Functional constraints of CAM leaf anatomy: tight cell packing is associated with increased CAM function across a gradient of CAM expression

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Abstract

Increased cell size, increased leaf succulence, reduced intercellular air space (IAS), and reduced surface of mesophyll exposed to IAS (Lmes/area) are traits associated with the Crassulacean acid metabolism (CAM) photosynthetic pathway. An examination was carried out to determine whether these anatomical and structural traits are related to the degree of CAM function in eight CAM species, as measured by CO₂ assimilation during the CAM and C₃ phases. Increased cell size and leaf succulence were not closely related to the degree of CAM function, indicating that succulence beyond a certain threshold does not enhance CAM function. Reduced IAS and Lmes/area were positively related to CAM function, and negatively related to C₃ function. These results indicate that reduced IAS and Lmes/area are beneficial for CAM function through the reduction of CO₂ efflux and the improvement of carbon economy. However, reduced IAS and Lmes/area limit C₃ photosynthesis, potentially mediating a bimodal distribution of weak and strong CAM species with high and low IAS and Lmes/area values, respectively.

Key words: Crassulacean acid metabolism, evolution, leaf anatomy, photosynthesis.

Introduction

Crassulacean acid metabolism (CAM) is found in >7% of vascular plant species arising from dozens of distinct phylogenetic lineages (Smith and Winter, 1996; Sayed, 2001). CAM species demonstrate pronounced variation in structure, adaptation to stressful environments, and degree of CAM photosynthesis (Roberts et al., 1997; Skillman and Winter, 1997; Borland et al., 1998, 2000; Griffiths et al., 2002). All CAM plants take up CO₂ at night via phosphoenolpyruvate carboxylase (PEPc) and store this carbon as malic acid in their cell vacuoles. This malic acid is decarboxylated during the day and the resulting CO₂ is made available to ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) for carbohydrate synthesis. However, CAM plants vary greatly in the degree of carbon uptake, throughout the night as well as during the day. C₃ photosynthesis, characterized by Rubisco-mediated uptake of atmospheric CO₂, occurs to varying degrees in most CAM plants (Osmond et al., 1999). Osmond et al. (1999) divide the photosynthetic behaviour of CAM species into four distinct phases. Phase I encompasses the night-time uptake of CO₂, while the stored malate is decarboxylated in phase III, when high partial pressures of CO₂ lead to stomatal closure (Cockburn et al., 1979; Osmond et al., 1999). This carbon-concentrating mechanism (CCM) generates high concentrations of CO₂ around Rubisco active sites, thus limiting the inhibitory process of photorespiration (Spalding et al., 1979; Maxwell et al., 1998). The transient phases II and IV encompass the shift in CO₂ uptake between enzymes in preparation for phases III and I, respectively, resulting in some level of competition between PEPc and Rubisco for CO₂ (Borland and Griffiths, 1996; Gillon et al., 1998; Maxwell et al., 1999). Phase II is dominated by PEPc CO₂ uptake, with a gradual increase in Rubisco activity (Maxwell et al., 1998, 1999; Roberts et al., 1997; Griffiths et al., 2002). Phase IV is initially dominated by C₃ photosynthesis via Rubisco-mediated atmospheric CO₂ uptake, and the
enzyme is at its highest activation state during this phase (Roberts et al., 1997; Maxwell et al., 1998, 1999; Griffiths et al., 2002). The products of phase IV are largely exported for growth and reproduction, and can greatly enhance overall plant productivity (Borland and Griffiths, 1996; Osmond et al., 1999; Borland et al., 2000).

CAM species vary considerably in the duration of each phase and the contribution of each phase to overall carbon balance. Constitutive CAM plants exhibit night-time uptake of CO₂ at all times, while facultative CAM plants balance. Constitutive CAM plants exhibit night-time phase and the contribution of each phase to overall carbon uptake during phase I can be considered strong (or ‘typical’) CAM species, whereas species with less than one-third dark fixation are considered weak CAM, and are indistinguishable from C₃ species on the basis of ¹³C discrimination values (Winter and Holtum, 2002). Species appear to be bimodally distributed into CAM-dominated or C₃-dominated modes, and the presence of intermediate C₃–CAM discrimination values is rare (Silvera et al., 2005).

