New Phytologist

Review

Tansley insight

Crassulacean acid metabolism: a continuous or discrete trait?

Author for correspondence:
Klaus Winter
Tel: +507 212 8131
Email: winterk@si.edu

Received: *21 January 2015*
Accepted: *22 March 2015*

Klaus Winter[1], Joseph A. M. Holtum[2] and J. Andrew C. Smith[3]

[1]Smithsonian Tropical Research Institute, PO Box 0843-03092, Balboa, Ancón, Republic of Panama; [2]Centre for Tropical Biodiversity and Climate Change, James Cook University, Townsville, QLD 4811, Australia; [3]Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK

Contents

New Phytologist (2015) **208:** 73–78
doi: 10.1111/nph.13446

Key words: C_3 photosynthesis, CAM photosynthesis, carbon-isotope ratio $\delta^{13}C$, crassulacean acid metabolism (CAM), evolution, phospho*enol*pyruvate carboxylase.

Summary

The key components of crassulacean acid metabolism (CAM) – nocturnal fixation of atmospheric CO_2 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 C_3 photosynthesis is highly variable. Depending upon species, ontogeny and environment, the contribution of nocturnal CO_2 fixation to 24-h carbon gain can range continuously from close to 0% to 100%. Nevertheless, not all possible combinations of light and dark CO_2 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 C_3-type isotope signatures. While questions remain concerning the adaptive significance of dark CO_2 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 C_3 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 C_4 are modifications of the basic C_3 pathway and represent CO_2-concentrating mechanisms that elevate $[CO_2]$ around Rubisco and suppress photorespiration. In CAM, this is achieved in two principal phases separated in time. At night, atmospheric CO_2 is incorporated by phospho*enol*pyruvate 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 CO_2 thus liberated is refixed by Rubisco and reduced in the Calvin cycle (Osmond, 1978; Winter & Smith, 1996). Decarboxylation of malate generates high intercellular $[CO_2]$ 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 μmol m^{-2} s^{-1}, which are challenging to resolve with conventional gas-exchange systems against respiratory background CO_2 effluxes of typically *c.* 1 μmol m^{-2} 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 $^{14}CO_2$ 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 NO_3^- 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 ^{13}C : ^{12}C), 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).

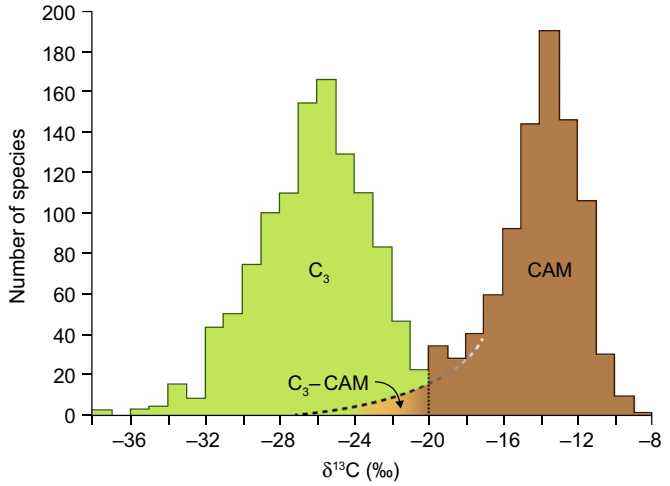

Fig. 1 Frequency histogram of $\delta^{13}C$ values of species of Bromeliaceae plotted in class intervals of 1‰ (re-plotted from data in Crayn *et al.*, 2015; copyright © 2015, John Wiley & Sons), showing the strongly bimodal distribution of isotope ratios with a frequency minimum at *c.* −20‰, as is typically observed in large-scale surveys of crassulacean acid metabolism (CAM) species. The two clusters of $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ value. At $\delta^{13}C$ 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 $\delta^{13}C$ 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

