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

Asaph B. Cousins*, Irene Baroli, Murray R. Badger, Alexander Ivakov, Peter J. Lea, Richard C. Leegood, and Susanne von Caemmerer

Molecular Plant Physiology Group (A.B.C., I.B., M.R.B., A.I., S.v.C) and Australian Research Council Centre of Excellence in Plant Energy Biology (A.B.C., M.R.B.), Research School of Biological Sciences, Australian National University, Canberra, Australian Capital Territory 2601, Australia; Department of Biological Sciences, Lancaster University, Lancaster LA1 4YQ, United Kingdom (P.J.L.); and Department of Animal and Plant Sciences, Robert Hill Institute, University of Sheffield, Sheffield S10 2TN, United Kingdom (R.C.L.)

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 13CO2 (Δ13C) and C18OO (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 (θ), the ratio 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; Izu et al., 2004). In higher plants, PEPC plays the anaplerotic role of replenishing the cytosolic pH regulation (Lepiniec et al., 1994; Britto and Kronzucker, 2005). In photosynthetic tissues, PEPC participates in the exchange of CO2 and water between a leaf and the atmosphere through its role in guard cell metabolism. Additionally, in C4 species PEPC catalyzes the first carboxylation reaction of C4 photosynthesis in the 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 severalfold higher than current atmospheric levels, significantly reducing the rates of photorespiration (Hatch, 1987).

Theoretical models of 13CO2 isotope discrimination (Δ13C) during C4 photosynthesis have been developed that link Δ13C to the ratio of intercellular to ambient pCO2 and bundle sheath leakage (θ) 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 θ 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 carboxylic anhydride (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; Kiihrs 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/pₑ) 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 (kₑ) (μmol m⁻² s⁻¹ bar⁻¹) was similar in all plants (Table I). The kₑ was determined from leaf extracts using mass spectrometry to measure the rates of ¹⁸O₂ exchange from labeled ¹³C₃O₂ to H₂¹⁸O (Badger and Price, 1989; Cousins et al., 2006a, 2006b). Leaf CA activity (CAₑ) determined as the product of kₑ and the mesophyll pCO₂ (pₑ), increased in the pp mutant due to the low rates of net CO₂ assimilation that caused pₑ to be greater in these plants (Table I). The values of pₑ were calculated as pₑ = pᵢ − A/Sₑ, where A is the net CO₂ assimilation rate and gₑ 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

<table>
<thead>
<tr>
<th>Measurements</th>
<th>WT</th>
<th>Pp</th>
<th>pp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosynthetic parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A$ (μmol m$^{-2}$ s$^{-1}$)</td>
<td>40.9 ± 1.6a</td>
<td>32.1 ± 1.7b</td>
<td>4.1 ± 0.4c</td>
</tr>
<tr>
<td>$p_{a}$ (bar)</td>
<td>0.39 ± 0.06a</td>
<td>0.45 ± 0.07a</td>
<td>0.82 ± 0.02b</td>
</tr>
<tr>
<td>$g_{s}$ (mol m$^{-2}$ s$^{-1}$)</td>
<td>0.28 ± 0.04a</td>
<td>0.25 ± 0.06a</td>
<td>0.09 ± 0.01b</td>
</tr>
<tr>
<td>$\Delta^{13}$C $\text{iso}$</td>
<td>3.3 ± 0.4a</td>
<td>3.0 ± 0.5a</td>
<td>16.2 ± 3.0b</td>
</tr>
<tr>
<td>$\Delta^{18}$O $\text{iso}$</td>
<td>16.8 ± 1.8a</td>
<td>20.9 ± 3.1a</td>
<td>207.4 ± 50.8b</td>
</tr>
<tr>
<td>Enzyme activities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEPC (μmol m$^{-2}$ s$^{-1}$)</td>
<td>144.9 ± 8.8a</td>
<td>61.0 ± 4.6b</td>
<td>3.8 ± 0.4c</td>
</tr>
<tr>
<td>Rubisco (μmol m$^{-2}$ s$^{-1}$)</td>
<td>44.2 ± 3.2a</td>
<td>nd</td>
<td>48.1 ± 3.2a</td>
</tr>
<tr>
<td>$k_{CA}$ (mol m$^{-2}$ s$^{-1}$ bar$^{-1}$)</td>
<td>6.1 ± 0.4a</td>
<td>5.3 ± 0.7a</td>
<td>6.4 ± 1.0a</td>
</tr>
<tr>
<td>$CA_{iso}$ * (μmol m$^{-2}$ s$^{-1}$)</td>
<td>783 ± 153a</td>
<td>831 ± 113a</td>
<td>2818 ± 64b</td>
</tr>
<tr>
<td>Isotopic equilibrium (θ)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Predicted (Eq. 12)</td>
<td>0.98 ± 0.01a</td>
<td>0.98 ± 0.01a</td>
<td>1.00 ± 0.00a</td>
</tr>
<tr>
<td>Measured (Eq. 11)</td>
<td>0.62 ± 0.02a</td>
<td>0.62 ± 0.07a</td>
<td>0.91 ± 0.05b</td>
</tr>
<tr>
<td>Leaf parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total leaf N (mmol m$^{-2}$)</td>
<td>132 ± 23a</td>
<td>120 ± 10a</td>
<td>130 ± 4a</td>
</tr>
<tr>
<td>LMA (g m$^{-2}$)</td>
<td>34.0 ± 2.9a</td>
<td>38.5 ± 3.2a</td>
<td>30.8 ± 1.6a</td>
</tr>
</tbody>
</table>

