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Oxygen Requirement and Inhibition of C_4 Photosynthesis[1]

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 phospho*enol*pyruvate carboxylase-deficient mutant), and a C_3-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_3 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), $^{18}O_2$ incorporation in glycolate in intact leaves (deVeau and Burris, 1989), and ^{14}C 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 $^{18}O_2$ 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

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Abbreviations: A, net CO_2 assimilation; αSSU, 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; F_s, steady-state fluorescence on an illuminated leaf; LSU, 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.

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 $^{14}CO_2$ from intact leaves of C_4 plants after a pulse with $^{14}CO_2$ 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 PSII 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 CO_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 from 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 *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 PSII 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 $^{14}CO_2$ 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_3 species (Ziem-Hanck and Heber, 1980; Dietz et al., 1985). However, the optimal O_2 partial pressure for photosynthesis is lower in C_3 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. Furthermore, 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 *A. edulis* (NAD-ME) that is deficient in PEPC activity (Dever et al., 1995), and the transgenic plant *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 *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 *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 μmol m^{-2} s^{-1} PAR. T_1 seeds from a self-fertilized *rbcS* antisense *Flaveria bidentis* plant (αSSU 141–6 with two independent antisense inserts; Furbank et al., 1996) were germinated under the same conditions as the *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 μmol m^{-2} s^{-1}.

Plant Screening and Enzyme Activity

Screening of PEPC activity in the F_2 seedlings of *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 *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 $NaHCO_3$, 5 mM $MgCl_2$, 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 $H^{14}CO_3^-$ into acid-stable products. The assay mixture (total volume of 150 μL) contained 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 20 mM $NaH^{14}CO_3$ (specific activity of 5.89×10^5 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 LS700, 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 incubation of 40 μL of the crude sample supernatant in 960 μL of absolute ethanol for 2 h in the dark, and then measured according to Wintermans and de Motts (1965).

SDS-PAGE and Western-Blot Analysis

The composition of soluble leaf protein was analyzed by SDS-PAGE in a 7.5 to 15% linear gradient polyacrylamide gel. Samples were prepared in SDS buffer and then boiled for 2 min. After centrifugation at 2000*g* for 2 min, 35 μg of protein was loaded per lane and run under constant current for 1 h at 15 mA and for 2.5 h at 30 mA. The gels were stained with Coomassie brilliant blue (Pierce) and dried in a vacuum gel drier (model 583, Bio-Rad).

Photosynthetic enzymes, PEPC, Rubisco (LSU and SSU), and carbonic anhydrase were identified by western immunoblotting. Maize PEPC antibody was courtesy of R. Chollet (University of Nebraska, Lincoln), and barley Rubisco SSU and LSU antibodies were courtesy of N.H. Chua (Rockefeller University, New York, NY). After SDS-PAGE, protein was electrotransferred to a nitrocellulose membrane overnight in transfer buffer (150 mM Tris-HCl, pH 8.0, 20 mM Gly, 3 mM SDS, and 5% methanol) at 4°C and 250 mA, with final transfer for 1 h at 800 mA. The membrane was blocked with 5% fat-free dry milk in TBS buffer (20 mM Tris-HCl, pH 7.5, and 0.5 M NaCl) and incubated with shaking for 2 h at room temperature with the antibodies (1:6000 dilution) in the same solution. After washing with TBS buffer, the membrane was incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase for 1 h at room temperature. The immunolocalized bands were then revealed by incubation of the membrane in alkaline phosphatase reaction medium containing 5 mM Tris-HCl, pH 9.5, 0.325 mg/mL nitroblue tetrazolium, and 0.165 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (all reagents and alkaline phosphatase-conjugated secondary antibody were obtained from Bio-Rad).