CAM species share anatomical traits that may reflect functional constraints. A general feature of CAM anatomy is leaf succulence, characterized by large, undifferentiated mesophyll cells dominated by large vacuoles (Gibson, 1982; Smith et al., 1996; Winter and Smith, 1996). Large vacuoles provide capacitance for C₄ acid accumulation and water storage in CAM photosynthetic tissues (Osmond et al., 1999; Borland et al., 2000). Borland et al. (1998) demonstrated an increase in cell succulence across a gradient of increasing CAM expression in three Clusia species. Teeri et al. (1981) and Winter et al. (1983) reported positive relationships between leaf thickness and CAM-like ¹³C discrimination values in samples of Crassulacean species and Australian ephiphytes, respectively. When CAM and C₃ species are compared, CAM species have relatively reduced intercellular air space, or IAS, in photosynthetic tissues (Smith and Heuer, 1981, Maxwell et al., 1997; Nelson et al., 2005). Nelson et al. (2005) also demonstrated a reduction in the surface of mesophyll exposed to IAS, or Lmes/area, in CAM species.

Reduced IAS and Lmes/area are causally linked to reduced internal CO₂ conductance, or gᵢ (Gillon et al., 1998; Evans and von Caemmerer, 1996; Evans and Loreto, 2000). These anatomical traits influence conductance in separate but complementary ways: reduced IAS results in more tortuous path lengths for CO₂ diffusing from inside the stomata, and Lmes/area acts as a gateway into the cell and represents access to the sites of carboxylation. Internal conductance is often portrayed as a modest player in plant carbon balance, given the large influence of stomatal conductance on CO₂ delivery to the sites of carboxylation (Friemert et al., 1986; Evans and von Caemmerer, 1996; Evans and Loreto, 2000). However, there is extensive evidence that CO₂ concentrations around sites of carboxylation are well below values expected in succulent tissues, despite high stomatal conductances (Maxwell et al., 1997, 1998; Evans and Loreto, 2000; Maxwell, 2002; Griffiths et al., 2007). In the CAM species Kalanchoë daigremontiana, Maxwell et al. (1997) speculate that tight cell packing and low IAS are responsible for one of the lowest values of gᵢ recorded, 0.05 mol CO₂ m⁻² s⁻¹ bar⁻¹. Evidence of reduced gᵢ has also been noted in a number of CAM studies: Friemert et al. (1986) and Robinson et al. (1993) recorded gradients of ¹³C discrimination values in succulent CAM leaves, an indication of greatly reduced gᵢ in these species. Gillon et al. (1998) demonstrated a decrease in gᵢ concomitant with an increase in succulence across a gradient of CAM in the genus Clusia, providing evidence of a causal relationship between CAM anatomy and low internal conductances to CO₂. Reduced gᵢ is known to limit photosynthetic capacity during C₃ photosynthesis (Evans and von Caemmerer, 1996; Evans and Loreto, 2000), and is therefore likely to limit CO₂ uptake during phase IV of CAM (Maxwell et al., 1999; Maxwell, 2002). Griffiths et al. (2007) utilized measurements of instantaneous CO₂ discrimination for both ¹³C and ¹⁸O to demonstrate diffusion limitations within K. daigremontiana leaves during phase IV. Dark fixation and daytime decarboxylation of PEPC-mediated CO₂ uptake is not limited by atmospheric CO₂ availability, however, and will therefore not be affected by reduced delivery of CO₂ to the sites of carboxylation (Borland et al., 1998, 2000; Gillon et al., 1998).

Although reduced IAS and Lmes/area have been identified previously in CAM photosynthetic tissues, these anatomical traits were considered an inevitable consequence of large cells with high storage requirements (Maxwell et al., 1997). However, IAS and Lmes/area across 18 CAM species were only weakly correlated with cell size, suggesting that they may be the result of an independent CAM selection pressure to restrict CO₂ efflux during phase III in strong CAM plants (Nelson et al., 2005). This may result in a trade-off between CAM and C₃ carbon gain, as tight cell packing would enhance CAM efficiency by reducing CO₂ leakage in phase III, but restrict access of CO₂ during C₃-dominated phase IV. This study examined four anatomical traits that have been associated with CAM photosynthesis: increased cell size and succulence, and reduced IAS and Lmes/area. This study tested the hypothesis that these anatomical traits enhance the degree of CAM photosynthesis and limit the degree of C₃ photosynthesis across CAM species. The degree of CAM photosynthesis was determined using two indicators: CO₂ uptake in phase I and duration of phase III. The degree of C₃ photosynthesis was quantified through parallel measurements within the same diel cycle: CO₂ uptake and duration of phase IV.
Materials and methods

Species selected

Eight CAM species were selected for this study: Ananas comosus (L.) Merr.; Calathea fragrans (Lindl.) Woods.; Clusia rosea Jaq.; Cissus rotundifolia (Forssk.) Vahl; Hoya carnosa (L. f.) R. Br.; Kalanchoë daigremontiana Hamet and Perr.; Peperomia obtusifolia (L.) A. Dietr.; and Vanilla fragrans G. Jackson. These species were chosen to represent the phylogenetic, anatomical, ecological, and functional diversity of CAM.

