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Oxygen Requirement and Inhibition of C_4 Photosynthesis

An Analysis of C_4 Plants Deficient in the C_3 and C_4 Cycles

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The basis for O_2 sensitivity of C_4 photosynthesis was evaluated using a C_4-cycle-limited mutant of *Amaranthus edulis* (a phosphoenolpyruvate carboxylase-deficient mutant), and a C_4-cycle-limited transformant of *Flaveria bidentis* (an antisense ribulose-1,5-bisphosphate carboxylase/oxygenase [Rubisco] small subunit transformant). Data obtained with the C_4-cycle-limited mutant showed that atmospheric levels of O_2 (20 kPa) caused increased inhibition of photosynthesis as a result of higher levels of photorespiration. The optimal O_2 partial pressure for photosynthesis was reduced from approximately 5 kPa O_2 to 1 to 2 kPa O_2, becoming similar to that of C_4 plants. Therefore, the higher O_2 requirement for optimal C_4 photosynthesis is specifically associated with the C_4 function. With the Rubisco-limited *F. bidentis*, there was less inhibition of photosynthesis by supraoptimal levels of O_2 than in the wild type. When CO_2 fixation by Rubisco is limited, an increase in the CO_2 concentration in bundle-sheath cells via the C_4 cycle may further reduce the oxygenase activity of Rubisco and decrease the inhibition of photosynthesis by high partial pressures of O_2 while increasing CO_2 leakage and overcycling of the C_4 pathway. These results indicate that in C_4 plants the investment in the C_3 and C_4 cycles must be balanced for maximum efficiency.

Although in C_3 plants the decrease of the O_2 partial pressures from ambient levels (approximately 20 kPa) to approximately 2 kPa can increase the net rate of CO_2 fixation by up to 50% as a result of reduced photorespiration, in C_4 plants no significant effect is generally observed (Edwards and Walker, 1983). This apparent lack of response of C_4 photosynthesis to O_2 led to the early conclusion that C_4 plants are O_2 insensitive and that photorespiration is not apparent. C_4 plants are capable of concentrating CO_2 in the bundle-sheath cells (where Rubisco is exclusively localized) to levels that have been estimated to exceed 3 to 20 times the atmospheric CO_2 concentration (Jenkins et al., 1989; Dai et al., 1993; Hatch et al., 1995; He and Edwards, 1996). Therefore, the ratio of [CO_2] to [O_2] increases in the bundle-sheath cells, and photorespiration is considered insignificant because of the suppression of the oxygenase reaction of Rubisco (Edwards and Walker, 1983; Edwards et al., 1985; Hatch, 1987; Byrd et al., 1992; Dai et al., 1993; Hatch et al., 1995). Even so, measurable rates of photorespiration have been observed in C_4 plants: in maize, from studies of Gly metabolism in leaf discs (Marek and Stewart, 1983), ^18O_2 incorporation in glycolate in intact leaves (deVeau and Burris, 1989), and ^14C incorporation in Gly and Ser in isolated bundle-sheath cells (Farineau et al., 1984); and in *Amaranthus edulis*, from studies of NH_4^+ production (Lacuesta et al., 1997). In other studies it may be partially responsible for ^18O_2 uptake in C_4 plants (Furbank and Badger, 1982; Badger, 1985).

Rates of photorespiration in C_4 plants under ambient atmospheric conditions have been estimated at 3 to 7% of the rate of CO_2 fixation (Farineau et al., 1984; deVeau and Burris, 1989; Dever et al., 1995; Lacuesta et al., 1997), and even higher under low CO_2 and/or higher O_2 partial pressures (Farineau et al., 1984; Dai et al., 1993, 1995). Because of the high resistance of the bundle-sheath cells to gas diffusion (Furbank et al., 1989; Jenkins et al., 1989; Byrd et al., 1992; He and Edwards, 1996), it is generally accepted that CO_2 released during photorespiration will be partially refixed by Rubisco. However, estimates of leakage rates of CO_2 from the bundle sheath vary from 10 to 50% of the C_4

Abbreviations: *A*, net CO_2 assimilation; ssSU, antisense Rubisco small subunit; Chl, chlorophyll; F_m, maximum fluorescence level after a saturating light pulse on a dark-adapted leaf; F_m′, maximum fluorescence after a saturating light pulse from a leaf during steady-state photosynthesis; F_o, basal fluorescence level on a dark-adapted leaf; F_o′, minimum fluorescence from a leaf following steady-state illumination and quickly dark adapted under a pulse of far-red light to fully oxidize PSI; PSII, steady-state fluorescence on an illuminated leaf; LSI, Rubisco large subunit; ME, malic enzyme; PEPC, PEP carboxylase; SSU, Rubisco small subunit; Φ_{CO_2}, quantum yield of CO_2 fixation; Φ_{PSII}, quantum yield of PSII activity.

