CSIRO PUBLISHING
Review
www.publish.csiro.au/journals/fpb
Functional Plant Biology, 2010, 37, 995–1010

Evolution along the crassulacean acid metabolism continuum

Katia Silvera[A], Kurt M. Neubig[B], W. Mark Whitten[B], Norris H. Williams[B], Klaus Winter[C] and John C. Cushman[A,D]

[A]Department of Biochemistry and Molecular Biology, MS200, University of Nevada, Reno, NV 89557-0200, USA.
[B]Florida Museum of Natural History, University of Florida, Gainesville, FL 32611-7800, USA.
[C]Smithsonian Tropical Research Institute, PO Box 0843-03092, Balboa, Ancón, Republic of Panama.
[D]Corresponding author. Email: jcushman@unr.edu

This paper is part of an ongoing series: '*The Evolution of Plant Functions*'.

Abstract. Crassulacean acid metabolism (CAM) is a specialised mode of photosynthesis that improves atmospheric CO_2 assimilation in water-limited terrestrial and epiphytic habitats and in CO_2-limited aquatic environments. In contrast with C_3 and C_4 plants, CAM plants take up CO_2 from the atmosphere partially or predominantly at night. CAM is taxonomically widespread among vascular plants and is present in many succulent species that occupy semiarid regions, as well as in tropical epiphytes and in some aquatic macrophytes. This water-conserving photosynthetic pathway has evolved multiple times and is found in close to 6% of vascular plant species from at least 35 families. Although many aspects of CAM molecular biology, biochemistry and ecophysiology are well understood, relatively little is known about the evolutionary origins of CAM. This review focuses on five main topics: (1) the permutations and plasticity of CAM, (2) the requirements for CAM evolution, (3) the drivers of CAM evolution, (4) the prevalence and taxonomic distribution of CAM among vascular plants with emphasis on the Orchidaceae and (5) the molecular underpinnings of CAM evolution including circadian clock regulation of gene expression.

Additional keywords: phospho*enol*pyruvate carboxylase, photosynthesis, $\delta^{13}C$.

Introduction

Crassulacean acid metabolism (CAM) is one of three modes of photosynthetic assimilation of atmospheric CO_2, along with C_3 and C_4 photosynthesis. The net result of CAM is an improvement in water use efficiency (WUE; CO_2 fixed per unit water lost) generally 6-fold higher than for C_3 plants and 3-fold higher than for C_4 plants under comparable conditions (Nobel 1996). Thus, CAM is an important ecophysiological metabolic adaptation that permits plants to occupy semiarid habitats and habitats with intermittent or seasonal water availability (Winter and Smith 1996; Cushman 2001). In this review, we examine the permutations and plasticity of CAM in the context of evolution, discuss the metabolic and genetic requirements for CAM – including leaf succulence – and consider the likely drivers for the evolution of CAM. We next review the current surveys of the taxonomic distribution of CAM species and the several survey methods used to estimate the prevalence of CAM. We then discuss the molecular evolution of CAM, including its origins, describe molecular markers used to study the evolutionary progression of gene family changes and to analyse circadian clock control. Finally, we address future directions for research.

Phases of CAM

The physiological and biochemical temporal sequence of events that constitute CAM have been described in detail as being separable into four discrete phases (Osmond 1978; Winter 1985; Lüttge 1987; Griffiths 1988). Phase I is typically characterised by nocturnal stomatal opening, CO_2 uptake and fixation by phospho*enol*pyruvate carboxylase (PEPC) in the cytosol and the formation of C_4 organic acids (usually malic acid), which are stored in the vacuole (Fig. 1). The rate of nocturnal CO_2 assimilation is governed by mesophyll processes, such as regulation of carbohydrate storage reserves (Cushman *et al*. 2008*a*) or vacuolar storage capacity, rather than stomatal conductance (Winter 1985; Winter *et al*. 1985). Depending on the CAM species, a variety of storage carbohydrates (e.g. starch, glucans, soluble hexoses) might be catabolised to produce phospho*enol*pyruvate (PEP), the substrate for carboxylation (Christopher and Holtum 1996, 1998; Holtum *et al*. 2005). Phase I reflects the fundamental adaptation of CAM that results in reduced transpiration and improved water economy due to lower night-time evapotranspirational demands and associated water losses (Griffiths 1988). Phase II describes the transition from PEPC to ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO)-mediated carboxylation during the early light period leading to carbohydrate production. During this phase, CO_2 is derived from both organic acid decarboxylation and direct uptake from the atmosphere. Phase III encompasses the period of major efflux of organic acids from the vacuole and their subsequent decarboxylation (Fig. 1). This decarboxylation can lead to

© CSIRO 2010
10.1071/FP10084
1445-4408/10/110995

Fig. 1. Postulated major CAM metabolite fluxes and transport requirements during the night and day. During night-time (CAM phase I, black arrows), while stomata are open, atmospheric CO_2 enters the cell and is fixed along with respired CO_2 as bicarbonate (HCO_3^-) by PEPC leading to the formation of oxaloacetate and then malate, which undergoes protonation and is stored in the vacuole as malic acid. During day-time (CAM phase III, yellow arrows), while stomata are closed, malic acid exits the vacuole and is decarboxylated. CO_2 released in the cytosol or mitochondria is then refixed by RUBISCO through the PCR cycle within the chloroplast. Pyruvate produced in the cytosol or mitochondria moves into the plastid to serve as the substrate for the gluconeogenic pathway where carbohydrates are regenerated and stored as starch. For simplicity, only key reactions of metabolic flux are shown with key enzymes indicated in red font. Cofactors, charge state and allosteric interactions of metabolites are not shown for simplicity. Model shown is for a starch-forming CAM plant such as *Mesembryanthemum crystallinum*, which uses NADP-ME and NAD-ME for decarboxylation of malate. Some CAM species utilise PEP carboxykinase (PEPCK), which decarboxylates OAA resulting in the formation of PEP (not shown). Hexose-storing CAM species, such as *Ananas comosus* store hexose in the vacuole that is transported to the cytosol (see Holtum *et al*. 2005). G6P, glucose-6-phosphate; TP, triose phosphate; PGA, 3-phosphoglycerate; PEP, phospho*enol*pyruvate; OAA, oxaloacetate; MAL, malate; FUM, fumarate; PYR, pyruvate; TCA cycle, tricarboxylic acid cycle; PCR cycle, photosynthetic carbon reduction cycle. Dashed arrows indicate multiple metabolic steps or diffusion. Key enzymes include CA, carbonic anhydrase; PEPC, cytosolic phospho*enol*pyruvate carboxylase; PPCK, PEPC kinase; NADP-ME, cytosolic NADP-dependent malic enzyme; NAD-ME, mitochondrial NAD-dependent malic enzyme; NAD-MDH, cytosolic and mitochondrial NAD-dependent malate dehydrogenase; PPDK, plastidic pyruvate orthophosphosphate dikinase; RUBISCO, plastidic ribulose-1,5-bisphosphate carboxylase/oxygenase. Transporters indicated by numbered squares include: (1) pGlcT, plastidic glucose transporter; (2) GPT, glucose-6-phosphate/phosphate transporter; (3) TPT, triose-phosphate/phosphate transporter; (4) PYT, plastidic pyruvate transporter; (5) PPT, plastidic phospho*enol*ypyruvate/phosphate transporter; (6) mDCT, putative mitochondrial dicarboxylate carriers; (7) putative mitochondrial pyruvate transporter; (8) inward-rectifying anion-selective malate channel; (9) V-ATPase, tonoplast vacuolar H^+-ATPase complex; (10) V-PP_iase, tonoplast vacuolar PP_iase complex; ?) unknown protein proposed to mediate malic acid efflux, possibly a tonoplast dicarboxylate transporter. This model is derived, in part, from information summarised by Holtum *et al*. (2005).

generation of internal leaf CO_2 partial pressures greater than 100 times atmospheric levels (Cockburn *et al*. 1979; Spalding *et al*. 1979), reduction in stomatal opening and transpiration and sometimes even release of CO_2 from the leaf despite low stomatal conductance (Frimert *et al*. 1986). Decarboxylation is catalysed by either cytosolic PEP carboxykinase (PEPCK) or cytosolic $NADP^+$- and/or mitochondrial NAD^+-malic enzymes (ME) (Smith and Bryce 1992; Christopher and Holtum 1996; Holtum *et al*. 2005). This CO_2-concentrating mechanism or 'CO_2 pump' effectively suppresses photorespiration during this phase.

Phase IV is a second transitional phase marked by the depletion of organic acid stores, slower rates of decarboxylation, reduction in internal partial pressure of CO_2 and increases in stomatal conductance, depending on the prevailing environmental conditions. CO_2 fixation during early phase IV is a mixture of CO_2 assimilation derived mainly from organic acid decarboxylation and direct atmospheric uptake and assimilation via RUBISCO; however, carboxylation into C_4 acids by PEPC may commence as the dark period approaches (Ritz *et al*. 1986). Because CAM plants perform both CO_2 fixation steps within the same cell, futile cycling of CO_2 is minimised by temporal control of the kinetic properties of PEPC in response to malic acid (Winter 1982) and of RUBISCO in response to light (Maxwell *et al*. 1999; Griffiths *et al*. 2002). The diel changes in kinetic properties of PEPC (Winter 1982) are triggered by reversible phosphorylation events catalysed by a dedicated protein kinase (Hartwell *et al*. 1996, 1999; Taybi *et al*. 2000), whose expression is controlled by the circadian clock (Hartwell 2005*a*, 2005*b*).

Permutations of CAM

Although the four CAM phase definitions appear adequate to describe all observed acid metabolism phenomena (Lüttge 1987), additional terminologies have been suggested to describe CAM in some astomatal aquatic species and in the astomatal green aerial roots of epiphytic orchids (Cockburn 1985). Furthermore, environmental conditions can modulate the extent to which each phase is manifested (see also next section) (Cushman 2001; Cushman and Borland 2002). For example, water deficit stress can reduce or eliminate phase IV and light and temperature can regulate the appearance or onset of phases II and III (Griffiths 1988). Under severe water deficit stress, phase I net nocturnal CO_2 uptake can be eliminated completely along with virtually all stomatal conductance across the four phases. This phenomenon, termed 'CAM idling', results in small, sustained diel fluctuations in organic acids with essentially all of the CO_2 fixed into malate being derived from internally recycled respiratory CO_2 (Szarek *et al*. 1973; Ting 1985). CAM idling might play an important role in the prevention of photoinhibition by maintaining photosystem stability (Osmond 1982). The phenomena of 'CAM cycling' or 'weak CAM' have also been described, wherein organic acid fluctuations are observed, but with little or no net nocturnal CO_2 fixation by PEPC (Sipes and Ting 1985; Ting 1985). In the context of evolution, CAM cycling has been interpreted to be a basal form of CAM, while increasing reliance on nocturnal CO_2 fixation has been associated with an increasingly advanced state among the Crassulaceae (Teeri 1982*a*, 1982*b*) and the Bromeliaceae (Smith *et al*. 1986). The ecophysiological significance of CAM cycling might be to keep plants poised to engage in full CAM once drought conditions end by maintaining the capacity for organic acid fluctuation (Ting 1985). Similarly, the evolutionary importance of weak CAM might be that it serves as a genetic reservoir for CAM radiations in the context of changing environmental conditions or habitat exploitation, such as epiphytism (Silvera *et al*. 2005, 2009). Finally, the term 'latent CAM' has been used to describe an intermediate form of CAM wherein organic acid concentrations remain high but constant throughout the diel cycle (Schuber and Kluge 1981). As with CAM cycling or weak CAM, latent CAM might be regarded as a step along the progression from C_3 to CAM (Lee and Griffiths 1987).

CAM plasticity

The degree to which CAM operates can vary greatly depending on the evolutionary history of a given species and its environmental context, resulting in a continuum of differences in the degree to which nocturnal net uptake of CO_2 occurs in relation to day-time net CO_2 uptake (Cushman and Bohnert 1999; Cushman 2001; Cushman and Borland 2002; Dodd *et al*. 2002). For example, many CAM species engage in 'obligate' or 'constitutive' CAM in fully mature photosynthetic organs (i.e. leaves and stems), although the extent of gas exchange and nocturnal acidification might be modulated by prevailing environmental conditions (Griffiths 1988). Many members of the Cactaceae and Crassulaceae provide excellent examples of this type of CAM. In contrast, 'facultative', 'inducible', or 'optional' CAM or C_3-CAM intermediate species engage in CAM in response to environmental stimuli such as drought stress (Winter 1985; Griffiths 1988; Winter *et al*. 2008). The expression of CAM in such C_3-CAM species varies dynamically with experimentally manipulated conditions, such as photoperiod (Brulfert and Queiroz 1982), water status, light, temperature, nutritional status, salinity, anoxia, or atmospheric CO_2 concentration (Winter 1985; Lüttge 1987; Griffiths 1988; Roberts *et al*. 1997). The common ice plant, *Mesembryanthemum crystallinum* L., a member of the Aizoaceae, is a well studied example of inducible CAM under strict environmental control (Winter 1985; Winter and Holtum 2007; Cushman *et al*. 2008*a*, 2008*b*).