Plant growth conditions

Plants were grown in 5 inch clay pots in a soil mix of 25% perlite; 25% sand; 50% 3-in-1 landscaping soil. Vanilla fragrans could not be potted due to a large number of aerial roots, and were placed horizontally on highly absorbent burlap mats. Plants were potted and grown in the University of Toronto Botany greenhouse facilities until plants possessed a minimum of three fully expanded leaves. Plants were then transferred to growth chambers (Enconair Model GC-20; Winnipeg, Ontario) and maintained under identical experimental conditions. Experimental conditions consisted of a 12 h light period at 27 °C and a 12 h dark period at 20 °C, which closely matched previous growing conditions. Plants were watered every other day, 6 h before the onset of the dark period. Plants were given a minimum of 14 d to acclimate to this photoperiod and water regimen before experimentation began. Light levels in the growth chamber were maintained between 350 μmol photons m⁻² s⁻¹ and 400 μmol photons m⁻² s⁻¹. Plants were fertilized 2 weeks before experimentation, and were maintained in the greenhouse with one fertilization treatment every 6 months.

CAM function

The degree of CAM was determined in well-watered plants over a 40 h time-course (n=3 plants for each species), and then checked against dusk–dawn titratable acidity and ¹³C carbon isotope discrimination values for the same individuals.

Gas exchange measurements: Forty hour gas exchange was measured on one leaf per plant using a LI-6400 Photosynthesis System with a 6400-05 Conifer Chamber attachment (Li-Cor Biosciences, Lincoln, NE, USA). In the case of A. comosus and C. fragrans, the leaf was positioned so that a longitudinal portion of the leaf was inside the chamber, while portions of the leaf remained on either side of the chamber. In all other cases, whole leaves were placed inside the chamber. The flow rate was kept constant at 500 μmol s⁻¹, while the CO₂ concentration of incoming air was kept at ambient levels (400 ppm). A measurement was taken every 5 min for 40 h. Following the sample time, the leaves were removed and leaf area was measured as projected leaf area using a LI-3100C Area Meter (Li-Cor Biosciences).

Each 4 h measurement was divided into CAM phases. The 40 h measurements began at 0 h, the onset of the dark period. Day 1 phase I began at 0 h and ended at 12 h. Phase II began at 12 h, the onset of the light period. Phase II ended when CO₂ uptake was negligible, at which point phase III began. An exception to the phase II definition was made for C. rosea which exhibited a clear phase II peak in CO₂ uptake but did not have a functional phase III. In this case, phase II began at the onset of the light period, and ended when the initial CO₂ uptake spike had reached average CO₂ uptake levels.

CO₂ uptake was considered negligible when CO₂ uptake was <1% of maximum CO₂ uptake (determined for each leaf separately), at which point phase III began. In consideration of measurement noise, CO₂ uptake was not considered negligible unless three consecutive measurements of CO₂ uptake were <1% of maximum CO₂ uptake. CO₂ uptake was considered positive when a measurement was >1% maximum CO₂ uptake, and was followed by two consecutive measurements of positive CO₂ uptake. Phase IV began when CO₂ uptake was no longer negligible, and continued until the onset of the dark period at 24 h. Day 2 phase I began at 24 h and ended at 36 h.

Leaf titratable acidity: Titratable acidity measurements were determined on leaf samples collected at dusk and dawn from well-watered plants (n=3 plants for each species). Samples were frozen in liquid nitrogen and then placed in an opaque container in the −80 °C freezer until assay.

Leaf samples were prepared for titratable acidity assay by placing frozen leaf punches in hot 80% methanol for 40 min. The resulting solution was titrated against 5 mM NaOH using phenolphthalein as the acid–base indicator.

¹³C carbon isotope discrimination: Three samples from each plant (n=3 for each species) were analysed at the UC Davis Stable Isotope Facility using a continuous flow isotope ratio mass spectrometer (IRMS).

Anatomical measurements

Leaves were fixed in formaldehyde–acetic–alcohol (FAA), dehydrated in an ethanol series, embedded in Spurr’s resin, sectioned, and stained. Photosynthetically active tissue was then analysed for cell size, %IAS, and L₅₀/area as in Nelson et al. (2005). Leaf succulence was determined by removing three leaf discs from well-watered plants (n=3 plants per species), recording their fresh weight, and dividing by the total area.

