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Crassulacean acid metabolism: a continuous or discrete trait?

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Summary

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The key components of crassulacean acid metabolism (CAM) – nocturnal fixation of atmospheric CO₂ and its processing via Rubisco in the subsequent light period – are now reasonably well understood in terms of the biochemical reactions defining this water-saving mode of carbon assimilation. Phenotypically, however, the degree to which plants engage in the CAM cycle relative to regular C₃ photosynthesis is highly variable. Depending upon species, ontogeny and environment, the contribution of nocturnal CO₂ fixation to 24-h carbon gain can range continuously from close to 0% to 100%. Nevertheless, not all possible combinations of light and dark CO₂ fixation appear equally common. Large-scale surveys of carbon-isotope ratios typically show a strongly bimodal frequency distribution, with relatively few intermediate values. Recent research has revealed that many species capable of low-level CAM activity are nested within the peak of C₃-type isotope signatures. While questions remain concerning the adaptive significance of dark CO₂ fixation in such species, plants with low-level CAM should prove valuable models for investigating the discrete changes in genetic architecture and gene expression that have enabled the evolutionary transition from C₃ to CAM.

I. Introduction

Crassulacean acid metabolism (CAM) is a water-conserving mode of photosynthesis and one of three photosynthetic pathways in vascular plants. CAM and C₄ are modifications of the basic C₃ pathway and represent CO₂-concentrating mechanisms that elevate [CO₂] around Rubisco and suppress photorespiration. In CAM, this is achieved in two principal phases separated in time. At night, atmospheric CO₂ is incorporated by phosphoenolpyruvate carboxylase (PEPC) via oxaloacetate into malic acid, which

accumulates in the large vacuoles of chloroplast-containing mesophyll cells. During the following light period, malic acid is released from the vacuoles and decarboxylated, and the CO₂ thus liberated is refixed by Rubisco and reduced in the Calvin cycle (Osmond, 1978; Winter & Smith, 1996). Decarboxylation of malate generates high intercellular [CO₂] and is associated with stomatal closure, minimizing water loss in the middle of the day when evaporative demand is highest.

In common garden experiments to compare growth rates under identical conditions, plant biomass production per unit water

utilized was 6 or 2 times higher for CAM than for C_3 or C_4 , respectively (Winter *et al.*, 2005). Maximum CO_2 uptake rates per unit surface area are generally lower in CAM plants than in C_3 and C_4 plants, but this is partially offset by the fact that almost the entire shoot surface is photosynthetic in typical CAM plants, as in agaves and cacti. Furthermore, in terms of the overall energetics of carbon assimilation, CAM is estimated to represent a fairly small marginal cost of *c.* 10% relative to the C_3 pathway (Winter & Smith, 1996), and indeed CAM plants typically grow in open or exposed habitats in which incident light energy is not the primary factor limiting growth. Thus, annual productivities of CAM plants can be considerable (Nobel, 1988), and agaves, platyopuntias and other CAM species have been proposed as potential biofuel crops on land not suitable for conventional C_3 and C_4 crops (Borland *et al.*, 2009; Davis *et al.*, 2011; Holtum *et al.*, 2011). The effect of leaf or photosynthetic-stem area ratio on the relative growth rate of CAM species has never been rigorously quantified. A comparison of agaves and platyopuntias with arborescent CAM species that allocate substantial biomass to a woody, nonphotosynthetic stem (e.g. in the genus *Clusia*; Lüttge, 2006) would be informative.

Amongst the estimated 350 000 species of vascular plants, *c.* 6% are believed capable of CAM photosynthesis, belonging to at least 35 families and over 400 genera, and outnumbering C_4 species approximately two-fold (Winter & Smith, 1996; Yang *et al.*, 2015). Most of the longer lived stem-succulent CAM species of large stature inhabit warm, seasonally dry habitats such as semi-deserts with little, but relatively predictable, seasonal rainfall (Ellenberg, 1981). The extant diversity of these and other major terrestrial CAM lineages seems to have arisen largely during the global expansion of arid environments in the late Miocene (Arakaki *et al.*, 2011; Horn *et al.*, 2014). Among epiphytic lineages, CAM is considered to have enabled diversification of the more extreme epiphytic life-forms occupying arid microhabitats in forest canopies, notably in the tropical Bromeliaceae and Orchidaceae (Crayn *et al.*, 2004, 2015; Silvera *et al.*, 2009).

