The Role of Phosphoenolpyruvate Carboxylase during C₄ Photosynthetic Isotope Exchange and Stomatal Conductance[OA]

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Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) plays a key role during C₄ photosynthesis and is involved in anaplerotic metabolism, pH regulation, and stomatal opening. Heterozygous (Pp) and homozygous (pp) forms of a PEPC-deficient mutant of the C₄ dicot Amaranthus edulis were used to study the effect of reduced PEPC activity on CO₂ assimilation rates, stomatal conductance, and δ¹³CO₂ (δ¹³C) and δ¹⁸O (δ¹⁸O) isotope discrimination during leaf gas exchange. PEPC activity was reduced to 42% and 3% and the rates of CO₂ assimilation in air dropped to 78% and 10% of the wild-type values in the Pp and pp mutants, respectively. Stomatal conductance in air (531 µbar CO₂) was similar in the wild-type and Pp mutant but the pp mutant had only 41% of the wild-type steady-state conductance under white light and the stomata opened more slowly in response to increased light or reduced CO₂ partial pressure, suggesting that the C₄ PEPC isoform plays an essential role in stomatal opening. There was little difference in Δ¹³C between the Pp mutant (3.0% ± 0.4%) and wild type (3.3% ± 0.4%), indicating that leakage (δ) of CO₂ leak rate out of the bundle sheath to the rate of CO₂ supply by the C₄ cycle, a measure of the coordination of C₄ photosynthesis, was not affected by a 60% reduction in PEPC activity. In the pp mutant Δ¹³C was 16% ± 3.2%, indicative of direct CO₂ fixation by Rubisco in the bundle sheath at ambient CO₂ partial pressure. Δ¹⁸O measurements indicated that the extent of isotopic equilibrium between leaf water and the CO₂ at the site of oxygen exchange (θ) was low (0.6) in the wild-type and Pp mutant but increased to 0.9 in the pp mutant. We conclude that in vitro carbonic anhydrase activity overestimated θ as compared to values determined from Δ¹⁸O in wild-type plants.

The enzyme phosphoenolpyruvate (PEP) carboxylase (PEPC) utilizes bicarbonate (HCO₃⁻) to catalyze the β-carboxylation of PEP, to form the four-carbon acid oxaloacetate (Andreo et al., 1987; Chollet et al., 1996; Lepiniec et al., 2003; Izui et al., 2004). In higher plants, PEPC plays the anaplerotic role of replenishing the citric acid cycle intermediates, oxaloacetate and malate, which are required for nitrogen assimilation and amino acid biosynthesis. The synthesis of malate, which are required for nitrogen assimilation and the citric acid cycle, allows for the accumulation of four-carbon acids that subsequently diffuse into the cytosol of mesophyll cells (Kanai and Edwards, 1999). C₄ plants generally have high PEPC activity in the cytosol of mesophyll cells, allowing for the accumulation of four-carbon acids that subsequently diffuse into the bundle sheath cells (BSCs) for decarboxylation (Kanai and Edwards, 1999; Furbank et al., 2000). The specialized biochemistry and leaf anatomy of C₄ plants results in CO₂ partial pressure (pCO₂) around the site of Rubisco several fold higher than current atmospheric levels, significantly reducing the rates of photosynthesis (Hatch, 1987).

Theoretical models of δ¹³CO₂ isotope discrimination (Δ¹³C) during C₄ photosynthesis have been developed that link Δ¹³C to the ratio of intercellular to ambient pCO₂ and bundle sheath leakiness (δ) defined as the fraction of CO₂ fixed by PEPC that subsequently leaks out of the BSCs and is not fixed by Rubisco (Farquhar, 1983). Leakiness is a measure of the efficiency of C₄ photosynthesis and has been estimated with concurrent measurements of leaf gas exchange and Δ¹³C in a number of C₄ species (Henderson et al., 1992). It has been shown that δ increases in transgenic Flaveria...
bidentis with reduced Rubisco content, demonstrating that the balance between C₃ and C₄ cycle activity influences φ (von Caemmerer et al., 1997a, 1997b; Cousins et al., 2006a). C¹³O₂ discrimination (Δ¹³O) during C₄ photosynthesis, which is largely determined by the residence time of CO₂ within a leaf and the number of hydration reactions per CO₂ molecule, is influenced by changes in the carbonic anhydrase (CA) activity (Cousins et al., 2006b) and the capacities of the C₄ and C₃ cycles.

In higher plants, independent of the photosynthetic pathway, PEPC participates in guard cell metabolism. Stomatal opening is achieved through the accumulation of high levels of solutes in guard cell vacuoles. The accumulation of potassium ions requires anions (such as malate or chloride) to provide charge balance and to maintain the membrane potential. Malate produced via PEPC is believed to contribute substantially to the maintenance of the proton and charge balance in these cells during stomatal opening (Allaway, 1973; Outlaw and Lowry, 1977). The production of malate in guard cells is thought to be directly linked to carbon metabolism as PEP, the substrate for carboxylation, originates mainly from carbon skeletons derived from starch breakdown in the guard cell chloroplast (Vavasseur and Raghavendra, 2005). Additionally, the amount of PEPC in guard cells of C₃ plants has been shown to be an order of magnitude greater than in mesophyll cells when expressed on a protein basis (Cotelle et al., 1999).

The concentration of malate inside guard cells correlates with stomatal aperture in epidermal strips but it was also shown that the influence of malate is dependent on the availability of chloride (van Kirk and Raschke, 1978; Willmer and Fricker, 1996). The role of PEPC in stomatal opening was also confirmed in epidermal strips of C₃ plants using a PEPC inhibitor (Parvathi and Raghavendra, 1997; Asai et al., 2000). At the whole leaf level, the use of antisense and overexpression of PEPC in Solanum tuberosum also suggested that malate accumulation is involved in stomatal function (Gehlen et al., 1996). This showed that rates of stomatal opening increased in plants overexpressing PEPC and decreased in plants with reduced levels of PEPC. However, low PEPC levels had no effect on steady-state stomatal conductance and overexpression of PEPC had only a marginal effect (Gehlen et al., 1996).

Isotope analysis of atmospheric carbon CO₂ has become an important tool for monitoring changes in the global exchange of CO₂ (Flanagan and Ehleringer, 1998; Yakir and Sternberg, 2000). However, to interpret the atmospheric CO₂ isotopic signature requires an understanding of the isotopic fractionation steps associated with specific processes during leaf gas exchange (Yakir and Sternberg, 2000). Here we used the PEPC-deficient mutants of the C₄ dicot Amaranthus edulis (Dever et al., 1995, 1996, 1997) to assess the contribution of PEPC activity on photosynthetic isotope exchange and stomatal conductance. Previous work on these plants has shown that the heterozygous (Pp) and the homozygous (pp) PEPC mutants contain approximately 50% and 2% of wild-type PEPC activity, respectively (Dever et al., 1996, 1997; Maroco et al., 1997, 1998a, 1998b; Kiriats et al., 2002). These mutants are a nice comparison to earlier work on the C₄ dicot F. bidentis that had high rates of PEPC and low CA due to antisense silencing of CA (Cousins et al., 2006a, 2006b). We have used the PEPC-deficient A. edulis mutants to address three distinct questions. (1) How does a reduction in PEPC activity affect Δ¹³C and bundle sheath leakiness? (2) Does the increased ratio of CA to PEPC activity affect the isotopic equilibrium between leaf water and CO₂ and hence Δ¹⁸O? (3) What are the effects of reduced PEPC activity on stomatal conductance?

