

Viewpoints

CAM photosynthesis: the acid test

Summary

There is currently considerable interest in the prospects for bioengineering crassulacean acid metabolism (CAM) photosynthesis – or key elements associated with it, such as increased water-use efficiency – into C_3 plants. Resolving how CAM photosynthesis evolved from the ancestral C_3 pathway could provide valuable insights into the targets for such bioengineering efforts. It has been proposed that the ability to accumulate organic acids at night may be common among C_3 plants, and that the transition to CAM might simply require enhancement of pre-existing fluxes, without the need for changes in circadian or diurnal regulation. We show, in a survey encompassing 40 families of vascular plants, that nocturnal acidification is a feature entirely restricted to CAM species. Although many C_3 species can synthesize malate during the light period, we argue that the switch to night-time malic acid accumulation requires a fundamental metabolic reprogramming that couples glycolytic breakdown of storage carbohydrate to the process of net dark CO_2 fixation. This central element of the CAM pathway, even when expressed at a low level, represents a biochemical capability not seen in C_3 plants, and so is better regarded as a discrete evolutionary innovation than as part of a metabolic continuum between C_3 and CAM.

Introduction

Crassulacean acid metabolism (CAM) and C_4 photosynthesis represent the two modifications of the basic C_3 pathway of CO_2 assimilation frequently found in vascular plants, especially those associated with warmer and/or more water-limited environments. Crassulacean acid metabolism and C_4 photosynthesis are complex genetic traits, but both have arisen independently multiple times in evolution, now being found in an estimated 10% of vascular plants in total. As our understanding of the metabolic and molecular-genetic basis of these traits has developed, interest has grown in the prospect of bioengineering these pathways – or key elements of them – into C_3 crop plants to enhance their productivity and stress tolerance (e.g. Borland *et al.*, 2014; Lim *et al.*, 2019; Töpfer *et al.*, 2020; Schiller & Bräutigam, 2021). This requires a detailed understanding of the core components of the respective pathways and the genetic architecture of their regulation. Progress towards these goals is being aided by recent findings from comparative

evolutionary studies, whole-genome sequencing and detailed molecular-genetic studies of tractable model species.

Crassulacean acid metabolism plants, in particular, have long been attractive targets for studies of drought tolerance in semiarid and water-limited habitats. Their ability to fix CO_2 at night and to close their stomata during the day – associated with the accumulation and remobilization, respectively, of vacuolar malic acid – results in high water-use efficiency and resilience to episodic drought. Many CAM plants are also phenotypically plastic, fixing considerable amounts of CO_2 during the daytime when conditions are favourable, but restricting gas exchange to the night-time under increasing water limitation. This continuous spectrum of phenotypes has led to recent suggestions that the evolution of CAM might merely require the upregulation of a low-level acidification–deacidification cycle already present in C_3 plants, and moreover that the circadian and diurnal rhythms in CAM plants are probably similar to those in C_3 plants (Bräutigam *et al.*, 2017; Schiller & Bräutigam, 2021). However, we believe this scenario overlooks the fundamental ways in which vacuolar acidification and CO_2 metabolism differ in CAM plants and C_3 plants, and in so doing fails to recognize the metabolic reprogramming required for the evolutionary transition from C_3 to CAM. Here, we highlight the defining characteristics of the day–night cycle of carbon fixation and acid metabolism in CAM plants, describe how these contrast with C_3 plants, and demonstrate that diel changes in titratable acidity are entirely lacking in a wide range of C_3 species. Our results are not consistent with the existence of a low-level CAM cycle in C_3 plants and serve to highlight the discrete nature of the evolutionary innovation involved in the acquisition of CAM photosynthesis.

The CAM cycle

In essence, the CAM cycle consists of two sequential metabolic components occurring in the chlorenchymatous mesophyll cells of the leaves or stem that are temporally separated across the 24 h diel cycle, as summarized schematically in Fig. 1(a).