Results

The eight selected CAM species ranged from strong to weak CAM (Fig. 1). Both V. fragrans and H. carnosa were identified as strong obligate constitutive CAM species, due to their complete dependence on phase I and II for net CO₂ gain (Fig. 1). Both species had negative phase IV CO₂ assimilation rates and positive phase I CO₂ assimilation rates, although H. carnosa had >3 times the CO₂ assimilation rate and subsequent CO₂ uptake than that of V. fragrans. The CO₂ uptake in phase II was a minor contribution in both species, although the CO₂ assimilation rate in phase II was higher in H. carnosa.

Kalanchoë daigremontiana, A. comosus, and C. rotundifolia were identified as strong constitutive CAM species (Fig. 1). All three species took up some CO₂ during phase IV, but this phase contributed only a small amount of carbon over the 24 h cycle. Cissus rotundifolia had the highest rate of CO₂ assimilation during phase II, during a brief burst of rapid CO₂ uptake, but still took up the majority of CO₂ during phase I. Both K. daigremontiana and A. comosus exhibited reductions in phase II and IV CO₂ fixation when compared with previously published studies. These reductions may reflect some degree of nutrient or water stress, imposed by earlier growth conditions, and matched by later experimental conditions. Leaf structure remained relatively constant throughout the
study, therefore, the relationship between CO₂ fixation and leaf structure in these two species is unlikely to be affected by minor nutrient or water stress.

Clusia rosea and C. fragrans were identified as weak constitutive CAM species, as neither species had a functional phase III, but both species had positive phase I CO₂ uptake (Fig. 1). Callisia fragrans took up a very small amount of CO₂ during the last few hours of the dark period, while C. rosea maintained a positive CO₂ assimilation rate for the duration of the dark period. Clusia rosea also took up a considerable amount of CO₂ during a brief phase II, while C. fragrans did not have an identifiable phase II signal.

Peperomia obtusifolia was identified as a C₃ species under well-watered conditions, as this species had negative phase I uptake (Fig. 1). Further experiments under drought treatments demonstrated weak CAM activity, consistent with published results of facultative inducement of CAM cycling (Helliker and Martin, 1997). Given the importance of CAM cycling species in the evolution of CAM (Griffiths, 1989; Guralnick and Jackson, 2001), this species was included in the study as a weak facultative CAM species.

Proportion of CO₂ uptake by phase

To control for different CO₂ assimilation rates, the proportion of CO₂ that was taken up in each phase was determined for each species (Table 1). The proportion of CO₂ taken up in phase I indicates the degree of CAM photosynthesis in each species. These results confirmed that V. fragrans, H. carnosa, A. comosus, and C. rotundifolia were strong CAM species, with all species exhibiting >70% of their total carbon uptake during phase I. Clusia rosea, C. fragrans, and P. obtusifolia exhibited weak CAM activity, with less than one-third daily CO₂ uptake during the night-time phase.

To determine whether %IAS and L_mes/area limit the degree of CAM photosynthesis, the proportion of phase I CO₂ uptake was plotted against these anatomical traits (Fig. 2). The proportion of phase I CO₂ uptake showed a significantly negative relationship with %IAS (P=0.049) and a negative trend when plotted against L_mes/area (P=0.097). This result indicates that species with high CAM function have reduced IAS, while weak CAM species exhibit C₃-like IAS values. The clear delineation between strong and weak CAM species on the basis of IAS provides evidence for an anatomical boundary of 17% IAS between strong and weak CAM modes, previously noted by Nelson et al. (2005).

To determine whether %IAS and L_mes/area limit the degree of C₃ photosynthesis, the proportion of phase IV CO₂ uptake was also plotted against these anatomical traits (Fig. 2). The proportion of phase IV CO₂ uptake is significantly related to %IAS (P=0.023) and shows a positive trend when plotted against L_mes/area (P=0.078). This result demonstrates that CAM species with high C₃ function (and therefore reduced CAM function) have increased IAS, and that there is a threshold of 17% IAS for high C₃ function.

The proportion of phase I CO₂ uptake was not significantly related to cell size or leaf succulence, indicating that enlarged cells and succulent photosynthetic tissues are not closely related to CAM function (data not shown).

Duration of each phase

The duration of each phase over a 24 h period was determined for each species (Table 2). Phase III duration was considered an indication of the degree of CAM photosynthesis, whereas phase IV duration was an indication of the degree of C₃ photosynthesis in each species. Strong CAM species had appreciable phase III
durations, ranging from 5.0 h to 11.7 h over the diel cycle. Weak CAM species exhibited negligible phase III durations, but lengthy phase IV durations ranging from 10.4 h to the full 12 h light period.