RESULTS

Steady-State Gas-Exchange and Enzyme Activities

Under our growth conditions, which contained 9.8 mbar of CO₂, both the heterozygous (Pp) and homozygous (pp) PEPC mutants had similar total nitrogen per leaf area and leaf mass per area as compared to wild-type plants (Table I). Concurrent measurements of Δ¹³C and Δ¹⁸O and gas exchange were made by directly coupling a mass spectrometer to the outlet of a portable leaf gas-exchange system via a gas permeable silicone membrane (Cousins et al., 2006a, 2006b). This allowed simultaneous measurements of leaf gas exchange and the ¹³C/¹²C or ¹⁸O/¹⁶O ratios of the CO₂ in the air stream without prior purification of the CO₂. Under ambient CO₂ concentrations (531 μbar), net CO₂ assimilation rates in the Pp and pp mutants were 78% and 10% of wild-type plants, respectively (Table I). Compared to the wild type, the ratio of intercellular to atmospheric pCO₂ (pᵢ/pᵢ) was higher and stomatal conductance (gₛ) lower in the pp mutant at high light and ambient CO₂ (531 μbar), whereas the differences between the wild-type and Pp mutant were not significant. PEPC activity determined on whole leaf extracts was 42% and 3% of wild-type in Pp and pp mutants, respectively, whereas Rubisco activity was not significantly different between the wild-type and pp mutant (Table I). The total extractable CA activity expressed as the rate constant kᵦA (μmol m⁻² s⁻¹ bar⁻¹) was similar in all plants (Table I). The kᵦA was determined from leaf extracts using mass spectrometry to measure the rates of ¹⁸O₂ exchange from labeled ¹³C₁₈O₂ to H₁₆O (Badger and Price, 1989; Cousins et al., 2006a, 2006b). Leaf CA activity (CAᵦ), determined as the product of kᵦA and the mesophyll pCO₂ (pᵢ), increased in the pp mutant due to the low rates of net CO₂ assimilation that caused pᵢ to be greater in these plants (Table I). The values of pᵢ were calculated as pᵢ = pᵢᵦ - A/sᵦ, where A is the net CO₂ assimilation rate and sᵦ is the CO₂ conductance from the intercellular air space to the site of PEPC carboxylation (assumed to equal 1 mol m⁻² s⁻¹ bar⁻¹; Cousins et al., 2006a).
Table 1. Gas exchange and leaf characteristics of wild-type A. edulis and PEPC mutants

| Measurements               | WT           | Pp           | pp           |
|----------------------------|--------------|--------------|--------------|
| Photosynthetic parameters  |              |              |              |
| $A$ (μmol m$^{-2}$ s$^{-1}$) | 40.9 ± 1.6a  | 32.1 ± 1.7b  | 4.1 ± 0.4c   |
| $p/n$ (mol m$^{-2}$ s$^{-1}$)| 0.39 ± 0.06a | 0.45 ± 0.07a | 0.82 ± 0.02b |
| $\Delta^{13}C$ (‰)         |              |              |              |
| $\Delta^{18}O$ (‰)         |              |              |              |
| Rubisco activity           |              |              |              |
| PEPC (μmol m$^{-2}$ s$^{-1}$) | 144.9 ± 8.8a | 61.0 ± 4.6b  | 3.8 ± 0.4c   |
| Rubisco (μmol m$^{-2}$ s$^{-1}$) | 44.2 ± 3.2a  | 5.3 ± 0.7a   | 6.4 ± 1.0a   |
| $k_{CA}$ (mol m$^{-2}$ s$^{-1}$) | 6.1 ± 0.4a   | 631 ± 113a   | 2818 ± 64b   |
| CAleaf (μmol m$^{-2}$ s$^{-1}$) | 783 ± 153a   | 831 ± 113a   | 2818 ± 64b   |
| Isotopic equilibrium ($\theta$) |              |              |              |
| Predicted (Eq. 12) | 0.98 ± 0.01a | 0.98 ± 0.01a | 1.00 ± 0.00a |
| Measured (Eq. 11) | 0.62 ± 0.02a | 0.62 ± 0.07a | 0.91 ± 0.05b |
| Leaf parameters            |              |              |              |
| Total leaf N (mmol m$^{-2}$) | 132 ± 23a   | 120 ± 10a    | 130 ± 4a     |
| LMA (g m$^{-2}$)            | 34.0 ± 2.9a  | 38.5 ± 3.2a  | 30.8 ± 1.6a  |

$^{13}$CO$_2$ and $^{18}$OO Discrimination

Carbon isotope discrimination ($\Delta^{13}C$) decreased slightly but not significantly in the Pp mutant as compared with the wild type (Table I). In the pp mutant the value of $\Delta^{13}C$ was approximately 5-fold higher than in wild-type and Pp plants, and $p/n$ was 0.82 compared to 0.39 in the wild type (Table I; Fig. 1). The values of $\Delta^{18}O$ were not significantly different between the wild-type and the Pp plants but $\Delta^{18}O$ was 12-times higher in the pp mutant compared to the wild type (Table I; Fig. 2). The proportion of CO$_2$ in isotopic equilibrium with water at the site of oxygen exchange ($\theta$) predicted from in vitro CA assays (Eq. 12) was substantially higher in the wild-type and Pp plants than the $\theta$ values estimated from $\Delta^{18}O$ measurements (Eq. 11; Table I). However, $\theta$ calculated from in vitro CA assays (Eq. 12) and $\Delta^{18}O$ measurements (Eq. 11) were similar in the pp mutant (Table I). The $\Delta^{18}O$ increased with $p/n$ as predicted from Equation 8, but the measured values of $\Delta^{18}O$ were less than those predicted at full isotopic equilibrium for the wild-type and Pp mutant (Fig. 2). The pp mutant had a high $p/n$ but wild-type levels of extractable CA activity and the measured values of $\Delta^{18}O$ were closer to the predicted values of $\Delta^{18}O$, compared to wild-type plants (Table I; Fig. 2). The theoretical line of full isotopic equilibrium in Figure 2 was calculated using Equation 8, assuming an average value of the oxygen isotope composition of CO$_2$ at the site of exchange during photosynthesis ($\Delta_{\theta}$), taken from all plants in Table II, of 39.2‰. The values of $\Delta_{\theta}$ were not significantly different between the plants (Table II).

The $\delta^{18}O$ of water at the site of evaporation ($\delta_e$) was similar in the wild-type and Pp plants but significantly

![Figure 1. Carbon isotope discrimination ($\Delta^{13}C$) as a function of the ratio of intercellular to ambient pCO$_2$ ($p/p_a$) in wild-type and mutant A. edulis plants. The dashed line represents the theoretical relationship of $\Delta^{13}C_i$ and $p/p_a$ during CA photosynthesis where $\phi = 0.24$, using Equation 6. The solid lines represent the theoretical relationships of $\Delta^{13}C_i$ and $p/p_a$ using the C$_3$ model (Eq. 3). Gas-exchange conditions are as in Table I. Each point represents the means ± SE of measurements made on three to five leaves from separate plants from wildtype (), Pp mutant (A), and pp mutant (O).](image-url)
Table I. Shown are the means of the theoretical relationship of equilibrium and different letters indicate significant differences between plants at ratio of mesophyll cytosolic to ambient CO2 partial pressure (p_{m}/p_{a}). p_{m} was calculated with p_{m} = 1 mol m^{-2} s^{-1} bar^{-1}. The line represents the theoretical relationship of Δ^{18}O and p_{m}/p_{a} at full isotopic equilibrium where a = 7.7^{18}_{16} and Δ_{a} = 33.7^{18}_{16} (Eq. 8) and the CO2 supplied to the leaf had a Δ^{18}O of 24^{18}_{16} relative to VSMOW. Symbols are as in Figure 1 and measurement conditions are as in Table I. The inset shows the expanded scale of Δ^{18}O for the wild-type and Pp plants.

more enriched in the pp plants (Table II). The 18O enrichment of CO2 compared to the atmosphere at the site of exchange in full oxygen isotope equilibrium (Δe), the ratio of the water vapor pressure in the atmosphere to the leaf intercellular spaces (e_{s}/e_{a}) were also similar in the wild-type and Pp plants but were different in the pp plants (Table II). The residence time of CO2 in the aqueous phase within the leaf (τ = p_{m}/F_{Pp}) and the intercellular pCO2 were greater in the pp plants compared to the wild-type and Pp plants (Table II).