(1) At night, atmospheric CO_2 is fixed, following its conversion to HCO_3^- by carbonic anhydrase, via the cytosolic enzyme phosphoenolpyruvate (PEP) carboxylase (PEPC). The three-carbon acceptor (PEP) for this reaction is supplied by glycolytic breakdown of storage carbohydrate (either chloroplastic starch/glucan or vacuolar hexose, depending on the plant species), initially forming oxaloacetate, which is immediately reduced to malate. To remove it from its site of synthesis, this cytosolic malate is transported into the large central vacuole of the mesophyll cells in a process energized by one or both of the two tonoplast-localized H^+ pumps, the V-ATPase and the V-PP₄ase. The net result is vacuolar accumulation of malic acid, as each mol of malate accumulated can be shown by titration to be balanced by 2 mol H^+ (Lüttge & Ball, 1980), a stoichiometry observed even in the submerged aquatic lycopod

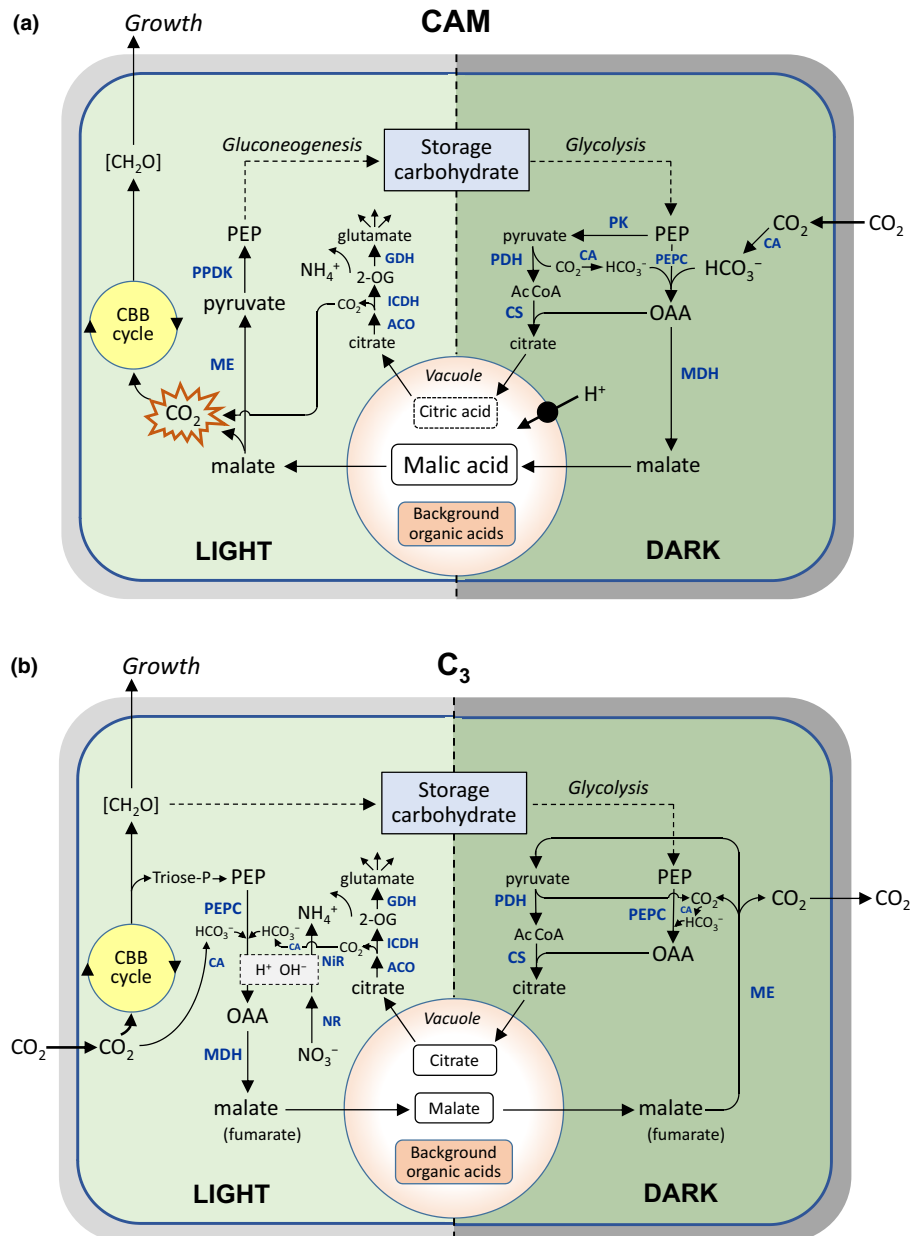


Fig. 1 Principal day–night carbon fluxes and associated carboxylate metabolism in photosynthetic mesophyll cells, highlighting similarities and differences between crassulacean acid metabolism (CAM) plants and C_3 plants. The schemes are modelled on the format used by Schiller & Bräutigam (2021) and show steady-state operation of the cycles over the 24 h period. For simplicity, details of the energetics of the pathways, subcellular compartmentation and charges (dissociation states) of the metabolites have been omitted, but further information can be found in Winter & Smith (1996a); Holtum *et al.* (2005) and Shameer *et al.* (2018). Additional internal sources of CO_2 , such as the tricarboxylic acid (TCA) cycle, oxidative pentose phosphate pathway and photorespiration, are not shown explicitly. The dashed lines to and from storage carbohydrate indicate multi-step pathways. The central vacuole is shown schematically, but in mature mesophyll cells can typically occupy $\geq 90\%$ of the cell volume. (a) In CAM plants, net nocturnal CO_2 fixation via phosphoenolpyruvate (PEP) carboxylase (PEPC) results in vacuolar accumulation of malic acid (2 H^+ per