Requirements for CAM

Because the basic enzymatic machinery essential for CAM operation is assumed to be present in the chloroplast-containing cells of all green plant species, what are the evolutionary changes that must occur in order for CAM to function? The first and foremost diagnostic indicator of CAM is nocturnal CO_2 uptake (Fig. 2). Second, diel fluctuations in organic acids and reciprocal fluctuations of storage carbohydrates such as starch, glucans, or soluble hexoses typically occur as features of the CAM cycle (Ting 1985). Third, associated transport activities across the tonoplast (e.g. vacuolar H^+-ATPase), mitochondrial (Holtum *et al*. 2005; Cushman *et al*. 2008*b*), and chloroplast envelope membranes (Häusler *et al*. 2000; Kore-eda *et al*. 2005) are needed to support these fluctuations (Fig. 1). Fourth, enhanced expression of PEPC and decarboxylating (e.g. PEPCK or $NADP^+$-/NAD^+-ME) enzymes is necessary. Fifth, enhanced expression of enzymes of both the glycolytic and gluconeogenic pathways is required to support the synthesis of large (typically 40–60% of available reserves) reciprocating pools of carbohydrates (Paul *et al*. 1993; Borland and Dodd 2002; Dodd *et al*. 2003). Discrete isogenes appear to be recruited selectively in order to conduct the activities necessary for CAM function (Kore-eda *et al*. 2005; Cushman *et al*. 2008*b*). Sixth, some degree of leaf succulence, characterised by increased mesophyll cell size due to large storage vacuoles and increased mesophyll tissue and leaf thickness are often characteristic of CAM species. Such large cell volumes per unit leaf or stem area

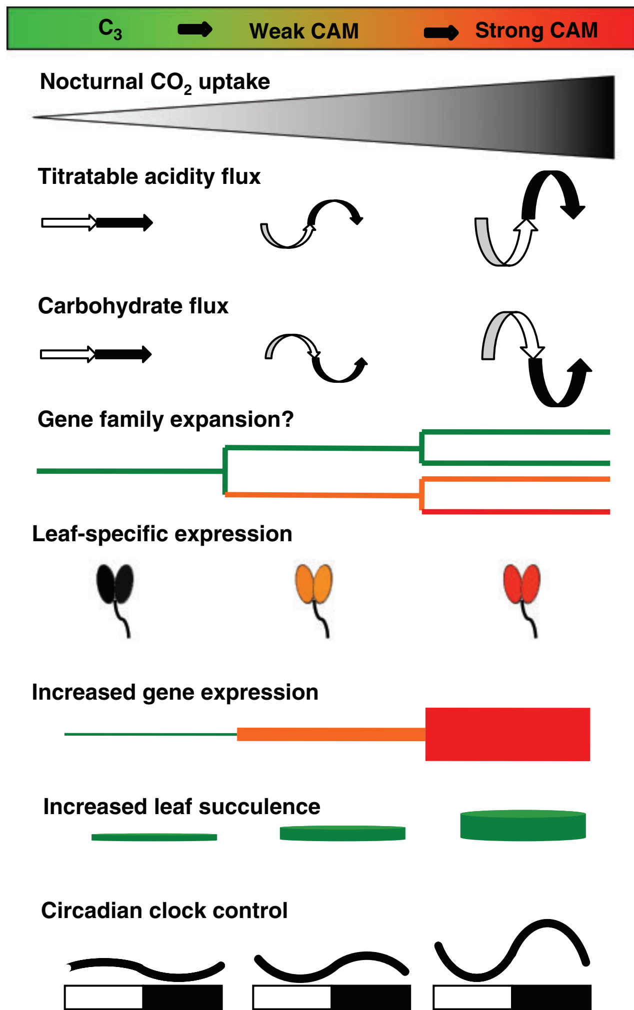

Fig. 2. Major requirements hypothesised for the evolution of CAM along the evolutionary progression from C_3 photosynthesis to weak CAM and strong CAM indicated by colour transitions from green to orange and red, respectively. The relative contribution of nocturnal CO_2 uptake increases as indicated by increased dark shading and height of triangle. Fluctuations in titratable acidity increase due to diel fluctuations in organic acid accumulation and reciprocal fluctuations in storage carbohydrates such as starch, glucans, or soluble hexoses as indicated by white (day-time) and black (night-time) arrows demonstrating increasing diel flux dynamics. These fluctuations are accompanied by associated increases in transport activities across the tonoplast (e.g. vacuolar H^+-ATPase), mitochondrial and chloroplast envelope membranes (not shown). Putative expansion in the complexity of gene families due to gene duplication events accompanied by selective recruitment of CAM-specific isogenes is indicated by orange and red lines. Diagnostic indicators of gene recruitment include a progressive increase in leaf-specific or leaf-preferential mRNA and protein expression and overall increased expression of PEPC, decarboxylating (e.g. PEPCK or $NADP^+$-/NAD^+-ME) enzymes and enzymes of both the glycolytic and gluconeogenic pathways indicated by orange and red coloured leaves and bars, respectively. CAM evolution is well correlated with increased leaf succulence characterised by mesophyll cells with increased size, increased mesophyll tissue and leaf thickness due to large storage vacuoles as indicated by the increased thickness of leaf models drawn in side view. Finally, circadian clock control is thought to progress by both shifts in the phase of circadian clock output and increased magnitude of circadian expression patterns, particularly at the level of mRNA expression. White shaded box, day-time, black shaded box, night-time.

ensure a high capacity for nocturnal organic acid storage and water storage (Gibson 1982; Nelson *et al.* 2005). Lastly, circadian clock control of CO_2 fixation, and mRNA and post-translational regulatory events, such as the reversible phosphorylation of PEPC by PEPC kinase (Fig. 1), are required to ensure that reciprocating organic acid and carbohydrate pools are properly synchronised along the diel CAM cycle (Borland *et al.* 1999; Hartwell *et al.* 1999; Taybi *et al.* 2000; Dodd *et al.* 2003; Cushman *et al.* 2008*a*, 2008*b*).

The differential enzyme-mediated discrimination against $^{13}CO_2$ during photosynthetic carbon assimilation between C_3 photosynthesis and CAM results in different whole-tissue carbon isotope ratios ($\delta^{13}C$) (Ehleringer and Osmond 1989). Species exhibiting pronounced CAM typically show $\delta^{13}C$ values less negative than –20‰; whereas, for C_3 plants, $\delta^{13}C$ values may range from about –33 to –22‰ (Ehleringer and Osmond 1989; Griffiths 1992) depending, for example, on interspecific variation in the chemical and diffusional processes that contribute to the carbon isotopic signature, plant water status, altitude and plant position within a forest canopy. Thus, $\delta^{13}C$ values have become widely used as a rapid and relatively inexpensive screening method for determining the presence of strong CAM. However, low level CAM activity or 'weak CAM' species have $\delta^{13}C$ values that overlap with those of C_3 species because the majority of CO_2 is being fixed by the C_3 pathway (Table 1) (Winter and Holtum 2002). Small changes in integrated tissue $\delta^{13}C$ values caused by small amounts of dark CO_2 fixation typical of weak CAM species are generally not significantly different from the wide range of $\delta^{13}C$ values exhibited by C_3 species in large species surveys due to variations in plant biochemistry, plant-environment interactions, and the $^{13}C/^{12}C$ composition of the source air under field conditions (Griffiths 1992; Winter and Holtum 2002).

Convergence of leaf succulence in CAM species

A general anatomical feature of CAM plants and apparent evolutionary co-requisite for CAM is leaf succulence (Fig. 2) with vacuoles occupying 90–95% of the volume of cells with

Table 1. $\delta^{13}C$ values, nocturnal fluctuation in titratable acidity, and leaf traits from 173 orchid species

Titratable acidity and leaf traits are represented by the mean ± s.d. from 86 C_3 species, 42 weak CAM, and 45 strong CAM species (Silvera *et al.* 2005). Species with $\delta^{13}C$ values less negative than –22‰ were designated strong CAM and species with $\delta^{13}C$ values more negative than –22‰ were designated weak CAM or C_3 depending on whether or not significant nocturnal increases in acidity were present (weak CAM) or absent (C_3). Specific leaf area (SLA equals area per unit dry mass); ratio of fresh mass to dry mass (FM/DM). Values in parentheses represent the fold-change in titratable acidity and corresponding leaf traits in weak CAM compared with C_3 photosynthesis or strong CAM compared with weak CAM

	C_3	Weak CAM	Strong CAM
$\delta^{13}C$ (‰) mean ± s.d.	–27.7 ± 1.8	–26.7 ± 2.3	–16.1 ± 2.6
Titratable acidity (ΔH^+) (μmol H^+ g^{-1} FW)	2.0 ± 3.2	12.2 ± 9.5 (+6.1)	75.8 ± 60.2 (+6.2)
Leaf thickness (mm)	0.5 ± 0.4	0.5 ± 0.3 (0)	2.0 ± 2.01 (+4.1)
SLA (cm^2 g^{-1})	167 ± 78	145 ± 59 (–0.8)	72 ± 31 (–2.0)
FM/DM	6.1 ± 2.1	6 ± 1.8 (0)	10.5 ± 4.1 (+1.8)

dimensions of greater than 100 µm (Gibson 1982; Smith 1984). A tight correlation between greater tissue succulence and increased magnitude of CAM has been observed within the Crassulaceae (Teeri *et al*. 1981; Kluge *et al*. 1991, 1993), in the Orchidaceae (Winter *et al*. 1983; Silvera *et al*. 2005), as well as in many other diverse CAM families (Nelson *et al*. 2005; Nelson and Sage 2008). Large cell size leads to a tightly packed chlorenchyma with reduced intercellular air spaces (IAS) and reduced surface area exposure of mesophyll cells to IAS (L_{mes}/area), which likely results in low internal conductance of CO_2 (g_i) and restriction of CO_2 efflux (particularly internal CO_2 leakage during phase III), thereby enhancing CAM carbon economy. Moreover, in CAM plants, C_3 photosynthetic CO_2 uptake during phases II and IV is believed to be limited more strongly by low g_i than PEPC mediated nocturnal CO_2 uptake during phase I (Maxwell *et al*. 1997; Maxwell 2002; Nelson and Sage 2008), increasing the reliance on CAM in highly succulent CAM species.

If the above anatomical traits associated with leaf succulence enhance the degree of CAM photosynthesis and limit the degree of C_3 photosynthesis, then all CAM species, regardless of their evolutionary lineage, would be expected to converge to a common succulent leaf anatomy. Supporting evidence for this hypothesis has been obtained by comparing the degree of leaf succulence, indicated by leaf thickness, to leaf $\delta^{13}C$ values (Winter *et al*. 1983; Zotz and Ziegler 1997). For example, a survey of leaf thickness and leaf $\delta^{13}C$ values in 173 tropical orchid species (Table 1) revealed that in species with leaf $\delta^{13}C$ values commonly observed for C_3 plants (–32 to –22‰), leaf thickness averaged 0.5 ± 0.4 mm, whereas in species with $\delta^{13}C$ values typical of strong CAM (–21.9 to –12‰), leaf thickness averaged 2.03 ± 2.01 mm. In weak CAM species with $\delta^{13}C$ values between –32 and –22‰, leaf thickness averaged 0.5 mm $\pm$ 0.3 mm (Silvera *et al*. 2005).

Thus, relative leaf thickness might serve as a useful, surrogate indicator for the presence of CAM activity provided that hydrenchyma (without chloroplast containing cells) is not the largest contributor of leaf thickness (Fig. 2). Just as increased leaf thickness and concomitant increase in storage capacity for malic acid confer a selective advantage for committing to CAM, if, in CAM plants, increased leaf succulence and associated decreases in g_i indeed affect CO_2 uptake in the light more than in the dark (Nelson and Sage 2008), then, evolutionary progression from the C_3 to CAM state would appear to favour either retention of C_3 photosynthesis or full conversion to CAM, but not the intermediate state. Indeed, such a pattern is reflected in differences in high and low IAS and L_{mes}/area values between weak and strong CAM species (Nelson and Sage 2008), as well as the bimodal distribution of $\delta^{13}C$ values observed in large surveys of plant families with mixtures of C_3/weak CAM and CAM species (see text below) (Zotz and Ziegler 1997; Pierce *et al*. 2002; Crayn *et al*. 2004; Holtum *et al*. 2004; Silvera *et al*. 2005, 2009, 2010).