Gas Exchange and Chl *a* Fluorescence

Newly expanded leaves of 35- to 40-d-old plants were used to measure simultaneously *A* and Chl *a* fluorescence. Gas-exchange rates were determined with a computer-controlled gas-exchange system (Bingham Interspace, Logan, UT) using the formulae of Zeiger et al. (1987), as described previously (Maroco et al., 1997). Measurements were made at leaf temperatures of 30.0 ± 0.1°C, a leaf-to-air vapor pressure deficit of 19.1 ± 0.1 Pa/kPa, and a PPFD of 1000 ± 25 μmol m^{-2} s^{-1}. O_2 was decreased from 20 kPa to about 0 kPa at ambient (34 Pa), low (9.3 Pa), and high (93 Pa) CO_2 partial pressures. Simultaneous Chl *a* fluorescence measurements were made with a pulse-amplitude fluorometer (OS-500, Opti-Sciences, Tyngsboro, MA) with the probe positioned above the cuvette at a 45° angle to avoid shading the leaf.

The quantum yield of PSII was calculated as $\Phi_{PSII} = (F'_m - F_s)/F'_m$ (Genty et al., 1989), and the state of reduction of the Q_A pool was estimated as $1 - q_P$, where $q_P = (F'_m - F_s)/(F'_m - F'_o)$ is the photochemical quenching (Dietz et al., 1985). The efficiency of PSII open centers for electron transport was calculated as $(F'_m - F'_o)/F'_m$ (Öquist and Chow, 1992). The quantum yield of CO_2 fixation ($\Phi_{CO_2} = A$/absorbed PPFD) was calculated as the ratio of net CO_2 fixation to PPFD absorbed, assuming a leaf absorptivity of 85% for C_4 plants (Oberhuber et al., 1993; Oberhuber and Edwards, 1993). Dark-type mitochondrial respiration was not included in the calculation because it is not known how this changes in the light under varying O_2.

Statistical Analysis

All measurements shown are the averages of three or four independent replicates. Statistically significant effects were studied by one-way or two-way analysis of variance and Fisher LSD values at $\alpha = 0.05$ for the differences between the means. The significance of the PEPC and Rubisco contents estimated from densitometric analysis was studied with a general linear model analysis of variance.

RESULTS

Enzyme Activity, SDS-PAGE, and Western Blotting

The measured activities of PEPC in the F_2 *A. edulis* plants obtained from the PEPC mutant plant LaC_4 2.16 (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 I. *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 SE 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 $\alpha = 0.05$.

Plant	Total Soluble Protein		Total Chl		PEPC Content			PEPC Activity		Rubisco Content			Rubisco Activity	
	g/m²	*%WT*	*g/m²*	*%WT*	*g/m²*	*%WT*	*% total protein*	*μmol m⁻² s⁻¹*	*%WT*	*g/m²*	*%WT*	*% total protein*	*μmol m⁻² s⁻¹*	*%WT*
WT	4.04 (0.75)a	100.0	0.52 (0.04)a	100.0	0.50	100.0	10.4	90.4 (4.1)a	100.0	1.365	100.0	33.8	53.7 (1.5)a	100.0
Pp	3.42 (0.61)a	84.6	0.41 (0.04)a	79.9	0.34	68.2	12.0	43.8 (4.6)b	48.6	1.254	91.8	36.7	48.2 (2.0)b	89.7
pp	2.24 (0.30)b	55.5	0.31 (0.03)b	59.7	0.03	5.4	1.2	2.0 (0.1)c	2.2	0.762	55.8	34.0	26.7 (1.7)c	49.7

(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 C_4 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 T_1, 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 O_2 on the net assimilation rates of the C_4 NAD-ME-type *A. edulis* wild-type plants was observed under both ambient (33 Pa) and approximately three times