malate); this is mobilized in the following daytime and decarboxylated (either via NAD(P)-ME, as shown, or in some CAM plants via PEP carboxykinase), with the CO_2 released behind closed stomata at high concentration being refixed by Rubisco in the Calvin–Benson–Bassham (CBB) cycle. In some CAM plants there is significant nocturnal synthesis and vacuolar accumulation of citric acid: this is not associated with net fixation of CO_2 , but on remobilization in the following daytime, the citrate supplies carbon skeletons for NH_4^+ assimilation via glutamate dehydrogenase (GDH) and glutamine synthetase (the latter not shown explicitly). Nocturnal acid accumulation requires net influx of H^+ into the vacuole, which is driven by the tonoplast H^+ pump(s) (see main text). The vacuole also contains a background pool of organic acids that does not oscillate during the day–night cycle. Carbon skeletons required to support nocturnal acid accumulation are provided by glycolytic breakdown of storage carbohydrate (usually chloroplastic starch/glucon, but significant vacuolar hexose in some species), which is regenerated by gluconeogenesis in the light period. (b) In C_3 plants, citrate synthesis and vacuolar accumulation also occur at night, but malate accumulation (and fumarate in some species) typically occurs during the daytime, which provides charge- and pH-balancing for light-dependent nitrate assimilation (light grey box). In some plants such as *Arabidopsis thaliana*, citrate and malate fluxes can be of similar magnitude, as shown here to achieve flux balance across the vacuolar membrane, but diel carboxylate accumulation and remobilization are not charge-balanced by H^+ and so do not result in vacuolar pH changes during the day–night cycle (see Table 1). AcCoA, acetyl-CoA; ACO, aconitase; CA, carbonic anhydrase; $[CH_2O]$, sugars/carbohydrate; CS, citrate synthase; ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; NiR, nitrite reductase; NR, nitrate reductase; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; PDH, pyruvate dehydrogenase; PK, pyruvate kinase; PPK, pyruvate, P_i kinase.