CAM is not only multifaceted in terms of diversity of species and habitats. There is also continuous variation in the extent to which species engage in CAM relative to C_3 photosynthesis. The fact that the engagement of CAM is not an all-or-nothing phenomenon raises fundamental questions about both the ecological significance and evolutionary origins of CAM.

II. Phenotypic diversity

CAM comes in many variants, differing, for example, in the enzymes of malate decarboxylation, and the type and compartmentation of storage carbohydrates that fluctuate reciprocally with malic acid (Holtum *et al.*, 2005). Above all, CAM provides some of the best examples of phenotypic diversity and plasticity in the plant kingdom, for example in the form of facultative CAM species such as the annuals *Mesembryanthemum crystallinum* (Aizoaceae) and *Calandrinia polyandra* (Montiaceae), or some perennial species of *Clusia* (Clusiaceae), in all of which CAM is drought-inducible (Winter *et al.*, 2008; Winter & Holtum, 2014). Over a relatively short period, these plants have the ability to transition progressively from full C_3 to full CAM and vice versa.

Even in obligate CAM species such as desert cacti, there is an ontogenetic progression from C_3 to CAM as tissues mature (Winter *et al.*, 2008, 2011). Indeed, most species regarded as constitutive CAM plants also incorporate, to varying degrees, atmospheric CO_2 directly in the light via Rubisco. Depending on species, environmental conditions and developmental status, the contribution of nocturnal CO_2 gain to total daily carbon gain may range from 100% to close to 0% (Winter & Holtum, 2002, 2014). In its weakest form, CAM is merely evident as a small nocturnal increase in tissue titratable acidity. Nocturnal H^+ increases of *c.* 1 mmol kg⁻¹ fresh mass 12 h⁻¹ currently represent the limit of detection for low-level CAM. They may correspond to average CO_2 fixation rates of < 0.05 $\mu\text{mol m}^{-2} \text{s}^{-1}$, which are challenging to resolve with conventional gas-exchange systems against respiratory background CO_2 effluxes of typically *c.* 1 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

CO_2 fixation via PEPC at night is by no means exclusive to CAM. All C_3 plants presumably have the capacity to fix CO_2 in the dark, as can be detected in ¹⁴CO₂ tracer studies (Kunitake *et al.*, 1959). While the nocturnal fixation of CO_2 and the linked stoichiometric accumulation of malic acid (Medina & Osmond, 1981) may be unique to CAM, PEP carboxylation and accumulation and decarboxylation cycles of the malate anion are not; these are integral to the maintenance of charge balance during processes such as NO₃⁻ reduction and changes in stomatal aperture (Smith *et al.*, 1996; Martinoia *et al.*, 2012). Moreover, the essential enzyme and transport reactions needed for CAM are all seemingly present in C_3 plants. Given the fundamental nature of these metabolic processes, the questions arise: where does C_3 end and CAM begin; are the differences between C_3 and CAM of a qualitative or merely quantitative nature; is CAM a continuous or a discrete trait?

III. Ecological context

On the basis of the wide spectrum of CAM phenotypes recognizable in CO_2 exchange studies, it is tempting to conclude that CAM is a continuous trait. However, it is notable that in large-scale surveys of $\delta^{13}C$ values (a measure of the ratio of ¹³C : ¹²C), which serve as integrated long-term proxies of the ratio of CO_2 fixed during the day to that fixed during the night by plants in their natural habitat (Cernusak *et al.*, 2013), $\delta^{13}C$ values are not equally distributed over the whole range of possible values (Fig. 1). Rather, their distribution is typically bimodal, with a minimum in the frequency distribution at *c.* -20‰. Based on the linear relationship observed between the $\delta^{13}C$ value and the proportion of CO_2 fixed at night, this value would correspond to a *c.* 40% contribution of dark CO_2 fixation to total carbon gain (Winter & Holtum, 2002). As yet, the ecological significance of this minimum is not fully understood, but it might somehow reflect a fitness cost of intermediate phenotypes, or a paucity of habitats in which such an intermediate phenotype is favoured. Ongoing research, especially in the Orchidaceae, is revealing the occurrence of low-level CAM in many species with C_3 -type $\delta^{13}C$ values, possibly indicating a second frequency peak of species capable of CAM nested within the C_3 cluster of isotope values (Silvera *et al.*, 2005).