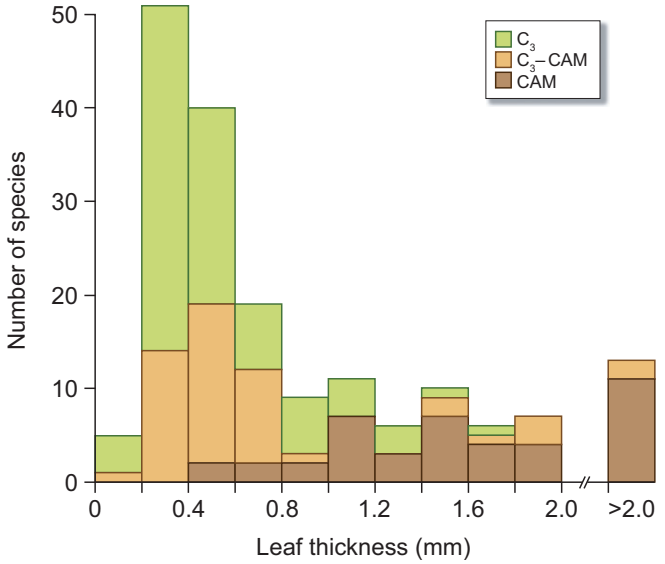

Fig. 2 Frequency histogram of leaf thickness, plotted in class intervals of 0.2 mm, of largely Panamanian species of Orchidaceae for which $\delta^{13}C$ values and nocturnal changes in leaf titratable acidity were determined (based on data in Silvera *et al.*, 2005). Following the nomenclature introduced in Fig. 1, species with $\delta^{13}C$ values less negative than −20‰ were considered crassulacean acid metabolism (CAM) species, while species with $\delta^{13}C$ values more negative than −20‰ were considered C_3 species or C_3–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 C_3 species. For instance, pyruvate, orthophosphate dikinase, first discovered in the plant kingdom in C_4 and CAM plants (Hatch & Slack, 1968; Kluge & Osmond, 1971), was briefly considered a C_4- and CAM-specific novelty, but the enzyme was soon shown to be present in C_3 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 C_4 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 CO_2 fixation and minimizing the futile cycling of CO_2 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 C_3 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 CO_2 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 C_3-type $\delta^{13}C$ values, yet showing small and significant rates of net dark CO_2 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 C_3-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 CO_2-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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 ($\delta^{13}C$ 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.

Acknowledgements

This work was supported by the Smithsonian Tropical Research Institute.

References

Aoyagi K, Bassham JA. 1984. Pyruvate orthophosphate dikinase of C_3 seeds and leaves as compared to the enzyme from maize. *Plant Physiology* **75**: 387–392.

Arakaki M, Christin P-A, Nyffeler R, Lendel A, Eggli U, Ogburn RM, Spriggs E, Moore MJ, Edwards EJ. 2011. Contemporaneous and recent radiations of the world's major succulent plant lineages. *Proceedings of the National Academy of Sciences, USA* **108**: 8379–8384.

Aubry S, Knerová J, Hibberd JM. 2014. Endoreduplication is not involved in bundle-sheath formation in the C_4 species *Cleome gynandra*. *Journal of Experimental Botany* **65**: 3557–3566.

Baresch A, Smith JAC, Winter K, Valerio AC, Jaramillo C. 2011. *Karatophyllum bromelioides* L.D. Gómez revisited: a probable CAM fossil bromeliad. *American Journal of Botany* **98**: 1905–1908.

Borland AM, Griffiths H, Hartwell J, Smith JAC. 2009. Exploiting the potential of plants with crassulacean acid metabolism for bioenergy production on marginal lands. *Journal of Experimental Botany* **60**: 2879–2896.

Borland AM, Zambrano VAB, Ceusters K, Shorrock K. 2011. The photosynthetic plasticity of crassulacean acid metabolism: an evolutionary innovation for sustainable productivity in a changing world. *New Phytologist* **191**: 619–633.

von Caemmerer S, Griffiths H. 2009. Stomatal responses to CO_2 during a diel Crassulacean acid metabolism cycle in *Kalanchoe daigremontiana* and *Kalanchoe pinnata*. *Plant, Cell & Environment* **32**: 567–576.