Isoetes (Keeley, 1998), believed to be the earliest-diverging CAM lineage. Sustained activity of the tonoplast H^+ pumps during the night is essential to maintain cytoplasmic pH within physiological bounds in the face of a substantial acid load. For example, for a *Kalanchoë* leaf accumulating malic acid at an average rate of 10 mM h^{-1} (e.g. Lüttge *et al.*, 1981), in mesophyll cells consisting of *c.* 97% vacuole and 1% cytosol by volume (Stuedle *et al.*, 1980), and assuming a typical cytoplasmic buffering capacity of $30 \text{ mmol H}^+ \text{ l}^{-1} (\text{pH unit})^{-1}$ (Kurkdjian & Guern, 1989), cytosolic pH would drop by 1 pH unit in just under a minute (54 s) in the absence of any transtonoplast H^+ pumping. By the end of the night, the vacuolar sap in these assimilatory cells can reach pH 4 or even lower.

(2) During the following daytime, the nocturnally accumulated malic acid is released from the vacuole and decarboxylated in the cytoplasm, either by NAD(P)-malic enzyme acting in concert with pyruvate, P_i dikinase, or by PEP carboxykinase (again depending on the plant species). During the main phase of malate decarboxylation, the liberated CO_2 accumulates to high internal concentrations behind closed stomata and is refixed by Rubisco in the Calvin–Benson–Bassham cycle, with the remaining three-carbon moiety (pyruvate or PEP, respectively) being recycled back into the pool of storage carbohydrate via gluconeogenesis. Once the decarboxylation phase has finished, and environmental conditions permitting (e.g. favourable water status), the stomata may open in the latter part of the day and the plant engages in normal C_3 photosynthesis, which contributes to the phenotypic plasticity in gas-exchange patterns seen in many CAM species.

The primary benefits of the CAM pathway are seen as two-fold. First, when the initial carboxylation reaction is mediated by PEPC, CAM plants benefit (as do C_4 plants) from the favourable kinetic properties of this enzyme compared with Rubisco (namely a higher effective affinity for CO_2 , a faster turnover number, and lack of an oxygenase activity); additionally, because PEPC in CAM plants is active at night, this means that the transpirational water loss associated with uptake of atmospheric CO_2 is even further reduced. And second, the high internal CO_2 concentration generated during the decarboxylation phase while stomata are closed helps to ensure that the carboxylase activity of Rubisco is maximized and its photorespiratory oxygenase minimized. These benefits come with an additional metabolic cost, mainly incurred in energizing vacuolar malic acid accumulation at night and gluconeogenesis during the day, but CAM plants generally grow in high-light environments and so are not usually photon-limited (Lüttge *et al.*, 1981; Winter & Smith, 1996a; Cheung *et al.*, 2014). Underpinning the entire CAM rhythm is thus the diel cycle of malic acid accumulation and degradation, the manifestation of which was memorably captured in the early report by Heyne (1815), who noted that: ‘The leaves of the *Cotyledon calycina* [*Kalanchoë pinnata*] . . . are in the morning as acid as sorrel, if not more so; as the day advances, they lose their acidity, and are tasteless about noon.’

It is worth emphasizing that net nocturnal fixation of CO_2 by CAM plants can be shown – under optimal conditions with minimal recycling of respiratory CO_2 – to be stoichiometrically

linked to the accumulation of malic acid (e.g. Medina & Osmond, 1981). Downregulation of mitochondrial NAD-malic enzyme activity at night in CAM plants will help to minimize futile cycling of malate synthesized via PEPC, and presumably will also mean that most respiratory substrate supplied by glycolysis feeds into the tricarboxylic acid (TCA) cycle through pyruvate kinase. Suggestions have occasionally been made for the involvement of other acids in the CAM cycle, most notably citric acid, which accumulates at night in significant amounts in some CAM species (Lüttge, 1988). However, nocturnal citric acid accumulation is not associated with net fixation of CO_2 , as provision of acetyl-CoA for the citrate synthase reaction via oxidative decarboxylation of pyruvate itself releases CO_2 . The other major vacuolar carboxylate anion found in some CAM plants is isocitrate, but this is primarily involved in charge-balancing inorganic cations and generally does not fluctuate significantly during the day–night cycle (Vickery, 1952; Milburn *et al.*, 1968; Phillips, 1980; Ceusters *et al.*, 2019). Other hypothetical schemes that might account for malate synthesis in CAM plants, such as operation of a glyoxylate cycle (Tcherkez, 2017) or a reverse citric acid cycle (Töpfer *et al.*, 2020), have not so far received empirical support. The overwhelming body of evidence is consistent with net dark CO_2 fixation in CAM plants being mediated via the canonical pathway of malic acid accumulation and degradation proposed in the late 1940s.