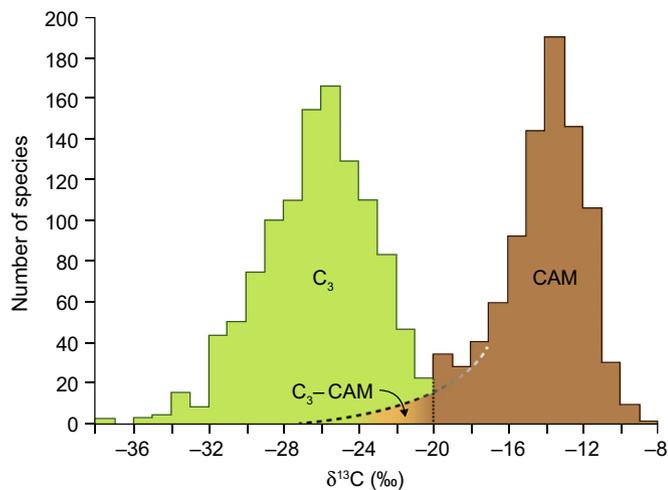


Fig. 1 Frequency histogram of $\delta^{13}\text{C}$ values of species of Bromeliaceae plotted in class intervals of 1‰ (re-plotted from data in Crayn *et al.*, 2015; copyright © 2015, John Wiley & Sons), showing the strongly bimodal distribution of isotope ratios with a frequency minimum at $c. -20\text{‰}$, as is typically observed in large-scale surveys of crassulacean acid metabolism (CAM) species. The two clusters of $\delta^{13}\text{C}$ values more negative and less negative than -20‰ are principally composed of C_3 species and CAM species, respectively. However, nested within the C_3 cluster are species that exhibit some degree of CAM activity, based on measurements of nocturnal increases in tissue titratable acidity and nocturnal net CO_2 uptake. These we define as C_3 -CAM species, although their exact number is not yet known accurately because, to date, nocturnal acidification has been tested in relatively few species of Bromeliaceae with $\delta^{13}\text{C}$ values more negative than -20‰ (Pierce *et al.*, 2002). While this representation of the frequency of C_3 -CAM species is schematic, it illustrates that they may be part of a progressive trend of increasing contribution of dark CO_2 fixation to total carbon gain with increasing $\delta^{13}\text{C}$ value. At $\delta^{13}\text{C}$ values above -20‰ , this trend merges with the cluster of CAM plants that show nocturnal fixation as their dominant mode of carbon assimilation. Analysis of the original data for goodness of fit (G -test) shows that the C_3 cluster of $\delta^{13}\text{C}$ values does not differ significantly from a normal frequency distribution, whereas the CAM cluster does ($P < 0.05$), reflecting the slightly higher than expected abundance of species with values in the range -20 to -17‰ .

IV. Structure–function context

CAM is in essence a single-cell phenomenon. This contrasts with the greater structural complexity of the dual-cell anatomy typical of C_4 plants, which necessitates close metabolic coordination between mesophyll and bundle-sheath cells and has been linked to as many as 25 forms of Kranz anatomy (Aubry *et al.*, 2014). But by virtue of being confined to individual chlorenchyma cells, operation of the CAM cycle must be underpinned by strict temporal control of the carboxylation and decarboxylation reactions if carbon assimilation is to be optimized and futile cycling kept to a minimum (Smith & Bryce, 1992; Borland *et al.*, 2011).