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cycle flux, depending on the method of analysis or assumptions used in modeling (Farquhar, 1983; Evans et al., 1986; Henderson et al., 1992; Hatch et al., 1995; He and Edwards, 1996). The release of \(^{14}\text{C}\)O\(_2\) from intact leaves of C\(_4\) plants after a pulse with \(^{14}\text{CO}_2\) was also shown to be consistently higher under 20 kPa O\(_2\) than 2 kPa O\(_2\) (about 8%; see fig. 4 in Hatch et al., 1995). Additionally, O\(_2\) partial pressures in the bundle-sheath cells may be even higher than the atmospheric levels in C\(_4\) plants having PSI activity in the bundle-sheath cells (Furbank et al., 1989), thus increasing the rate of photorespiration.

Generally, there are no significant differences in photosynthetic rates of C\(_4\) plants at 2 versus 20 kPa O\(_2\), even when O\(_2\) is limiting for photosynthesis (Dai et al., 1993, 1995; Maroco et al., 1997). Even if photorespired CO\(_2\) is partially refixed by Rubisco in the bundle-sheath cells, or by PEPC in the mesophyll cells, when CO\(_2\) is limiting, some inhibition of photosynthesis by O\(_2\) should occur. Because that is not the case (Edwards and Walker, 1983; Edwards et al., 1985; Byrd et al., 1992), some other inhibitory mechanism must operate. Indeed, when the response of net CO\(_2\) fixation is measured under different O\(_2\) partial pressures in 20 kPa to 5 to 10 kPa, a measurable increase in net photosynthesis is observed. Below this O\(_2\) partial pressure, net photosynthesis is then inhibited, with rates at 2 kPa being essentially the same as those at 20 kPa.

This phenomenon was first observed by Ku et al. (1983) in \textit{Flaveria trinervia} and was then studied in some detail in maize, both NADP-ME species (Dai et al., 1993, 1995). Recently, we have shown that this dual response of O\(_2\) is common to all C\(_4\) photosynthetic plants, including both monocots and dicots (Maroco et al., 1997). Simultaneous gas-exchange and Chl fluorescence measurements under different CO\(_2\) partial pressures suggested that above the optimal O\(_2\) partial pressure, the inhibition of net photosynthesis is associated with photorespiration. Below the optimum, O\(_2\) inhibition is associated with reduced PSI activity and efficiency of electron transport of open centers and possibly with a decrease in ATP supply to the C\(_4\) cycle (Maroco et al., 1997).

Incorporation of \(^{14}\text{CO}_2\) in C\(_4\) acids in several C\(_4\) species has previously been shown to be stimulated by increasing O\(_2\) partial pressures (Glacoleva and Zalensky, 1978), and an O\(_2\) requirement for maximum CO\(_2\) assimilation has also been observed in C\(_4\) species (Ziem-Hanck and Heber, 1980; Dietz et al., 1985). However, the optimal O\(_2\) partial pressure for photosynthesis is lower in C\(_4\) plants than for the C\(_3\)-C\(_4\) intermediate and C\(_4\) photosynthetic types: 1, 2, and 9 kPa, respectively (Dai et al., 1993, 1996). Taken together, these results suggest that compared with C\(_3\) photosynthesis, C\(_4\) photosynthesis requires a higher O\(_2\) partial pressure for maximum photosynthetic CO\(_2\) assimilation. However, it was not understood why C\(_4\) plants have a higher O\(_2\) requirement than C\(_3\) plants (5–10 kPa versus 1–2 kPa), although we speculated that this could be because of the higher ATP demand for operating the C\(_4\) cycle. Because pseudo-cyclic electron transport may at least in part provide extra ATP for the C\(_4\) cycle (Edwards and Walker, 1983; Hatch, 1987; Furbank et al., 1990), a decrease of the O\(_2\) partial pressure could impair this energy supply. Further-

more, increased reduction of electron carriers of the cyclic pathway may also be achieved under near-anaerobic conditions, limiting the production of ATP by cyclic electron transport (Ziem-Hanck and Heber, 1980; Suzuki and Ikawa, 1984a, 1984b, 1993).