Caird MA, Richards JH, Donovan LA. 2007. Nighttime stomatal conductance and transpiration in C_3 and C_4 plants. *Plant Physiology* **143**: 4–10.

Cernusak LA, Ubierna N, Winter K, Holtum JAM, Marshall JD, Farquhar GD. 2013. Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. *New Phytologist* **200**: 950–965.

Christin P-A, Arakaki M, Osborne CP, Bräutigam A, Sage RF, Hibberd JM, Kelly S, Covshoff S, Wong GK-S, Hancock L *et al.* 2014. Shared origins of a key enzyme during the evolution of C_4 and CAM metabolism. *Journal of Experimental Botany* **65**: 3609–3621.

Crayn DM, Winter K, Schulte K, Smith JAC. 2015. Photosynthetic pathways in Bromeliaceae: the phylogenetic and ecological significance of CAM and C_3 based on carbon isotope ratios for 1893 species. *Botanical Journal of the Linnean Society* **178**: 169–221.

Crayn DM, Winter K, Smith JAC. 2004. Multiple origins of crassulacean acid metabolism and the epiphytic habit in the Neotropical family Bromeliaceae. *Proceedings of the National Academy of Sciences, USA* **101**: 3703–3708.

Darwin F. 1898. Observations on stomata. *Philosophical Transactions of the Royal Society of London Series B* **190**: 531–621.

Davis SC, Dohleman FG, Long SP. 2011. The global potential for *Agave* as a biofuel feedstock. *Global Change Biology Bioenergy* **3**: 68–78.

Dodd AN, Kusakina J, Hall A, Gould PD, Hanaoka M. 2014. The circadian regulation of photosynthesis. *Photosynthesis Research* **119**: 181–190.

Edwards EJ, Ogburn RM. 2012. Angiosperm responses to a low-CO_2 world: CAM and C_4 photosynthesis as parallel evolutionary trajectories. *International Journal of Plant Sciences* **173**: 724–733.

Ellenberg H. 1981. Ursachen des Vorkommens und Fehlens von Sukkulenten in den Trockengebieten der Erde. *Flora* **171**: 114–169.

Gibson AC. 1982. The anatomy of succulence. In: Ting IP, Gibbs M, eds. *Crassulacean acid metabolism*. Rockville, MD, USA: American Society of Plant Physiologists, 1–17.

Griffiths H, Cousins AB, Badger MR, von Caemmerer S. 2007. Discrimination in the dark. Resolving the interplay between metabolic and physical constraints to phospho*enol*pyruvate carboxylase activity during the crassulacean acid metabolism cycle. *Plant Physiology* **143**: 1055–1067.

Hancock L, Edwards EJ. 2014. Phylogeny and the inference of evolutionary trajectories. *Journal of Experimental Botany* **65**: 3491–3498.

Harris FS, Martin CE. 1991. Plasticity in the degree of CAM-cycling and its relationship to drought stress in five species of *Talinum* (Portulacaceae). *Oecologia* **86**: 575–584.

Hartwell J. 2006. The circadian clock in CAM plants. In: Hall A, McWatters H, eds. *Endogenous plant rhythms*. Oxford, UK: Blackwell, 211–236.

Hartwell J, Smith LH, Wilkins MB, Jenkins GI, Nimmo HG. 1996. Higher plant phosphoenolpyruvate carboxylase kinase is regulated at the level of translatable mRNA in response to light or a circadian rhythm. *Plant Journal* **10**: 1071–1078.

Hatch MD, Slack CR. 1968. A new enzyme for the interconversion of pyruvate and phosphopyruvate and its role in the C_4 dicarboxylic acid pathway of photosynthesis. *Biochemical Journal* **106**: 141–146.