To determine whether %IAS and L mes/area limit the degree of CAM photosynthesis, phase III duration was plotted against these anatomical traits (Fig. 3). The duration of phase III was not related to %IAS, but showed a significant negative relationship when plotted against L mes/area (P = 0.033). This result indicates that species which conduct CAM photosynthesis for the majority of the day have reduced L mes/area, and that commitment to CAM over the diel cycle decreases as L mes/area increases.

To determine whether %IAS and L mes/area limit the degree of C3 photosynthesis, phase IV duration was also plotted against these anatomical traits (Fig. 3). The duration of phase IV showed a positive trend when plotted against %IAS (P = 0.075) and showed a significantly positive relationship when plotted against L mes/area (P = 0.029). This result demonstrates that CAM species with reduced time commitment to CAM photosynthesis (and therefore limited CAM function) are likely to have increased L mes/area, and that CAM commitment increases as L mes/area decreases.

**Strong versus weak CAM species**

To determine whether the anatomical traits of strong CAM species are significantly different from those of weak CAM species, A. comosus, C. rotundifolia, H. carnosa, K. daigremontiana, and V. fragrans were compared against C. fragrans, C. rosea, and P. obtusifolia (Fig. 4). Strong CAM species did not have significantly different cell size or leaf succulence from weak CAM species. However, strong CAM species did have lower

<table>
<thead>
<tr>
<th>Species</th>
<th>CAM</th>
<th>Proportion of CO₂ uptake</th>
<th>Phase I</th>
<th>Phase II</th>
<th>Phase III</th>
<th>Phase IV</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. fragrans</td>
<td>Strong</td>
<td>0.887±0.041</td>
<td>0.113±0.041</td>
<td>0</td>
<td>0</td>
<td>0.113±0.041</td>
<td>0.887±0.041</td>
</tr>
<tr>
<td>H. carnosa</td>
<td>Strong</td>
<td>0.881±0.049</td>
<td>0.119±0.049</td>
<td>0</td>
<td>0</td>
<td>0.119±0.049</td>
<td>0.881±0.049</td>
</tr>
<tr>
<td>K. daigremontiana</td>
<td>Strong</td>
<td>0.957±0.006</td>
<td>0.037±0.002</td>
<td>0</td>
<td>0</td>
<td>0.037±0.002</td>
<td>0.957±0.006</td>
</tr>
<tr>
<td>A. comosus</td>
<td>Strong</td>
<td>0.961±0.010</td>
<td>0.010±0.001</td>
<td>0</td>
<td>0</td>
<td>0.010±0.001</td>
<td>0.961±0.010</td>
</tr>
<tr>
<td>C. rotundifolia</td>
<td>Strong</td>
<td>0.703±0.063</td>
<td>0.216±0.037</td>
<td>0.002±0.001</td>
<td>0</td>
<td>0.216±0.037</td>
<td>0.703±0.063</td>
</tr>
<tr>
<td>C. rosea</td>
<td>Weak</td>
<td>0.164±0.077</td>
<td>0.150±0.119</td>
<td>0.004±0.001</td>
<td>0</td>
<td>0.150±0.119</td>
<td>0.164±0.077</td>
</tr>
<tr>
<td>C. fragrans</td>
<td>Weak</td>
<td>0.019±0.005</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.019±0.005</td>
<td>0.019±0.005</td>
</tr>
<tr>
<td>P. obtusifolia</td>
<td>Weak</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.006±0.009</td>
<td>0.006±0.009</td>
</tr>
</tbody>
</table>

**Fig. 2.** The relationship between the proportion of phase I and phase IV CO₂ uptake and the anatomical traits %IAS and L mes/area (mean ±SE, n=3). See Fig. 1 for abbreviations.
%IAS ($P < 0.01$) and a trend towards lower $L_{mes/area}$ ($P < 0.07$). These results indicate that cell size and leaf succulence are not good indicators of strong and weak CAM modes. These results further indicate that the divergence between strong and weak CAM modes is mediated by %IAS and $L_{mes/area}$.

**Twenty-four hour CO$_2$ assimilation rate**

To determine whether %IAS and $L_{mes/area}$ limit atmospheric CO$_2$ uptake over the diel cycle in constitutive CAM plants, these anatomical traits were compared against a CO$_2$ assimilation rate over a 24 h period which included both day and night phases. *Peperomia obtusifolia* was excluded from this analysis because it did not exhibit any CAM uptake under well-watered conditions, and was therefore not comparable with the focal species.

The 24 h CO$_2$ assimilation rate is not correlated with %IAS ($P=0.32$), but has a highly significant relationship with $L_{mes/area}$ ($P=0.003$; Fig. 5) when only constitutive CAM species are included in the analysis. These results indicate that reduced $L_{mes/area}$ limits CO$_2$ uptake over the diel cycle, and probably limits overall plant productivity.