Further structural hallmarks of CAM are manifested at the higher morphological levels of tissues and organs, most characteristically in the succulent leaves and stems that endow the shoot with a low surface area : volume ratio and high water-storage capacitance. The tightly packed, thin-walled, highly vacuolated cells that make up the chlorenchyma tissue maximize the storage capacity for malic acid per unit surface area of shoot across which uptake of

atmospheric CO_2 occurs (Smith *et al.*, 1996). In fact, CAM plants in general are characterized by relatively low stomatal densities on their shoot surfaces and low maximal stomatal conductances (Pfeffer, 1897; Kluge & Ting, 1978; Gibson, 1982; Zambrano *et al.*, 2014), which although helping to restrict water loss in transpiration also act as a partial constraint on maximum daily carbon gain.

Increased succulence of photosynthetic tissues may be one of the key preconditioning traits for CAM (Ogburn & Edwards, 2010; Edwards & Ogburn, 2012). This is convincingly seen in clades possessing a full spectrum of intermediate phenotypes such as the Orchidaceae, in which the trend from C_3 through C_3 -CAM species to strongly expressed CAM is associated with progressively increasing succulence (Fig. 2). A similar relationship is also seen in other families in which a wide range of leaf morphologies have been studied, such as the Polypodiaceae (Winter *et al.*, 1983), Bromeliaceae (Baresch *et al.*, 2011), Clusiaceae (Zambrano *et al.*, 2014) and Crassulaceae (Teeri *et al.*, 1981; Kluge *et al.*, 1993). Another correlate of succulence and tight cell packing in the chlorenchyma is the reduction in intercellular air spaces and internal conductance to CO_2 , which it has been argued favours PEPC-mediated nighttime (phase I) fixation relative to daytime (principally phase IV) fixation directly via Rubisco, as the latter is strongly diffusion-limited (Maxwell *et al.*, 1999; Nelson & Sage, 2008). The transition between the C_3 and CAM pathways is thus associated with a complex suite of biochemical and structural tradeoffs that may determine the optimal niches for these plants in their natural environments (cf. Fig. 1).

CAM plants are not unique in having their stomata open at night, as many C_3 plants maintain stomata partially open in the dark (Darwin, 1898; Caird *et al.*, 2007). In CAM plants, nocturnal stomatal opening is largely mediated by the decline in CO_2 concentration in the intercellular air spaces caused by activation of PEPC (Griffiths *et al.*, 2007; von Caemmerer & Griffiths, 2009). Thus, CAM plants do not fix CO_2 at night because their stomata open at night; instead, stomatal opening is driven by nocturnal CO_2 fixation. The pattern of diel CO_2 exchange of *Kalanchoë daigremontiana* leaves with the lower epidermis removed still resembles that of fully intact leaves (Kluge & Fischer, 1967). Furthermore, net CO_2 exchange of roots of leafless CAM orchids exhibits all four phases of CAM gas exchange as defined by Osmond (1978), even though the roots lack stomata (Winter *et al.*, 1985). It remains to be seen whether or not stomata of CAM plants have acquired CAM-specific features that help optimize the physiology of CAM.

Similarly, there has been considerable discussion about the role played by endogenous circadian rhythms in CAM plants and the requirement for a CAM-specific oscillator for a functional CAM cycle. When studied under constant conditions, many biological processes including CO_2 fixation show circadian behaviour related to the action of endogenous oscillators, which are formed from a series of interlocked transcription/translation feedback loops (Dodd *et al.*, 2014). While certain key features of the CAM cycle have long been known to show endogenous circadian rhythmicity under constant conditions (Wilkins, 1959; Hartwell, 2006), the extent to which the operation of the CAM cycle and the growth of