$^{13}$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 1). In the $pp$ mutant the value of $\Delta^{13}$C was approximately 5-fold higher than in wild-type and $Pp$ plants, and $p_{a}$/$p_{a}$ was 0.82 compared to 0.39 in the wild type (Table 1; 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 (θ) predicted from in vitro CA assays (Eq. 12) was substantially higher in the wild-type and $Pp$ plants than the θ values estimated from $\Delta^{18}$O measurements (Eq. 11; Table I). However, θ 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 $p_{a}$/$p_{a}$ 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 $p_{a}$/$p_{a}$ 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 ($\Delta_{d}$), taken from all plants in Table II, of 39.2‰. The values of $\Delta_{d}$ 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$_2$ ($p_{a}$/$p_{a}$) in wild-type and mutant A. edulis plants. The dashed line represents the theoretical relationship of $\Delta^{13}$C$_3$ and $p_{a}$/$p_{a}$ during $C_3$ photosynthesis where $\phi = 0.24$, using Equation 6. The solid lines represent the theoretical relationship of $\Delta^{13}$C$_4$ and $p_{a}$/$p_{a}$ using the $C_4$ model (Eq. 3). Gas-exchange conditions are as in Table I. Each point represents the means ± s.e. of measurements made on three to five leaves from separate plants from wild type ( ), $Pp$ mutant ( ), and $pp$ mutant ( ).
Table I. Shown are the means where a theoretical relationship of ANOVA and different letters indicate significant differences between plants at a ratio of mesophyll cytosolic to ambient CO2 partial pressure (leaf had a 1 and measurement conditions are as in Table I. The inset shows the was higher in the atmosphere to the leaf intercellular spaces (Fig. 2).

Table II. Stomatal Response

<table>
<thead>
<tr>
<th>Plants</th>
<th>δt</th>
<th>Δt</th>
<th>e/e</th>
<th>s−1</th>
<th>μ mol mol−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>24.0 ± 0.8a</td>
<td>20.6 ± 0.6a</td>
<td>36.9 ± 1.4a</td>
<td>0.41 ± 0.03a</td>
<td>1.9 ± 0.1a</td>
</tr>
<tr>
<td>Pp</td>
<td>24.7 ± 0.9a</td>
<td>36.7 ± 1.5a</td>
<td>0.39 ± 0.03a</td>
<td>2.0 ± 0.2a</td>
<td>199 ± 29a</td>
</tr>
<tr>
<td>pp</td>
<td>29.9 ± 0.9b</td>
<td>38.8 ± 1.1b</td>
<td>43.2 ± 1.7a</td>
<td>0.21 ± 0.03b</td>
<td>16.5 ± 2.7b</td>
</tr>
</tbody>
</table>