Do C_3 plants accumulate organic acids at night?

The notion that nocturnal acid accumulation is a unique feature of CAM photosynthesis has been challenged in two recent publications by Bräutigam *et al.* (2017) and Schiller & Bräutigam (2021). In considering the evolutionary changes necessary for the emergence of CAM, they argue that, because ‘ C_3 plants store organic acids at night to fuel daytime amino acid synthesis . . . the framework of the CAM cycle actually is already in place in C_3 species and . . . it is not a question of rewiring metabolism but of selecting for increased flux’ (Bräutigam *et al.*, 2017). Indeed, the flow diagrams presented by Bräutigam *et al.* (2017) depicting night-time and daytime metabolite fluxes in C_3 plants and CAM plants are essentially identical, both showing nocturnal storage of malate and citrate, and differing only in the greater magnitude of the fluxes involved in CAM plants. The authors go on to propose that, because there is ‘a continuum from C_3 photosynthesis to CAM . . . [there are] likely no general barriers to trait engineering’ (Schiller & Bräutigam, 2021). However, we believe this interpretation overlooks the fundamental differences between organic acid metabolism in C_3 and CAM plants, is not consistent with a substantial body of experimental evidence, and may deflect attention away from the prime targets of metabolic engineering needed in order to express CAM-like traits in C_3 plants.

We initially address the assertion that C_3 plants can reversibly accumulate organic acids in the vacuole at night, which, if true, would invalidate the use of nocturnal acidification as a diagnostic criterion for CAM. The term ‘organic acids’ is often used loosely in the literature when referring to carboxylate metabolism in plant cells, but the nature of the cation involved in charge-balancing the

carboxylate anion is important, as this has profound implications for pH regulation and ion homeostasis. In CAM, as explained earlier, the night-time acidification resulting from net CO₂ fixation is mechanistically attributable to malic acid (even if some species show significant accumulation of citric acid as well), but this differs in two important respects from the situation in C₃ plants, as summarized in Fig. 1(b). First, while C₃ species such as *Arabidopsis* may show nocturnal accumulation of citrate, there is no evidence in C₃ plants for nocturnal accumulation of malate (which, by contrast, tends to accumulate during the daytime); and second, this process does not involve the free acid (citric acid), as in CAM plants, but rather the conjugate base (the citrate anion). We shall return to the question of the contrasting diel rhythms in malate content seen in CAM plants and C₃ plants, but will consider first the issue of acid accumulation and its implications for cell metabolism and pH regulation.

The vacuolar sap of plant cells is typically acidic on account of its substantial content of hydroxycarboxylic acids, and there are some well-known examples of extreme acid accumulation in C₃ plants – such as oxalic acid in leaves of *Begonia*, *Oxalis* and sorrel (*Rumex acetosa*), and citric acid in *Citrus* fruits – that can result in vacuolar pH values as low as 2 (Bennet-Clark, 1933; Hurd-Karrer, 1939). But in most plants the vacuolar carboxylate anions are largely charge-balanced by the strong inorganic cations K⁺, Na⁺, Ca²⁺ and Mg²⁺, with the result that the vacuolar contents are only mildly acidic, with free H⁺ concentrations in the micromolar range (pH 5–6). Moreover, there do not appear to have been any reports that vacuolar pH in C₃ plants changes during the day–night cycle in a manner similar to CAM plants, as would be the case if C₃ plants could reversibly store organic acids in the vacuole at night (Bräutigam *et al.*, 2017).