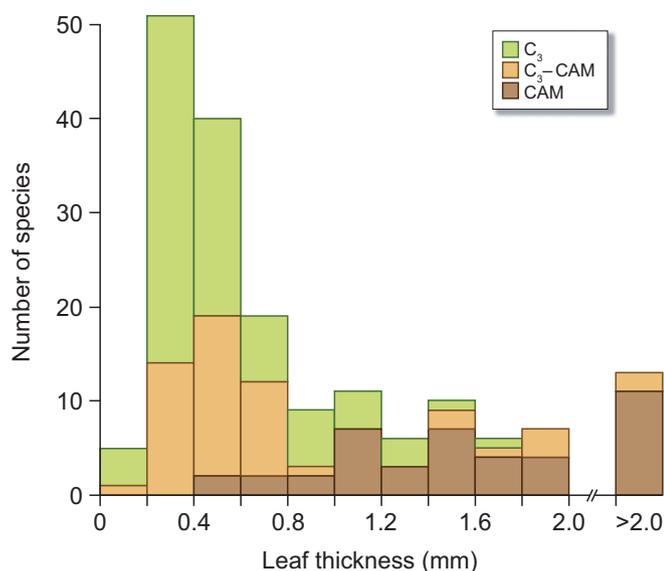


Fig. 2 Frequency histogram of leaf thickness, plotted in class intervals of 0.2 mm, of largely Panamanian species of Orchidaceae for which $\delta^{13}\text{C}$ values and nocturnal changes in leaf titratable acidity were determined (based on data in Silvera *et al.*, 2005). Following the nomenclature introduced in Fig. 1, species with $\delta^{13}\text{C}$ values less negative than -20‰ were considered crassulacean acid metabolism (CAM) species, while species with $\delta^{13}\text{C}$ values more negative than -20‰ were considered C₃ species or C₃-CAM species on the basis of the absence or presence, respectively, of nocturnal increases in tissue acidity.

CAM plants under natural day : night cycles is dependent upon, or optimized by, a CAM-specific oscillator will be a key issue for future research.

V. Biochemical–genomics context

The enzymes and transporters involved in the CAM cycle appear to be homologues of proteins ubiquitous in C₃ species. For instance, pyruvate, orthophosphate dikinase, first discovered in the plant kingdom in C₄ and CAM plants (Hatch & Slack, 1968; Kluge & Osmond, 1971), was briefly considered a C₄- and CAM-specific novelty, but the enzyme was soon shown to be present in C₃ plants as well (Aoyagi & Bassham, 1984).

There is some evidence that the gain of CAM is associated with gene duplication and neofunctionalization, allowing the novel isoforms to fulfil CAM-specific functions. Discrete changes may include alterations in expression patterns involving changes in *cis*-regulatory elements and transcription factors, and/or changes in kinetic properties of key enzymes resulting from adaptive amino acid substitutions. For example, analysis of PEPC genes in the Caryophyllales, an order containing multiple C₄ and CAM lineages, indicates that an early genome-wide duplication event before the emergence of land plants was followed by another whole-genome duplication that gave rise to two PEPC gene lineages now found in most eudicots; one of these (*ppc-1E1*) was then repeatedly duplicated, leading to several gene lineages containing CAM-specific isoforms of PEPC in Aizoaceae, Cactaceae, Portulacaceae and Didiereaceae

(Christin *et al.*, 2014). Gene duplication events have also been implicated in the evolution of CAM-specific PEPC isoforms in the monocot family Orchidaceae (Silvera *et al.*, 2014). CAM-specific posttranslational regulation optimizes CAM functioning through diel control of the kinetic properties of CAM PEPC, stimulating dark CO₂ fixation and minimizing the futile cycling of CO₂ in the light (Winter, 1982; Nimmo, 2000). These day–night changes in PEPC kinetics are brought about through reversible phosphorylation of the enzyme by a specific protein kinase (Hartwell *et al.*, 1996). In principle, these and other changes to CAM could have been initiated by random *de novo* mutation, or by exploiting the standing genetic variation already present in populations (West-Eberhard *et al.*, 2011).

VI. Transitional states?

Thus far, it is not known whether weakly expressed CAM and facultative CAM represent transitional states along an ordered stepwise evolutionary trajectory from C₃ to strong CAM (Hancock & Edwards, 2014). Low-level and facultative CAM species are frequently found in the same lineages as species with fully expressed CAM, suggesting common ecological, anatomical and genomic predispositions. However, compared with the extensive carbon-isotope surveys of herbarium material, relatively few species have been tested physiologically for their mode of photosynthesis, so our knowledge of the true extent of low-level CAM is probably very incomplete. The most extensive and systematic information of this sort to date comes from the Orchidaceae, in which living material of 173 species has been studied for day–night changes in titratable acidity (Fig. 2; Silvera *et al.*, 2005).