Figure 2. Oxygen isotope discrimination (Δ18O) as a function of the ratio of mesophyll cytosolic to ambient CO2 partial pressure (p)/p). p was calculated with p = 1 mol m−2 s−1 bar−1. The line represents the theoretical relationship of ANOVA and different letters indicate significant differences between plants at a ratio of mesophyll cytosolic to ambient CO2 partial pressure (leaf had a 1 and measurement conditions are as in Figure 1 and measurement conditions as are in Table I. The inset shows the expanded scale of Δ18O 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 isotopic equilibrium (Δe) and the ratio of the water vapor pressure in the atmosphere to the leaf intercellular spaces (e/e) 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 = Pm/Fp) 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 Δ13C and Δ18O 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) 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 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 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 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 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 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 pCO2 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 pCO2 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...
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

$^{13}$CO$_2$ Isotope Discrimination in the PEPC Mutants

The low activity of PEPC caused rates of net CO$_2$ 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 CO$_2$ (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 C$_4$ photosynthesis causes a decrease in the initial CO$_2$ carboxylation reaction and reduces the capacity of the C$_4$ pump to concentrate CO$_2$ within the BSCs (von Caemmerer, 2000; Cousins et al., 2006a). In the model of C$_4$ carbon isotope discrimination (Eq. 6 in “Materials and Methods”) the main factors that influence $\Delta^{13}$C are changes in the intercellular to ambient CO$_2$ partial pressures ($p_i/p_a$), the fraction of CO$_2$ fixed by PEPC that subsequently leaks out of the BSC ($\phi$), and the combined fractionation of PEPC and the isotopic equilibrium during dissolution of CO$_2$ and conversion to bicarbonate ($b_4$), which is dependent on the ratio of PEPC to CA activity (Farquhar, 1983). During C$_4$ photosynthesis decreases in $\phi$ and $b_4$ are both predicted to cause values of $\Delta^{13}$C to decrease (Farquhar, 1983). The values of $\Delta^{13}$C and $p_i/p_a$ in the Pp mutant were not significantly different from the wild-type plants even though the Pp plants had lower rates of net CO$_2$ assimilation (Table I). This implies that neither $\phi$ nor the $b_4$ 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 $\Delta^{13}$C remained constant because the balance in the C$_3$ and C$_4$ cycles was not altered.

The very low PEPC activity in the pp plants (Table I) severely inhibited the initial carboxylation step of the C$_4$ photosynthetic pathway causing the rates of net CO$_2$ assimilation to decrease considerably relative to wild type (Table I; Fig. 4B). The value of $p_i/p_a$ increased in the pp plants compared to wild type and the Pp plants (Table I) and according to the $\Delta^{13}$C model during C$_4$ photosynthesis (Eq. 6) a decrease in $p_i/p_a$ leads to a decrease in $\Delta^{13}$C at $\phi$ values of less than 0.6 and 32.4 μmol 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 CO$_2$ concentration was 360 μbar instead of 9.8 μbar. The leaf chamber humidity was maintained at 30.01% 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 (●).

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 CO$_2$ 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 (●).

Figure 4. Light induction of stomatal conductance (A) and net CO$_2$ assimilation (B) in wild-type and the pp mutant. Plants were grown under 9.8 μbar CO$_2$ and then transferred to ambient CO$_2$ 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$^{-2}$), respectively. The ambient CO$_2$ partial pressure was maintained at 364 μbar for the duration of the measurements. Data are the means of measurements of five 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 $\Delta^{13}$C 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$_3$-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 ($g_i$) 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 ($g_i$) of $33 \pm 10$ mmol m$^{-2}$ s$^{-1}$ in the pp plants (Eq. 5). This value of $g_i$ 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 $g_i$, we used discrimination factors for photorespiration ($f = 10\%$) and for respiration ($e = -6\%$) reported from the literature (Gillon and Griffiths, 1997; Ghashghaie et al., 2003; Igamberdiev et al., 2004). Our measurements were made at low O$_2$ partial pressures so that

![Figure 5](image5.jpg)