Herrera A. 2009. Crassulacean acid metabolism and fitness under water deficit stress: if not for carbon gain, what is facultative CAM good for? *Annals of Botany* **103**: 645–653.

Holtum JAM, Chambers D, Morgan D, Tan DKY. 2011. *Agave* as a biofuel feedstock in Australia. *Global Change Biology Bioenergy* **3**: 58–67.

Holtum JAM, Smith JAC, Neuhaus HE. 2005. Intracellular transport and pathways of carbon flow in plants with crassulacean acid metabolism. *Functional Plant Biology* **32**: 429–449.

Horn JW, Xi Z, Riina R, Peirson JA, Yang Y, Dorsey BL, Berry PE, Davis CC, Wurdack KJ. 2014. Evolutionary bursts in *Euphorbia* (Euphorbiaceae) are linked with photosynthetic pathway. *Evolution* **68**: 3485–3504.

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

Kluge M, Fischer K. 1967. Über Zusammenhänge zwischen dem CO_2-Austausch und der Abgabe von Wasserdampf durch *Bryophyllum daigremontianum* Berg. *Planta* **77**: 212–223.

Kluge M, Osmond CB. 1971. Pyruvate P_i dikinase in crassulacean acid metabolism. *Naturwissenschaften* **8**: 414–415.

Kluge M, Ting IP. 1978. *Crassulacean acid metabolism. Analysis of an ecological adaptation*. Berlin, Germany: Springer.

Kunitake G, Stitt C, Saltman P. 1959. Dark fixation of CO_2 by tobacco leaves. *Plant Physiology* **34**: 123–127.

Lüttge U. 2006. Photosynthetic flexibility and ecophysiological plasticity: questions and lessons from *Clusia*, the only CAM tree, in the neotropics. *New Phytologist* **171**: 7–25.

Martinoia E, Meyer S, De Angeli A, Nagy R. 2012. Vacuolar transporters in their physiological context. *Annual Review of Plant Biology* **63**: 183–213.

Maxwell K, Borland AM, Haslam RP, Helliker BR, Roberts A, Griffiths H. 1999. Modulation of Rubisco activity during the diurnal phases of the crassulacean acid metabolism plant *Kalanchoë daigremontiana*. *Plant Physiology* **121**: 849–856.

Medina E, Osmond CB. 1981. Temperature dependence of dark CO_2 fixation and acid accumulation in *Kalanchoë daigremontiana*. *Australian Journal of Plant Physiology* **8**: 641–649.

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.

Nimmo HG. 2000. The regulation of phosphoenolpyruvate carboxylase in CAM plants. *Trends in Plant Science* **5**: 75–80.

Nobel PS. 1988. *Environmental biology of agaves and cacti*. Cambridge, UK: Cambridge University Press.

Ogburn RM, Edwards EJ. 2010. The ecological water-use strategies of succulent plants. *Advances in Botanical Research* **55**: 179–225.

Osmond CB. 1978. Crassulacean acid metabolism: a curiosity in context. *Annual Review of Plant Physiology* **29**: 379–414.

Pfeffer W. 1897. *Pflanzenphysiologie. Ein Handbuch der Lehre vom Stoffwechsel und Kraftwechsel in der Pflanze, Erster Band: Stoffwechsel, 2nd edn.* Leipzig, Germany: Wilhelm Engelmann.

Pierce S, Winter K, Griffiths H. 2002. Carbon isotope ratio and the extent of daily CAM use in Bromeliaceae. *New Phytologist* **156**: 75–83.

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.

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

Silvera K, Winter K, Rodriguez BL, Albion RL, Cushman JC. 2014. Multiple isoforms of phospho*enol*pyruvate carboxylase in the Orchidaceae (subtribe Oncidiinae): implications for the evolution of crassulacean acid metabolism. *Journal of Experimental Botany* **65**: 3623–3636.