Stomatal Response

Online measurements of Δ^{13}C and Δ^{18}O leaf gas exchange at 531 μbar CO2 partial pressure indicated that steady-state leaf conductance in the homozygous pp mutant was reduced compared to the wild type under these conditions (Table I). Further analysis showed that stomatal conductance (g_{s}) under growth conditions (9.8 mbar CO2, 400 μmol quanta m^{-2} s^{-1}, air humidity 29–32 mmol mol^{-1}, leaf temperature of 30°C) was higher in the pp mutant (0.7 ± 0.1 mol m^{-2} s^{-1}) compared to wild type (0.3 ± 0.1 mol m^{-2} s^{-1}; Fig. 3). However, g_{s} declined in the pp mutant and increased in the wild type when plants were rapidly shifted from growth conditions to similar conditions with low CO2 (364 μbar; Fig. 3). To compare the response of g_{s} under laboratory conditions the pp and wild-type plants were dark adapted overnight in ambient CO2 (364 μbar) and gas exchange was measured the following day. The two genotypes showed no difference in g_{s} under steady-state conditions in the dark before the onset of illumination (Fig. 4). However, the homozygous pp mutant had an approximately 3-times lower rate of stomatal opening (Fig. 4) in response to light under 364 μbar CO2 than the wild type and g_{s} after 90 min in the light was only 41% of wild-type values (Fig. 4). In spite of the difference in steady-state conductance, both the wild-type and the pp mutant reached half their maximal conductance within approximately 13 min of the onset of illumination (Fig. 4). Long-term (5 h) measurements of g_{s} under the same conditions showed that the difference in stomatal conductance between the wild-type and pp mutant plants was maintained (data not shown).

To examine whether the guard cells in the pp mutant were sensitive to changes in pHCO2, leaf gas exchange was measured under steady-state conditions (364 μbar CO2, 2,000 μmol quanta m^{-2} s^{-1} and a vapor-pressure difference of 10 mbar) and then the pHCO2 was dropped to 48 μbar. Compared to the wild type, the pp mutant had a lower initial rate of stomatal opening and steady-state conductance reached only half of wild-type values (Fig. 5). As in the response to light, the halftime of stomatal opening to CO2 was similar in both types of plants (14.1 ± 0.9 min and 11.2 ± 0.7 min, for wild-type and pp mutant, respectively; Fig. 5).

Epidermal PEPC Content and Stomatal Density

In agreement with previous reports (Dever et al., 1995, 1996, 1997; Maroco et al., 1998b) and our activity measurements (Table I), leaf tissue of the pp mutant showed a large decrease in the content of PEPC, detected either by Coomassie staining or by immunoblot (Fig. 6). An epidermal fraction showed a protein profile similar to that of whole leaves and was identical in wild-type and pp mutant with the exception of

| Plants | Δ_{e} | Δ_{a} | e_{s}/e_{a} | τ s^{-1} | μ |
|--------|-------|-------|-------------|----------|---|
| WT     | 24.0 ± 0.8a | 20.6 ± 0.6a | 36.9 ± 1.4a | 0.41 ± 0.03a | 1.9 ± 0.1a | 166 ± 19a |
| pp     | 24.7 ± 0.9a | 21.6 ± 3.0a | 37.6 ± 1.5a | 0.39 ± 0.03a | 2.8 ± 0.2a | 199 ± 29a |
| Pp     | 29.9 ± 0.9b | 38.8 ± 1.1b | 43.2 ± 1.7a | 0.21 ± 0.03b | 16.5 ± 2.7b | 431 ± 14b |

Table II. Oxygen isotope exchange parameters of wild-type A. edulis and PEPC mutants

The δ^{18}O of water at the site of evaporation (δ_{i}), δ^{18}O enrichment of CO2 compared to the atmosphere at the site of exchange in full oxygen isotope equilibrium (Δ_{ae}), the oxygen isotope composition of CO2 at the site of exchange during photosynthesis (Δ_{wa}), the ratio of the vapor pressure in the atmosphere to the leaf intercellular spaces (e_{s}/e_{a}), the residence time of CO2 in the aqueous phase within the leaf (τ s^{-1}), and the intercellular pCO2 (p) in wild-type (WT) and homozygous (Pp) mutant A. edulis plants. Source water was −5.3 ± 0.3. Measurement conditions as in Table I. Shown are the means ± se of measurements made on three to five leaves from separate plants. Statistical analysis was conducted using an ANOVA and different letters indicate significant differences between plants at P < 0.05.
D data are the means of measurements of four different wild-type and five different pp mutants, error bars represent SE; wild type (○) and pp mutant (●).

Figure 3. Stomatal conductance for wild-type and pp mutant plants under growth conditions (indicated by arrow) at 400 μmol quanta m⁻² s⁻¹, leaf temperature of 30°C, and the leaf chamber humidity was 29.6 ± 0.6 and 32.4 ± 0.4 mmol mol⁻¹ for the wild-type and pp mutant, respectively. Plants were subsequently transferred from the growth cabinets at time zero and a leaf was immediately placed into the gas-exchange chamber under growth conditions except the CO₂ concentration was 360 μbar instead of 9.8 μbar. The leaf chamber humidity was maintained at 30.01 ± 0.01 for both the wild-type and pp mutant. Data are the means of measurements of four different wild-type and five different pp mutants, error bars represent se; wild type (○) and pp mutant (●).

the band corresponding to PEPC. The pp mutant epidermis contained 53% ± 4% of wild-type PEPC as determined by western analysis on three wild-type and four pp mutant plants (Fig. 6 shows one such representative western). The number of stomata per unit leaf area was greater in the pp mutant (63% and 77% greater than in the wild type on the adaxial and abaxial sides, respectively); however, the stomatal index remained unchanged (Table III).

DISCUSSION

1³CO₂ Isotope Discrimination in the PEPC Mutants

The low activity of PEPC caused rates of net CO₂ assimilation in the heterozygous (Pp) and the homozygous (pp) PEPC mutant to be significantly less than wild-type plants (Table I) when measured under ambient CO₂ (531 μbar) concentrations as previously reported (Dever et al., 1997, 1998; Maroco et al., 1998a, 2000; Kiirats et al., 2002). Limited activity of PEPC during C₄ photosynthesis causes a decrease in the initial CO₂ carboxylation reaction and reduces the capacity of the C₄ pump to concentrate CO₂ within the BSCs (von Caemmerer, 2000; Cousins et al., 2006a). In the model of C₄ carbon isotope discrimination (Eq. 6 in “Materials and Methods”) the main factors that influence Δ¹³C are changes in the intercellular to ambient CO₂ partial pressures (pᵢ/pₐ), the fraction of CO₂ fixed by PEPC that subsequently leaks out of the BSC (φ), and the combined fractionation of PEPC and the isotopic equilibrium during dissolution of CO₂ and conversion to bicarbonate (b₄), which is dependent on the ratio of PEPC to CA activity (Farquhar, 1983). During C₄ photosynthesis decreases in φ and b₄ are both predicted to cause values of Δ¹³C to decrease (Farquhar, 1983). The values of Δ¹³C and pᵢ/pₐ in the Pp mutant were not significantly different from the wild-type plants even though the Pp plants had lower rates of net CO₂ assimilation (Table I). This implies that neither φ nor the b₄ value was significantly different between wild-type and Pp plants. We conclude that although photosynthetic rates were lower in the Pp mutant compared to wild type, the Δ¹³C remained constant because the balance in the C₃ and C₄ cycles was not altered.

The very low PEPC activity in the pp plants (Table I) severely inhibited the initial carboxylation step of the C₄ photosynthetic pathway causing the rates of net CO₂ assimilation to decrease considerably relative to wild type (Table I; Fig. 4B). The value of pᵢ/pₐ increased in the pp plants compared to wild type and the Pp plants (Table I) and according to the Δ¹³C model during C₄ photosynthesis (Eq. 6) a decrease in pᵢ/pₐ leads to a decrease in Δ¹³C at φ values of less than

Figure 4. Light induction of stomatal conductance (A) and net CO₂ assimilation (B) in wild-type and the pp mutant. Plants were grown under 9.8 mbar CO₂ and then transferred to ambient CO₂ in the dark overnight. Leaf gas exchange was measured for several minutes prior to the start of illumination at 2,000 μmol quanta m⁻² s⁻¹ (indicated by the arrow). The rate of stomatal opening for the wild-type and pp mutant was 7.8 ± 1.9 and 2.4 ± 0.5 (mmol water m⁻² s⁻¹), respectively. The ambient CO₂ partial pressure was maintained at 364 μbar for the duration of the measurements. Data are the means of measurements of six different plants, error bars represent se; wild type (○) and pp mutant (●).
approximately 30% (Fig. 1). However, values of $\Delta^{13}C$ were dramatically higher in the $pp$ plant compared to the wild-type and $Pp$ plants (Figs. 1) even though $p_{i}/p_{a}$ was higher in the $pp$ plants and the $b_{i}$ would be close to $-5.7$ (Table I; Eq. 7). This implies that the simplistic model of $C_{4}$ isotope exchange presented here does not provide an accurate description of the processes contributing to $\Delta^{13}C$ in the $pp$ plants (see below).