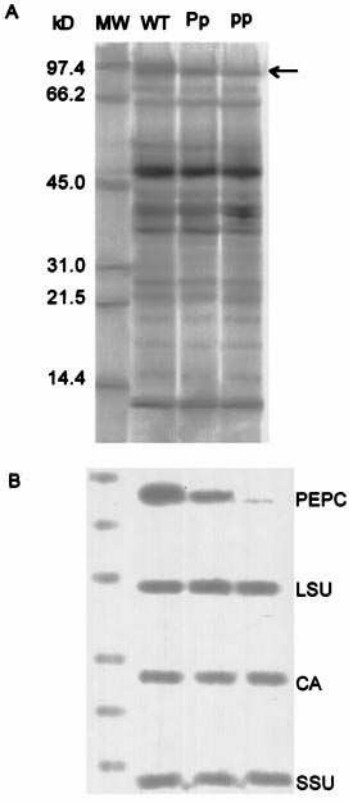

Figure 1. A, Coomassie blue-stained SDS-PAGE gel of soluble leaf protein of *A. edulis*. WT, Wild type; Pp, heterozygous PEPC mutant; pp, homozygous PEPC mutant; MW, molecular mass in kilodaltons (kD). Thirty-five micrograms of protein was loaded per lane. Arrow indicates the PEPC band. B, Western blot of PEPC, LSU, carbonic anhydrase (CA), and SSU. Twenty-five micrograms of protein was loaded per lane.

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 SE 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 $\alpha = 0.05$.

Plant	Total Soluble Protein		Total Chl		PEPC Content			PEPC Activity		Rubisco Content			Rubisco Activity	
	g/m²	*%WT*	*g/m²*	*%WT*	*g/m²*	*%WT*	*% total protein*	*μmol m⁻² s⁻¹*	*%WT*	*g/m²*	*%WT*	*% total protein*	*μmol m⁻² s⁻¹*	*%WT*
WT	3.35 (0.13)a	100.0	0.63 (0.02)a	100.0	0.33	100.0	10.0	97.8 (1.6)a	100.0	0.68	100.0	20.3	55.0 (4.4)a	100.0
αSSU	2.20 (0.03)b	65.7	0.66 (0.03)a	105.4	0.19	59.2	8.9	73.2 (1.0)b	74.8	0.27	39.4	12.2	18.2 (0.4)b	33.1

ambient (93 Pa) CO_2 partial pressures (Fig. 3a). Maximum photosynthetic rates occurred between 2.5 and 5 kPa O_2, below and above which *A* was reduced. Statistical analysis revealed that the O_2 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 O_2 effect was dependent on the CO_2 partial pressure at O_2 partial pressures above the optimum ($P = 0.03$).

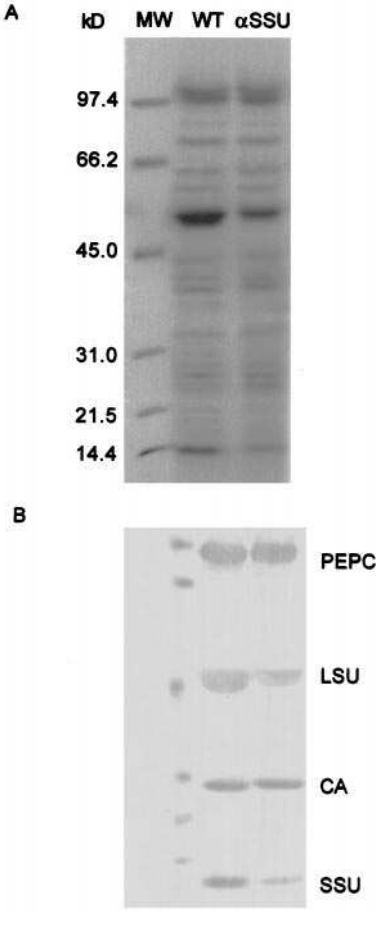

Figure 2. A, Coomassie blue-stained SDS-PAGE gel of soluble leaf protein of *F. bidentis*. WT, Wild type; MW, molecular mass in kilodaltons (kD). Thirty-five micrograms of protein was loaded per lane. B, Western blot of PEPC, LSU, carbonic anhydrase (CA), and SSU. Twenty-five micrograms of protein was loaded per lane.

For ambient CO_2 (33 Pa) and O_2 (20 kPa) partial pressures, inhibition of *A* by O_2 was approximately 13% of the maximum. Increasing the CO_2 partial pressures to approximately three times ambient levels (93 Pa) greatly reduced the O_2 inhibition to approximately 6% of the maximum (Fig. 3b). Below the optimal O_2 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 Q_A pool (Fig. 3e) and decreased efficiency of the remaining PSII open centers (Fig. 3f) can explain the observed reduction of the Φ_{PSII} at suboptimal O_2 levels. The ratio of Φ_{CO_2}/Φ_{PSII}, which reflects the efficiency of CO_2 fixation relative to PSII activity (Fig. 3d), decreased slightly at supraoptimal O_2 and increased exponentially at low O_2 partial pressures. Thus, the most efficient use of electron flow for CO_2 assimilation is at the lowest O_2 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 O_2 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 CO_2 and at ambient CO_2 partial pressures (33 Pa). The optimal O_2 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 O_2 partial pressures (20 kPa) and ambient CO_2 (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 CO_2 ($P = 0.2$).