Smith JAC, Bryce JH. 1992. Metabolite compartmentation and transport in CAM plants. In: Tobin AK, ed. *Plant organelles: compartmentation of metabolism in photosynthetic cells*. Cambridge, UK: Cambridge University Press, 141–167.

Smith JAC, Ingram J, Tsiantis MS, Barkla BJ, Bartholomew DM, Bettey M, Pantoja O, Pennington AJ. 1996. Transport across the vacuolar membrane in CAM plants. In: Winter K, Smith JAC, eds. *Crassulacean acid metabolism: biochemistry, ecophysiology and evolution*. Berlin, Germany: Springer, 53–71.

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

West-Eberhard MJ, Smith JAC, Winter K. 2011. Photosynthesis, reorganized. *Science* **332**: 311–312.

Wilkins MB. 1959. An endogenous rhythm in the rate of carbon dioxide output of *Bryophyllum* I. Some preliminary experiments. *Journal of Experimental Botany* **10**: 377–390.

von Willert DJ, Armbrüster N, Drees T, Zaborowski M. 2005. *Welwitschia mirabilis*: CAM or not CAM – what is the answer? *Functional Plant Biology* **32**: 389–395.

Winter K. 1982. Properties of phosphoenolpyruvate carboxylase in rapidly prepared, desalted leaf extracts of the crassulacean acid metabolism plant *Mesembryanthemum crystallinum*. *Planta* **154**: 298–308.

Winter K, Aranda J, Holtum JAM. 2005. Carbon isotope composition and water-use efficiency in plants with crassulacean acid metabolism. *Functional Plant Biology* **32**: 381–388.

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.

Winter K, Garcia M, Holtum JAM. 2011. Drought-stress-induced up-regulation of CAM in seedlings of a tropical cactus, *Opuntia elatior*, operating predominantly in the C_3 mode. *Journal of Experimental Botany* **62**: 4037–4042.

Winter K, Holtum JAM. 2002. How closely do the δ^{13}C values of CAM plants reflect the proportion of CO_2 fixed during day and night? *Plant Physiology* **129**: 1843–1851.

Winter K, Holtum JAM. 2011. Induction and reversal of crassulacean acid metabolism in *Calandrinia polyandra*: effects of soil moisture and nutrients. *Functional Plant Biology* **38**: 576–582.

Winter K, Holtum JAM. 2014. Facultative crassulacean acid metabolism (CAM) plants: powerful tools for unravelling the functional elements of CAM photosynthesis. *Journal of Experimental Botany* **65**: 3425–3441.

Winter K, Lüttge U, Winter E, Troughton JH. 1978. Seasonal shift from C_3 photosynthesis to crassulacean acid metabolism in *Mesembryanthemum crystallinum* growing in its natural environment. *Oecologia* **34**: 225–237.

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

Winter K, Smith JAC, eds. 1996. *Crassulacean acid metabolism: biochemistry, ecophysiology and evolution*. Berlin, Germany: Springer.

Winter K, Wallace BJ, Stocker GC, Roksandic Z. 1983. Crassulacean acid metabolism in Australian vascular epiphytes and some related species. *Oecologia* **57**: 129–141.

Winter K, Ziegler H. 1992. Induction of crassulacean acid metabolism in *Mesembryanthemum crystallinum* increases reproductive success under conditions of drought and salinity stress. *Oecologia* **92**: 475–479.

Yang X, Cushman JC, Borland AM, Edwards EJ, Wullschleger SD, Tuskan GA, Owen NA, Griffiths H, Smith JAC, De Paoli HC *et al.* 2015. A roadmap for research on crassulacean acid metabolism (CAM) to enhance sustainable food and bioenergy production in a hotter, drier world. *New Phytologist* **207**: 491–504.

Zambrano VAB, Lawson T, Olmos E, Fernández-García N, Borland AM. 2014. Leaf anatomical traits which accommodate the facultative engagement of crassulacean acid metabolism in tropical trees of the genus *Clusia*. *Journal of Experimental Botany* **65**: 3513–3523.