**Determination of CAM function via alternative methods**

Values derived from 40 h gas exchange measurements were compared against dusk–dawn titratable acidity and $^{13}$C carbon isotope discrimination values for the same individuals. Values of overnight acid accumulation were closely related to phase I uptake (Table 3; $r^2=0.98$; $P<0.0001$), with any discrepancies probably due to recycling of respiratory CO$_2$ (Borland and Griffiths, 1996). The proportion of phase I uptake was closely

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**Table 2. Phase duration over a 24 h period for each species (mean ± SE, n=3)**

<table>
<thead>
<tr>
<th>Species</th>
<th>CAM</th>
<th>Phase duration (h)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phase I</td>
<td>Phase II</td>
<td>Phase III</td>
<td>Phase IV</td>
<td>24 h</td>
<td></td>
</tr>
<tr>
<td>V. fragrans</td>
<td>Strong</td>
<td>12</td>
<td>0.3±0.1</td>
<td>11.7±0.1</td>
<td>0</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>H. carnosa</td>
<td>Strong</td>
<td>12</td>
<td>1.6±0.7</td>
<td>10.4±0.7</td>
<td>0</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>K. daigremontiana</td>
<td>Strong</td>
<td>12</td>
<td>0.6±0.04</td>
<td>10.4±0.7</td>
<td>1.0±0.7</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>A. conosus</td>
<td>Strong</td>
<td>12</td>
<td>0.3±0.04</td>
<td>6.5±0.3</td>
<td>5.2±0.3</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>C. rotundifolia</td>
<td>Strong</td>
<td>12</td>
<td>2.9±1.0</td>
<td>5.0±0.9</td>
<td>4.1±0.3</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>C. rosea</td>
<td>Weak</td>
<td>12</td>
<td>1.6±0.04</td>
<td>0</td>
<td>10.4±0.04</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>C. fragrans</td>
<td>Weak</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12.0</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>P. obtusifolia</td>
<td>Weak</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>12.0</td>
<td>24</td>
<td></td>
</tr>
</tbody>
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**Fig. 3.** The relationship between phase III and phase IV duration and the anatomical traits %IAS and $L_{mes/area}$ (mean ± SE, n=3). See Fig. 1 for abbreviations.
related to $^{13}$C carbon isotope discrimination values (Table 3; $r^2=0.93; \ P<0.0001$), consistent with the results of Winter and Holtum (2002). These strong relationships demonstrate that values derived from gas exchange measurements are consistent with other methods of characterizing CAM expression, although gas exchange measurements are better able to describe the varied phase behaviour of a wide range of CAM species.

Discussion

This study examined four anatomical traits that have been associated with CAM photosynthesis: cell size, leaf succulence, %IAS, and $L_{mes}/area$. The hypothesis that these traits are correlated with the degree of CAM photosynthesis was tested. Cell size and leaf succulence are known to act as functional traits in CAM photosynthesis due to their importance in overnight acid storage (Osmond et al., 1999; Borland et al., 2000). Reduced IAS and $L_{mes}/area$ have been previously identified as common CAM anatomical traits, but have been regarded as side-effects of large cells in succulent leaves (Smith and Heuer, 1981; Maxwell et al., 1997). Increased leaf succulence, decreased IAS, and decreased $L_{mes}/area$ are all associated with declines in internal CO$_2$ conductance, or $g_i$ (Gillon et al., 1998; Evans and von Caemmerer, 1996; Evans and Loreto, 2000).

Cell size and leaf succulence as CAM functional traits

This study indicates that cell size and leaf succulence are not closely related to the degree of CAM function, despite their prevalence amongst CAM species. The species examined in this study all had succulent leaves, which supports the existing CAM paradigm that succulence and large cell size are pre-conditions for the development of CAM photosynthesis (Griffiths, 1989; Borland et al., 2000; Guralnick and Jackson, 2001). Results from Nelson et al. (2005) demonstrated that there was an extensive overlap between CAM, C$_3$, and C$_4$ species with respect to cell size, indicating a wide variation in this trait amongst diverse plant groups. Cell size and leaf succulence are therefore unlikely to mediate the divergence of CAM species from their C$_3$ ancestors, and thus are not significantly different between weak and strong CAM species.

Reduced IAS as a CAM functional trait

This study indicates that %IAS is related to the proportion of carbon taken up by both CAM and C$_3$ phases, with CAM photosynthesis favouring IAS values <17%, and C$_3$ photosynthesis favouring leaves with >17% air space. The proportion of IAS is also significantly different between weak and strong CAM species, thus indicating the presence of a constraint between optimal anatomy for high CAM function and the internal structure ideal for C$_3$ photosynthesis.