**Figure 5.** A, The net change in stomatal conductance over time after a step change from 364 μbar to 48 μbar CO$_2$, normalized by the initial conductance in 364 μbar CO$_2$. Steady-state conductance at low CO$_2$ was $0.83 \pm 0.08$ (wild-type) and $0.41 \pm 0.05$ (pp mutant) mol water m$^{-2}$ s$^{-1}$ and the rate of stomatal opening for the wild-type and pp mutant was $20.2 \pm 4.5$ and $11.9 \pm 2.3$ mmol water m$^{-2}$ s$^{-1}$, respectively. Illumination was kept at 2,000 μmol quanta m$^{-2}$ s$^{-1}$ for the duration of the experiment. B, The change in CO$_2$ assimilation rate over time at low CO$_2$ partial pressure is shown for comparison. Data are the means of measurements of four and five different plants, respectively, for the wild-type (○) and homozygous pp mutant (●). Error bars represent se.

![Figure 6](image6.jpg)

**Figure 6.** Soluble protein profile of leaf discs and epidermis of A. edulis wild-type (WT) and pp mutant. A, Coomassie Blue-stained SDS-PAGE gel of soluble leaf protein from leaf and epidermal (epi) fraction. B, Immunoblot of PEPC and the large subunit of Rubisco (RbcL), shown as a loading control. Thirty micrograms of total protein were loaded per lane. The epidermal PEPC protein content of the pp mutant was $53\% \pm 4\%$ of wild type. The relative abundance of epidermal PEPC protein in the pp mutant compared to wild type was determined from immunoblot labeling of three wild-type and four pp mutant epidermal extractions. Shown here is one representative blot.
Table III. Stomatal density and index of wild-type A. edulis and PEPC pp mutants

<table>
<thead>
<tr>
<th>Leaf Side</th>
<th>Plant</th>
<th>Stomatal Density</th>
<th>Stomatal Index (Stomata/Total Epidermal Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adaxial</td>
<td>WT</td>
<td>144 ± 11a</td>
<td>0.22 ± 0.01a</td>
</tr>
<tr>
<td>Abaxial</td>
<td>WT</td>
<td>123 ± 7a</td>
<td>0.23 ± 0.01a</td>
</tr>
<tr>
<td></td>
<td>pp</td>
<td>218 ± 35b</td>
<td>0.22 ± 0.01a</td>
</tr>
</tbody>
</table>

the expected fractionation from photosynthesis is only about 0.17‰ at a 1 atm of 9.25 μbar. The effect of respiratory fractionation is between −1.67‰ and 1‰ on leaves from wild type and heterozygous mutant A. edulis. Shown are the means ± se of counts taken on leaves from three (wild type) or four (pp mutant) plants. Statistical analysis was conducted using a Student’s t test and different letters indicate significant differences between wild type (WT) and pp plants at P < 0.1.

Stomatal Conductance in Response to Light and Atmospheric CO2

The pp mutant had low stomatal conductance (g<sub>s</sub>) during steady-state gas-exchange conditions (at 531 μbar CO<sub>2</sub>) relative to the Pp mutant and wild-type plants (Table I). Additionally, g<sub>s</sub> in the pp mutant decreased but increased in the wild type when leaves were rapidly transferred from the high CO<sub>2</sub> (9.8 mbar) growth conditions into air (364 μbar CO<sub>2</sub>) at a constant leaf chamber humidity (Fig. 3). The higher g<sub>s</sub> under elevated CO<sub>2</sub> reported here is consistent with previous reports that g<sub>s</sub> is generally greater under superelevated conditions.

C<sup>18</sup>O<sub>2</sub> Isotope Discrimination and CO<sub>2</sub>/Water Isotopic Equilibrium