Drivers of CAM evolution

Numerous reports have postulated that C_3 photosynthesis is the evolutionary ancestral or progenitor state for CAM, with a progression towards strong CAM taking place in several incremental steps (Teeri 1982*a*, 1982*b*; Pilon-Smits *et al*. 1996; Crayn *et al*. 2004; Silvera *et al*. 2009). Reversion from CAM to the C_3 state is also possible (Teeri 1982*a*, 1982*b*) and evidence for likely reversal events, associated with radiations into less xeric habitats, has come from large-scale isotopic surveys within the Bromeliaceae (Crayn *et al*. 2004) and the Orchidaceae (Silvera *et al*. 2009). The reversion of CAM to C_3 photosynthesis points to the complex evolutionary histories within these taxa. Although the main driver for CAM evolution remains unclear, several hypotheses have been put forward. Water limitation and the resulting limitation of CO_2 brought about by stomatal closure and reductions in atmospheric CO_2 concentrations during the late Tertiary (Pearson and Palmer 2000) might have provided the selective pressures for the evolution of CAM over the last 40–100 million years (Monson 1989; Ehleringer and Monson 1993; Raven and Spicer 1996). It is difficult, however, to determine the first origin of CAM in plants, especially because the majority of families in which CAM is present originated recently and fossil evidence of CAM has not been discovered (Raven and Spicer 1996). Indeed, *Dendrobium* and *Earina* (Epidendroideae) macrofossils of orchid specimens from the early Miocene (23–20 MYA) have been described, however, these were not investigated for the presence of CAM-related characters (Conran *et al*. 2009). Based on the broad diversity of taxa showing CAM compared with species exhibiting C_4, CAM likely evolved first, and because of the presence of CAM in ancient groups such as the isoetids and cycads, CAM might have appeared as early as the Triassic (Griffiths 1992; Ehleringer and Monson 1993). In any event, CAM likely evolved in response to selection for increased carbon gain and increased water use efficiency (Ehleringer and Monson 1993) after the global reduction in atmospheric CO_2 concentration during the Miocene and early Pleistocene or perhaps even earlier during the Oligocene (Edwards *et al*. 2010). Notably, a large CAM radiation event in the most species-rich epiphytic clade in orchids (Fig. 3), the Epidendroideae, was predicted to have originated ~65 MYA and linked to the decline of atmospheric CO_2 during the Tertiary (Silvera *et al*. 2009). CAM has contributed to the exploitation of wider epiphytic habitat ranges, from low elevation sites where CAM orchids are mostly present, to mid-elevation tropical forest sites of around 1000 m, where moist suitable microenvironments exist for epiphytic orchid colonisation (Silvera *et al*. 2009).

Taxonomic distribution of CAM

Numerous past studies have estimated the phylogenetic distribution of CAM (Moore 1982; Winter 1985; Lüttge 1987; Griffiths 1988; Ehleringer and Monson 1993). CAM is widespread within the plant kingdom across at least 343 genera in 35 plant families comprising ~6% of flowering plant species (Table 2; JAC Smith, unpubl. data) (Griffiths 1989; Smith and Winter 1996; Holtum *et al*. 2007). In contrast, C_4 photosynthesis occurs in only 19 families and accounts for ~3% of plant species comprising mainly grasses and sedges and some dicots (Sage 2001, 2004). The oldest lineage with CAM described to date is represented by *Isoetes*, a mostly aquatic or semi-aquatic group distributed in oligotrophic lakes or mesotrophic shallow seasonal pools (Keeley 1998). The retention of CAM in this group is hypothesised to be due to the chronically low or day-time

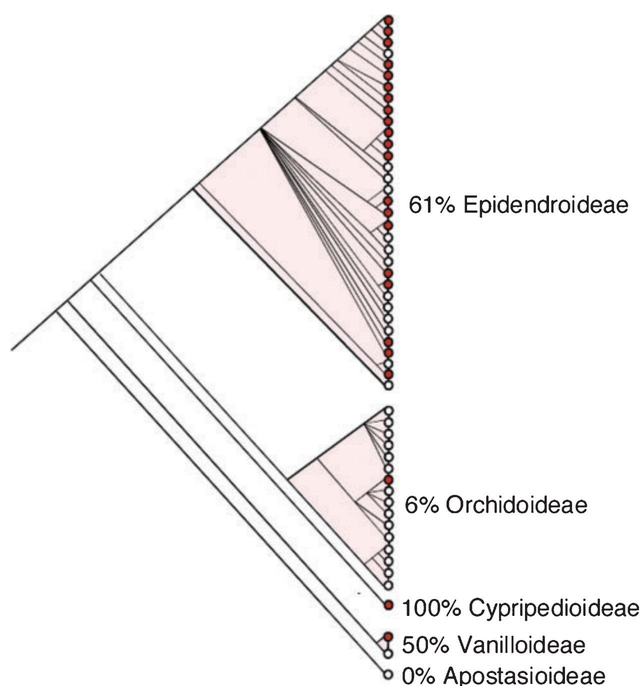

Fig. 3. Summary tree for the classification of the family Orchidaceae. Names on the clades represent the five orchid subfamilies where pink shading highlights large speciation events. Circles represent the number of subtribes identified within each subfamily. Red and white shaded circles represent subtribes in which CAM is either present or absent, respectively. The percentage of subtribes with CAM is given for each subfamily. For additional details see Silvera *et al.* (2009).

decline in levels of dissolved CO_2 in these aquatic environments (Keeley 1996). CAM has also been documented within the Gnetales, *Welwitschia mirabilis* Hook. F. (Welwischiaceae) (von Willert *et al.* 2005), the Cycadales, *Dioon edule* Lindl. (Zamiaceae) (Vovides *et al.* 2002) and in several epiphytic families of ferns within the Polypodiaceae (Holtum and Winter 1999) and the Vittariaceae (Martin *et al.* 2005).

The widespread taxonomic distribution of both C_4 and CAM plants indicates that C_4 and CAM plants must have evolved independently multiple times, even within a single genus (Monson 1989, 1999; Ehleringer and Monson 1993; Kellogg 1999; Silvera *et al.* 2009). Studies with limited taxon sampling by δ^{13}C analysis have been reported for the Crassulaceae (*Kalanchoë*) (Kluge *et al.* 1991), *Sedum* and *Aeonium* (Pilon-Smits *et al.* 1996), Clusiaceae (Gehrig *et al.* 2003; Gustafson *et al.* 2007), and Orchidaceae (*Cymbidium*) (Motomura *et al.* 2008). More extensive combined taxon and isotopic sampling has been completed within the Bromeliaceae (Crayn *et al.* 2004) and the Orchidaceae (Silvera *et al.* 2009, 2010).

Table 2. Taxonomic distribution of CAM plants, including family, order and exemplar species

The list has been updated from that of Smith and Winter (1996) and Holtum *et al.* (2007). Family nomenclature follows those provided by the Angiosperm Phylogeny website (Stevens 2008). Major groups are represented in capital letters

Family	Order	Examples
LYCOPODIOPHYTA		
Isoetaceae	Isoetales	*Isoetes* (quillworts)
PTERIDOPHYTA		
Polypodiaceae	Polypodiales	*Pyrrosia*
Vittariaceae	Polypodiales	*Vittaria*
MAGNOLIOPHYTA		
Agavaceae = Asparagaceae	Asparagales	*Agave*
Aizoaceae	Caryophyllales	*Mesembryanthemum*
Alismataceae	Alismatales	*Sagittaria*
Anacampserotaceae	Caryophyllales	*Grahamia*
Araceae	Alismatales	*Zamioculcas*
Asphodelaceae = Xanthorrhoeaceae	Asparagales	*Aloe*, *Haworthia*
Apiaceae	Apiales	aquatic *Lilaeopsis*
Apocynaceae	Gentianales	*Pachypodium*
Asteraceae	Asterales	*Senecio*
Bromeliaceae	Poales	*Aechmea*, *Tillandsia*
Commelinaceae	Commelinales	*Callisia*
Cactaceae	Caryophyllales	*Opuntia*
Clusiaceae	Malpighiales	*Clusia*
Crassulaceae	Saxifragales	*Crassula*, *Kalanchoë*
Cucurbitaceae	Cucurbitales	*Xerosicyos*
Didiereaceae	Caryophyllales	*Didierea*
Euphorbiaceae	Malpighiales	*Euphorbia*, *Monadenium*
Geraniaceae	Geraniales	*Sarcocaulon*
Gesneriaceae	Lamiales	*Codonanthe*
Hydrocharitaceae	Alismatales	*Vallisneria*
Lamiaceae	Lamiales	*Plectranthus*
Montiaceae	Caryophyllales	*Calandrinia*
Orchidaceae	Asparagales	*Oncidium*, *Phalaenopsis*
Oxalidaceae	Oxalidales	*Oxalis*
Passifloraceae	Malpighiales	*Adenia*
Piperaceae	Piperales	*Peperomia*
Plantaginaceae	Lamiales	*Littorella*
Portulacaceae	Caryophyllales	*Portulaca*, *Portulacaria*
Rubiaceae	Gentianales	*Myrmecodia*
Ruscaceae = Asparagaceae	Asparagales	*Sansevieria*, *Dracaena*
Vitaceae	Vitales	*Cissus*
Welwitschiaceae	Gnetales	*Welwitschia*
Zamiaceae	Cycadales	*Dioon*

Estimating the prevalence of CAM

CAM species are widely distributed throughout semiarid tropical and subtropical environments, including epiphytes in the humid tropics that must endure frequent reductions in water availability. Excluding the Orchidaceae, ~9000 species are estimated to perform CAM (Winter and Smith 1996). However, the Orchidaceae alone could contribute an additional 7800 species assuming that of the estimated 26 000 orchid species (Pfahl *et al.* 2008), 75% are epiphytic, and that ~10% of these engage in strong CAM, and 30% engage in weak CAM (Silvera *et al.* 2005). Thus, ~16 800 species or close to 6% of an estimated 300 000 vascular plant species (Kreft and Jetz 2007) might engage in CAM to varying degrees. The extent of CAM expression generally correlates with the degree of adaptation to more xeric ecological niches (Kluge *et al.* 2001; Pierce *et al.* 2002; Zotz 2004). A recent survey of 1022 orchid species from Panama and Costa Rica using stable isotopic measurements documented that the number of CAM species increases with decreasing precipitation with the majority of CAM species occurring at

sites between sea level and 500 m and no CAM species occurring above 2400 m (Silvera *et al*. 2009). At two lowland forest sites in Panama, 36% and 42% of epiphytic orchid species displayed CAM isotopic values (Zotz and Ziegler 1997; Zotz 2004), and these percentages were 26% and over 60% among tropical epiphytic orchids collected in Papua New Guinea (Earnshaw *et al*. 1987) and Australia (Winter *et al*. 1983), respectively. Also, within a single site, the percentage of CAM epiphytes tended to increase from shaded understory sites to exposed canopy sites. For example, in a moist tropical forest in Panama, CAM was more prevalent in emergent layers and exposed tree canopies than in understory sites (Zotz and Ziegler 1997).

A recent study of how closely $\delta^{13}C$ values reflect the proportion of CO_2 fixed during day and night revealed that 'the typical CAM plant' gains ~71–77% of its carbon through nocturnal fixation (Winter and Holtum 2002). However, surveys using only $\delta^{13}C$ values to determine the number of CAM-equipped species do not take into account CAM species obtaining less than one-third of their carbon in the dark (Winter and Holtum 2002). Recent surveys that include measurements of nocturnal tissue acidification have identified a greater number of CAM species than surveys using isotopic composition measurements alone (Pierce *et al*. 2002; Silvera *et al*. 2005). Furthermore, surveys conducted during the rainy season might not reveal the presence of facultative CAM species that exhibit CAM only under water deficit stress conditions. Thus, estimates of the taxonomic distribution of CAM using only stable isotopes measurements are likely to underestimate the prevalence of CAM.

Integrative studies that attempt to map carbon-isotopic ratio surveys with molecular phylogenies remain limited. Although a well-resolved and comprehensively sampled molecular phylogeny of the Aizoaceae exists (Klak *et al*. 2003, 2004), the occurrence of CAM has not been mapped onto the available phylogenetic tree. Similarly, an incomplete molecular phylogeny has been established for about two-thirds of species within the Agavaceae (Good-Avila *et al*. 2006); however, most of those species are expected to perform CAM. A detailed molecular phylogenetic reconstruction of the Vanilloideae with emphasis on the genus *Vanilla*, which surveyed 47 of the 110 different species, has also been constructed using four plastid genes (Bouetard *et al*. 2010). However, no attempt was made in this study to map the occurrence of CAM. DNA-based molecular phylogenies are well established for the Bromeliaceae (Crayn *et al*. 2004; Barfuss *et al*. 2005; Jabaily and Sytsma 2010). Carbon-isotopic ratios collected from 1873 of 2885 bromeliad species revealed that CAM photosynthesis and the epiphytic habit evolved a minimum of three times in this family (Crayn *et al*. 2004). Molecular phylogenies have also been established for 87 of the estimated 400 species within the Clusiaceae (Gustafson *et al*. 2007), revealing CAM arose independently within two of the three major groups of *Clusia* species with multiple reversal events as determined by carbon isotope ratio analysis (Vaasen *et al*. 2002; Gehrig *et al*. 2003; Gustafson *et al*. 2007).