### $C_{4}$-Like Isotope Discrimination in the $pp$ Mutant

As reported previously, the defective $C_{4}$ cycle in the $pp$ plants necessitates the direct diffusion of atmospheric $CO_{2}$ into the BSC for $CO_{2}$ assimilation (Dever et al., 1995, 1997; Maroco et al., 1998a, 1998b; Kiirats et al., 2002) and the instantaneous $\Delta^{13}C$ in these plants is more accurately described by the $\Delta^{13}C_{3}$ model for $C_{3}$ photosynthesis with a low conductance of $CO_{2}$ diffusion to the site of Rubisco carboxylation ($g_{b}$) within the BSC (see below). Assuming that the discriminations associated with photorespiration and respiration are negligible (see Eq. 1), we estimated an average $CO_{2}$ conductance from the intercellular air space to the site of Rubisco carboxylation within the BSC ($g_{b}$) of $33 \pm 10$ mmol m$^{-2}$ s$^{-1}$ in the $pp$ plants (Eq. 5). This value of $g_{b}$ is toward the high end of the range reported in the literature from various $C_{4}$ plants and approximately 4-times higher than the $g_{bs}$ value reported by Kiirats et al. (2002) in their experiments with the $pp$ plants. Mathematical modeling of the $C_{4}$ photosynthetic pathway suggests that such high conductance values need to be matched with high biochemical capacity to maintain low values of leakiness (for discussion see von Caemmerer, 2003).

To estimate the effect of photorespiratory and respiratory fractionation on our estimates of $g_{b}$, we used discrimination factors for photorespiration ($f = 10\%$) and for respiration ($e = -6\%$) reported from the literature (Gillon and Griffiths, 1997; Ghoshhaieh et al., 2003; Igamberdiev et al., 2004). Our measurements were made at low $O_{2}$ partial pressures so that
the expected fractionation from photorespiration is only about 0.17$\%$ at a $\Gamma$, of 9.25 $\mu$bar. The effect of respiratory fractionation is between $-1.67\%$ to $1\%$. It therefore appears that neither photorespiration nor respiration can easily account for the high $\Delta_{13}C$ values measured in the $pp$ plants and the major contributing factor to the high $\Delta_{13}C$ values in these plants is the direct diffusion of CO$_2$ from the intercellular air spaces to the site of Rubisco carboxylation with the BSC.

Determining absolute values of $g_i$ in C$_4$ plants and BSC leakiness is difficult as these parameter are effected by numerous factors including growth and measurement conditions (Henderson et al., 1992; Cousins et al., 2006a; Kubásek et al., 2007). Potentially $g_i$ can be influenced by changes in the diffusivity of CO$_2$ across cell walls, the cytoplasm in the mesophyll and BSC, and across plasma and chloroplast membranes (von Caemmerer and Furbank, 2003). Additionally, the BSC surface area to leaf area ratio can also influence $g_i$; however, we saw no differences in leaf vein density in the $pp$ plants (data not shown), indicating that the BSC surface area on a leaf area basis had not increased. However, because the $pp$ plants rely on the direct diffusion of CO$_2$ into the BSC to sustain photosynthesis, the internal conductance of CO$_2$ may be greater than in wild-type plants, increasing the CO$_2$ concentration within the BSC.

C$^{18}$O Isotope Discrimination and CO$_2$/Water Isotopic Equilibrium

In a leaf, the oxygen isotope composition of CO$_2$ is determined by the isotope composition of leaf water at the site of evaporation ($\delta_o$) and CA activity. The exchange of $^{18}$O between CO$_2$ and water is facilitated by CA, which catalyzes the interconversion of CO$_2$ and bicarbonate (HCO$_3$), and high CA activity will increase the proportion of CO$_2$ in isotopic equilibrium with the water. Based on calculated values of $\delta_o$ (Table II) the C$^{18}$O discrimination ($\Delta_{18}O$) was low in the wild-type plants compared to the high levels of leaf CA (CA$_{leaf}$, Table I). The extent of isotopic equilibrium ($\theta$) measured from $\Delta_{18}O$ (Eq. 11) was also low in the wild-type plants relative to $\theta$ estimated from CA activity using Equation 12 (Table I). These findings are similar to previous work with another C$_4$ dicot, F. bidentis, which also had low $\Delta_{18}O$ and $\theta$ measured from $\Delta_{18}O$ compared to the high rates of CA$_{leaf}$ (Cousins et al., 2006b). This further suggests that the total leaf CA activity in C$_4$ dicots does not represent the CA activity associated with the CO$_2$-water oxygen exchange that influences $\Delta_{18}O$ (Cousins et al., 2006b).

The value of $\theta$ is related to the mean number of hydration reactions a CO$_2$ molecule experiences inside a leaf. This in turn is the product of residence time ($\tau = p_m/F_m$) and the hydration constant of leaf CA ($k_{CA}$), where $p_m$ is the mesophyll pCO$_2$ and $F_m$ is the gross flux of CO$_2$ into the leaf (Eq. 12). The low photosynthetic rates increase the residence time of CO$_2$ as $p_m$ increases (Tables I and II). Therefore, the number of hydration reactions per CO$_2$ increases when rates of net CO$_2$ assimilation are reduced by low PEPC activity. The amount of CA, expressed as the rate constant, was similar in the wild-type and PEPC mutants (Table I); however, under similar gas-exchange conditions the CA$_{leaf}$ activity in the PEPC mutants were higher (Table I). The increase in CA$_{leaf}$ in these plants is attributed to an increase in substrate availability for CA due to the lack of photosynthetic CO$_2$ drawdown caused by the low PEPC activity. The increase in $\theta$ in the $pp$ plants suggests that in wild-type A. edulis the CA$_{leaf}$ activity does not allow full isotopic equilibrium between the CO$_2$ and water within the leaf under steady-state conditions.

Our study suggests that in C$_4$ species leaf CA activity cannot readily be used as an indicator of the extent of $^{18}$O equilibration as has had been suggested by Gillon and Yakir (2001). This might be because not all of the CA located in mesophyll cytosol in C$_4$ species is available at the site of oxygen exchange compared to C$_3$ species where CA is located in chloroplasts that appress intercellular airspaces (Poincelot, 1972; Ku and Edwards, 1975). Alternatively, the extent of $^{18}$O equilibration may be miscalculated if the estimated values of $\delta_o$ do not accurately describe the isotopic composition of water at the site of oxygen exchange between leaf water and CO$_2$.

### Table III. Stomatal density and index of wild-type A. edulis and PEPC $pp$ mutants

| Leaf Side | Plant  | Stomatal Density | Stomatal Index (Stomata/Total Epidermal Cells) |
|-----------|--------|-----------------|-----------------------------------------------|
|           |        | mm$^{-2}$       |                                               |
| Adaxial   | WT     | 144 ± 11a       | 0.22 ± 0.01a                                  |
|           | $pp$   | 233 ± 35b       | 0.22 ± 0.01a                                  |
| Abaxial   | WT     | 123 ± 7a        | 0.23 ± 0.01a                                  |
|           | $pp$   | 218 ± 35b       | 0.22 ± 0.01a                                  |