In a leaf, the oxygen isotope composition of CO<sub>2</sub> is determined by the isotope composition of leaf water at the site of evaporation (δ<sub>water</sub>) and CA activity. The exchange of δ<sub>18</sub>O between CO<sub>2</sub> and water is facilitated by CA, which catalyzes the interconversion of CO<sub>2</sub> and bicarbonate (HCO<sub>3</sub>−), and high CA activity will increase the proportion of CO<sub>2</sub> in isotopic equilibrium with the water. Based on calculated values of δ<sub>water</sub> (Table II) the 18O discrimination (Δδ<sub>18</sub>O) was low in the wild-type plants compared to the high levels of leaf CA (CA<sub>leaf</sub>, Table I). The extent of isotopic equilibrium (θ) measured from Δδ<sub>18</sub>O (Eq. 11) was also low in the wild-type plants relative to θ estimated from CA activity using Equation 12 (Table I). These findings are similar to previous work with another C<sub>4</sub> dicot, F. bidentis, which also had low Δδ<sub>18</sub>O and θ measured from Δδ<sub>18</sub>O compared to the high rates of CA<sub>leaf</sub> (Cousins et al., 2006b). This further suggests that the total leaf CA activity in C<sub>4</sub> dicots does not represent the CA activity associated with the CO<sub>2</sub>-water oxygen exchange that influences Δδ<sub>18</sub>O (Cousins et al., 2006b).

The value of θ is related to the mean number of hydration reactions a CO<sub>2</sub> molecule experiences inside a leaf. This in turn is the product of residence time (τ = P<sub>m</sub>/F<sub>in</sub>) and the hydration constant of leaf CA (k<sub>CA</sub>), where P<sub>m</sub> is the mesophyll pCO<sub>2</sub> and F<sub>in</sub> is the mass flux of CO<sub>2</sub> into the leaf (Eq. 12). The low photosynthetic rates increase the residence time of CO<sub>2</sub> as P<sub>m</sub> increases (Tables I and II). Therefore, the number of hydration reactions per CO<sub>2</sub> increases when rates of net CO<sub>2</sub> 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<sub>leaf</sub> activity in the PEPC mutants were higher (Table I). The increase in CA<sub>leaf</sub> in these plants is attributed to an increase in substrate availability for CA due to the lack of photosynthetic CO<sub>2</sub> drawdown caused by the low PEPC activity. The increase in θ in the pp plants suggests that in wild-type A. edulis the CA<sub>leaf</sub> activity does not allow full isotopic equilibrium between the CO<sub>2</sub> and water within the leaf under steady-state conditions.

Our study suggests that in C<sub>4</sub> species leaf CA activity cannot readily be used as an indicator of the extent of 18O equilibration as has been suggested by Gillon and Yakir (2001). This might be because not all of the CA located in mesophyll cytosol in C<sub>4</sub> species is available at the site of Rubisco carboxylation with the BSC. The value of θ also varies with the leaf water δ<sub>18</sub>O (Cousins et al., 2006b). This suggests that in C<sub>4</sub> species leaf CA activity associated with CO<sub>2</sub>-water oxygen exchange that influences Δδ<sub>18</sub>O (Cousins et al., 2006b)
CO₂ (above 4.0 mbar) compared to air CO₂ concentrations (see review and references within Wheeler et al., 1999). Low $g_s$ 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 $g_s$ 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 $g_s$ 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 $g_s$ 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, $g_s$ 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 $g_s$ in response to conditions that normally stimulate $g_s$. As with the light response, even though $g_s$ in the pp mutant did not reach similar values to the wild-type plants, the values of $g_s$ increased about three times in response to low CO₂ availability in both plants (Fig. 5).

The shifts in $g_s$ 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 $g_s$ 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 $g_s$ was approximately 3-times greater under the lower CO₂ concentrations (Fig. 5). Although it has been demonstrated that there is a tight correlation between $g_s$ 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 $g_s$ 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 $g_s$ 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 (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 909 mbar dry N2 and 48 μbar O2. A portion of the nitrogen/oxygen air was used to zero the mass spectrometer to correct for N2O and other contaminants contributing to the 44, 45, and 46 peaks. Pure CO2 (5.0 mbar) and δ13CO2 (δ13CO2 = −28.8‰) and δ18O (δ18O = −2.8‰) was added to the remaining air stream to obtain a CO2 partial pressure of approximately 531 μbar. The isotopic CO2 used for measurements was similar to that used in the controlled environments. Low oxygen (48 mbar) was used to minimize contamination of the 46 (mass-to-charge ratio) signal caused by the interaction between carbon dioxide and oxygen. The isotopic 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, b is the combined fractionation due to dissolution and diffusion of CO2 in water (1.8‰), and the fractionation by Rubisco is b = 30‰ (Roeseke and Ollery, 1984). Fv is the CO2 compensation point in the absence of day respiration. Rb is the rate of mitochondrial respiration, a and b are the discrimination factors of respiration and photorespiration with respect to the average carbon composition associated with respiration and photorespiration, 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

\[ \Delta_c = a + (b - a)p_o/p_a \]  