To further understand the roles of the C\(_4\) versus the C\(_3\) cycle in the O\(_2\) requirement and inhibition of C\(_4\) photosynthesis, we used a mutant of the C\(_4\) plant \textit{A. edulis} (NAD-ME) that is deficient in PEPC activity (Dever et al., 1995), and the transgenic plant \textit{Flaveria bidentis} (NADP-ME), which has reduced levels of Rubisco (Furbank et al., 1996). In this study we show that the higher O\(_2\) requirement of C\(_4\) photosynthesis is associated with the C\(_4\) cycle, since plants deficient in the C\(_4\) isoform of PEPC have O\(_2\) requirements similar to those of C\(_3\) plants (about 1 kPa). Results obtained with the two species also provide further evidence that the inhibition of C\(_4\) photosynthesis by supraoptimal O\(_2\) partial pressures is a result of photorespiration. Transgenic \textit{F. bidentis} plants with reduced Rubisco activity and increased bundle-sheath CO\(_2\) concentration (von Caemmerer et al., 1997) are less sensitive, whereas PEPC mutants are more sensitive to supraoptimal O\(_2\) partial pressures.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

F\(_2\) seeds of the \textit{Amaranthus edulis} Speg. mutant LaC\(_4\) 2.16 deficient in PEPC activity (Dever et al., 1995) were germinated and grown in a commercial soil mixture containing 2:1:1 peat-moss-vermiculite in a temperature-controlled growth chamber under a 1% CO\(_2\) atmosphere. Night/day temperatures were 25/35°C with a 12-h photoperiod of 600 \textmu mol m\(^{-2}\) s\(^{-1}\) PAR. F\(_1\) seeds from a self-fertilized \textit{rbcS} antisense \textit{Flaveria bidentis} plant (αSSU 141–6 with two independent antisense inserts; Furbank et al., 1996) were germinated under the same conditions as the \textit{A. edulis} plants but in a temperature-controlled greenhouse under ambient CO\(_2\) partial pressures (33 Pa). Night/day temperatures were 25/35°C, and maximum daily PAR was 1200 \textmu mol m\(^{-2}\) s\(^{-1}\).

**Plant Screening and Enzyme Activity**

Screening of PEPC activity in the F\(_2\) seedlings of \textit{A. edulis} was done by measuring the PEPC activity of fully expanded young leaves. Three 1-cm\(^2\) leaf discs (approximately 0.1 g fresh weight), each from a different fully expanded young leaf, were harvested from each plant and homogenized in 1.5 mL of cold (4°C) grinding medium containing 50 mM Tris-HCl, pH 7.5, 1 mM MgCl\(_2\), 5 mM DTT, 1 μM leupeptin, 2% (w/v) insoluble PVP, 10% (v/v) glycerol, and 0.1% (v/v) Triton X-100 (Sigma). Total extraction of Rubisco from the \textit{A. edulis} wild-type plants grown under 1% CO\(_2\) required up to 1% Triton X-100 in the grinding medium. The extract was centrifuged at 14,000 g for 10 min at 4°C, and the supernatant was used for determination of enzyme activity, total soluble proteins, and total Chl.

PEPC activity was determined at 30°C by following the carboxylation of PEP to oxaloacetate and its reduction to
malate by malate dehydrogenase coupled with NADH oxidation. The assay medium (total volume of 1 mL) contained 50 mm Tris-HCl, pH 8.0, 10 mm NaHCO₃, 5 mm MgCl₂, 0.1 mm NADH, 2 units of malate dehydrogenase, and 25 μL of the enzyme extract. The reaction was initiated by the addition of 50 μL of 50 mm PEP (final concentration of 2.5 mm) (Sigma).

Rubisco activity was measured radiometrically by the incorporation of H¹⁴CO₃⁻ into acid-stable products. The assay mixture (total volume of 150 μL) contained 50 mm Tris-HCl, pH 8.0, 10 mm MgCl₂, 5 mm DTT, 20 mm NaH¹⁴CO₃ (specific activity of 5.89 × 10⁶ cpm/μmol), and 15 μL of enzyme extract. The assay mixture was incubated in 20-mL glass scintillation vials for 2 min at 30°C, and the reaction was started by the addition of 20 μL of 10 mm ribulose bisphosphate (final concentration of 1.3 mm). After 1 min at 30°C the reaction was stopped with 50 μL of tricarboxylic acid (20%), and the samples were left at room temperature for 10 min and then thoroughly flushed with mild air for 10 min. Ten milliliters of scintillation liquid (Bio-Safe II, Research Products International, Mount Prospect, IL) was added to the samples and the activity counted in a liquid scintillation counter (model LS7000, Beckman). Enzyme activity was calculated after correction for background counts and counting efficiency.