Orchids as a model for the study of CAM evolution

The Orchidaceae is the largest family of flowering plants with >800 genera and ~26 000 species worldwide, of which ~75% are estimated to be epiphytic (Atwood 1986; Dressler 1993; Gravendeel *et al*. 2004). Orchids exhibit a large number of morphological, anatomical, ecological and physiological characteristics that allow them to exist within diverse ecosystems and ecological niches with the greatest diversity in mountainous regions of the tropics (Cribb and Govaerts 2005). One such characteristic is the expression of CAM. Orchids are a large and taxonomically well studied family of CAM plants. For example, from 1994–2004 DNA sequences from 4262 orchids had been deposited in GenBank (Cameron 2005). Chase *et al*. (2003) proposed an updated classification for the family based upon many recent and ongoing molecular phylogenetic studies. Orchids contain a mixed distribution of C_3 and CAM species, a feature that is useful for tracing the occurrence of CAM within discrete lineages (Silvera *et al*. 2010). In contrast, nearly all species within some other families, such as the Agavaceae, Cactaceae and the Didiereaceae, display CAM and, thus, do not permit the evaluation of CAM evolutionary progression. Of families that display a mixture of both C_3 and CAM species (e.g. Aizoaceae, Bromeliaceae, Clusiaceae, Crassulaceae), the Orchidaceae has an advanced, well-resolved molecular phylogeny as summarised in the five volumes of Genera Orchidacearum (Pridgeon *et al*. 1999, 2009).

The diversity expressed by orchids is crucial in linking CAM expression to vegetative morphology such as leaf thickness, to habitat specialisation such as epiphytism, and to adaptive radiation spanning moisture gradients (Dressler 1993; Williams *et al*. 2001*a*). Silvera *et al*. (2005) used a combination of $\delta^{13}C$ isotopic ratios and titratable acidity measurements to survey for the presence of CAM in 200 native Panamanian orchid species. The survey produced a bimodal distribution of $\delta^{13}C$ values with peaks around –15‰ (signifying strong CAM) and –28‰ (signifying C_3 photosynthesis), comparable to other broad surveys employing $\delta^{13}C$ value measurements. Within the peak of C_3 photosynthesis $\delta^{13}C$ values, titratable acidity measurements revealed a second CAM cluster indicative of species with low capacities for nocturnal CO_2 fixation (weak CAM). Taking into account both $\delta^{13}C$ values and titratable acidity measurements, CAM appears to be widespread among tropical epiphytic orchids. However, fewer than 4% of all known orchid species have been sampled for isotope analysis to date (Silvera *et al*. 2009, 2010).

Mapping the occurrence of CAM within the Orchidaceae

A key prerequisite for the phylogenetic reconstruction of the evolutionary origins of CAM is a sufficiently robust and densely sampled phylogeny for the family based on molecular and morphological characters. The subtribe Oncidiinae is one of the most highly derived clades of orchids of the New World, with great variation in chromosome number, vegetative features and floral characteristics (Chase *et al*. 2005). Oncidiinae is the second largest orchid subtribe and comprises ~69 genera and ~1600 species, most of which are epiphytic (Williams *et al*. 2001*a*, 2001*b*). This subtribe is one of the most intensively sampled clades within the Orchidaceae. Approximately 600 of 1600 (37%) species have been sampled, employing data from both nuclear and plastid DNA sequences as well as morphological characters (Williams *et al*. 2001*a*, 2001*b*) to provide an excellent

basis from which to study CAM evolution. The monophyly of the Oncidiinae and phylogenetic relationships of related genera have been evaluated by combined data from the internal transcribed spacer of nuclear rDNA (nrITS) and three plastid regions (*matK*, *trnL* intron, and the *trnL-F* intergenic spacer) producing highly resolved cladograms (Williams *et al*. 2001*a*, 2001*b*). Members of the Oncidiinae occupy a wide variety of epiphytic sites, from large limbs that are exposed in the canopy of tropical forests, to densely shaded sites in the understory (Chase 1988; Chase *et al*. 2005). Leaf morphology is also highly variable, with species exhibiting a gradient from thick-succulent terete or conduplicate leaves to species showing thin conduplicate leaves. Species within Oncidiinae also show a gradient of CAM expression, from C_3 photosynthesis to weak and strong CAM. Ancestral state reconstruction of the occurrence of CAM onto a phylogeny of orchids shows multiple independent origins of CAM with several reversal events (Fig. 3) and a positive cross-genera relationship between epiphytism and photosynthetic pathways, indicating that divergence of photosynthetic pathways has been correlated through evolutionary time (Silvera *et al*. 2009). CAM is prevalent in low-elevation epiphytes, especially in those from habitats with a strong dry season, and less prevalent in those from cooler habitats with a more even moisture regime that includes both rainfall and fog (Silvera *et al*. 2009). Ancestral state reconstruction of CAM onto a phylogeny of Oncidiinae species indicates at least eight independent origins of CAM within the clade (Fig. 4).

Molecular evolution of CAM

The progression of photosynthetic pathways has been shown consistently to be from C_3 ancestors to CAM photosynthesis. However, the genetic changes required for this progression (and reversion) remain unclear. The multiple independent evolutionary origins of CAM and the observation that presumably all of the enzymatic requirements to perform CAM already exist in most plant cells, particularly stomatal guard cells, might suggest that CAM evolution involves relatively few genetic changes. The available molecular data from C_4 cycle enzymes support this view in that none of the C_4 or CAM cycle enzymes or corresponding genes are unique to these plants (Westhoff and Gowik 2004). However, given the large number of anatomical and biochemical requirements for CAM (Fig. 2) and the complexity of the regulatory changes associated with modulation of stomatal behaviour and gene expression patterns associated with CAM (Cushman *et al.* 2008*b*), we suggest that the number of genetic changes necessary for CAM to arise are likely to be many.

Molecular markers for studying CAM evolution

The cytosolic enzyme phospho*enol*pyruvate carboxylase (EC 4.1.1.31; PEPC) catalyses the β-carboxylation of phospho*enol*pyruvate, with oxaloacetate and inorganic phosphate as products, and serves various functions in plants (Chollet *et al*. 1996; Nimmo 2000). In addition to its anaplerotic roles in leaves and nonphotosynthetic tissues, PEPC catalyses the initial fixation of atmospheric CO_2 into C_4-dicarboxylic acids in CAM and C_4 photosynthesis. For the PEPC gene family, both non-photosynthetic and photosynthetic isoforms are present in C_3, C_4 and CAM species. These non-photosynthetic, 'C_3 isoforms' might have served as the starting point for the evolution of the C_4 and CAM isogenes. In C_4 plants, key determinants for the evolution of the C_4 cycle include duplication of ancestral non-photosynthetic or C_3 isogenes, followed by the acquisition of increased mRNA and protein expression, with organ- and cell-type-specific expression patterns of the C_4 photosynthetic isogenes largely due to transcriptional changes in gene expression (Furumoto *et al*. 2000; Westhoff and Gowik 2004; Hibberd and Covshoff 2010). As in C_4 plants, CAM-specific isoforms of PEPC are distinguished by their elevated mRNA and protein expression in leaf tissues. Evidence from comparative analysis of C_3, C_3-C_4 intermediates, and C_4 *Flaveria* species suggests that C_4 photosynthetic PEPC isoforms have evolved from ancestral non-photosynthetic or C_3 isoforms by gene duplication, and have acquired distinct kinetic and regulatory properties mediated by discrete amino acid changes (Bläsing *et al*. 2000, 2002; Engelmann *et al*. 2002, 2003; Westhoff and Gowik 2004).

In CAM plants, an evolutionary progression of gene family changes similar to that described for C_4 plants is thought to have occurred in PEPC gene families from C_3, weak CAM and strong CAM orchid species (Fig. 5; K Silvera, unpubl. data). This assumption is based on the premise that the molecular mechanisms that drive evolutionary changes in gene family structure are conserved throughout the plant kingdom regardless of the type of photosynthetic pathway. Among CAM species, multiple PEPC isogenes have been described in various species, with CAM-specific PEPC isoforms exhibiting enhanced mRNA expression relative to the expression of C_3 PEPC isoforms (Cushman *et al*. 1989; Gehrig *et al*. 1995, 2001). For example, comparison of four *Clusia* species (one C_3, two C_3-CAM intermediates with water-deficit stress-inducible CAM and one strong, constitutive CAM species), revealed that the ability to conduct nocturnal CO_2 fixation was well correlated with PEPC quantity and activity (Borland *et al*. 1998) and that PEPC mRNA and protein expression was a major factor underpinning the genotypic capacity for CAM (Taybi *et al*. 2004).

Detailed comparisons of PEPC isoforms from C_3 photosynthesis, weak CAM and strong CAM species within the Orchidaceae are underway. As in C_4 plants, distinct kinetic and regulatory properties might be expected to be conferred by discrete amino acid changes in CAM-specific isoforms of PEPC, which do show distinct amino acid differences from housekeeping or C_3 photosynthesis isoforms (Fig. 5; K Silvera, unpubl. data). In addition, genes of CAM isoforms for PEPC and PEPC kinase appear to have evolved unique expression patterns that are under circadian clock control with expression patterns that are distinct from those in C_3 plants (Taybi *et al*. 2004; Boxall *et al*. 2005; Cushman *et al*. 2008*b*). However, the evolutionary recruitment of gene family members must extend beyond those involved in C_4 acid metabolism and include those genes that control the large reciprocating pools of storage carbohydrates, which can account for up to 20% of the total leaf dry weight (Winter and Smith 1996; Dodd *et al*. 2002, 2003). Some CAM plants can accumulate soluble sugars (e.g. sucrose, glucose, fructose) and polysaccharides (e.g. fructan,

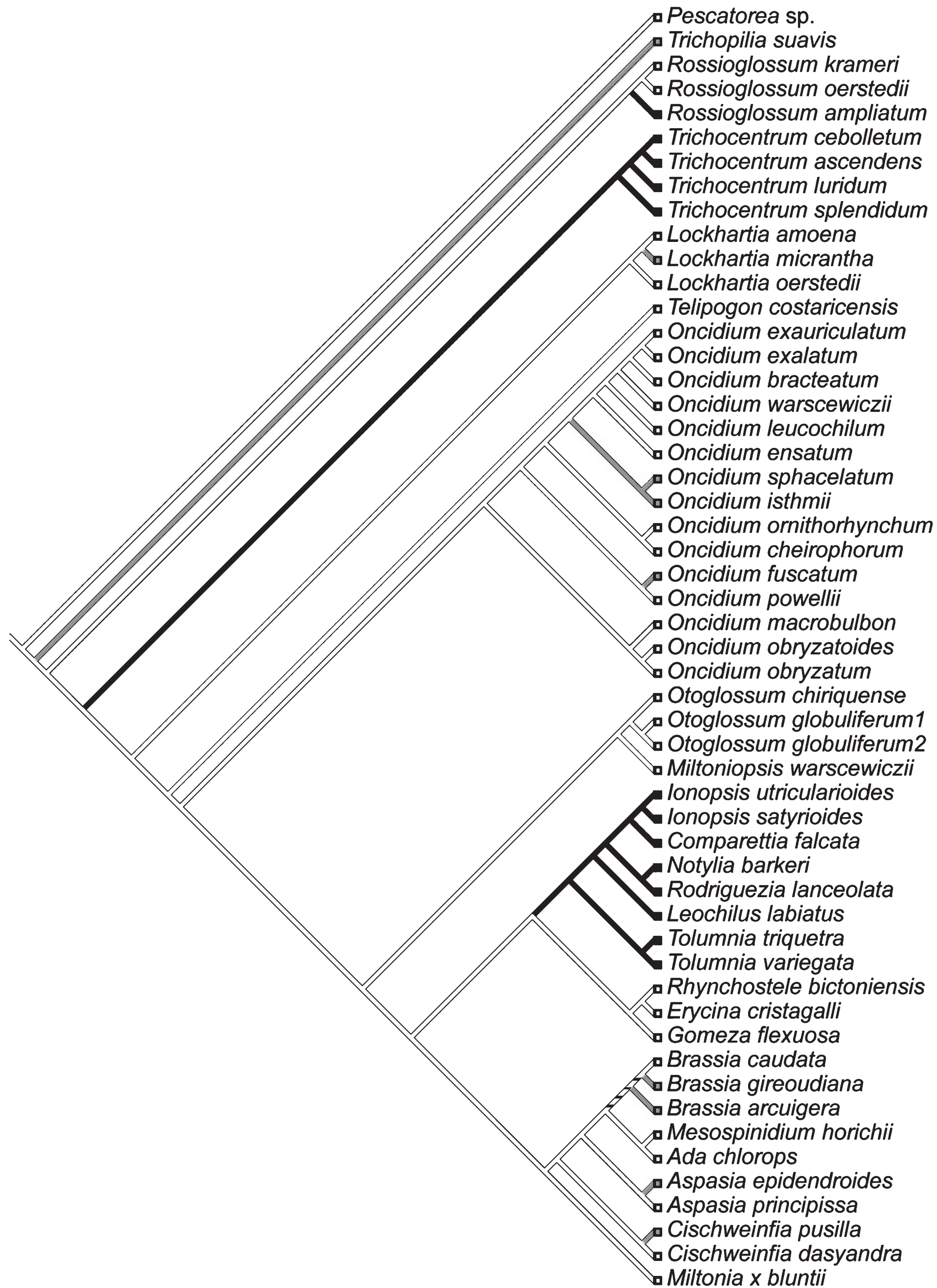

Fig. 4. Summary tree for the appearance of weak and strong CAM among Oncidiinae (Orchidaceae) species. The cladogram is derived from Williams *et al.* (2001*a*); generic concepts follow Pridgeon *et al.* (2009). Photosynthetic pathway based on $\delta^{13}C$ and titratable acidity was mapped onto the cladogram using MacCLADE V.4.08 (ACCTRAN optimization) using *Pescatorea* (Zygopetalinae) as outgroup. White and black bars represent those lineages with either C_3 photosynthesis or CAM, respectively. Gray bars represent weak CAM and striped bars represent those with equivocal results.