As described for the wild-type plants, a decrease of *A* at below-optimal O_2 partial pressures in this mutant was associated with the decrease in the Φ_{PSII} (Fig. 4c). However, low O_2 was not as inhibitory to *A* and Φ_{PSII} in the mutant as it was in the wild-type plants. The reduction state of the Q_A pool (Fig. 4e) was similar to the reduction state in the wild-type plants at three times ambient CO_2 partial pressures, but was higher at ambient CO_2 partial pressures. No statistically significant differences were observed in the Φ_{CO_2}/Φ_{PSII} ratio (Fig. 4d) or in the efficiency of the PSII open centers (Fig. 4f) under varying O_2 at the two CO_2 partial pressures.

The almost total suppression of PEPC in the *A. edulis* homozygous mutant (Table I) resulted in negative *A* rates under ambient CO_2 partial pressures (Fig. 5a). At this CO_2

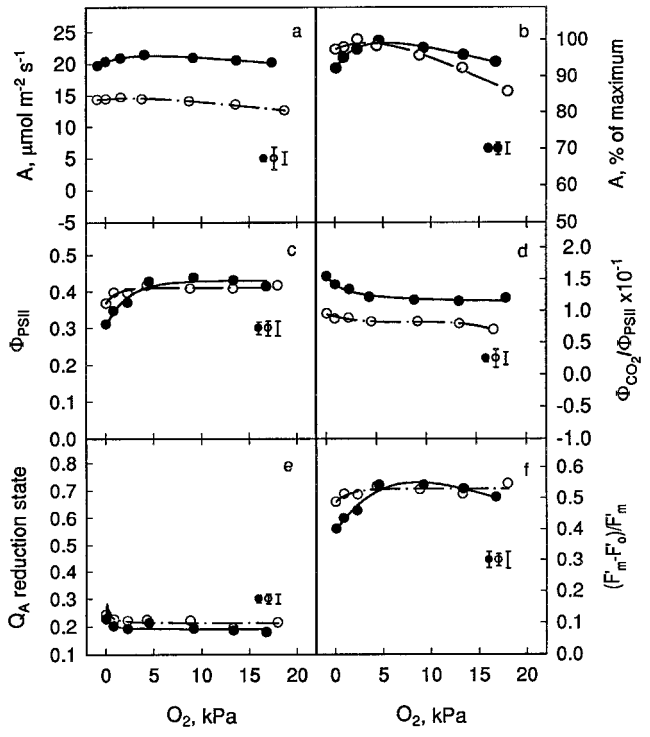

Figure 3. O_2 effects on the net CO_2 assimilation (a), net CO_2 assimilation as a percentage of the maximum rates (b), quantum yield of PSII (c), electron use efficiency for CO_2 assimilation (d), reduction state of the Q_A pool (e), and efficiency of PSII open centers (f) in *A. edulis* wild-type plants. Measurements were made at an ambient CO_2 concentrations 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 $O_2 \times CO_2$ 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 Φ_{CO_2}/Φ_{PSII} ratio increased by more then 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 Φ_{CO_2} to Φ_{PSII} (Fig. 5d). The trend observed in the Φ_{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 Φ_{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.

αSSU 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 Φ_{PSII} (Fig. 6c), increased reduction state of the Q_A pool (Fig. 6e), and decreased efficiency of open PSII centers (Fig. 6f).