Stomatal Conductance in Response to Light and Atmospheric CO$_2$

The $pp$ mutant had low stomatal conductance ($g_s$) during steady-state gas-exchange conditions (at 531 $\mu$bar CO$_2$) relative to the $Pp$ mutant and wild-type plants (Table I). Additionally, $g_s$ in the $pp$ mutant decreased but increased in the wild type when leaves were rapidly transferred from the high CO$_2$ (9.8 mbar) growth conditions into air (364 $\mu$bar CO$_2$) at a constant leaf chamber humidity (Fig. 3). The higher $g_s$ under elevated CO$_2$ reported here is consistent with previous reports that $g_s$ is generally greater under supererelevated
CO₂ (above 4.0 mbar) compared to air CO₂ concentrations (see review and references within Wheeler et al., 1999). Low gs in the pp mutant at ambient CO₂ (364 or 531 μbar) is different from previous publications where stomatal conductance was generally not affected when the photosynthetic capacity was reduced due to antisense silencing of either Rubisco or Rubisco activase in the C₃ dicot F. bidentis (von Caemmerer et al., 1997b, 2005). In C₄ plants PEPC initiates carbon fixation in mesophyll cells but also plays an important role in providing malate as a counter ion and an osmoregulator in the guard cells to help counterbalance the large influx of potassium ions during stomatal opening (Vavasseur and Raghavendra, 2005). The content of PEPC in the pp mutant was reduced at both the whole leaf level as well as in the epidermal tissue, indicating that the C₄ PEPC gene is the same as the guard cell gene in A. edulis (Table I; Fig. 6). Therefore, reduced PEPC activity in the pp mutant not only limited photosynthetic rates but likely impaired the accumulation of malate in the guard cells. Studies with epidermal peels have demonstrated strong correlations between stomatal opening and malate accumulation in guard cells (Allaway, 1973; Pearson, 1973; van Kirk and Raschke, 1978) and with the use of a PEPC inhibitor (DCDP) it has also been shown that stomatal opening is restricted when PEPC activity is reduced in epidermal strips (Parvathi and Raghavendra, 1997). In epidermal strips, the importance of malate as a counter ion to K⁺ is influenced by the availability of chloride (van Kirk and Raschke, 1978; Schnabl and Raschke, 1980; Willmer and Fricker, 1996). The low gs and epidermal PEPC content in the pp mutants show that maximum stomatal opening is dependent on PEPC activity and presumably malate in the guard cells in vivo.

In the dark gs was similar in pp mutant and wild type at air CO₂ concentrations (364 μbar), but the rate of opening during the light induction was slow in the pp mutant and the stomata were able to maintain only a third of the conductance under steady-state conditions compared to wild-type plants (Fig. 4). These findings provide further support that PEPC is necessary for stomatal opening in response to light (Asai et al., 2000). Interestingly, both the wild-type and pp mutants reached half their maximal gs rate at similar times (Fig. 4), indicating that the perception of changing light conditions was not inhibited in the pp mutant, only that the stomata could not open as quickly and were unable to establish high rates of conductance.

Stomatal conductance increased in both the wild-type and the pp mutant in response to lowering pCO₂ (Fig. 5). However, gs was slower to respond to the shift in CO₂ in the pp mutants and did not reach similar rates as in the wild-type plants (Fig. 5). The pp mutant can therefore sense the change in CO₂ availability but lacks the ability to achieve maximal values of gs in response to conditions that normally stimulate gs. As with the light response, even though gs in the pp mutant did not reach similar values to the wild-type plants, the values of gs increased about three times in response to low CO₂ availability in both plants (Fig. 5).

The shifts in gs in response to changing light and pCO₂ did not correlate with the changes in net CO₂ assimilation in the pp plants (Fig. 5). For example, there was only a slight increase in net CO₂ assimilation from the dark to light transition in the pp plants but stomatal conductance increased about 8 times (Fig. 4). This increase in gs during the light induction was less than in the wild-type plants but was still significant. Changes in net CO₂ assimilation were also minor in response to CO₂ in the pp plants (Fig. 5) but gs was approximately 3-times greater under the lower CO₂ concentrations (Fig. 5). Although it has been demonstrated that there is a tight correlation between gs and photosynthetic capacity in both C₃ and C₄ plants (Wong et al., 1985), the use of antisense and photosynthetic mutants indicates that under certain conditions this relationship may not hold (for review, see von Caemmerer et al., 2004a).

The low gs in the pp mutant could have been attributed to reduced stomatal density compared to wild-type plants; however, stomatal density was approximately 1.5-times greater in the pp mutant, both adaxial and abaxial, than in the wild type (Table III). In fact stomatal conductance was higher in the pp mutant compared to the wild type under the 9.8 mbar CO₂ growth conditions (Fig. 3), which may in part be due to the alleviation of PEPC limitation on gs in the pp mutant by high CO₂ availability coupled with the higher stomatal density in the pp mutant (Table II). The stomatal index in these two plants was similar, indicating that the increase in stomata in the pp plants was due to a general increase in the number of total epidermal cells (Table III). The increase in stomatal density may help alleviate the BSC CO₂ limitation in the pp plants that rely on direct fixation of atmospheric CO₂ by Rubisco.

CONCLUSION

The reduction in PEPC activity in A. edulis reduced rates of net CO₂ assimilation and Δ¹³C and Δ¹⁸O were dramatically increased in the homozygous PEPC mutant (pp). The high Δ¹³C value in the pp plants is likely caused by the direct diffusion of CO₂ from the intercellular air spaces to the site of Rubisco carboxylation within the BSC. The isotopic equilibrium between leaf water and the intercellular pCO₂ appears to be overestimated by in vitro measurements of total leaf CA activity compared to isotopic equilibrium determined from Δ¹⁸O measurements in wild-type plants. Lower stomatal conductance under steady-state conditions and the slower responses of stomata to changing light and CO₂ conditions in the pp mutant corresponded with reduced PEPC content in the epidermal tissue, implicating the C₄ isoform of PEPC in controlling stomatal movement.
MATERIALS AND METHODS

Growth Conditions

Seeds from the F2 population of *Amaranthus edulis* LaC4 2.16 mutant deficient in PEPC activity (Dever et al., 1995, 1997) and from the corresponding wild type were grown under 9.8 mbar of CO2 in a controlled environment growth cabinet at an irradiance of 400 μmol quanta m−2 s−1 at plant height and air temperature of 27°C during the day and 18°C at night, with a day length of 14 h. Plants were grown in 5 L pots in garden mix with 2:4 to 4 g Osmocote/L soil (15/48/10.8/12 N/P/K/Mg + trace elements: B, Cu, Fe, Mn, Mo, Zn; Scotts Australia Pty Ltd.) and watered daily. The mutant plants were screened by gas exchange and PEPC activity (see below).

Gas-Exchange Measurements

Online 13CO2 and C18OO Discrimination

The uppermost fully expanded leaves were placed into the leaf chamber of the LI-6400 portable gas-exchange system (LI-COR) and equilibrated under measurement conditions for a minimum of 1.5 h (Cousins et al., 2006a, 2006b). Air entering the leaf chamber was prepared by using mass flow controllers (MKS instruments) to obtain a gas mix of 900 μbar dry N2 and 48 μbar O2. A portion of the nitrogen/oxygen air was used to zero the mass spectrometer to control O2 and N2 to produce NO2 with the mass spectrometer source. Simultaneously, measured CO2 of the air surrounding the leaf, in the intercellular air spaces and at the site of Rubisco carboxylation, respectively, a (4.4%) is the fractionation during diffusion of CO2 in air, α is the combined fractionation due to dissolution and diffusion of CO2 in water (1.8%), and the fractionation by Rubisco is (b) = 30°C (Reeske and Oleary, 1984). G is the CO2 compensation point in the absence of day respiration. G is the rate of mitochondrial respiration, α and δ are the discrimination factors of respiration and photorespiration with respect to the average carbon composition associated with respiration and photorespiration, respectively, and s is the Rubisco carboxylation efficiency (Farquhar et al., 1982; Evans et al., 1986). The carboxylation efficiency is given by

\[ k = V_{max} \frac{(p_i + K_i)(1 + O/K_i)}{p_i} \]

where \( V_{max} \) denotes the maximal Rubisco activity, \( K_i \) and \( K_o \) are the Michaelis Menten constants for CO2 and O2, respectively, and \( O \) stands for O2 partial pressure.