galactomannan) in extra-chloroplastic compartments, while other species store both plastidic starch and cytoplasmic glucose (Christopher and Holtum 1996). Detailed studies have revealed at least eight distinct combinations of malate decarboxylation and carbohydrate storage strategies in CAM plants (Christopher and Holtum 1996). These various

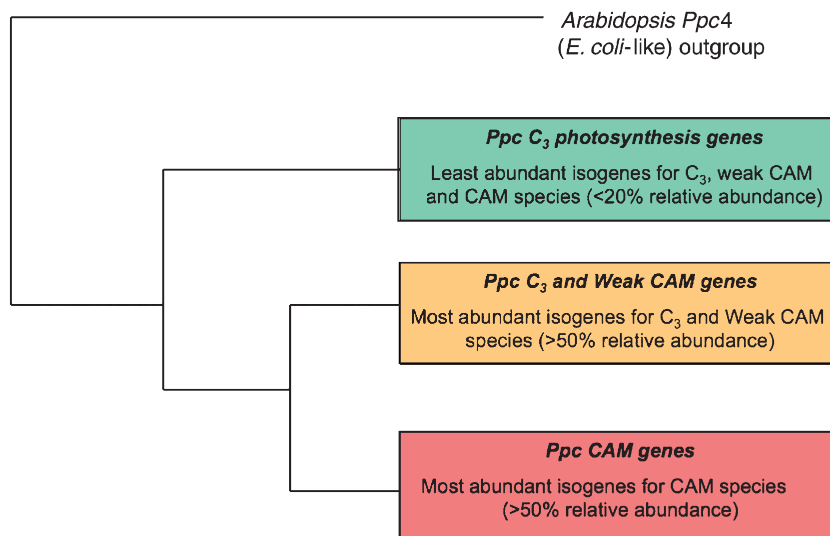

Fig. 5. Proposed model for the evolutionary progression of paralogous PEPC genes (*Ppc*) in the Orchidaceae. CAM-*Ppc* genes from CAM species cluster together (red shading), and belong to a sister group of weak CAM (orange shading) and C_3 (green shading) most abundant *Ppc*-genes. The least abundant isoforms from C_3, weak CAM and CAM species cluster separately and presumably represent *Ppc* genes having anaplerotic, as opposed to photosynthetic functions (K Silvera, unpublished data). The *E. coli*-like *Ppc4* gene from *Arabidopsis* was used as outgroup.

carbohydrate accumulation patterns likely reflect the evolutionary history of the species, rather than the carbon flow constraints of the pathway (Winter and Smith 1996; Winter and Holtum 2002).

Studies of developmental and spatial expression patterns using partial nucleotide sequences of PEPC have provided valuable molecular tools for understanding the evolution of metabolic pathways in which PEPC is involved. PEPC sequences are useful, not only because the gene is ubiquitous in prokaryotes and plants, but also because the marker can provide information about the tissue-specific expression patterns and metabolic roles of specific gene family members (Gehrig *et al*. 2001). For example, Gehrig *et al*. (2001) used expression changes during leaf development to infer potential CAM-related isogenes of PEPC relative to non-CAM isoforms expressed predominantly in non-photosynthetic roots. However, tissue-specific expression alone is inadequate to infer a CAM-related function. The relative abundance of each isoform in CAM-performing tissue must be confirmed in order to designate the most abundantly expressed isoform as CAM-specific (Cushman *et al*. 1989; Gehrig *et al*. 1995, 2005; Taybi *et al*. 2004). However, this does not necessarily preclude the possibility that multiple isoforms contribute to CAM-specific function. Interestingly, three leafless orchid species with chloroplast-containing, CAM-performing aerial roots (Winter *et al*. 1985) also expressed PEPC isoforms that clustered with PEPC isoforms recovered from CAM-performing leaves of other species, but not with PEPC isoforms from nonphotosynthetic aerial roots (Gehrig *et al*. 2001). This observation suggests that such 'shootless' species do not make use of the root-inherent isoform for photosynthetic carbon assimilation, but express either an ancestral leaf, CAM-derived or an additional PEPC isoform that conducts the initial fixation of CO_2 needed for CAM.

Characterisation of PEPC isogenes from *Kalanchoë pinnata* (Lam.) Pers. revealed seven distinct PEPC isogenes: four in leaves and three in roots. Sequence similarity comparisons and distance neighbour-joining calculations separate the seven PEPC isoforms into two clades, one of which contains the three PEPCs found in roots (Gehrig *et al*. 2005). The second clade contains the four isoforms found in leaves and is divided into two branches, one of which contains two PEPCs most similar to previously described CAM isoforms. Of these two isoforms, however, only one exhibited abundant expression in CAM-performing leaves, but not in very young leaves, which do not exhibit CAM, suggesting that this isoform encodes a CAM-specific PEPC. Protein sequence comparison and phylogenetic analysis using the neighbour joining method suggest that all isogenes are likely derived from a common ancestor gene, presumably by serial gene duplication events (Sánchez and Cejudo 2003). In addition to plant-type PEPCs, higher plant genomes also encode a bacterial-type, non-phosphorylatable form of PEPC that phylogenetic analysis suggests diverged early during the evolution of plants from a common ancestral PEPC gene probably from γ-proteobacteria (Sánchez and Cejudo 2003).

Comprehensive examination of PEPC gene families together with phylogenetic tree construction from C_3, weak CAM and strong CAM orchids, suggests an emerging model for the evolutionary appearance of paralogous PEPC genes (Fig. 5) Abundantly expressed CAM-*PPC* genes from CAM species cluster together and belong to a sister group of weak CAM and C_3-most-abundant *PPC*-genes. Less abundantly expressed isoforms from C_3, weak CAM, and CAM species cluster separately and presumably belong with *PPC* genes involved in

anaplerotic function (Fig. 5). Although this model requires additional experimental support to confirm these proposed evolutionary events, it should be possible to discern CAM isoforms not only from developmental and tissue-specific expression pattern data, but also by comparing nucleotide or amino acid sequences.

Circadian clock-regulated markers

In addition to PEPC, two other genes have been characterised recently that are likely to be excellent markers for tracing CAM evolution within the context of circadian clock biology: PEPC kinase and the glucose-6-phosphate/Pi translocator. In CAM plants, PEPC is activated at night via phosphorylation of a Ser residue near the *N*-terminus, which renders the enzyme more sensitive to PEP and the positive effectors glucose-6-phosphate (G6P) and triose-phosphate (TP) and less sensitive to the allosteric inhibitor, malate (Chollet *et al*. 1996; Nimmo 2000). This phosphorylation is carried out by PEPC kinase (PPCK), a dedicated, calcium-independent Ser/Thr protein kinase, the steady-state transcript abundance and activity of which is controlled by the circadian clock (Hartwell *et al*. 1999; Taybi *et al*. 2000). However, the circadian regulation of *PPCK* mRNA abundance can also be regulated by metabolic signals, such as malate accumulation (Borland *et al*. 1999). Thus, both circadian and metabolic signals appear to modulate *PPCK* transcript abundance, which in turn regulates PPCK activity, the phosphorylation/activation state of PEPC, and the degree of nocturnal carbon assimilation (Nimmo 2000). In a comparison of four *Clusia* species including a C_3 species, two C_3-CAM intermediates and a strong, constitutive CAM species, the circadian modulation of *PPCK* mRNA abundance correlated with the performance of CAM and with day/night changes in malate and soluble sugar content. However, circadian fluctuations in *PPCK* mRNA abundance were not evident in the C_3 species and one of the C_3-CAM intermediates (Taybi *et al*. 2004).

In a more recent study in the C_3-CAM plant, *M. crystallinum*, the expression of the glucose-6-phosphate/Pi translocator gene (*Gpt2*) was undetectable in plants performing C_3, but was preferentially enhanced in leaves of CAM-induced plants (Kore-eda *et al*. 2005) and was under circadian clock control (Cushman *et al*. 2008*b*). In summary, three major changes appear to have occurred during the evolution of progenitor genes in order to function in CAM (Fig. 2): (1) high expression in plants performing CAM, (2) leaf-specific or leaf-preferential expression patterns and (3) expression patterns coming under circadian clock control. We know very little about the *cis*-regulatory elements and cognate *trans*-factors involved in controlling the CAM-specific expression of PEPC, PEPC kinase and glucose-6-phosphate/Pi translocator and other CAM-related genes. We also do not know whether there are common regulatory genes responsible for directing the expression of coordinately regulated sets of genes within these regulatory networks.

Circadian clock specialisation during CAM evolution

CAM represents an noteworthy example of circadian clock specialisation and is one of the best-characterised physiological rhythms in plants (Wilkins 1992; Lüttge 2003; Wyka *et al*. 2004). The presence of the CAM enzymatic machinery within a single cell requires strict temporal control of the competing carboxylation reactions by PEPC and RUBISCO. PEPC activity is regulated by reversible protein phosphorylation by PEPC kinase, whose expression is under circadian clock control (Hartwell *et al*. 1999; Taybi *et al*. 2000). RUBISCO activity also appears to be modulated, with peak activity apparent during the mid-to-late part of the light period, which would reduce the likelihood that RUBISCO and PEPC would compete for CO_2 during the early morning (Maxwell *et al*. 1999; Griffiths *et al*. 2002). Indeed, cytosolic malate (or a related metabolite) concentration appears to exert a negative effect on PEPC kinase gene expression or mRNA stability and override its circadian control in *K. daigremontiana* (Borland *et al*. 1999; Borland and Taybi 2004). This observation has led to the suggestion that circadian control of PEPC kinase expression is a secondary response to malate transport across the tonoplast membrane of the vacuole (Nimmo 2000). However, it should be noted that this metabolite override mechanism has only been reported in this single CAM species. Circadian control of the large, reciprocating pools of carbohydrates (Dodd *et al*. 2003), changes in mRNA abundance for starch synthesis and degradation enzymes (Dodd *et al*. 2003; Cushman *et al*. 2008*b*), diurnal or circadian expression of genes encoding plastidic triose phosphate/Pi and glucose-6-phosphate/Pi translocators (Häusler *et al*. 2000; Kore-eda *et al*. 2005; Cushman *et al*. 2008*b*) and the partitioning of isotopically distinct, C_3- or C_4-derived classes of carbon pools are also likely to be critical for the optimal performance of CAM (Borland and Dodd 2002; Ceusters *et al*. 2008).

Comparison of the steady-state mRNA abundance patterns of seven circadian clock components in the facultative CAM plant *M. crystallinum* operating in either C_3 or CAM mode, indicated that its central clock is very similar to that in *Arabidopsis* and is not perturbed by development or salinity stress (Boxall *et al*. 2005). However, various clock components can be used in different ways to alter clock outputs. Evidence for alternate clock component functions comes from the observation that ZEITLUPE (*ZTL*), a gene that does not exhibit an oscillating pattern of mRNA expression abundance in *Arabidopsis*, does so in *M. crystallinum.* Furthermore, the circadian abundance profile of *McZTL* transcripts exhibits a slightly more prolonged period of expression. Additional support for alterations in the circadian clock control outputs in *M. crystallinum* comes from oligonucleotide-based microarray experiments that document that the shift from C_3 to CAM is accompanied by shifts in the phase at which peak expression occurs (Cushman *et al*. 2008*b*). A large proportion (70%) of *Arabidopsis* genes that exhibit circadian fluctuations in transcript abundance also respond to environmental stress (i.e. low temperature, salt, and drought; Kreps *et al*. 2002). Such rhythmic expression of stress-adaptive genes might prepare the plant to better withstand a stress or exploit a limiting resource (Eriksson and Millar 2003). Given that water deficit stress is likely to be one of the driving forces behind CAM evolution (Raven and Spicer 1996), ancestral C_3 progenitors of CAM plants might have evolved clocks which exerted pervasive control over metabolism as a means of maintaining metabolic homeostasis under stressful environments (Borland and Taybi 2004).

