fossil material.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Sorghum (Sorghum bicolor L. Moench. cv CSH-1) plants were grown in controlled environment cabinets (model SGC097, Fitotron, Sanyo-Gallenkamp, UK) at either 350 μmol m⁻² s⁻¹ at plant height. A 12-h photoperiod was maintained throughout the experiment with day and night temperatures of 30°C and 23°C, respectively, and vapor-pressure differences of 1.7 and 1.1 kPa, respectively.

Plants were grown in washed sand and irrigated with 40% full strength Long Ashton solution modified such that N was at 20% (0.5 mol m⁻³ NH₄NO₃) via an automatic drip-irrigation system. Initially plants received 48 cm² of nutrient solution each per day, this was increased to 96, 132, and 240 cm² at 4, 6, and 8 weeks after sowing, respectively.

Gas Exchange and Chl a Fluorescence

Net CO₂ assimilation rates and Chl a fluorescence characteristics were determined simultaneously, using the youngest, fully expanded leaf of 45- to 50-d-old plants. An open gas-exchange system was used with a Parkinson-type leaf chamber (PLC-3, ADC, Hoddesdon, UK). Actinic light was supplied, via a fiber optic bundle, from a KL 1500 light source (Schott, Mainz, Germany), and the same fiber optic bundle was connected to two other KL 1500 light sources to provide the saturating pulses for determination of the Chl a fluorescence parameters F_m and F_v. Input gases (N₂, O₂, and CO₂) were mixed using mass flow controllers (AFC 260, ASM, Bithoven, The Netherlands). Prior to the addition of CO₂, N₂ and O₂ were bubbled through water and then dripped to a set humidity using a condenser coil immersed in a temperature controlled water bath. Differences in the concentrations of CO₂ and H₂O entering and leaving the leaf chamber were measured with an IRGA (LCA-3, ADC, Hoddesdon, UK) and gas-exchange parameters were calculated using the equations of von Caemmerer and Farquhar (1981). Measurements were made at a leaf temperature of 30°C and a leaf to air vapor-pressure difference of 1.7 kPa.

Chl a fluorescence was determined using a pulse amplitude modulated fluorometer (PAM 103, Walz, Effeltrich, Germany). The quantum yield of PSII in the light (ΦPSII) was calculated as ΦPSII = (F_m' - F_o')/F_m' (Genty et al., 1989). The quantum yield of CO₂ fixation (ΦCO₂) was calculated as ΦCO₂ = A/absorbed PFD, assuming a leaf absorptivity of 85% (Oberhuber and Edwards, 1993).

The response of A to cᵢ was assessed by varying the concentration of CO₂ entering the leaf chamber (O₂ was maintained at 210 μmol L⁻¹). Measurements for the A/cᵢ response were made at a PFD of 1,200 μmol m⁻² s⁻¹. Light response curves of photosynthesis were measured at a cᵢ of 350 μmol L⁻¹ and a range of PFDs. Curve fitting software (Sigma Plot for Windows 4.0) was used to analyze both the A/cᵢ and PFD responses using a three component exponential function of the form:

\[ A = a(1 - e^{-bx}) + c \]  

where A = steady-state assimilation rate and x = cᵢ or PFD. Using this equation, the Asat was calculated as a
+ c and the CE as the slope at A = 0 (calculated as b[a + c]). The quantum yield of photosynthesis was calculated in a similar fashion to CE.

SDS-PAGE and Western Blotting

Proteins were extracted from the same leaves that had been used for gas-exchange measurements. Leaf discs (0.56 cm²) were collected, immediately frozen in liquid N₂, and then ground in 300 μL of extraction buffer (180 mol m⁻³ Bicine [N,N’-bis(2-hydroxyethyl)glycine]-KOH, pH 9.0, 5.0 mol m⁻³ DTT (dithiothreitol), and 1.0% [w/v] SDS). The extracts were centrifuged at 14,000 g for 2 min then solubilization buffer (62.5 mol m⁻³ Tris [Tris(hydroxymethyl)-aminomethane]-HCl, pH 6.8, 20% [v/v] glycerol, 2.5% [w/v] SDS, and 5% [v/v] 2-mercaptoethanol) was combined with an aliquot of the supernatant in a ratio of 1:1 (v/v) and boiled in a water bath for 90 s. Proteins were separated using SDS-PAGE. The separated proteins were transferred from gels to polyvinydene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Following transfer, membranes were blocked in 4% milk/Tris-buffered saline (TBS; 20 mol m⁻³ Tris and 140 mol m⁻³ NaCl, pH 7.4) for 1 h and then probed with antisera to either Rubisco (1:1,000 in 4% milk/TBS) or PEPC (1:10,000 in 4% milk/TBS) for 45 min. Membranes were washed several times with TBS and then probed with the secondary antibody, antirabbit IgG peroxidase complex (Sigma, Poole, Dorset, UK). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL Kit, Amersham Life Sciences, Buckinghamshire, UK) and recorded on x-ray film (X-Omat, Kodak Eastman, Rochester, NY). Band densities on the exposed film were quantified by computerized video imaging. Previous determinations indicated that band densities were within the linear range.