Another challenging question is whether an adaptive benefit or fitness advantage of low-level CAM activity can be convincingly demonstrated. Even if CAM suffices only to minimize the loss of respiratory CO₂ at night, as is the case for plants displaying ‘CAM cycling’ (Harris & Martin, 1991; Herrera, 2009), mortality could be reduced during drought stress. However, persuasive evidence for the adaptive significance of low-level CAM is still missing. Some species of *Oncidium* (Orchidaceae) with C₃-type $\delta^{13}\text{C}$ values, yet showing small and significant rates of net dark CO₂ fixation under well-watered and droughted conditions, are highly tolerant to water deficit stress (Katia Silvera, personal communication). An adaptive advantage is more clearly evident for the facultative CAM expressed in species such as *M. crystallinum* (Winter *et al.*, 1978) and *C. polyandra* (Winter & Holtum, 2011). Facultative CAM in these annuals combines C₃-driven growth after germination in the wet season with prolonged, CAM-based carbon gain at low water cost during the subsequent dry season, thereby aiding reproduction. Drought-stressed plants of *M. crystallinum* exposed to CO₂-free air at night have drastically reduced seed set compared with drought-stressed plants that can take full advantage of CAM (Winter & Ziegler, 1992). Facultative CAM may be an optimal strategy for these annuals in their characteristic habitats, and it is difficult to envision that these plants would be merely transitional forms on their way to a perennial life-style with full CAM.

VII. Conclusions – what is a CAM plant?

Attempts to reconstruct the evolutionary origins of CAM photosynthesis necessarily involve decisions about the most appropriate character or trait to map onto the phylogenetic trees. A restrictive approach would be to code the presence or absence of strongly expressed CAM as a binary character state, for example when surveys of the study group reveal a clear bimodal distribution of $\delta^{13}\text{C}$ values (cf. Fig. 1). A more inclusive approach would be to code for the occurrence in a taxon of any degree of CAM activity, however small, as detected by measurements of nocturnal CO_2 fixation or associated diel acid fluctuations. In its most minimalistic form, a complete CAM cycle could theoretically operate with just a single molecule of atmospheric CO_2 being fixed by PEPC at night, leading to the storage of a single molecule of malic acid, and generating 1 CO_2 during the following day for refixation via Rubisco. This highlights the need for systematic collection of living material to obtain a much more complete picture of lineages possessing the capacity for nocturnal CO_2 fixation via the CAM cycle. Furthermore, if low-level CAM is only facultatively expressed, its detection may depend on investigating the species under the precise conditions (e.g. of water deficit stress) that induce this activity.

Based on the distinct bimodal distribution of $\delta^{13}\text{C}$ values in taxa where CAM is present (Fig. 1), we propose that the terminology in this field can be rationalized by reserving the simple, unqualified designation ‘CAM species’ or ‘CAM plant’ for taxa that are part of the strong CAM cluster in the frequency histogram of $\delta^{13}\text{C}$ values. These plants will have isotopic signatures less negative than c. -20‰ and will correspond to species such as agaves and platyopuntias in which CAM makes a substantial, and typically the major, contribution to carbon acquisition. We propose classifying as ‘ C_3 -CAM species’ all taxa in the C_3 cluster ($\delta^{13}\text{C}$ values more negative than -20‰) for which some capacity to engage in CAM has been demonstrated, as determined by CO_2 exchange and/or nocturnal H^+ increase. In these species the CAM cycle is demonstrably present, but C_3 photosynthesis clearly remains the principal mechanism of carbon gain (e.g. the gymnosperm *Welwitschia mirabilis*; von Willert *et al.*, 2005). Future research will show whether C_3 -CAM species are transitional intermediates (phylogenetically and/or in the metabolic complexity of the CAM cycle) along the evolutionary trajectory from C_3 to full CAM.

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