Concluding remarks

The varying degrees to which CAM is expressed reflect a continuum of photosynthetic metabolism from C_3 photosynthesis to weakly expressed CAM to fully expressed CAM arising from the unique evolutionary history of a particular species. The plasticity of CAM is governed by the evolutionary disposition of each species, whether under developmental control in a constitutive CAM species or under environmental control in a facultative CAM species. Our understanding of the CAM photosynthetic pathway is advancing, especially at the molecular genetic level. Gene sequence information has proliferated quickly and will provide a solid foundation for future research into CAM evolution. For example, transcriptome sequencing has been performed in a strong CAM orchid species (*Rossioglossum ampliatum* (Lindl.) M.W.Chase & N.H.Williams) along with the fabrication of a custom oligonucleotide microarray (K Silvera and JC Cushman, unpublished data), which will permit mRNA expression patterns to be compared within closely related C_3 photosynthesis and weak CAM species as a way to define large-scale gene expression changes associated with CAM evolution. Future projects aimed at analysing the presence or absence of *cis*-regulatory elements responsible for circadian clock-controlled expression patterns will determine whether or not CAM-specific expression patterns are regulated by evolutionary changes within 5′ flanking or other control regions. Weak CAM species are of particular interest because a reservoir of duplicated genes that have undergone neofunctionalisation from C_3 ancestral genes is expected to be present, in addition to C_3 genes. Gene duplication events followed by neofunctionalisation and subfunctionalisation are likely to occur through the differentiation of *cis*-regulatory elements that control tissue- and clock-specific patterns of expression (Monson 2003). However, the major distinction for CAM evolution is that temporal regulation of gene expression patterns will have greater importance than the cell-specific expression patterns found in C_4 plants. In both cases, differentiation within the coding regions can also be expected to produce modified functional domains within proteins.

Undoubtedly, the presence of CAM in evolutionary lineages must be defined at the molecular level, in order to understand the genetic changes responsible for the evolutionary progression from C_3 to strong CAM, and possible reversal events linked to a changing environment. Molecular analyses will also provide insights into whether or not weak CAM in certain lineages might have served as a genetic reservoir for adaptive radiations leading to strong CAM. The use of phylogenetic comparative methods will be particularly useful for the testing of correlated evolutionary changes of multiple CAM traits (e.g. molecular, physiological, anatomical and environmental traits). Larger carbon isotope ratio surveys should be attempted and these should be performed in conjunction with titratable acidity measurements from live specimens under defined water status. Future surveys should be conducted under both well watered and water deficit conditions to discover facultative CAM species that might be missed by carbon-isotope ratio surveys alone. High throughput RNA/DNA sequencing strategies should be used to compare gene expression patterns in closely related C_3, weak CAM and strong CAM species. Such information will provide important insights into the molecular genetic requirements for CAM evolution within discrete lineages. Such large-scale sequencing strategies can also be applied in a comparative genomics context in order to investigate the convergent evolution of CAM in lineages that evolved CAM independently. The ultimate goal of this approach will be to define the molecular ‘parts list’ required for CAM. Complementary proteomic studies targeting temporal changes in protein abundance or post-translational modifications are also expected to improve our understanding of circadian regulation, especially when coupled with mRNA expression profiling of both coding mRNAs and non-coding micro RNAs (miRNAs) in selected CAM species. Ultimately, integrated approaches that combine molecular genetic strategies, genetic approaches, phylogenetic analysis, ecophysiology, and bioinformatics will aid in our understanding of the molecular evolution of CAM.

Acknowledgements

This work was supported by funding from the USA Environmental Protection Agency under the Greater Research Opportunities Graduate Program (Agreement no. MA 91685201 to KS), National Science Foundation NSF IOB-0543659 (to JCC), and Smithsonian Tropical Research Institute (to KW). We are indebted to Dr J Andrew C Smith (Oxford University) for his contributions to Table 2. We would also like to thank the two anonymous reviewers for their helpful comments and Mary Ann Cushman for her critical reading of the manuscript. EPA has not formally reviewed this publication. The views expressed in this publication are solely those of the authors and the EPA does not endorse any products or commercial services mentioned in this publication. This publication was also made possible by NIH Grant Number P20 RR-016464 from the INBRE Program of the National Center for Research Resources through its support of the Nevada Genomics, Proteomics and Bioinformatics Centers.

References

Atwood JT (1986) The size of the Orchidaceae and the systematic distribution of epiphytic orchids. *Selbyana* **7**, 171–186.

Barfuss MHJ, Samuel R, Till W, Stuessy TF (2005) Phylogenetic relationships in subfamily Tillandsioideae (Bromeliaceae) based on DNA sequence data from seven plastid regions. *American Journal of Botany* **92**, 337–351. doi:10.3732/ajb.92.2.337

Bläsing O, Westhoff P, Svensson P (2000) Evolution of C_4 phospho*enol*pyruvate carboxylases in *Flaveria*, a conserved serine residue in the carboxyl-terminal part of the enzyme is a major determinant for C_4-specific characteristics. *Journal of Biological Chemistry* **275**, 27 917–27 923.

Bläsing O, Ernst K, Streubel M, Westhoff P, Svensson P (2002) The non-photosynthetic phospho*enol*pyruvate carboxylases of the C_4 dicot *Flaveria trinervia* – implications for the evolution of C_4 photosynthesis. *Planta* **215**, 448–456. doi:10.1007/s00425-002-0757-x

Borland AM, Dodd AN (2002) Carbohydrate partitioning in crassulacean acid metabolism plants: reconciling potential conflicts of interest. *Journal of Experimental Botany* **29**, 707–716.

Borland AM, Taybi T (2004) Synchronization of metabolic processes in plants with crassulacean acid metabolism. *Journal of Experimental Botany* **55**, 1255–1265. doi:10.1093/jxb/erh105

Borland AM, Tecsi LI, Leegood RC, Walker RP (1998) Inducibility of crassulacean acid metabolism (CAM) in *Clusia* species; physiological/biochemical characterisation and intercellular localisation of carboxylation processes in three species which show different degrees of CAM. *Planta* **205**, 342–351. doi:10.1007/s004250050329

Borland AM, Hartwell J, Jenkins GI, Wilkins MB, Nimmo HG (1999) Metabolite control overrides circadian regulation of phospho*enol*pyruvate carboxylase kinase and CO_2 fixation in crassulacean acid metabolism. *Plant Physiology* **121**, 889–896. doi:10.1104/pp.121.3.889

Bouetard A, Lefeuvre P, Gigant R, Bory S, Pignal M, Besse P, Grisoni M (2010) Evidence of transoceanic dispersion of the genus *Vanilla* based on plastid DNA phylogenetic analysis. *Molecular Phylogenetics and Evolution* **55**, 621–630. doi:10.1016/j.ympev.2010.01.021

Boxall SF, Foster JM, Bohnert HJ, Cushman JC, Nimmo HG, Hartwell J (2005) Conservation and divergence of circadian clock operation in a stress-inducible crassulacean acid metabolism species reveals clock compensation against stress. *Plant Physiology* **137**, 969–982. doi:10.1104/pp.104.054577

Brulfert J, Queiroz O (1982) Photoperiodism and crassulacean acid metabolism. *Planta* **154**, 339–343. doi:10.1007/BF00393912

Cameron KM (2005) Molecular systematics of Orchidaceae: a literature review and an example using five plastid genes. In 'Proceedings of the 17th World Orchid Conference 2002 – Sustaining orchids for the future'. (Eds H Nair, J Arditti) pp. 80–96. (Kota Kinabalu, Natural History Publications: Borneo)

Ceusters J, Borland AM, Londers E, Verdoodt V, Godts C, De Proft MP (2008) Diel shifts in carboxylation pathway and metabolite dynamics in the CAM bromeliad *Aechmea* 'Maya' in response to elevated CO_2. *Annals of Botany* **102**, 389–397. doi:10.1093/aob/mcn105

Chase MW (1988) Obligate twig epiphytes: a distinct subset of Neotropical orchidaceous epiphytes. *Selbyana* **10**, 24–30.

Chase MW, Cameron KM, Barrett RL, Freudenstein JV (2003) DNA data and Orchidaceae systematics: a new phylogenetic classification. In 'Orchid conservation'. (Eds KW Dixon, SP Kell, RL Barrett, PJ Cribb) pp. 69–89. (Natural History Publications: Borneo)

Chase MW, Hanson L, Albert VA, Whitten WM, Williams NH (2005) Life history evolution and genome size in subtribe Oncidiinae (Orchidaceae). *Annals of Botany* **95**, 191–199. doi:10.1093/aob/mci012

Chollet R, Vidal J, O'Leary MH (1996) Phospho*enol*pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 273–298. doi:10.1146/annurev.arplant.47.1.273

Christopher JT, Holtum JAM (1996) Patterns of carbohydrate partitioning in the leaves of crassulacean acid metabolism species during deacidification. *Plant Physiology* **112**, 393–399.

Christopher JT, Holtum JAM (1998) Carbohydrate partitioning in the leaves of Bromeliaceae performing C_3 photosynthesis or crassulacean acid metabolism. *Australian Journal of Plant Physiology* **25**, 371–376. doi:10.1071/PP98005

Cockburn W (1985) Variation in photosynthetic acid metabolism in vascular plants: CAM and related phenomena. *New Phytologist* **101**, 3–24. doi:10.1111/j.1469-8137.1985.tb02815.x

Cockburn W, Ting IP, Sternberg LO (1979) Relationships between stomatal behavior and internal carbon dioxide concentration in crassulacean acid metabolism plants. *Plant Physiology* **63**, 1029–1032. doi:10.1104/pp.63.6.1029

Conran JG, Bannister JM, Lee DE (2009) Earliest orchid macrofossils: early Miocene *Dendrobium* and *Earina* (Orchidaceae: Epidendroideae) from New Zealand. *American Journal of Botany* **96**, 466–474. doi:10.3732/ajb.0800269

Crayn DM, Winter K, Smith JAC (2004) Multiple origins of crassulacan acid metabolism and the epiphytic habit in the neotropical family Bromeliaceae. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3703–3708. doi:10.1073/pnas.0400366101

Cribb P, Govaerts R (2005) Just how many orchids are there? In 'Proceedings of the 18th World Orchid Conference'. (Eds A Raynal-Roques, A Rogeuenant, D Prat) pp. 161–172. (Naturalia Publications: Dijon, France)

Cushman JC (2001) Crassulacean acid metabolism. A plastic photosynthetic adaptation to arid environments. *Plant Physiology* **127**, 1439–1448. doi:10.1104/pp.010818

Cushman JC, Bohnert HJ (1999) Crassulacean acid metabolism: molecular genetics. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 305–332. doi:10.1146/annurev.arplant.50.1.305

Cushman JC, Borland AM (2002) Induction of crassulacean acid metabolism by water limitation. *Plant, Cell & Environment* **25**, 295–310. doi:10.1046/j.0016-8025.2001.00760.x

Cushman JC, Meyer G, Michalowski CB, Schmitt JM, Bohnert HJ (1989) Salt stress leads to differential expression of two isogenes of phospho*enol*pyruvate carboxylase during crassulacean acid metabolism induction in the common ice plant. *The Plant Cell* **1**, 715–725.

Cushman JC, Agarie S, Albion RL, Elliot SM, Taybi T, Borland AM (2008*a*) Isolation and characterization of mutants of ice plant, *Mesembryanthemum crystallinum*, deficient in crassulacean acid metabolism. *Plant Physiology* **147**, 228–238. doi:10.1104/pp.108.116889

Cushman JC, Tillett RL, Wood JA, Branco JM, Schlauch KA (2008*b*) Large-scale mRNA expression profiling in the common ice plant, *Mesembryanthemum crystallinum*, performing C_3 photosynthesis and crassulacean acid metabolism (CAM). *Journal of Experimental Botany* **59**, 1875–1894. doi:10.1093/jxb/ern008

Dodd AN, Borland AM, Haslam RP, Griffiths H, Maxwell K (2002) Crassulacean acid metabolism: plastic, fantastic. *Journal of Experimental Botany* **53**, 569–580. doi:10.1093/jexbot/53.369.569

Dodd AN, Griffiths H, Taybi T, Cushman JC, Borland AM (2003) Integrating diel starch metabolism with the circadian and environmental regulation of crassulacean acid metabolism in *Mesembryanthemum crystallinum. Planta* **6**, 789–797.