The discrimination that would occur if the partial pressure of CO2 in the chloroplast equals the intercellular pCO2 and ignoring fractionations associated with respiration and photorespiration is usually given by

\[ \Delta_e = a_i(b_i-a_i)p_i/p_f \]

Subtracting Equation 3 from Equation 1 shows that the difference between the \( \Delta_e \) and the measured \( \Delta^{13}C \) is inversely proportional to the conductance to CO2 diffusion from the intercellular airspace to the site of Rubisco carboxylation (g, Evans et al., 1986; von Caemmerer and Evans, 1991; Evans and von Caemmerer, 1996):

\[ \Delta_e - \Delta^{13}C = \frac{(b_i - a_i)a_i/p_i}{p_i} \]

\[ g_e = \frac{(b_i - a_i)a_i/p_i}{\Delta_e - \Delta^{13}C - (eR_d/k + fT_i)} \]

Thus the measurements were made under the elevated CO2 growth conditions as described above by bringing the gas-exchange system into the growth chamber. Leaves were then turned on to give an irradiance of 2,000 μmol m−2 s−1 and maintained at that level for the rest of the experiment. After the initial 90 min in the light the CO2 concentration in the leaf chamber was decreased to 48 μbar and maintained for a further 90 min. Leaf chamber humidity and temperature were kept at 18 to 20 mmol mole−1 and 25°C, respectively, for the duration of the measurements. Flow rate over the leaf was 500 μmol s−1.

Stomatal Responses

To characterize the stomatal response of *A. edulis* plants, gas-exchange measurements were made with the LI-6400 portable gas-exchange system on two different sets of plants grown under identical conditions, young plants with an average of 10 leaves/plant and older plants with 20 to 25 leaves/plant. Data from the two sets of plants were combined in the final figures and tables. Light was provided by a red/blue LED light source (LI-6400-02B, LI-COR). Plants were dark adapted overnight at ambient CO2 conditions and equilibrated under measurement conditions for a minimum of 1.5 h (Cousins et al., 2006a, 2006b). Gas-exchange measurements, from silicone rubber impressions taken by gas exchange and PEPC activity (see below), were determined from both sides of the leaves (von Caemmerer et al., 2004a). Stomata and epidermal cells were counted from positives made from the impressions with nail polish, in 10 different fields of view per leaf, with a compound microscope using a magnification of 200-fold. Digital photographs of each field were taken and cells counted with the publicly available Image J software (http://rsb.info.nih.gov/ij/).

Calculations of 13CO2 Discrimination

To calculate the conductance to CO2 diffusion from intercellular airspace to the site of Rubisco carboxylation in the BSCs in the pp mutant we used the model of C4 carbon isotope discrimination (Δ4C) developed by Farquhar et al. (1982). This model is given as:

\[ \Delta^{13}C_i = a_i(p_i - p_a)/p_i + a_i(p_i - p_f)/p_i + b_i p_f/p_i - (eR_d/k + fT_i)/p_i \]

where \( p_i \) and \( p_a \) represent the pCO2 of the air surrounding the leaf, in the intercellular air spaces and at the site of Rubisco carboxylation, respectively, and \( \Delta^{13}C \) is the discrimination that would occur if the partial pressure of CO2 in the chloroplast equals the intercellular pCO2 and ignoring fractionations associated with respiration and photorespiration is usually given by

\[ \Delta_e = a_i(b_i-a_i)p_i/p_f \]

\[ g_e = \frac{(b_i - a_i)a_i/p_i}{\Delta_e - \Delta^{13}C - (eR_d/k + fT_i)} \]

such that \( g_e \) can be estimated after rearranging Equation 4 from

\[ \Delta_e = \Delta^{13}C_i - a_i(b_i-a_i)a_i/p_i \]

\[ g_e = \frac{(b_i - a_i)a_i/p_i}{\Delta_e - \Delta^{13}C - (eR_d/k + fT_i)} \]

The model of C4 carbon isotope discrimination (Δ4C) from Farquhar (1983) was used to determine which factors in the model would influence \( \Delta^{13}C \) consistent with our experimental data in the pp mutant and wild-type plants. The simplified model predicts that

\[ \Delta^{13}C_i = a_i + b_i(a_i - \frac{30°C}{9} \times \frac{s}{18°C}) \times p_i/p_f \]

where \( s \) (1.8%) is the fractionation due to dissolution and diffusion of CO2 in water (1.8%), and the fractionation by Rubisco is (b) = 30°C (Reeske and Oleary, 1984). G is the CO2 compensation point in the absence of day respiration. G is the rate of mitochondrial respiration, α and δ are the discrimination factors of respiration and photorespiration with respect to the average carbon composition associated with respiration and photorespiration, respectively, and s is the Rubisco carboxylation efficiency (Farquhar et al., 1982; Evans et al., 1986). The carboxylation efficiency is given by

\[ k = V_{max} \frac{(p_i + K_i)(1 + O/K_i)}{p_i} \]

where \( V_{max} \) denotes the maximal Rubisco activity, \( K_i \) and \( K_o \) are the Michaelis Menten constants for CO2 and O2, respectively, and \( O \) stands for O2 partial pressure.

The discrimination that would occur if the partial pressure of CO2 in the chloroplast equals the intercellular pCO2 and ignoring fractionations associated with respiration and photorespiration is usually given by

\[ \Delta_e = a_i(b_i-a_i)p_i/p_f \]

\[ g_e = \frac{(b_i - a_i)a_i/p_i}{\Delta_e - \Delta^{13}C - (eR_d/k + fT_i)} \]

such that \( g_e \) can be estimated after rearranging Equation 4 from

\[ \Delta_e = \Delta^{13}C_i - a_i(b_i-a_i)a_i/p_i \]

\[ g_e = \frac{(b_i - a_i)a_i/p_i}{\Delta_e - \Delta^{13}C - (eR_d/k + fT_i)} \]

Calculations of C18OO Isotope Discrimination

Discrimination against C18OO (Δ^18O) when water and CO2 at the site of exchange are at full isotopic equilibrium (θ = 1) can be predicted as (Farquhar and Lloyd, 1993)
where \( a' \) is the differential discrimination (7.7\(^\circ\)) and \( e \) is calculated as
\[
\Delta^{18}O = \frac{a' + c_{\text{a}}}{1 - c_{\text{e}}} \tag{8}
\]
where \( \Delta^{18}O \) is the equilibrium fractionation between water and CO\(_2\) (Cernusak et al., 2004). The equilibrium fractionation between water and CO\(_2\) is determined as the product of the CA hydration rate constant (Price, 1989; von Caemmerer et al., 2004b; Cousins et al., 2006a, 2006b).

\[
\Delta^{18}O = \frac{\Delta P_{\text{H}2\text{O}}(\text{a}) + \epsilon_{\text{a}}}{1 - \epsilon_{\text{e}}} \tag{9}
\]

The equilibrium fractionation between liquid water and water vapor (\( \epsilon_{\text{a}} \) and \( \epsilon_{\text{e}} \)) is determined as the product of the CA hydration rate constant (Cernusak et al., 2004) and Cousins et al. (2006b).

\[
\delta_{\text{a}} = \delta_{\text{i}} + \epsilon_{\text{a}} + \epsilon_{\text{e}} + (\delta_{\text{a}} - \delta_{\text{i}} - \epsilon_{\text{e}}) \frac{\epsilon_{\text{a}}}{\epsilon_{\text{e}}} \tag{10}
\]

The proportion of CO\(_2\) in isotopic equilibrium with water at the site of oxygen exchange (\( \theta \)) can be estimated from

\[
\theta = \frac{\Delta^{18}O + a(1 + \epsilon_{\text{e}})}{\Delta^{18}O + a(1 + \epsilon_{\text{e}})} \tag{11}
\]

where \( \Delta^{18}O \) is the oxygen isotope composition of CO\(_2\) at the site of exchange during photosynthesis (Gillon and Yakir, 2000a, Cousins et al., 2006b).

It has been suggested that the extent of \( \theta \) in a leaf can also be calculated from in vitro CA assays coupled with the unidirectional flux of CO\(_2\) into the leaf (Gillon and Yakir, 2000a, 2000b, 2001) from the equation initially developed by Mills and Urey (1940):

\[
\theta = 1 - \frac{e^{-f_{\text{CA}}}}{1 + e^{-f_{\text{CA}}}} \tag{12}
\]

where \( f_{\text{CA}} \) represents the mean number of hydration reactions for each CO\(_2\) molecule inside the leaf (Gillon and Yakir, 2001). Leaf CA activity (\( f_{\text{CA}} \)) is determined as the product of the CA hydration rate constant (\( k_{\text{CA}} \), mol m\(^{-2}\) s\(^{-1}\) bar\(^{-1}\)) and the mesophyll pCO\(_2\) (\( p_{\text{a}} \)). The rate constant \( k_{\text{CA}} \) is calculated from in vitro measurements of CA activity in leaf extracts (see below). The gross influx of CO\(_2\) into a leaf \( f_{\text{CA}} \), where \( g_{\text{a}} \) is the total conductance of CO\(_2\) from the atmosphere to the site of CO\(_2\)-water exchange; Gillon and Yakir, 2000a), as well as \( p_{\text{a}} \), determine the residence time (\( \tau = p_{\text{a}}/f_{\text{CA}} \)) of CO\(_2\) within the leaf. The relationship of \( f_{\text{CA}}/f_{\text{A}} \) indicates that conditions that influence \( p_{\text{a}} \) or \( f_{\text{A}} \) will alter the value of \( \theta \).