To test for the possibility that nocturnal acidification is a widespread phenomenon in C₃ plants that has been previously overlooked, we conducted a macroevolutionary common-garden experiment with 70 species of vascular plants representing 40 families growing under field conditions in Panama (Table 1). Measurements of titratable acidity were made on tissue extracts sampled at the end of the dark period and the end of the light period, a technique capable of resolving differences in dawn–dusk acidity of the order of 1 mmol H⁺ kg⁻¹ fresh mass (the smallest statistically significant dawn–dusk difference in acidity demonstrated to date appears to be 1.7 mmol H⁺ kg⁻¹ fresh mass in two orchid species, *Coryanthes hunteriana* and *Oncidium schroederianum*; Silvera *et al.*, 2005.) All species known to exhibit CAM (30 species from 14 different families) showed nocturnal acidification, to different degrees, with dawn–dusk differences in titratable acidity ranging from 452 mmol H⁺ kg⁻¹ in *Clusia witana* to 7.9 mmol H⁺ kg⁻¹ in *Werauhia sanguinolenta*. By contrast, none of the C₃ plants tested (40 species from 31 different families) showed a significant difference in dawn–dusk acidity (Table 1). Several C₃ species (notably *Clusia* spp.) had high values of tissue acidity, but did not show diel fluctuations. These results do not support the notion that nocturnal increases in organic acid content are a common feature of C₃ plants, and confirm that nocturnal acidification can be regarded as a hallmark of CAM plants.

Diel malate and citrate metabolism in C₃ plants

Even if they do not show diel rhythms in acidity, leaves of C₃ plants often exhibit significant day–night changes in the bulk tissue concentration of malate, which is one of the most ubiquitous carboxylate anions found in plant vacuoles. However, in photosynthetic tissues of C₃ plants, carbon metabolism is widely associated with increases in malate content during the daytime (Fig. 1b), not during the night-time as implied in the metabolic schemes of Bräutigam *et al.* (2017) and Schiller & Bräutigam (2021). Daytime malate production is linked to maintenance of charge balance during light-dependent assimilation of nitrate, as the negative charge from NO₃⁻ can be transferred to malate²⁻ during the synthesis of reduced (and approximately net-neutral) nitrogen compounds (Osmond, 1976; Raven & Smith, 1976). In some species, such as several members of the Brassicaceae, there is also concurrent accumulation of fumarate, which in *Arabidopsis thaliana* is attributable to the existence of a cytosolic isoform of fumarase, FUM2 (Pracharoenwattana *et al.*, 2010). (An ortholog of FUM2 is present in the CAM plant *Agave americana*, and some nocturnal fumarate accumulation is detectable in parallel with malate in Phase I of the CAM cycle, but the bulk-tissue fumarate concentrations are two orders of magnitude lower than malate: Abraham *et al.*, 2016.) Even in C₃ plants, the quantities of carboxylates involved are large enough that almost all the malate and fumarate must be localized in the vacuole (Medeiros *et al.*, 2017), but how the diurnal accumulation of these anions is charge-balanced in C₃ plants has been less commonly investigated. A role for inorganic cations such as K⁺ is possible: for example, malate is a major balancing ion for K⁺ accumulated in stomatal guard cells during stomatal opening. This process is in fact associated with a slight increase in vacuolar pH (Willmer & Fricker, 1996; Andrés *et al.*, 2014), because the accumulated malate, acting as a conjugate base, increases the pH-buffering capacity of the vacuole. Another possibility is that daytime accumulation of malate (and fumarate) in the vacuole may be counterbalanced by efflux of nocturnally accumulated citrate, as measurements of bulk tissue concentrations of these metabolites suggest these fluxes can be of similar magnitude (Zell *et al.*, 2010; Annunziata *et al.*, 2017). A carboxylate exchange of this type would obviate the need for movement of charge-balancing cations and could be mediated by the tonoplast dicarboxylate transporter (tDT), which can counter-exchange malate and citrate anions in their divalent forms (Frei *et al.*, 2018).