Dressler R (1993) 'Phylogeny and classification of the orchid family.' (Dioscorides Press: Portland, OR)

Earnshaw MJ, Winter K, Ziegler H, Sticher W, Cruttwell NEG, *et al*. (1987) Altitudinal changes in the incidence of crassulacean acid metabolism in vascular epiphytes and related life forms in Papua New Guinea. *Oecologia* **73**, 566–572. doi:10.1007/BF00379417

Edwards EJ, Osborne CP, Strömberg CAE, Smith SA, C_4 Grasses Consortium (2010) The origins of C_4 grasslands: integrating evolutionary and ecosystem science. *Science* **328**, 587–591. doi:10.1126/science.1177216

Ehleringer JR, Monson RK (1993) Evolutionary and ecological aspects of photosynthetic pathway variation. *Annual Review of Ecology and Systematics* **24**, 411–439. doi:10.1146/annurev.es.24.110193.002211

Ehleringer JR, Osmond CB (1989) Stable isotopes. In 'Plant physiological ecology'. (Ed. P Rundel) pp. 255–280. (Chapman and Hall: London)

Engelmann S, Bläsing OE, Westhoff P, Svensson P (2002) Serine 774 and amino acids 296 to 437 comprise the major C_4 determinants of the C_4 phospho*enol*pyruvate carboxylase of *Flaveria trinervia. FEBS Letters* **524**, 11–14. doi:10.1016/S0014-5793(02)02975-7

Engelmann S, Bläsing OE, Gowik U, Svensson P, Westhoff P (2003) Molecular evolution of C_4 phospho*enol*pyrvate carboxylase in the genus *Flaveria* – a gradual increase from C_3 to C_4 characteristics. *Planta* **217**, 717–725. doi:10.1007/s00425-003-1045-0

Eriksson ME, Millar AJ (2003) The circadian clock. A plant's best friend in a spinning world. *Plant Physiology* **132**, 732–738. doi:10.1104/pp.103.022343

Frimert V, Kluge M, Smith JAC (1986) Net CO_2 output by CAM plants in the light: the role of leaf conductance. *Physiologia Plantarum* **68**, 353–358. doi:10.1111/j.1399-3054.1986.tb03365.x

Furumoto T, Hata S, Izui K (2000) Isolation and characterization of cDNAs for differentially accumulated transcripts between mesophyll cells and bundle sheath strands of maize leaves. *Plant & Cell Physiology* **41**, 1200–1209. doi:10.1093/pcp/pcd047

Gehrig HH, Taybi T, Kluge M, Brulfert J (1995) Identification of multiple PEPC isogenes in leaves of the facultative crassulacean acid metabolism (CAM) plant *Kalanchoë blossfeldiana* Poelln. cv. Tom Thumb. *FEBS Letters* **377**, 399–402. doi:10.1016/0014-5793(95)01397-0

Gehrig HH, Heute V, Kluge M (2001) New partial sequences of phospho*enol*pyruvate carboxylase as molecular phylogenetic markers. *Molecular Phylogenetics and Evolution* **20**, 262–274. doi:10.1006/mpev.2001.0973

Gehrig HH, Aranda J, Cushman MA, Virgo A, Cushman JC, Hammel BE, Winter K (2003) Cladogram of Panamanian *Clusia* based on nuclear DNA: implications for the origins of crassulacean acid metabolism. *Plant Biology* **5**, 59–70. doi:10.1055/s-2003-37983

Gehrig HH, Wood JA, Cushman MA, Virgo A, Cushman JC, Winter K (2005) Large gene family of phospho*enol*pyruvate carboxylase in the crassulacean acid metabolism plant *Kalanchoë pinnata* (Crassulaceae). *Functional Plant Biology* **32**, 467–472. doi:10.1071/FP05079

Gibson AC (1982) The anatomy of succulence. In 'Crassulacean acid metabolism'. (Eds I Ting, M Gibbs) pp. 1–17. (American Society of Plant Physiologists: Rockville, MD)

Good-Avila SV, Souza V, Gaut BS, Eguiarte LE (2006) Timing and rate of speciation in *Agave* (Agavaceae). *Proceedings of the National Academy of Sciences of the United States of America* **103**, 9124–9129. doi:10.1073/pnas.0603312103

Gravendeel B, Smithson A, Slik FJW, Schuiteman A (2004) Epiphytism and pollinator specialization: drivers for orchid diversity? *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **359**, 1523–1535. doi:10.1098/rstb.2004.1529

Griffiths H (1988) Crassulacean acid metabolism: a re-appraisal of physiological plasticity in form and function. *Advances in Botanical Research* **15**, 43–92. doi:10.1016/S0065-2296(08)60044-0

Griffiths H (1989) Carbon dioxide concentrating mechanisms and the evolution of CAM in vascular epiphytes. In 'Vascular plants as epiphytes: evolution and ecophysiology'. (Ed. U Lüttge) pp. 42–86. (Springer-Verlag: Berlin)

Griffiths H (1992) Carbon isotope discrimination and the integration of carbon assimilation pathways in terrestrial CAM plants. *Plant, Cell & Environment* **15**, 1051–1062. doi:10.1111/j.1365-3040.1992.tb01655.x

Griffiths H, Helliker B, Roberts A, Haslam RP, Girnus J, Robe WE, Borland AM, Maxwell K (2002) Regulation of Rubisco activity in crassulacean acid metabolism plants: better late than never. *Functional Plant Biology* **29**, 689–696. doi:10.1071/PP01212

Gustafson M, Winter K, Bittrich V (2007) Diversity, phylogeny and classification of *Clusia*. In '*Clusia*: a woody neotropical genus of remarkable plasticity and diversity'. (Ed. U Lüttge) pp. 95–116. (Springer-Verlag: Berlin)

Hartwell J (2005*a*) The circadian clock in CAM plants. In 'Endogenous plant rhythms. Annual Plant Reviews. Vol. 21'. (Eds AJW Hall, HG McWatters) pp. 211–236. (Blackwell: Oxford)

Hartwell J (2005*b*) The co-ordination of central plant metabolism by the circadian clock. *Biochemical Society Transactions* **33**, 945–948. doi:10.1042/BST20050945

Hartwell J, Smith LH, Wilkins MB, Jenkins GI, Nimmo HG (1996) Higher plant phospho*enol*pyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. *The Plant Journal* **10**, 1071–1078. doi:10.1046/j.1365-313X.1996.10061071.x

Hartwell J, Gill A, Nimmo GA, Wilkins MB, Jenkins GI, Nimmo HG (1999) Phospho*enol*pyruvate carboxylase kinase is a novel protein kinase regulated at the level of gene expression. *The Plant Journal* **20**, 333–342. doi:10.1046/j.1365-313X.1999.t01-1-00609.x

Häusler RE, Baur B, Scharte J, Teichmann T, Elcks M, Fischer KL, Flugge U, Schubert S, Weber A, Fischer K (2000) Plastidic metabolite transporters and their physiological functions in the inducible crassulacean acid metabolism plant *Mesembryanthemum crystallinum. The Plant Journal* **24**, 285–296. doi:10.1046/j.1365-313x.2000.00876.x

Hibberd JM, Covshoff S (2010) The regulation of gene expression required for C_4 photosynthesis. *Annual Reviews of Plant Biology* **61**, 181–207. doi:10.1146/annurev-arplant-042809-112238

Holtum JAM, Winter K (1999) Degrees of crassulacean acid metabolism in tropical epiphytic and lithophytic ferns. *Australian Journal of Plant Physiology* **26**, 749–757. doi:10.1071/PP99001

Holtum JAM, Aranda J, Virgo A, Gehrig HH, Winter K (2004) $\delta^{13}C$ values and crassulacean acid metabolism in *Clusia* species from Panama. *Trees – Structure and Function* **18**, 658–668.

Holtum JAM, Smith JAC, Neuhaus H (2005) Intracellular transport and pathways of carbon flow in plants with crassulacean acid metabolism. *Functional Plant Biology* **32**, 429–449. doi:10.1071/FP04189

Holtum JAM, Winter K, Weeks MA, Sexton TR (2007) Crassulacean acid metabolism of the ZZ plant, *Zamioculcas zamiifolia* (Araceae). *American Journal of Botany* **94**, 1670–1676. doi:10.3732/ajb.94.10.1670

Jabaily RS, Sytsma KJ (2010) Phylogenetics of *Puya* (Bromeliaceae): placement, major lineages, and evolution of Chilean species. *American Journal of Botany* **97**, 337–356. doi:10.3732/ajb.0900107

Keeley JE (1996) Aquatic CAM photosynthesis. In 'Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution'. (Eds K Winter, JAC Smith) pp. 281–295. (Springer-Verlag: Berlin)

Keeley J (1998) CAM photosynthesis in submerged aquatic plants. *Botanical Review* **64**, 121–175. doi:10.1007/BF02856581

Kellogg EA (1999) Phylogenetic aspects of the evolution of C_4 photosynthesis. In 'C_4 plant biology'. (Eds RF Sage, RK Monson) pp. 411–444. (Academic Press: San Diego)

Klak C, Khunou A, Reeves G, Hedderson T (2003) A phylogenetic hypothesis for the Aizoaceae (Caryophyllales) based on four plastid DNA regions. *American Journal of Botany* **90**, 1433–1445. doi:10.3732/ajb.90.10.1433

Klak C, Reeves G, Hedderson T (2004) Unmatched tempo of evolution in Southern African semi-desert ice plants. *Nature* **427**, 63–65. doi:10.1038/nature02243

Kluge M, Brulfert J, Ravelomanana D, Lipp J, Ziegler H (1991) Crassulacean acid metabolism in *Kalanchoë* species collected in various climatic zones of Madagascar: a survey by $\delta^{13}C$ analysis. *Oecologia* **88**, 407–414. doi:10.1007/BF00317586

Kluge M, Brulfert J, Lipp J, Ravelomanana D, Ziegler H (1993) A comparative study of $\delta^{13}C$ analysis of crassulascean acid metabolism (CAM) in *Kalanchoë* (Crassulaceae) species of Africa and Madagascar. *Botanica Acta* **106**, 320–324.

Kluge M, Razanoelisoa B, Brulfert J (2001) Implications of genotypic diversity and plasticity in the ecophysiological success of CAM plants, examined by studies on the vegetation of Madagascar. *Plant Biology* **3**, 214–222. doi:10.1055/s-2001-15197

Kore-eda S, Noake C, Ohishi M, Ohnishi J, Cushman JC (2005) Transcriptional profiles of organellar metabolite transporters during induction of crassulacean acid metabolism in *Mesembryanthemum crystallinum. Functional Plant Biology* **32**, 451–466. doi:10.1071/FP04188

Kreft H, Jetz W (2007) Global patterns and determinants of vascular plant diversity. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 5925–5930. doi:10.1073/pnas.0608361104

Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for *Arabidopsis* in reponse to salt, osmotic and cold stress. *Plant Physiology* **130**, 2129–2141. doi:10.1104/pp.008532

Lee HSJ, Griffiths H (1987) Induction and repression of CAM in *Sedum telephium* L. in response to photoperiod and water stress. *Journal of Experimental Botany* **38**, 834–841. doi:10.1093/jxb/38.5.834

Lüttge U (1987) Carbon dioxide and water demand: crassulacean acid metabolism (CAM), a versatile ecological adaptation exemplifying the need for integration in ecophysiological work. *New Phytologist* **106**, 593–629. doi:10.1111/j.1469-8137.1987.tb00163.x

Lüttge U (2003) Circadian rhythmicity: is the 'biological clock' hardware or software? *Progress in Botany* **64**, 277–319.

Martin SL, Davis R, Protti P, Lin T-C, Lin S-H, Martin CE (2005) The occurrence of crassulacean acid metabolism in epiphytic ferns, with an emphasis on the Vittariaceae. *International Journal of Plant Sciences* **166**, 623–630. doi:10.1086/430334

Maxwell K (2002) Resistance is useful: diurnal patterns of photosynthesis in C_3 and crassulacean acid metabolism epiphytic bromeliads. *Functional Plant Biology* **29**, 679–687. doi:10.1071/PP01193

Maxwell K, von Caemmerer S, Evans JR (1997) Is a low internal conductance to CO_2 diffusion a consequence of succulence in plants with crassulacean acid metabolism? *Australian Journal of Plant Physiology* **24**, 777–786. doi:10.1071/PP97088

Maxwell K, Borland AM, Haslam RP, Helliker BR, Roberts A, Griffiths H (1999) Modulation of ribulose-1,5-bisphosphate carboxylase activity during the diurnal phases of the crassulacean acid metabolism plant *Kalanchoë daigremontiana. Plant Physiology* **121**, 849–856. doi:10.1104/pp.121.3.849

Monson RK (1989) On the evolutionary pathways resulting in C_4 photosynthesis and crassulacean acid metabolism (CAM). *Advances in Ecological Research* **19**, 57–110. doi:10.1016/S0065-2504(08)60157-9

Monson R (1999) The origins of C_4 genes and evolutionary patterns in the C_4 metabolic phenotype. In 'C_4 plant biology'. (Eds RF Sage, RK Monson) pp. 377–410. (Academic Press: San Diego)

Monson R (2003) Gene duplication, neofunctionalization, and the evolution of C_4 photosynthesis. *International Journal of Plant Sciences* **164**, S43–S54. doi:10.1086/368400

Moore PD (1982) Evolution of photosynthetic pathways in flowering plants. *Nature* **295**, 647–648. doi:10.1038/295647a0

Motomura H, Yukawa T, Ueno O, Kagawa A (2008) The occurrence of crassulacean acid metabolism in *Cymbidium* (Orchidaceae) and its ecological and evolutionary implications. *Journal of Plant Research* **121**, 163–177. doi:10.1007/s10265-007-0144-6