Subtracting Equation 3 from Equation 1 shows that the difference between the Δ and the measured Δ13C 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):

\[ \Delta_c - \Delta^{13}C = (b - a)A/(g_p \cdot p_o + \epsilon R_d / k + f \Gamma) / p_a \]  

such that g can be estimated after rearranging Equation 4 from

\[ g_o = \Delta_c - \Delta^{13}C - (b - a)p_o / \epsilon R_d / k + f \Gamma \]

The model of C4 carbon isotope discrimination (Δ13C4) from Farquhar (1983) was used to determine which factors in the model would influence Δ13C4 consistent with our experimental data in the pp mutant and wild-type plants. The simplified model predicts that

\[ \Delta^{13}C_4 = a + (b_3 + (b_2 - s) \times \phi - a) \times \epsilon / p_a \]

indicating that the fractionation when CO2 and HCO3− are not at equilibrium depends on the rate of CO2 hydration (VH4) and the rate of PEP carboxylation (VP).

Calculations of C18OO Isotope Discrimination

Discrimination against C18OO (Δ18O) when water and CO2 at the site of exchange are at full isotopic equilibrium (θ = 1) can be predicted as (Farquhar and Lloyd, 1993)

\[ \Delta^{18}O = \Delta^{13}C_4 \times \theta \]

Determination of Stomatal Numbers

Stomatal numbers were determined from the same or similar leaves as used for gas-exchange measurements, from silicone rubber impressions taken 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/).
\[ \Delta^18O = \delta^18O - \delta^18O_a \]

where \( \delta^18O \) is the diffusional discrimination (7.7\%/a) and \( \epsilon \) is calculated as \( \Delta^18O / \Delta^18O_a \) and \( \epsilon = \frac{1}{1 - \epsilon} \).

The equilibrium fractionation between water and CO2 was calculated as

\[ \Delta^18O = \frac{\delta^18O_a + \epsilon \delta^18O_{ea}}{1 + \epsilon} \]

(Cernusak et al., 2004). The equilibrium fractionation between water and CO2 from in vitro measurements of CA activity in leaf extracts (see below). The relationship of CAleaf/C\textsubscript{176} was 40.17\% at 30°C (Cernusak et al., 2004).

The \( \delta^18O \) of water at the sites of evaporation within a leaf (\( \delta_a \)) can be estimated from the Craig and Gordon model of evaporative enrichment (Craig and Gordon, 1965; Farquhar and Lloyd, 1993)

\[ \delta_a = \delta_e + \epsilon a + \epsilon c (1 + \epsilon) \]

where \( \epsilon_a \) and \( \epsilon_c \) are the vapor pressures in the atmosphere and the leaf intercellular spaces. \( \delta_e \) and \( \delta_i \) are the isotopic composition of water vapor in the air and transpired by the leaf, respectively. The kinetic fractionation during diffusion of water from leaf intercellular air spaces to the atmosphere (\( \epsilon_c \)) and the equilibrium fractionation between liquid water and water vapor (\( \epsilon \)) was calculated according to Cernusak et al. (2004) and Cousins et al. (2006b). During the online gas-exchange measurements we let the leaves remain in steady-state conditions for a minimum of 1.5 h and under those conditions the value of \( \delta_a \) is equal to the isotopic composition of source water, the water taken up by the plant (\( \epsilon = 4.4 \pm 0.3 \); Harwood et al., 1998).