The absolute concentrations of tissue carboxylates are influenced by the growth conditions of the plants (particularly light intensity and nitrogen nutrition), but the rhythms of opposing diel changes in malate and citrate content have also been observed in the leaves of other well-studied C₃ species besides *A. thaliana*, such as tobacco (Pucher *et al.*, 1937; Scheible *et al.*, 2000), tomato (Niedziela *et al.*, 1993) and potato (Urbanczyk-Wochniak *et al.*, 2005). The widespread occurrence of daytime malate accumulation in C₃ plants is difficult to reconcile with the proposal of Bräutigam *et al.* (2017), based on the labelling studies of Szecowka *et al.* (2013), that C₃ plants possess a CAM-like cycle involving ‘malate decarboxylation . . . during the day’ and subsequent refixation of the liberated CO₂ by Rubisco. This would potentially represent a futile cycle of malate synthesis and degradation at a time when bulk tissue

Table 1 Titratable acidity measured at dusk and dawn in the photosynthetic tissues of 70 species of vascular plants: (a) species showing a statistically significant nocturnal increase in titratable acidity (for which dawn values are indicated in bold); species are ranked in descending order of dawn–dusk difference in acidity; (b) species showing no significant difference in dawn–dusk titratable acidity; species are arranged according to the phylogenetic position of their family (for angiosperms; APG IV, 2016).