Total soluble protein was measured using Coomassie Plus reagent (Pierce) according to the method of Bradford (1976). PEPC and Rubisco (LSU) contents were estimated by densitometric analysis of SDS-PAGE gels of total soluble protein using National Institutes of Health imaging software (Scion, Marlboro, MA). Total Chl was determined by spectrophotometric analysis of total soluble protein using National Institutes of Health imaging software (Zeiger et al., 1987).

**RESULTS**

**Enzyme Activity, SDS-PAGE, and Western Blotting**

The measured activities of PEPC in the F₃ A. edulis plants obtained from the PEPC mutant plant LaC₁₂.16 (Dever et al., 1995, 1997) revealed the normal Mendelian segregation pattern, with three statistically different groups of PEPC activity. Twenty-five percent of the total number of plants exhibited about 2% of maximum wild-type PEPC activity...
Table 1. Total soluble protein, Chl, PEPC, and Rubisco content, and PEPC and Rubisco activity in wild type (WT), heterozygous (Pp), and PEPC homozygous mutants (pp) of A. edulis

All values except the PEPC and Rubisco contents are the average of three or four replicates, with st values in parentheses. Rubisco and PEPC contents were estimated as described in “Materials and Methods.” Means with different letter suffixes are statistically significantly different at α = 0.05.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Total Soluble Protein</th>
<th>Total Chl</th>
<th>PEPC Content</th>
<th>PEPC Activity</th>
<th>Rubisco Content</th>
<th>Rubisco Activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>g/m²</td>
<td>%WT</td>
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<td>% total protein</td>
<td>μmol m⁻² s⁻¹</td>
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<td>WT</td>
<td>4.04 (0.75)a</td>
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<td>100.0</td>
<td>50.0</td>
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<tr>
<td>Pp</td>
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<td>0.41 (0.04)a</td>
<td>79.9</td>
<td>34.8</td>
<td>12.0</td>
</tr>
<tr>
<td>pp</td>
<td>2.24 (0.30)b</td>
<td>55.5</td>
<td>0.31 (0.03)b</td>
<td>59.7</td>
<td>5.4</td>
<td>1.2</td>
</tr>
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</table>

(2.02 ± 0.14 μmol m⁻² s⁻¹), 50% with approximately 50% of PEPC activity (43.88 ± 4.63 μmol m⁻² s⁻¹), and 25% with 100% activity of the wild-type A. edulis plants (90.34 ± 4.09 μmol m⁻² s⁻¹) (Table I). The total soluble protein content of PEPC homozygous mutants (pp) expressed on a leaf-area basis was approximately 56% of that in the wild type, whereas for the heterozygous plants (Pp) this percentage was 86% (Table I). The total Chl content followed the same trend. Consistent with the activity, the PEPC content in the leaves of the heterozygous plants was about one-half of that in the wild-type plants, whereas the homozygous mutants contained very low PEPC protein (5% of that in the wild type).

When expressed on a leaf-area basis, the Rubisco content of heterozygous plants was about 10% lower than that in the wild-type plants, and the Rubisco content of the homozygous mutants was about 50% of that in the wild-type plants (P < 0.05). However, when these values were expressed as a percentage of the total soluble protein, no significant differences were found (P > 0.1). SDS-PAGE and analysis of total soluble leaf protein (Fig. 1) for these enzymes confirmed the pattern of enzyme activity, with estimates of PEPC and Rubisco contents within the ranges reported for other C₄ species (Table I) (Schmitt and Edwards, 1981; Sugiyama et al., 1984; Baer and Schrader, 1985).

The segregation of the αSSU insert in F. bidentis was irregular, with a continuous range of Rubisco activity from less than 10% to 100% of that in the wild-type plants (55.0 ± 4.4 μmol m⁻² s⁻¹). This is consistent with a segregation of two independent antisense inserts in the T₄, giving a range of enzyme activities corresponding to 1, 2, 3, and 4 loci of the antisense insert. From this heterogeneous group, a subset of plants exhibiting normal growth and 33% of wild-type Rubisco activity was chosen for further studies. These αSSU plants showed an approximately 34% reduction of total soluble protein (expressed on a leaf-area basis) relative to the wild-type plants (P = 0.03) (Table II). However, no statistically significant difference was observed in total Chl content among the segregates. Both Rubisco and PEPC contents were significantly lower in αSSU plants than in the wild-type plants (P < 0.01). However, the Rubisco activity was 66% lower, whereas the PEPC activity was only 25% lower in the αSSU relative to the wild-type plants (P < 0.001). SDS-PAGE separation of total soluble protein and identification with western-blot analysis confirmed that both LSU and SSU were the main polypeptides significantly reduced in the αSSU plants used and that no significant changes were observed in carbonic anhydrase (Fig. 2).