Nelson EA, Sage RF (2008) Functional constraints of CAM leaf anatomy: tight cell packing is associated with increased CAM function across a gradient of CAM expression. *Journal of Experimental Botany* **59**, 1841–1850. doi:10.1093/jxb/erm346

Nelson EA, Sage TL, Sage RF (2005) Functional leaf anatomy of plants with crassulacean acid metabolism. *Functional Plant Biology* **32**, 409–419. doi:10.1071/FP04195

Nimmo HG (2000) The regulation of phospho*enol*pyruvate carboxylase in CAM plants. *Trends in Plant Science* **5**, 75–80. doi:10.1016/S1360-1385(99)01543-5

Nobel PS (1996) High productivities of certain agronomic CAM species. In 'Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution'. (Eds K Winter, JAC Smith) pp. 255–265. (Springer-Verlag: Berlin)

Osmond CB (1978) Crassulacean acid metabolism: a curiosity in context. *Annual Review of Plant Physiology* **29**, 379–414. doi:10.1146/annurev.pp.29.060178.002115

Osmond CB (1982) Carbon cycling and stability of the photosynthetic apparatus in CAM. In 'Crassulacean acid metabolism'. (Eds I Ting, M Gibbs) pp. 112–127. (American Society of Plant Physiologists: Rockville, MD)

Paul MJ, Loos K, Stitt M, Ziegler P (1993) Starch-degrading enzymes during the induction of CAM in *Mesembryanthemum crystallinum. Plant, Cell & Environment* **16**, 531–538. doi:10.1111/j.1365-3040.1993.tb00900.x

Pearson PN, Palmer MR (2000) Atmospheric carbon dioxide concentrations over the past 60 million years. *Nature* **406**, 695–699. doi:10.1038/35021000

Pfahl J, Campacci MA, Holland Baptista D, Tigges H, Shaw J, Cribb P, George A, Kreuz K, Wood J (2008) World checklist of the Orchidaceae. The Board of Trustees of the Royal Botanic Gardens, Kew. Available at: http://www.kew.org/wcsp/ [Accessed 13 April 2010]

Pierce S, Winter K, Griffiths H (2002) Carbon isotope ratio and the extent of daily CAM use by Bromeliaceae. *New Phytologist* **156**, 75–83. doi:10.1046/j.1469-8137.2002.00489.x

Pilon-Smits EAH, 't Hart H, van Brederode J (1996) Evolutionary aspects of crassulacean acid metabolism in the crassulaceae. In 'Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution'. (Eds K Winter, JAC Smith) pp. 349–359. (Springer-Verlag: Berlin)

Pridgeon A, Cribb P, Chase M, Rasmussen F (1999–2009) 'Genera Orchidacearum.' (Oxford University Press: Oxford)

Raven JA, Spicer RA (1996) The evolution of crassulacean acid metabolism. In 'Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution'. (Eds K Winter, JAC Smith) pp. 360–385. (Springer-Verlag: Berlin)

Ritz D, Kluge M, Veith HJ (1986) Mass-spectrometric evidence for the double-carboxylation pathway of malate synthesis by crassulacean acid metabolism plants in light. *Planta* **167**, 284–291. doi:10.1007/BF00391428

Roberts A, Borland AM, Griffiths H (1997) Discrimination processes and shifts in carboxylation during the phases of crassulacean acid metabolism. *Plant Physiology* **113**, 1283–1292.

Sage RF (2001) Environmental and evolutionary preconditions for the origin and diversification of the C_4 photosynthetic syndrome. *Plant Biology* **3**, 202–213. doi:10.1055/s-2001-15206

Sage RF (2004) The evolution of C_4 photosynthesis. *New Phytologist* **161**, 341–370. doi:10.1111/j.1469-8137.2004.00974.x

Sánchez R, Cejudo FJ (2003) Identification and expression analysis of a gene encoding a bacterial-type phospho*enol*pyruvate carboxylase from *Arabidopsis* and rice. *Plant Physiology* **132**, 949–957. doi:10.1104/pp.102.019653

Schuber M, Kluge K (1981) *In situ* studies on crassulacean acid metabolism in *Sedum acre* L. and *Sedum mite* Gil. *Oecologia* **50**, 82–87. doi:10.1007/BF00378797

Silvera K, Santiago LS, Winter K (2005) Distribution of crassulacean acid metabolism in orchids of Panama: evidence of selection of weak and strong modes. *Functional Plant Biology* **32**, 397–407. doi:10.1071/FP04179

Silvera K, Santiago LS, Cushman JC, Winter K (2009) Crassulacean acid metabolism and epiphytism linked to adaptive radiations in the Orchidaceae. *Plant Physiology* **149**, 1838–1847. doi:10.1104/pp.108.132555

Silvera K, Santiago LS, Cushman JC, Winter K (2010) The incidence of crassulacean acid metabolism in the Orchidaceae derived from carbon isotope ratios: a checklist of the flora of Panama and Costa Rica. *Botanical Journal of the Linnean Society* **163**, 194–222. doi:10.1111/j.1095-8339.2010.01058.x

Sipes DL, Ting IP (1985) Crassulacean acid metabolism and crassulacean acid metabolism modifications in *Peperomia camptotricha. Plant Physiology* **77**, 59–63. doi:10.1104/pp.77.1.59

Smith JAC (1984) Water relations in CAM plants. In 'Physiological ecology of CAM plants'. (Ed. E Medina) pp. 30–51. (CIET (Unesco-IVIC): Caracas, Venezuela)

Smith JAC, Bryce JH (1992) Metabolite compartmentation and transport in CAM plants. In 'Plant organelles'. (Ed. AK Tobin) pp. 141–167. (Cambridge University Press: Cambridge)

Smith JAC, Winter K (1996) Taxonomic distribution of crassulacean acid metabolism. In 'Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution'. (Ed. K Winter, JAC Smith) pp. 427–436. (Springer-Verlag: Berlin)

Smith JAC, Griffiths H, Lüttge U (1986) Comparative ecophysiology of CAM and C_3 bromeliads. I. The ecology of the Bromeliaceae in Trinidad. *Plant, Cell & Environment* **9**, 359–376. doi:10.1111/j.1365-3040.1986.tb01750.x

Spalding MD, Stumpf DK, Ku MSB, Burris RH, Edwards GE (1979) Crassulacean acid metabolism and diurnal variations in internal CO_2 and O_2 concentrations in *Sedum praealtum* DC. *Australian Journal of Plant Physiology* **6**, 557–567. doi:10.1071/PP9790557

Stevens P (2008) Angiosperm phylogeny website. Version 9. Available at: http://www.mobot.org/MOBOT/research/APweb/ [Accessed 28 June 2010]

Szarek SR, Johnson HB, Ting IP (1973) Drought adaptation in *Opuntia basilaris*. Significance of recycling carbon through crassulacean acid metabolism. *Plant Physiology* **52**, 539–541. doi:10.1104/pp.52.6.539

Taybi T, Patil S, Chollet R, Cushman JC (2000) A minimal Ser/Thr protein kinase circadianly regulates phospho*enol*pyruvate carboxylase activity in CAM-induced leaves of *Mesembryanthemum crystallinum. Plant Physiology* **123**, 1471–1482. doi:10.1104/pp.123.4.1471

Taybi T, Nimmo HG, Borland AM (2004) Expression of phospho*enol*pyruvate carboxylase and phospho*enol*pyruvate carboxylase kinase genes. Implications for genotypic capacity and phenotypic plasticity in the expression of crassulacean acid metabolism. *Plant Physiology* **135**, 587–598. doi:10.1104/pp.103.036962

Teeri JA (1982*a*) Photosynthetic variation in the Crassulaceae. In 'Crassulacean acid metabolism'. (Eds I Ting, M Gibbs) pp. 244–259. (American Society of Plant Physiologists: Rockville, MD)

Teeri JA (1982*b*) Carbon isotopes and the evolution of C_4 photosynthesis and crassulacean acid metabolism. In 'Biochemical aspects of evolutionary biology'. (Ed. MH Nitecki) pp. 93–130. (University of Chicago Press: Chicago, IL)

Teeri JA, Tonsor SJ, Turner M (1981) Leaf thickness and carbon isotope composition in the Crassulaceae. *Oecologia* **50**, 367–369. doi:10.1007/BF00344977

Ting I (1985) Crassulacean acid metabolism. *Annual Review of Plant Physiology* **36**, 595–622. doi:10.1146/annurev.pp.36.060185.003115

Vaasen A, Begerow D, Lüttge U, Hampp R (2002) The genus *Clusia* L.: molecular evidence for independent evolution of photosynthetic flexibility. *Plant Biology* **4**, 86–93. doi:10.1055/s-2002-20440

von Willert D, Armbrüster N, Drees T, Zaborowski M (2005) *Welwitschia mirabilis*: CAM or not CAM – what is the answer? *Functional Plant Biology* **32**, 389–393. doi:10.1071/FP01241

Vovides AP, Etherington JR, Dresser PQ, Groenhof A, Iglesias C, Ramirez JF (2002) CAM-cycling in the cycad *Dioon edule* Lindl. in is natural deciduous forest habitat in central Veracruz, Mexico. *Botanical Journal of the Linnean Society* **138**, 155–162. doi:10.1046/j.1095-8339.2002.138002155.x

Westhoff P, Gowik U (2004) Evolution of C_4 phospho*enol*pyruvate carboxylase. Gene and proteins: a case study with the genus *Flaveria. Annals of Botany* **93**, 13–23. doi:10.1093/aob/mch003

Wilkins MB (1992) Circadian rhythms: their origin and control. *New Phytologist* **121**, 347–375. doi:10.1111/j.1469-8137.1992.tb02936.x

Williams NH, Chase MW, Fulcher T, Whitten WM (2001*a*) Molecular systematics of the Oncidiinae based on evidence from four DNA regions: expanded circumscriptions of *Cyrtochilum, Erycina, Otoglossum* and *Trichocentrum* and a new genus (Orchidaceae). *Lindleyana* **16**, 113–139.

Williams NH, Chase MW, Whitten WM (2001*b*) Phylogenetic position of *Miltoniopsis*, *Caucaea*, a new genus, *Cyrtochiloides*, and relationship of *Oncidium phymatochilum* based on nuclear and chloroplast DNA sequence data (Orchidaceae: Oncidiinae). *Lindleyana* **16**, 95–114.

Winter K (1982) Properties of phospho*enol*pyruvate carboxylase in rapidly prepared, desalted leaf extracts of the crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. *Planta* **154**, 298–308. doi:10.1007/BF00393907

Winter K (1985) Crassulacean acid metabolism. In 'Photosynthetic mechanisms and the environment'. (Eds J Barber, NR Baker) pp. 329–387. (Elsevier: Amsterdam)

Winter K, Holtum JAM (2002) How closely do the $\delta^{13}C$ values of crassulacean acid metabolism plants reflect the proportion of CO_2 fixed during day and night? *Plant Physiology* **129**, 1843–1851. doi:10.1104/pp.002915

Winter K, Holtum JAM (2007) Environment or development? Lifetime net CO_2 exchange and control of the expression of crassulacean acid metabolism in *Mesembryanthemum crystallinum. Plant Physiology* **143**, 98–107. doi:10.1104/pp.106.088922

Winter K, Smith JAC (1996) An introduction to crassulacean acid metabolism. Biochemical principles and ecological diversity. In 'Crassulacean acid metabolism. Biochemistry, ecophysiology and evolution'. (Eds K Winter, JAC Smith) pp. 1–13. (Springer-Verlag: Berlin)

Winter K, Wallace B, Stocker G, Roksandic Z (1983) Crassulacean acid metabolism in Australian vascular epiphytes and some related species. *Oecologia* **57**, 129–141. doi:10.1007/BF00379570

Winter K, Medina E, Garcia V, Mayoral ML, Muniz R (1985) Crassulacean acid metabolism in roots of a leafless orchid, *Campylocentrum tyrridion* Caray & Dunsterv. *Journal of Plant Physiology* **118**, 73–78.

Winter K, Garcia M, Holtum JAM (2008) On the nature of facultative and constitutive CAM: environmental and developmental control of CAM expression during early growth of *Clusia*, *Kalanchoë*, and *Opuntia. Journal of Experimental Botany* **59**, 1829–1840. doi:10.1093/jxb/ern080

Wyka TB, Bohn A, Duarte HM, Kaiser F, Lüttge U (2004) Perturbations of malate accumulation and the endogenous rhythms of gas exchange in the crassulacean acid metabolism plant *Kalanchoë daigremontiana*: testing the tonoplast-as-oscillator model. *Planta* **219**, 705–713. doi:10.1007/s00425-004-1265-y

Zotz G (2004) How prevalent is crassulacean acid metabolism among vascular epiphytes? *Oecologia* **138**, 184–192. doi:10.1007/s00442-003-1418-x

Zotz G, Ziegler H (1997) The occurrence of crassulacean acid metabolism among vascular epiphytes from Central Panama. *New Phytologist* **137**, 223–229. doi:10.1046/j.1469-8137.1997.00800.x

Manuscript received 15 April 2010, accepted 2 August 2010