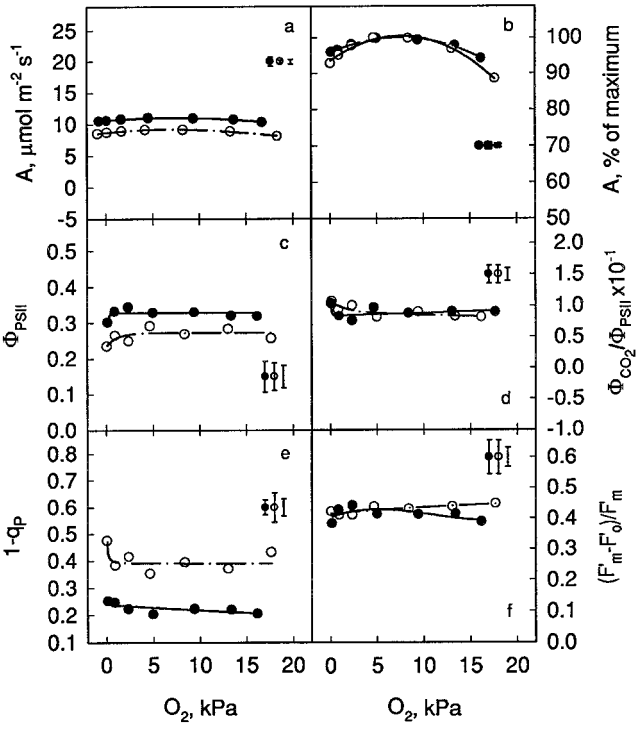

Figure 4. O_2 effects on the net CO_2 assimilation (a), net CO_2 assimilation as a percentage of the maximum rates (b), quantum yield of PSII (c), electron use efficiency for CO_2 assimilation (d), reduction state of the Q_A pool (e), and efficiency of PSII open centers (f) in the *A. edulis* PEPC heterozygous 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 $O_2 \times CO_2$ interaction.

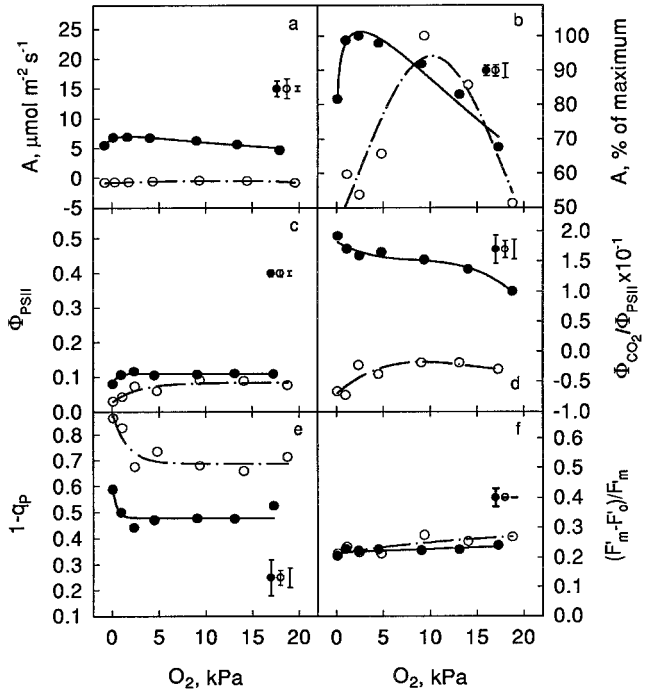

Figure 5. O_2 effects on the net CO_2 assimilation (a), net CO_2 assimilation as percentage of the maximum rates (b), quantum yield of PSII (c), electron use efficiency for CO_2 assimilation (d), reduction state of the Q_A pool (e), and efficiency of PSII open centers (f) in *A. edulis* PEPC homozygous mutants. Measurements were made at ambient CO_2 concentrations of 93 (●) and 33 Pa (○), with corresponding intercellular CO_2 values of 74.0 ± 2.1 and 34.1 ± 0.2 Pa, respectively. Error bars are the Fisher LSD values at $\alpha = 0.05$. Error bar without symbol is the Fisher LSD value for the $O_2 \times CO_2$ interaction.