Gas Exchange and Chl a Fluorescence

PEPC-Deficient A. edulis

A dual effect of O₂ on the net assimilation rates of the C₄ NAD-ME-type A. edulis wild-type plants was observed under both ambient (33 Pa) and approximately three times

Figure 1. A, Coomassie blue-stained SDS-PAGE gel of soluble leaf protein of A. edulis. WT, Wild type; Pp, heterozygous PEPC mutant; pp, homozygous PEPC mutant; MW, molecular mass in kilodaltons (kD). Thirty-five micrograms of protein was loaded per lane. Arrow indicates the PEPC band. B, Western blot of PEPC, LSU, carbonic anhydrase (CA), and SSU. Twenty-five micrograms of protein was loaded per lane.
ambient (93 Pa) CO₂ partial pressures (Fig. 3a). Maximum photosynthetic rates occurred between 2.5 and 5 kPa O₂, below and above which A was reduced. Statistical analysis revealed that the O₂ effect was significant only when the leaf-to-leaf variation was subtracted by expressing the data on a relative basis (as a percentage of the maximum; Fig. 3b) (P = 0.002). Furthermore, the magnitude of the O₂ effect was dependent on the CO₂ partial pressure at O₂ partial pressures above the optimum (P = 0.03).

For ambient CO₂ (33 Pa) and O₂ (20 kPa) partial pressures, inhibition of A by O₂ was approximately 13% of the maximum. Increasing the CO₂ partial pressures to approximately three times ambient levels (93 Pa) greatly reduced the O₂ inhibition to approximately 6% of the maximum (Fig. 3b). Below the optimal O₂ partial pressures, the reduction in A was associated with decreased efficiency of electron transport through PSII reaction centers (Fig. 3c). The increased reduction of the QA pool (Fig. 3e) and decreased efficiency of the remaining PSII open centers (Fig. 3f) can explain the observed reduction of the ΦPSII at suboptimal O₂ levels. The ratio of ΦCO₂/ΦPSII, which reflects the efficiency of CO₂ fixation relative to PSII activity (Fig. 3d), decreased slightly at supraoptimal O₂ and increased exponentially at low O₂ partial pressures. Thus, the most efficient use of electron flow for CO₂ assimilation is at the lowest O₂ partial pressures.

The decrease of PEPC content and activity in the heterozygous A. edulis plants to about 50% of the wild-type levels (Table I) did not change the dual O₂ effect on A (Fig. 4a). Maximum net photosynthesis rates in the heterozygous plants were approximately 55% of those in the wild-type plants, both at 93 Pa CO₂ and at ambient O₂ partial pressures (33 Pa). The optimal O₂ partial pressure for A was also shifted to 5 to 10 kPa (Fig. 4a), compared with 2.5 to 5 kPa in the wild type. The inhibition at supraoptimal O₂ partial pressures (20 kPa) and ambient CO₂ (33 Pa) was lower than the inhibition in the wild-type plants (11% versus 13%), but this difference was not statistically significant (P = 0.3) (Fig. 4a). No statistically significant difference was found at approximately three times ambient CO₂ (P = 0.2).

As described for the wild-type plants, a decrease of A at below-optimal O₂ partial pressures in this mutant was associated with the decrease in the ΦPSII (Fig. 4c). However, low O₂ was not as inhibitory to A and ΦPSII in the mutant as it was in the wild-type plants. The reduction state of the QA pool (Fig. 4e) was similar to the reduction state in the wild-type plants at three times ambient CO₂ partial pressures, but was higher at ambient CO₂ partial pressures. No statistically significant differences were observed in the ΦCO₂/ΦPSII ratio (Fig. 4d) or in the efficiency of the PSII open centers (Fig. 4f) under varying O₂ at the two CO₂ partial pressures.

The almost total suppression of PEPC in the A. edulis homozygous mutant (Table I) resulted in negative A rates under ambient CO₂ partial pressures (Fig. 5a). At this CO₂

Table II. Total soluble protein, Chl, PEPC, and Rubisco content, and PEPC and Rubisco activity in wild type (WT) and αSSU plants of *F. bidentis*

All values except the PEPC and Rubisco contents are the average of three or four replicates, with ± values in parentheses. Rubisco and PEPC contents were estimated as described in “Materials and Methods.” Means with different letter suffixes are statistically significantly different at α = 0.05.