**Enzyme Activities**

Enzyme activities were determined on approximately 1 cm\(^2\) discs taken from the same leaves used for gas exchange. Leaf samples were collected after the gas-exchange measurements and subsequently frozen in liquid nitrogen and stored at \(-80^\circ\)C. Tissue was ground on ice in 600 µL of extraction buffer (50 mM HEPES-KOH, pH 7.4, 10 mM diethiothreitol, 1% polyvinylpyrrolidone, 1 mM EDTA, 0.2 mM NADH, 5 mM Glc-6-P, 1 mM NaHCO\(_3\), and 2% (w/v) polyvinylpolypyrrolidone, 0.1% (v/v) Triton X-10, and 4% (v/v) of protease inhibitor cocktail, using a 2-mL glass homogenizer. Samples were centrifuged in a cooled microcentrifuge at maximum speed for 4 min. The green pellet was discarded and the supernatant was brought to a final concentration of SDS (2% (w/v) and heated to 65°C in a water bath for 10 min.

**Protein Extraction and Immunoblotting**

Soluble proteins from 1.28 cm\(^2\) leaf discs or 100-mg of epidermal fragments were extracted on ice in 0.7 mL of extraction buffer containing 50 mM HEPES-KOH, pH 7.4, 5 mM MgCl\(_2\), 2 mM EDTA, 5 mM diethiothreitol, 1% (v/v) polyvinylpyrrolidone, 0.1% (v/v) Triton X-10, and 4% (v/v) of protease inhibitor cocktail, using a cooled microcentrifuge at maximum speed for 4 min. The green pellet was discarded and the supernatant was brought to a final concentration of SDS (2% (w/v) and heated to 65°C in a water bath for 10 min.

**Statistical Analysis**

An ANOVA was conducted and Student’s t test in STATISTICA (version 6.0 StatSoft). Tukey’s honestly significant difference tests were used for post hoc comparisons.