The ratio of Φ_{CO_2} to Φ_{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 (αSSU) 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 CO_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, Φ_{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 Φ_{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 Φ_{CO_2} to Φ_{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.*

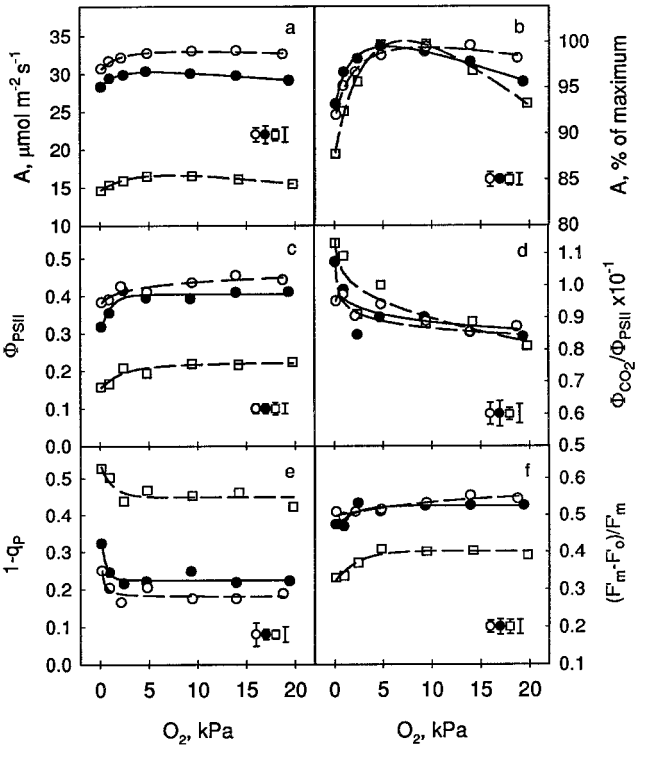

Figure 6. O_2 effects on the net CO_2 assimilation rates (a), net CO_2 assimilation as a percentage of the maximum rates (b), quantum yield of PSII (c), electron use efficiency for CO_2 assimilation (d), reduction state of the Q_A pool (e), and efficiency of PSII open centers (f) in *F. bidentis* wild-type plants. Measurements were done at ambient CO_2 concentrations of 9.3 (□), 33 (●), and 93 Pa (○), with corresponding intercellular CO_2 values of 2.4 ± 0.1, 11.0 ± 0.3, and 45.9 ± 1.4 Pa, respectively. Error bars are the Fisher LSD values at $\alpha = 0.05$. Error bar without symbol is the Fisher LSD value for the $O_2 \times CO_2$ interaction.

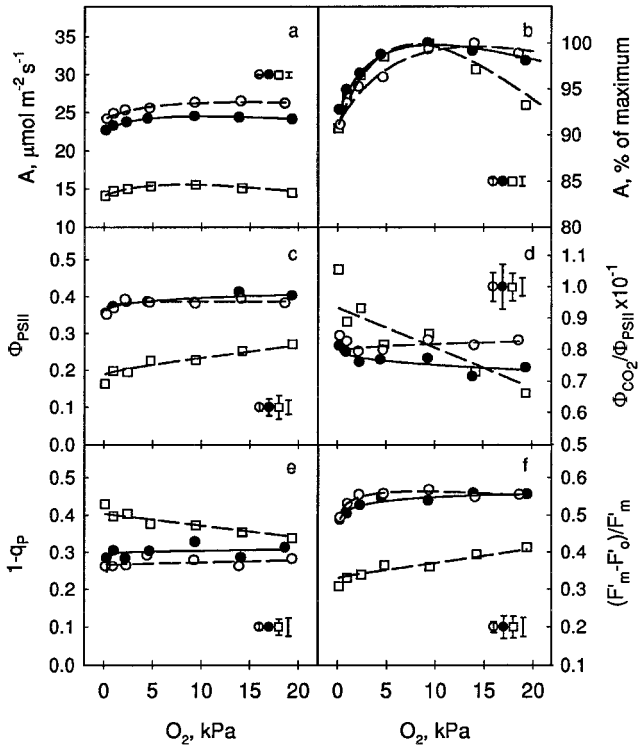

Figure 7. O_2 effects on the net CO_2 assimilation rates (a), net CO_2 assimilation as a percentage of the maximum rates (b), quantum yield of PSII (c), electron use efficiency for CO_2 assimilation (d), reduction state of the Q_A pool (e), and efficiency of PSII open centers (f) in *F. bidentis* αSSU plants. Measurements were done at ambient CO_2 concentrations of 9.3 (□), 33 (●), and 93 Pa (○), with corresponding intercellular CO_2 values of 3.1 ± 0.1, 15.3 ± 0.2, and 68.9 ± 2.0 Pa, respectively. Error bars are the Fisher LSD values at $\alpha = 0.05$. Error bar without symbol is the Fisher LSD value for the $O_2 \times CO_2$ interaction.