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<td>WT</td>
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<td>αSSU</td>
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<td>0.66 (0.03)a</td>
<td>105.4</td>
<td>0.19</td>
<td>59.2</td>
</tr>
</tbody>
</table>

Figure 2. A, Coomassie blue-stained SDS-PAGE gel of soluble leaf protein of *F. bidentis*. WT, Wild type; MW, molecular mass in kilodaltons (kD). Thirty-five micrograms of protein was loaded per lane. B, Western blot of PEPC, LSU, carbonic anhydrase (CA), and SSU. Twenty-five micrograms of protein was loaded per lane.
Figure 3. $O_2$ effects on the net $CO_2$ assimilation (a), net $CO_2$ assimilation as a percentage of the maximum rates (b), quantum yield of PSII (c), electron use efficiency for $CO_2$ assimilation (d), reduction state of the $Q_A$ pool (e), and efficiency of PSII open centers (f) in A. edulis wild-type plants. Measurements were made at an ambient $CO_2$ concentration of 93 (●) and 33 Pa (○), with corresponding intercellular $CO_2$ values of 28.3 ± 3.7 and 15.8 ± 0.9 Pa, respectively. Error bars are the Fisher LSD values at $\alpha = 0.05$. Error bar without symbol is the Fisher LSD value for the $O_2 × CO_2$ interaction.

concentration, reducing the $O_2$ partial pressures from 20 to 10 kPa increased $A$ by approximately 50% (Fig. 5b). However, at ambient $CO_2$, photorespiration was in excess of $CO_2$ fixation and so there was no net carbon gain at any $O_2$ partial pressure. At ambient $CO_2$ partial pressures the $\Phi_{CO_2}/\Phi_{PSII}$ ratio increased by more than 50% from ambient to 10 kPa $O_2$, and then decreased. $A$ also decreased at lower $O_2$ partial pressure, possibly because of photoinhibition (Fig. 5d).

At 93 Pa $CO_2$, ambient $O_2$ partial pressures caused an inhibition of net photosynthesis of about 30% of the maximum rate (Fig. 5b). Optimal $O_2$ partial pressures occurred between 1 and 2 kPa, below which a large decrease in $A$ was observed, as reported for $C_3$ species (Ziem-Hanck and Heber, 1980; Dietz et al., 1985; Dai et al., 1996). At 93 Pa $CO_2$, decreasing $O_2$ from ambient to approximately 1 kPa $O_2$ caused a statistically significant ($P < 0.01$), linear increase in $A$ that was also followed by an approximately 2-fold increase in the ratio of $\Phi_{CO_2}$ to $\Phi_{PSII}$ (Fig. 5d). The trend observed in the $\Phi_{PSII}$ response to low $O_2$ in the wild-type and heterozygous plants was also observed in the homozygous mutant (Fig. 5c). However, in the latter, the $\Phi_{PSII}$ values were three and four times lower than the values in the wild-type and heterozygous plants, respectively. In contrast, the reduction state of the $Q_A$ pool (Fig. 5e) was also much higher (up to four times) than that in the wild-type plants. No apparent effect of $O_2$ on the efficiency of open centers (Fig. 5f) was observed at 93 Pa $CO_2$, but a linear decrease was revealed at ambient $CO_2$ from 20 to about 0 kPa $O_2$.

aSSU F. bidentis

In wild-type F. bidentis, the optimal $O_2$ partial pressures for $A$ occurred at 5 to 10 kPa (Fig. 6, a and b). Again, the leaf-to-leaf variance masks the statistical significance of the $O_2$ effect on $A$ ($P = 0.09$). However, when this variation is eliminated by expressing the data as a percentage of the maximum rates, the $O_2$ effect becomes statistically significant ($P < 0.001$). At ambient $O_2$ partial pressure (20 kPa), increasing the $CO_2$ partial pressure from approximately one-third of ambient (9.3 Pa) to ambient (32 Pa) and to approximately three times ambient (93 Pa) decreased the inhibition of net photosynthesis from 8 to 5 to 2%, respectively, of its maximum rates (Fig. 6b). The $O_2$ inhibition at below-optimal $O_2$ partial pressures is associated with reduced $\Phi_{PSII}$ (Fig. 6c), increased reduction state of the $Q_A$ pool (Fig. 6e), and decreased efficiency of open PSII centers (Fig. 6f).