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LITERATURE CITED

Allaway WG (1973) Accumulation of malate in guard cells of Vicia faba during stomatal opening. Planta 110: 63–70
Andreo CS, Gonzales D, Inlesias A (1987) Higher plant phosphoenolpyruvate carboxylyase: structure and regulation. FEBS Lett 213: 1–8
Asai N, Nakajima N, Tamaoki M, Kamada H, Kondo N (2000) Role of malate synthesis mediated by phosphoenolpyruvate carboxylase in guard cells in the regulation of stomatal movement. Plant Cell Physiol 41: 10–15
Badger MR, Price GD (1989) Carbonic anhydrase activity associated with the cyanobacterium Synechococcus PCC7942. Plant Physiol 89: 51–60
Britto DT, Kronzucker HJ (2005) Nitrogen acquisition, PEP carboxylase, and cellular pH homeostasis: new news on old paradigms. Plant Cell Environ 28: 1396–1409
Cernusak LA, Farquhar GD, Wong SC, Stuart-Williams H (2004) Measurement and interpretation of the oxygen isotope composition of carbon dioxide inspired by leaves in the dark. Plant Physiol 136: 3350–3363
Chollet R, Vidal J, Oleary MH (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 273–298
Cotelle V, Pierre JP, Vavasseur A (1999) Potential strong regulation of guard cell phosphoenolpyruvate carboxylase through phosphorylation. J Exp Bot 50: 777–783
Cousins AB, Badger MR, von Caemmerer S (2006a) Carbonic anhydrase and its influence on carbon isotope discrimination during C4 photosynthesis: insights from antisense RNA in V. faba. Plant Biotech 141: 232–242
Cousins AB, Badger MR, von Caemmerer S (2006b) A transgenic approach to understanding the influence of carbonic anhydrase on C4 isotope discrimination during C4 photosynthesis. Plant Physiol 142: 662–672
Craig H, Gordon LI (1965) Deutrium and oxygen-18 variations in the ocean and the marine atmosphere. In E Torgiorgi, ed, Proceedings of a Conference on Stable Isotopes in Oceanographic Studies and Paleotemperatures. Consiglio Nazionale delle Ricerche, Laboratore Geologia Nucleare, Pisa, Italy, pp 9–130
Dever LV, Bailey KJ, Lacuesta M, Leegood RC, Lea PJ (1996) The isolation and characterization of mutants of the C4 plant Amaranthus edulis. C R Acad Sci Ser III Sci Vie 319: 951–959
Dever LV, Bailey KJ, Leegood RC, Lea PJ (1997) Control of photosynthesis in Amaranthus edulis mutants with reduced amounts of PEP carboxylase. Aust J Plant Physiol 24: 469–476
Dever LV, Blackwell RD, Fullwood NJ, Lacuesta M, Leegood RC, Onek LA, Pearson M, Lea PJ (1995) The isolation and characterization of mutants of the C4 photosynthetic pathway. J Exp Bot 46: 1363–1376
Dever LV, Pearson M, Ireland RJ, Leegood RC, Lea PJ (1998) The isolation and characterization of a mutant of the C4 plant Amaranthus edulis deficient in NAD-malic enzyme activity. Planta 206: 649–656
Evans JR, Sharkey TD, Berry JA, Farquhar GD (1986) Carbon isotope discrimination measured concurrently with gas-exchange to investigate CO2 diffusion in leaves of higher-plants. Aust J Plant Physiol 13: 281–292
Evans JR, vonCaemmerer S (1996) Carbon dioxide diffusion inside leaves. Plant Physiol 110: 339–346
Farquhar GD (1983) On the nature of carbon isotope discrimination in C4 species. Aust J Plant Physiol 10: 205–226
Farquhar GD, Lloyd J (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In GD Farquhar, ed, Stable Isotopes and Plant Carbon-Water Relations. Academic Press, New York, pp 47–70
Farquhar GD, Oleary MH, Berry JA (1982) On the relationship between carbon isotope discrimination and the inter-cellular carbon-dioxide concentration in leaves. Aust J Plant Physiol 9: 121–137
Flanagan LB, Ehleringer JR (1998) Ecosystem-atmosphere CO2 exchange: interpreting signals of change using stable isotope ratios. Trends Ecol Evol 13: 10–14
Furbank RT, Hatch MD, Jenkins CLD (2000) C4 photosynthesis: mechanism and regulation. In S von Caemmerer, ed, Photosynthesis: Physiology and Metabolism, Vol 9. Academic Press, San Diego, pp 435–457
Gehlen J, Panstruga R, Smets H, Merkelbach S, Kleinies M, Porsch P, Fladung M, Becker I, Radermacher T, Hauser RE, et al (1996) Effects of altered phosphoenolpyruvate carboxylase activities on transgenic C4 plant Solanum tuberosum. Plant Mol Biol 32: 831–848
Ghashghaie J, Badeck FW, Lanigan G, Nogues S, Tcherkez G, Deleens E, Comin G, Griffiths H (2003) Carbon isotope fractionation during dark respiration and photorespiration in C3 plants. Phytochem Rev 2: 145–161
Gillon JS, Griffiths H (1997) The influence of (photo)respiration on carbon isotope discrimination in plants. Plant Cell Environ 20: 1217–1230
Gillon JS, Yakir D (2000a) Internal conductance to CO2 diffusion and (CO2)-O3 discrimination in C4 leaves. Plant Physiol 123: 201–213
Gillon JS, Yakir D (2000b) Naturally low carbonic anhydrase activity in C4 plants limits discrimination against (CO2)-O3 during photorespiration. Plant Cell Environ 23: 903–915
Gillon JS, Yakir D (2001) Influence of carbonic anhydrase activity in terrestrial vegetation on the O3 content of atmospheric CO2. Science 291: 2584–2587
Griffiths H, Cousins AB, Badger MR, von Caemmerer S (2007) Discrimination in the dark: resolving the interplay between metabolic and physical constraints to phosphoenolpyruvate carboxylase activity during the crassulacean acid metabolism cycle. Plant Physiol 143: 1055–1067
Harwood KG, Gillon JS, Griffiths H, Broadmeadow MSJ (1998) Diurnal variation of delta(CO2)-C-13, delta(CO2)-O-18 and O-16 and evaporative site enrichment of delta(O18)-H2O-18 in Piper aduncum under field conditions in Trinidad. Plant Cell Environ 21: 269–283
Hatch MD (1987) C4 photosynthesis—a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta 895: 81–106
Henderson SA, von Caemmerer S, Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C4 species. Aust J Plant Physiol 19: 263–285
Igamberdiev AU, Mikelles TN, Ambus P, Bauwe H, Lea PJ, Gardestrom P (2004) Photorespiration contributes to stomatal regulation and carbon isotope fractionation: a study with barley, potato and Arabidopsis plants deficient in glycine decarboxylase. Photosynth Res 81: 139–152
Izui K, Matsumura H, Furumoto T, Kai Y (2004) Phosphoenolpyruvate carboxylase: a new era of structural biology. Annu Rev Plant Biol 55: 69–84
Kanai R, Edwards GE (1999) The biochemistry of C4 photosynthesis. In R Monson, ed, Plant Biology. Academic Press, San Diego, pp 49–87
Kiziris O, Lea PJ, Franceschi VR, Edwards GE (2002) Bundle sheath diffusive resistance to CO2 and effectiveness of C4 photosynthesis and relaxation of fotosynthetic CO2 in a C4 cycle mutant and wild-type Amaranthus edulis. Plant Physiol 130: 964–976
Kopka J, Proverb NJ, Müller Röber B (1997) Potato guard cells respond to drying soil by a complex change in the expression of genes related to carbon metabolism and turgor regulation. Plant J 11: 871–882
Ku MSB, Edwards GE (1975) Photosynthesis in mesophyll protoplasts and bundle sheath cells of various types of C4 plants. V. Enzymes of respiratory metabolism and energy utilizing enzymes of photosynthetic pathways. Z Pflanzenphysiol 77: 16–32
Kubásek J, Šetlik J, Dwyer S, Santruc J (2007) Light and growth temperature alter carbon isotope discrimination and estimated bundle sheath leakage in C4 grasses and dicots. Photosynth Res 91: 47–58
Lepiniec L, Thomas M, Vidal J (2003) From enzyme to plant biotechnology: 30 years of research on phosphoenolpyruvate carboxylase. Plant Physiol Biochem 47: 533–539
Lepiniec L, Vidal J, Chollet R, Gadai P, Cretin C (1994) Phosphoenolpyruvate carboxylase: structure, regulation and evolution. Plant Physiol 99: 111–124
Maroco JP, Ku MSB, Edwards GE (1997) Oxygen sensitivity of C4 photosynthesis: evidence from gas exchange and chlorophyll fluorescence analyses with different C4 subtypes. Plant Cell Environ 20: 1525–1533
Maroco JP, Ku MSB, Edwards GE (2000) Utilization of O2 in the metabolic optimization of C4 photosynthesis. Plant Cell Environ 23: 115–121
Maroco JP, Ku MSB, Furbank RT, Lea PJ, Leegood RC, Edwards GE (1998a) CO2 and O2 dependence of PSI II activity in C4 plants having genetically produced deficiencies in the C4 or C3 cycle. Photosynth Res 58: 91–101
Maroco JP, Ku MSB, Lea PJ, Dever LV, Leegood RC, Furbank RT, Edwards GE (1998b) Oxygen requirement and inhibition of C4 photosynthesis—an analysis of C4 plants deficient in the C4 and C3 cycles. Plant Physiol 116: 823–832
Mills G, Urey H (1940) The kinetics of isotopic exchange between carbon dioxide, bicarbonate ion, carbonate ion and water. J Am Chem Soc 62: 1019–1026
Outlaw WH Jr, Lowry OH (1977) Organic acid and potassium accumulation in guard cells during stomatal opening. Proc Natl Acad Sci USA 74: 4434–4438
Parvathi K, Raghavendra AS (1997) Both rubisco and phosphoenolpyruvate carboxylase are beneficial for stomatal function in epidermal strips of Commelina benghalensis. Plant Sci 124: 153–157
Pearson CJ (1973) Daily changes in stomatal aperture and in carbohydrates and malate within epidermis and mesophyll of leaves of Commelina cyanea and Vicia faba. Aust J Biol Sci 26: 1035–1044
Poincelot RP (1972) Intercellular distribution of carbonic anhydrase in spinach leaves. Biochim Biophys Acta 258: 637–642
Roelse CA, Oleary MH (1984) Carbon isotope effects on the enzyme-catalyzed carboxylation of ribulose bisphosphate. Biochemistry 23: 6275–6284
Schnabl H, Raschke K (1980) Potassium chloride as stomatal osmoticum in Allium cepa L., a species devoid of starch in guard cells. Plant Physiol 65: 88–93
van Kirk CA, Raschke K (1978) Presence of chloride reduces malate production in epidermis during stomatal opening. Plant Physiol 61: 361–364
Vavasseur A, Raghavendra AS (2005) Guard cell metabolism and CO₂ sensing. New Phytol 165: 665–682
von Caemmerer S (2000) Biochemical Models of Leaf Photosynthesis. CSIRO Publishing, Collingwood, Australia
von Caemmerer S (2003) C₄ photosynthesis in a single C₃ cell is theoretically inefficient but may ameliorate internal CO₂ diffusion limitations of C₄ leaves. Plant Cell Environ 26: 1191–1197
von Caemmerer S, Evans JR (1991) Determination of the average partial pressure of CO₂ in chloroplast from leaves of several C₄ plants. Aust J Plant Physiol 18: 287–305
von Caemmerer S, Furbank RT (2003) The C₄ pathway: an efficient CO₂ pump. Photosynth Res 77: 191–207
von Caemmerer S, Hendrickson L, Quinn V, Vella N, Millgate AG, Furbank RT (2005) Reductions of Rubisco activase by antisense RNA in the C₄ plant Flaveria bidentis reduces Rubisco carbamylation and leaf photosynthesis. Plant Physiol 137: 747–755
von Caemmerer S, Lawson T, Oxborough K, Baker NR, Andrews TJ, Raines CA (2004a) Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. J Exp Bot 55: 1157–1166
von Caemmerer S, Ludwig M, Millgate A, Farquhar GD, Price D, Badger MR, Furbank RT (1997a) Carbon isotope discrimination during C₄ photosynthesis: insights from transgenic plants. Aust J Plant Physiol 24: 487–494
von Caemmerer S, Millgate A, Farquhar GD, Furbank RT (1997b) Reduction of Ribulose-1,5-bisphosphate carboxylase/oxygenase by antisense RNA in the C₄ plant Flaveria bidentis leads to reduced assimilation rates and increased carbon isotope discrimination. Plant Physiol 113: 469–477
von Caemmerer S, Quinn V, Hancock NC, Price GD, Furbank RT, Ludwig M (2004b) Carbonic anhydrase and C₄ photosynthesis: a transgenic analysis. Plant Cell Environ 27: 697–703
Wheeler RM, Mackowiak CL, Yorio NC, Sager JC (1999) Effects of CO₂ on stomatal conductance: do stomata open at very high CO₂ concentrations? Ann Bot (Lond) 83: 243–251
Willmer C, Fricker M (1996) Stomata, Ed 2. Chapman & Hall, London
Wong SC, Cowan IR, Farquhar GD (1985) Leaf conductance in relation to rate of CO₂ assimilation.1. Influence of nitrogen nutrition, phosphorus-nutrition, photon flux-density, and ambient partial-pressure of CO₂ during ontogeny. Plant Physiol 78: 821–825
Yakir D, Sternberg LD L (2000) The use of stable isotopes to study ecosystem gas exchange. Oecologia 123: 297–311