The proportion of CO\textsubscript{2} in isotopic equilibrium with water at the site of oxygen exchange (\( \theta \)) can be estimated from

\[ \theta = \frac{\Delta^18O + \delta_t}{\Delta^18O + \epsilon (\epsilon + 1)} \]

where \( \Delta^18O \) is the oxygen isotope composition of CO\textsubscript{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\textsubscript{2} into the leaf (Gillon and Yakir, 2000a, 2006b, 2001) from the equation initially developed by Mills and Lrey (1940):

\[ \theta = 1 - \frac{1}{\epsilon_a + \Delta^18O_{/C_{18}}/\theta} \]

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

Enzyme Activities

Enzyme activities were determined on approximately 1 cm\textsuperscript{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°C. Tissue was ground on ice in 600 \( \mu \)L of extraction buffer (50 mM HEPES-KOH, pH 7.4, 10 mM dithiothreitol, 1% (v/v) polyvinylpyrrolidone, 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 of 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\textsuperscript{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 7.8, 5 mM MgCl\textsubscript{2}, 2 mM EDTA, 5 mM dithiothreitol, 1% (w/v) polyvinylpyrrolidone, 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 of 2% (w/v) and heated to 65°C in a water bath for 10 min.

Protein concentration in the samples was determined with the biocinchonic acid method (BCA Protein Assay kit, Pierce) prior to addition of SDS. Samples were prepared for gel loading by adding 0.25 volumes of Bio-Rad XT sample buffer (Bio-Rad). Thirty micrograms of total protein were loaded per gel well. Proteins were separated by electrophoresis on NuPAGE Bis-Tris precast gels (4%-12% acrylamide concentration, Novex) using the manufacturer-specified buffer system and blotted onto nitrocellulose membranes. Blots were probed with polyclonal antibodies raised against tobacco (Nicotiana tabacum) Rubisco and recombinant maize (Zea mays) PEPC, and with anti-Ig G alkaline phosphatase conjugate (Bio-Rad) as secondary antibody. Blots were developed using the AlitoPhos fluorescence substrate system (Promega). Epidermal PEPC in the pp mutant was compared to wild type using Image Quant (Molecular Dynamics) to determine the relative abundance of the PEPC protein labeled by immunoblotting from extractions prepared from three individual wild-type and four individual pp mutants.

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.

ACKNOWLEDGMENTS

We thank Dr. Spencer Whitney for the Rubisco and Dr. Tsuyoshi Furumoto for PEPC antibodies. We are thankful to Dr. Louisa Dever for the original isolation of the LaC\textsubscript{4} 2.16 PEPC-deficient mutant of A. edulis and Jessica Janek for her technical assistance.

Received June 5, 2007; accepted September 3, 2007; published September 7, 2007.

PEPC Influence on Isotope Exchange and Stomatal Conductance

Measurements of leaf extracts were made at 25°C with a subsaturating total carbon concentration of 1 mm. The hydration rates were calculated from the enhancement in the rate of \( ^3\text{O} \) loss over the uncatalyzed rate and the nonenzymatic first order rate constant was applied (pH 7.4, appropriate for the mesophyll cytosol). The CA activity was reported as a first order rate constant \( k_{ca} \) (mol m\textsuperscript{-2} s\textsuperscript{-1} bar\textsuperscript{-1}) and \( k_{ca}\text{pp} \) gives the in vivo CA activity at that particular cytosolic pCO\textsubscript{2}.

Epidermal Preparations

A fraction enriched in epidermal tissue was prepared by adapting the method of Kopka et al. (1997). A young expanding leaf was picked, the major veins were removed and discarded, and the rest was blended with 250 mL of chilled distilled water with a Sorvall Omni Mixer blender at maximum speed, with four pulses of 30 s each and waiting 30 s between pulses. The resulting epidermal fragments were rinsed with 300 mL of chilled distilled water on a 100 to 149 \( \mu \)m Nytal mesh to rid them of contaminating mesophyll cells. The epidermal fragments were drained of excess water, disrupted by grinding with mortar and pestle in liquid nitrogen for 3 min, and stored at –80°C until later use. The resulting fraction was highly enriched in epidermises compared to mesophyll cells (less than 1 mesophyll cell per mm\textsuperscript{2} epidermis) and \( k_{ca}\text{pp} \) was calculated under the observed compound microscope. Different blending regimes and filtration methods were ineffective in decreasing the contamination of epidermal fragments with bundle sheaths, so that the final preparations routinely contained a 10% to 20% contamination with bundle sheaths and vascular tissue as determined visually with the light microscope.
Cousins et al.

LITERATURE CITED