Nelson et al. (2005) demonstrated that CAM species in general have reduced IAS values relative to C$_3$ and C$_4$ species, with a threshold between the two of 17%. Given such clear delineations of IAS between CAM and non-CAM, and now between strong and weak CAM, this anatomical trait may represent a functional threshold between C$_3$ and CAM activity.

Given these results, it appears unlikely that reduced IAS is an unfortunate consequence of increased cell size and leaf succulence, as suggested by Maxwell et al. (1997) and Smith and Heuer (1981). The proportion of IAS is not closely related to either cell size or leaf succulence (Nelson et al., 2005), and the close association between IAS and CAM function indicates that low IAS is necessary for high CAM function. Given that strong
CAM photosynthesis and reduced IAS have arisen in parallel across several distinct evolutionary lineages, appears likely that the association between low IAS and high CAM reflects an evolutionary pre-requisite for high CAM function.

Reduced Lmes/area as a CAM functional trait

Lmes/area is an anatomical trait that was first characterized by this laboratory, and parallels the more common Smes measurements used elsewhere (Evans and Loreto, 2000). Smes measurements in CAM leaves are strongly biased by leaf thickness, and the results are difficult to interpret without making large assumptions about the influence of vertical pathlengths. Lmes/area removes this bias, and provides a simple ratio of total mesophyll surface perimeter to leaf area, which is more directly indicative of the cell wall resistance to CO2. S_c, which is the surface of chloroplasts exposed to IAS, is the anatomical measure which is most closely related to g_i (Evans and Loreto, 2000). However, Maxwell et al. (1997) observed that the intracellular space adjacent to the cell wall in the CAM plant K. daigremontiana was shared by both chloroplasts and mitochondria, and was not dominated by chloroplasts as is assumed by measurement of S_c, and is the norm in C_3 leaves. They suggested that the unique anatomy of CAM mesophyll cells was due to the differing sites of carboxylation: the cytosol for PEPC and the chloroplasts for Rubisco (Maxwell et al., 1997). Both carboxylating enzymes require access to CO2 in the IAS, and must share space along the cell surface. For these reasons, Lmes/area is the most relevant and least biased proxy measurement for cell wall resistance in CAM tissues.

This study indicates that reduced Lmes/area is closely related to the time committed to CAM photosynthesis, as well as the total amount of CO2 assimilated over the diel cycle. The highly significant positive relationship between Lmes/area and 24 h CO2 assimilation indicates a significant resistance to CO2 diffusion across the cell wall. This resistance is more costly during C_3 photosynthesis, given the additional resistance of the chloroplast membranes (Maxwell et al., 1997; Evans and von Caemmerer, 1996; Evans and Loreto, 2000). Consequently, the cost of reduced Lmes/area is reduced C_3 carbon uptake, probably resulting in reduced plant productivity (Maxwell et al., 1997; Borland et al., 2000; Maxwell, 2002).

Unlike IAS, CAM species were not completely distinct from C_3 and C_4 species on the basis of Lmes/area, and some overlap occurred between strong and weak CAM species (Nelson et al., 2005). Lmes/area does not represent a clear threshold between low and high CAM function, but a continuum of declining C_3 function across all CAM species. The loss of C_3 function may be a powerful impetus to enhance commitment to the CAM pathway. Therefore, the cost of reduced productivity mediated by Lmes/area may drive the divergence of CAM species from C_3 ancestors.

Reduced Lmes/area significantly increased the duration of phase III, as the cell wall resistance to CO2 operates in both directions. Lower Lmes/area limits CO2 efflux from the sites of carboxylation, enhancing the phase III CCM (Borland et al., 2000; Maxwell, 2002; Nelson et al., 2005). Limiting CO2 efflux enhances CAM efficiency by increasing the likelihood of CO2 recapture in all four phases, regardless of whether the carbon is derived from respiration or malate decarboxylation (Maxwell et al., 1997; Gillon et al., 1998; Nelson et al., 2005). Increasing the duration of the phase III CCM allows CAM plants to maximize photosynthetic yield and minimize photoinhibition during the time of highest light availability (Skillman and Winter, 1997; Osmond et al., 1999).