Figure 4. $O_2$ effects on the net $CO_2$ assimilation (a), net $CO_2$ assimilation as a percentage of the maximum rates (b), quantum yield of PSII (c), electron use efficiency for $CO_2$ assimilation (d), reduction state of the $Q_A$ pool (e), and efficiency of PSII open centers (f) in the A. edulis PEPC heterozygous plants. Measurements were made at ambient $CO_2$ concentrations of 93 (●) and 33 Pa (○), with corresponding intercellular $CO_2$ values of 20.5 ± 2.2 and 11.9 ± 0.7 Pa, respectively. Error bars are the Fisher LSD values at $\alpha = 0.05$. Error bar without symbol is the Fisher LSD value for the $O_2 × CO_2$ interaction.
The ratio of $\Phi_{\text{CO}_2}$ to $\Phi_{\text{PSII}}$ increased linearly from ambient down to the optimal $O_2$ partial pressures and then exponentially for suboptimal $O_2$ partial pressures (Fig. 6d). Decrease of Rubisco activity to 33% of that of the wild type in the antisense plants (aSSU) did not change the inhibition of net photosynthesis to below-optimal $O_2$ partial pressures ($P = 0.08; P < 0.001$ when the leaf-to-leaf variation is eliminated by expressing the rates in a relative term). Rather, it limits the effect of above-optimal $O_2$ partial pressures (Fig. 7a). At approximately one-third ambient $O_2$ partial pressures, the inhibition of $A$ by 20 kPa $O_2$ was about 7% of the maximum. However, at ambient $CO_2$ (32 Pa) this inhibition was only 2% (compared with 5% in the wild type), and at three times ambient $CO_2$ this inhibition was nonsignificantly reduced to 1% (Fig. 7b).

Contrary to what was observed in the wild-type plants, $\Phi_{\text{PSII}}$ decreased linearly from high to low $O_2$ at low $CO_2$ (9.3 Pa) and decreased just below the optimal $O_2$ partial pressures for ambient (32 Pa) and high (93 Pa) $CO_2$ (Fig. 7c). The efficiency of PSII open centers (Fig. 7f) showed the same trend as that described for the $\Phi_{\text{PSII}}$, whereas the reduction state of the $Q_A$ pool (Fig. 7e) was almost constant at ambient and high $CO_2$, but increased linearly with decreasing $O_2$ at low $CO_2$. At the lower $CO_2$ partial pressure, the ratio of $\Phi_{\text{CO}_2}$ to $\Phi_{\text{PSII}}$ increased linearly over the whole $O_2$ range, whereas at ambient and high $CO_2$ partial pressures this ratio was almost constant (Fig. 7d).

**DISCUSSION**

Because the net rates of photosynthetic $CO_2$ assimilation are essentially the same at 20 and 2 kPa $O_2$, it has been generally accepted that $C_4$ plants are insensitive to $O_2$. However, we have shown recently that $C_4$ photosynthesis exhibits a dual response to $O_2$ from 20 to near 0 kPa, with an optimum around 5 kPa. Below the optimum, the decrease in photosynthesis is associated with decreased PSII activity, whereas above the optimum, photorespiration accounts for the inhibition of photosynthesis (Dai et al., 1995; Maroco et al., 1997). In this study, we evaluated the basis for the dual response of $C_4$ photosynthesis to $O_2$ using genetic modifications that limit either the $C_3$ or the $C_4$ cycle.

**The $O_2$ Requirement of $C_4$ Photosynthesis and Its Association with the $C_4$ Cycle**

Increased reduction of the $Q_A$ pool at suboptimal partial pressures of $O_2$ was observed in wild-type plants of $A.$
A. Indeed, the $A$ rates in the homozygous mutant are negative at ambient CO$_2$ partial pressures, and it requires up to three times ambient CO$_2$ partial pressures to maintain a net gain of carbon that is increased by up to 30% with decreasing O$_2$. Under ambient conditions, $A$ in the mutant is limited by both photosynthesis and bundle-sheath diffusive resistance (increasing the CO$_2$ concentration up to 30 times the ambient level, 930 Pa, led to photosynthetic rates close to 60% of those observed in the wild-type plants at ambient CO$_2$ data not shown). At approximately three times ambient CO$_2$ partial pressure (93 Pa), enough CO$_2$ apparently diffuses into the bundle-sheath cells to maintain a positive $A$. Under these conditions, i.e. in a C$_3$ photosynthetic mode, the O$_2$ requirement for maximum rates of photosynthesis is similar to that required by C$_3$ plants. In addition, changes in both $A$, the reduction state of Q$_A$, and $\Phi_{PSII}$ in response to O$_2$ have the same form reported for the C$_3$ species spinach, sunflower, and Asarum europaeum (Dietz et al., 1985). Because mutant plants deficient in PEPC show O$_2$ requirements similar to those of C$_3$ plants, we conclude that the higher O$_2$ requirement of C$_3$ photosynthesis is specifically associated with the C$_4$ function.