edulis and *F. bidentis* (Figs. 3 and 6) and in PEPC homozygous mutant and αSSU plants (Figs. 5 and 7). The efficiency of PSII open centers was also often reduced under low O_2. Closure of some PSII centers (increased reduction of Q_A) and decreased efficiency of open centers both contributed to lower Φ_{PSI} under low O_2.

In wild-type *A. edulis* plants the reduction state of the Q_A pool was low and essentially the same at the CO_2 partial pressures studied (33 and 93 Pa), with the ratio of Φ_{CO_2} to Φ_{PSII} being substantially higher at the higher CO_2 concentration. This suggests that O_2 does act as an alternative electron sink at 33 Pa CO_2, either as the final acceptor of the electron-transport carriers (the Mehler peroxidase reaction) or in photorespiration. In *A. edulis* heterozygous PEPC plants, the optimal O_2 level for maximum rates of net photosynthesis is slightly higher than that in the wild type (compare Fig. 3, a and b, with Fig. 4, a and b). A higher O_2 requirement for functioning of the electron transport chain in heterozygous plants was also suggested by the linear decrease in the efficiency of PSII open centers (Fig. 4f), with the increased reduction of the Q_A pool occurring only at ambient CO_2 and 0 kPa O_2; however, these differences are probably not significant.

Suppression of the C_4 cycle by a decrease of PEPC activity in *A. edulis* to levels found in C_3 plants greatly reduces *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 photorespiration 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 Φ_{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_4 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 (Φ_{CO_2}/Φ_{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 Cornic and Briantais, 1991; Krall and Edwards, 1992), in *A. edulis*, a decrease in CO_2 or an increase in O_2 decreases the ratio Φ_{CO_2} to Φ_{PSII}, consistent with photorespiration (Fig. 3d). Similarly, increasing O_2 causes a decrease in Φ_{CO_2}/Φ_{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 Φ_{CO_2}/Φ_{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 Φ_{CO_2}/Φ_{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 CO_2 should be expected (Furbank et al., 1996). Indeed, von Caemmerer et al. (1997) observed a higher carbon isotope discrimination in T_1 αSSU *F. bidentis* plants with 40% less Rubisco, and concluded that the CO_2 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 Φ_{CO_2}/Φ_{PSII} response curves to O_2 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 CO_2 and overcycling of the C_4 cycle through pseudocyclic (the Mehler peroxidase reaction) ATP production.

In summary, the effect of O_2 on C_4 photosynthesis can be distinguished as two different components: (a) an O_2 requirement specifically associated with the C_4 cycle, and (b) an O_2 inhibition attributable to photorespiration. The strong requirement for O_2 in C_4 photosynthesis, which is apparent when the C_4 cycle is functional, provides support for the concept that this is linked to the O_2-dependent production of ATP by pseudocyclic/cyclic photophosphorylation. This O_2-dependent generation of ATP is probably associated with the extra energy required for regeneration of PEP, the primary substrate of the C_4 cycle. The inhibition of photosynthesis by supraoptimal partial pressures of O_2 may be accounted for largely, if not entirely, by photorespiration. The results of this study with two genetically modified C_4 plants indicate that when the C_4 cycle is deficient (i.e. ineffective in concentrating CO_2), there is an increase in photorespiration, and when the C_3 cycle is deficient, there is an increase in overcycling of the C_4 pathway and an increase in bundle-sheath CO_2 leakage. Thus, C_4 photosynthesis requires a coordinated function of the C_3 and C_4 cycles for maximum efficiency.

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