Chl and N Determination

Dried sorghum leaf tissue was analyzed for nitrogen using a modified Kjeldahl technique. Samples of dried tissue (50 mg) were digested in concentrated H₂SO₄-salicylic acid in the presence of a catalyst (CuSO₄·Li₂SO₄) for 5 h at 365°C. The resulting digest was diluted to a known volume with distilled H₂O and analyzed with a colorimetric assay using a flow injection analysis system (Tecator 5042 Detector and 5012 Analyzer, Tecator, UK). Leaf discs collected from the same leaves used for gas-exchange were analyzed for their Chl content using the method of Porra et al. (1989).

Stable Carbon Isotope Discrimination

Samples of dried and ground sorghum leaf tissue were analyzed for their stable carbon isotope composition. In each case about 1 mg of plant material was combusted and the relative abundance of $^{13}$C and $^{12}$C was determined using the mass spectrometer facilities at the University of Newcastle upon Tyne (UK; Europa Scientific 20/20 MS, interfaced with an ANCA SL prep unit, Europa Scientific, Crewe, UK). Gas samples from the growth cabinets were analyzed with a trace gas prep unit interfaced to the same mass spectrometer. Carbon isotope compositions of the plant material and source gas in the growth cabinets were determined relative to the Pee Dee Belemnite standard and discrimination against $^{13}$C ($\Delta$) was calculated using Equation 2.

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_i}$$

where $\delta_a$ is the $^{13}$C value of the source air in the growth cabinets and $\delta_p$ is the $^{13}$C value of the plant material. The $\delta_a$ values (means ± se) for the ambient and elevated CO₂ cabinets were -11.45‰ (±0.22) and -18.62‰ (±0.24), respectively. Sampling of gas in both cabinets was carried out over a single day, with 3 samples collected every 2 h between 9 AM and 5 PM. The same cylinder of CO₂ was used to enrich the elevated CO₂ cabinet throughout the experiment.

$\phi$ to CO₂ was estimated using the equations derived by Farquhar et al. (1989) for C₄ photosynthesis. Ideally, when using these equations, values of $\Delta$ and $c_i/c_s$ should be obtained from concurrent gas-exchange measurements. However, in this case we used the $\Delta$ obtained from the dried leaf material and the $c_i/c_s$ values measured during gas-exchange of the same plants (corresponding to growth-CO₂ concentrations) to provide an approximation of $\phi$ for the plants in our experiment. Using this approach, $\phi$ was estimated using Equation 3.

$$\phi = \frac{\Delta - a + (a - b_3)c_i/c_s}{b_3 - s}c_i/c_s$$

where $a$ (4.4‰) is the fractionation occurring during diffusion of CO₂ in air, $b_3$ (~5.7‰) is the combined fractionation due to PEPC (2.2‰) and the activity of carbonic anhydrase in the mesophyll, $b_3$ (30‰) is the fractionation by Rubisco and $s$ (1.8‰) is the fractionation associated with leakage of CO₂ from the bundle sheath to the mesophyll (von Caemmerer et al., 1997).

Electron Microscopy

Leaf tissue was collected from 3 plants at each CO₂ concentration at 54 d after sowing. In each case tissue samples were taken from a location one-half-way along the leaf and mid-way between the mid-vein and the leaf edge. Throughout the experiment leaf production rates were the same for plants in both CO₂ treatments, therefore, we believe samples were collected from leaves that were at the same developmental stage. Samples were fixed in Karnovsky’s solution (2% [w/v] paraformaldehyde and 2% [w/v] glutaraldehyde in 100 mol m⁻³ phosphate buffer) for 3 h at 4°C followed by three washes (30 min each) in 10% (w/v) Suc in 100 mol m⁻³ phosphate.
buffer. Secondary fixation was conducted at room temperature for 1 h in 2% (w/v) aqueous OsO4. Following secondary fixation, tissue samples were passed through an ethanol dehydration series (75%, 95%, and 100% [v/v] ethanol) with 15 min at each step and culminating in a final step at 100% ethanol dried over anhydrous CuSO4. The samples were then incubated twice (15 min each) in propylene oxide. Infiltration was achieved by incubation overnight in 1:1 propylene oxide:Araldite resin (Araldite resin; 1:1 CY212 resin:DDSA hardener, with accelerator 0.1 mL mL⁻¹ araldite resin). Specimens were left in full-strength Araldite resin for 6 to 8 h at room temperature and then embedded in fresh Araldite resin for 48 h at 60°C. Ultrathin sections (70–90 nm) were cut on an ultramicrotome (Ultracut E, Reichert, Austria) and stained for 15 min with 3% (w/v) uranyl acetate in 50% (v/v) aqueous ethanol followed by 2 min with Reynold's lead citrate. Secondary fixation, tissue samples were passed through an ethanol dehydration series (75%, 95%, and 100% [v/v] ethanol) with 15 min at each step and culminating in a final step at 100% ethanol dried over anhydrous CuSO4. The final step at 100% ethanol followed by 2 min with Reynold's lead citrate.

Data Analysis
Where appropriate, data were analyzed using two sample t tests (Mininet 11.0). The response of ΔCO2/ΔPSII to ci was analyzed using ANOVA and a Tukey Test (Zar, 1984).

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LITERATURE CITED


Wong SC, Osmond CB (1991) Elevated atmospheric partial pressure of CO₂ and plant growth: III. Interactions between Triticum aestivum (C₃) and Echinochloa frumentacea (C₄) during growth in mixed culture under different CO₂, N nutrition and irradiance treatments, with emphasis on below-ground responses estimated using the δ¹³C value of root biomass. Aust J Plant Physiol 18: 137–152