**Reduced CO$_2$ Fixation by Rubisco in C$_4$ Plants May Increase the CO$_2$ Concentration in the Bundle Sheath and Decrease Photorespiration**

The progressive decrease in $A$ at supraoptimal O$_2$ partial pressures both in *A. edulis* and *F. bidentis* can be explained by photorespiration, as suggested by the decreased inhibition of photosynthesis by O$_2$ with increasing CO$_2$ partial pressures (Figs. 3b and 6b). Furthermore, the progressive decrease of PSII electron transport efficiency for CO$_2$ assimilation ($\Phi_{CO2}/\Phi_{PSII}$) with increasing O$_2$ also supports the hypothesis of O$_2$ as an alternative electron sink through photorespiration or the Mehler peroxidase reaction at supraoptimal O$_2$ partial pressures. As for C$_3$ plants (see Corin and Briantais, 1991; Krall and Edwards, 1992), in *A. edulis*, a decrease in CO$_2$ or an increase in O$_2$ decreases the ratio $\Phi_{CO2}$ to $\Phi_{PSII}$, consistent with photorespiration (Fig. 3d). Similarly, increasing O$_2$ causes a decrease in $\Phi_{CO2}/\Phi_{PSII}$ ratio in *F. bidentis*, although there was no apparent effect on the ratio by changing CO$_2$ (Fig. 6d). Perhaps in this case, the O$_2$-dependent Mehler peroxidase reaction contributes to the decrease in the $\Phi_{CO2}/\Phi_{PSII}$ ratio with increasing O$_2$.

In the αSSU *F. bidentis* plants, whereas suboptimal partial pressures of O$_2$ cause a similar response to that observed in wild-type plants, supraoptimal partial pressures are not so inhibitory to $A$ as for the wild-type plants. Although at low CO$_2$ partial pressure, photorespiration apparently limits photosynthesis in the αSSU plants, at ambient and approximately three times ambient CO$_2$ partial pressures, photorespiration seems to be suppressed. At 20 kPa O$_2$, photosynthetic rates are not statistically significantly different from the rates at 5 kPa, with the $\Phi_{CO2}/\Phi_{PSII}$ ratio increasing only slightly from 20 to 5 kPa O$_2$ at 32 Pa CO$_2$.

If the rate of the C$_4$ cycle is not greatly affected in the αSSU plants (PEPC activity is only 25% less; Table II) and CO$_2$ fixation in the bundle sheath is reduced, then a
buildup of CO₂ should be expected (Furbank et al., 1996). Indeed, von Caemmerer et al. (1997) observed a higher carbon isotope discrimination in T₁ αSSU F. bidentis plants with 40% less Rubisco, and concluded that the CO₂ concentration in the αSSU plants was higher than that of the wild-type plants. In this scenario, photorespiration could indeed be reduced, as suggested by the current study. At the same time, the ΔCO₂/ΔP net response curves to O₂ are higher in the wild-type than in the αSSU plants (Figs. 6d and 7d). This suggests that with a decrease of Rubisco capacity in αSSU plants there may be some increase in other electron sinks. In part this could be linked to increased bundle-sheath leakage of CO₂ and overcycling of the C₄ cycle through pseudocyclic (the Mehler peroxidase reaction) ATP production.

In summary, the effect of O₂ on C₄ photosynthesis can be distinguished as two different components: (a) an O₂ requirement specifically associated with the C₄ cycle, and (b) an O₂ inhibition attributable to photorespiration. The strong requirement for O₂ in C₄ photosynthesis, which is apparent when the C₄ cycle is functional, provides support for the concept that this is linked to the O₂-dependent production of ATP by pseudocyclic/cyclic photophosphorylation. This O₂-dependent generation of ATP is probably associated with the extra energy required for regeneration of PEP, the primary substrate of the C₄ cycle. The inhibition of photosynthesis by supraoptimal partial pressures of O₂ may be accounted for largely, if not entirely, by photorespiration. The results of this study with two genetically modified C₄ plants indicate that when the C₄ cycle is deficient (i.e. ineffective in concentrating CO₂), there is an increase in photorespiration, and when the C₃ cycle is deficient, there is an increase in overcycling of the C₄ pathway and an increase in bundle-sheath CO₂ leakage. Thus, C₄ photosynthesis requires a coordinated function of the C₃ and C₄ cycles for maximum efficiency.

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