Implications of reduced IAS and Lmes/area

These results indicate that reduced IAS and Lmes/area are essential for increased CAM function, despite inevitable reductions in internal CO2 conductance. Reduced g_i limits

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**Table 3. Comparison of alternative methods of CAM function determination**

Phase I CO2 uptake as well as the proportion of phase I CO2 uptake were determined from leaf gas exchange, whereas overnight acid accumulation was determined via dusk–dawn titratable acidity, and 13C discrimination was determined by isotopic analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>40 h gas exchange</th>
<th>Dusk–dawn titratable acidity</th>
<th>13C isotope discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase I CO2 uptake (mmol CO2 m⁻²)</td>
<td>Proportion of phase I CO2 uptake</td>
<td>Acid accumulation (mmol H⁺ m⁻²)</td>
</tr>
<tr>
<td>V. fragrans</td>
<td>9.5±0.8</td>
<td>0.887±0.041</td>
<td>7.6±5.3</td>
</tr>
<tr>
<td>H. carnosa</td>
<td>80.2±34.2</td>
<td>0.881±0.049</td>
<td>139.4±24.3</td>
</tr>
<tr>
<td>K. daigremontiana</td>
<td>321.2±29.0</td>
<td>0.957±0.006</td>
<td>376.0±107.5</td>
</tr>
<tr>
<td>A. comosus</td>
<td>231.2±19.7</td>
<td>0.961±0.010</td>
<td>247.7±20.6</td>
</tr>
<tr>
<td>C. rotundifolia</td>
<td>77.1±15.8</td>
<td>0.703±0.063</td>
<td>112.9±53.0</td>
</tr>
<tr>
<td>C. rosea</td>
<td>64.4±25.8</td>
<td>0.164±0.077</td>
<td>68.9±49.1</td>
</tr>
<tr>
<td>C. fragrans</td>
<td>4.6±0.7</td>
<td>0.019±0.005</td>
<td>22.7±17.4</td>
</tr>
<tr>
<td>P. obtusifolia</td>
<td>0.0</td>
<td>0</td>
<td>22.7±4.0</td>
</tr>
</tbody>
</table>
the availability of CO2 to both PEPC and Rubisco, the two carboxylating enzymes. This limitation is only relevant if CO2 availability is the primary factor limiting the CO2 assimilation rate. CAM CO2 uptake through the action of PEPC is generally limited by PEPAvailability, which is determined by starch availability, which in turn depends upon light availability the previous day (Borland et al., 1998, 2000). Reduced g, is therefore not expected to affect CO2 uptake during phase I, and may reduce efflux of respiratory CO2, enabling carbon recycling by PEPC (Gillon et al., 1998; Griffiths et al., 2007). Phase III is not limited by the availability of CO2, as high concentrations of CO2 are generated behind closed stomates, and reduced g, may prevent the leakage of CO2 from the leaf (Gillon et al., 1998; Borland et al., 2000). Therefore, the two characteristic phases of strong CAM photosynthesis may benefit from reduced conductance due to the enhancement of carbon economy, a driving force of CAM evolution (Griffiths, 1989). Hence, low IAS and Lmes/area may reflect selection to promote the efficiency of CAM function (Maxwell et al., 1997; Nelson et al., 2005).

However, reduced conductance will limit the CO2 concentration around Rubisco active sites during Rubisco-mediated atmospheric CO2 uptake, thus reducing Rubisco photosynthetic efficiency (Evans and von Caemmerer, 1996; Maxwell et al., 1997, 1998; Evans and Loreto, 2000). This effect will be limited to phase IV, given high light availability but low CO2 delivery to Rubisco (Maxwell et al., 1999; Maxwell, 2002). CAM species are often dependent on phase IV-derived assimilates for gains in growth and productivity, with high phase IV CO2 assimilation rates allowing some CAM species to achieve C3-like productivities under ideal conditions (Nobel, 1996; Borland et al., 2000). Therefore, CAM leaf anatomy may limit productivity due to decreased efficiency of C3 photosynthesis (Maxwell, 2002). Hence, low IAS and Lmes/area may reflect a fundamental constraint between CAM carbon economy and C3 productivity.

In conclusion, both reduced IAS and Lmes/area are associated with the degree of CAM, and may serve an important functional role in CAM photosynthesis by preventing the efflux of previously fixed CO2 during CAM phases I and III. These anatomical traits may also act as fundamental constraints within CAM evolution, due to the lost potential carbon gain during Rubisco-mediated atmospheric CO2 uptake in phase IV. Functional anatomy may therefore mediate the bimodal distribution of CAM expression within putative CAM species, as this distribution may reflect the divergence between ideal leaf anatomies for each photosynthetic pathway (Silvera et al., 2005). Given the phylogenetic, anatomical, ecological, and functional diversity of the species selected for this study, and the degree of plasticity in leaf structure exhibited under varying age and growth conditions, the conservation of these basic anatomical traits is remarkable. Further examination of the relationship between CAM function and CAM anatomy within closely related species or subspecies may further clarify the fidelity of this relationship.

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