No.	Species	Family	Titratable acidity (mmol H ⁺ kg ⁻¹ fresh mass)	
			Dusk	Dawn
(a) Species with significant nocturnal H⁺ increase				
1.	<i>Clusia uvitana</i> Pittier	Clusiaceae	27.4 ± 11.0	479.7 ± 23.9
2.	<i>Clusia minor</i> L.	Clusiaceae	46.2 ± 30.3	436.5 ± 51.2
3.	<i>Furcraea cabuya</i> Trel.	Asparagaceae	22.9 ± 4.1	401.0 ± 28.5
4.	<i>Alluaudia humbertii</i> Choux	Didiereaceae	93.8 ± 5.7	457.6 ± 20.7
5.	<i>Clusia rosea</i> Jacq.	Clusiaceae	127.2 ± 26.0	454.0 ± 85.1
6.	<i>Agave americana</i> L.	Asparagaceae	18.1 ± 4.6	331.1 ± 28.9
7.	<i>Clusia pratensis</i> Seem.	Asparagaceae	99.2 ± 67.8	386.9 ± 126.6
8.	<i>Kalanchoë pinnata</i> (Lam.) Pers.	Crassulaceae	15.6 ± 3.9	283.5 ± 14.6
9.	<i>Agave angustifolia</i> Haw.	Asparagaceae	9.2 ± 2.3	273.1 ± 16.2
10.	<i>Epiphyllum phyllanthus</i> (L.) Haw.	Cactaceae	8.1 ± 3.4	239.8 ± 11.8
11.	<i>Kalanchoë daigremontiana</i> Raym.-Hamet & H.Perrier	Crassulaceae	17.8 ± 6.4	234.8 ± 22.8
12.	<i>Ananas comosus</i> (L.) Merr.	Bromeliaceae	10.9 ± 2.9	206.4 ± 33.6
13.	<i>Tillandsia elongata</i> Kunth	Bromeliaceae	9.1 ± 1.2	203.3 ± 38.1
14.	<i>Clusia fructiangusta</i> Cuatrec.	Clusiaceae	36.5 ± 11.3	217.3 ± 45.2
15.	<i>Tillandsia flexuosa</i> Sw.	Bromeliaceae	10.6 ± 2.2	173.8 ± 64.9
16.	<i>Xerosicyos danguyi</i> Humbert	Cucurbitaceae	7.9 ± 3.7	164.2 ± 6.2
17.	<i>Sansevieria zeylanica</i> (L.) Willd.	Asparagaceae	13.0 ± 4.4	157.2 ± 30.9
18.	<i>Coleus amboinicus</i> Lour.	Lamiaceae	6.3 ± 1.6	118.6 ± 5.7
19.	<i>Clusia peninsulae</i> Hammel	Clusiaceae	248.4 ± 84.1	355.0 ± 64.0
20.	<i>Vanilla pompona</i> Schiede	Orchidaceae	19.9 ± 2.1	125.9 ± 3.0
21.	<i>Opuntia cochenillifera</i> (L.) Mill.	Cactaceae	8.4 ± 0.7	110.2 ± 18.6
22.	<i>Crassula ovata</i> (Mill.) Druce	Crassulaceae	11.0 ± 2.8	99.5 ± 13.6
23.	<i>Pyrrosia longifolia</i> (Burm.f.) Morton	Polypodiaceae	21.6 ± 2.2	107.8 ± 12.3
24.	<i>Talinum fruticosum</i> (L.) Juss.	Talinaceae	3.6 ± 1.4	82.1 ± 13.8
25.	<i>Clusia quadrangula</i> Bartlett	Clusiaceae	125.6 ± 19.3	171.5 ± 20.1
26.	<i>Aloe vera</i> (L.) Burm.f.	Asphodelaceae	8.5 ± 0.8	48.1 ± 5.5
27.	<i>Opuntia ficus-indica</i> (L.) Mill.	Cactaceae	12.4 ± 1.2	40.3 ± 6.3
28.	<i>Basella alba</i> L.	Basellaceae	9.2 ± 1.1	29.9 ± 1.5
29.	<i>Pilea peperomioides</i> Diels	Urticaceae	10.5 ± 7.5	23.2 ± 5.4
30.	<i>Werauhia sanguinolenta</i> (Cogn. & Marchal) J.R.Grant	Bromeliaceae	8.0 ± 1.0	15.9 ± 5.3
(b) Species without significant nocturnal H⁺ increase				
31.	<i>Zamia furfuracea</i> L.f. ex Aiton	Zamiaceae	19.7 ± 1.3	20.7 ± 1.1
32.	<i>Gnetum leyboldii</i> Tul.	Gnetaceae	10.4 ± 1.4	9.7 ± 1.0
33.	<i>Piper arboreum</i> Aubl.	Piperaceae	19.0 ± 2.1	19.8 ± 2.5
34.	<i>Piper marginatum</i> Jacq.	Piperaceae	13.8 ± 1.8	14.5 ± 3.9
35.	<i>Persea americana</i> Mill.	Lauraceae	13.2 ± 0.7	13.7 ± 0.7
36.	<i>Monstera deliciosa</i> Liebm.	Araceae	17.9 ± 3.2	13.8 ± 4.0
37.	<i>Monstera</i> sp.	Araceae	20.6 ± 0.9	18.7 ± 3.6
38.	<i>Hymenocallis littoralis</i> (Jacq.) Salisb.	Amaryllidaceae	6.1 ± 2.3	5.7 ± 1.4
39.	<i>Hippeastrum puniceum</i> (Lam.) Voss	Amaryllidaceae	7.2 ± 1.6	8.4 ± 1.3
40.	<i>Heliconia vaginalis</i> Benth.	Heliconiaceae	8.1 ± 0.3	7.9 ± 0.6
41.	<i>Musa × paradisiaca</i> L.	Musaceae	5.5 ± 0.5	4.9 ± 0.4
42.	<i>Andira inermis</i> (W.Wright) DC.	Fabaceae	12.8 ± 1.5	11.3 ± 1.4
43.	<i>Dioclea guianensis</i> Benth.	Fabaceae	12.6 ± 2.4	12.9 ± 2.0
44.	<i>Inga spectabilis</i> (Vahl) Willd.	Fabaceae	22.6 ± 3.7	22.8 ± 3.1
45.	<i>Ormosia macrocalyx</i> Ducke	Fabaceae	10.0 ± 0.9	10.1 ± 1.3
46.	<i>Ficus insipida</i> Willd.	Moraceae	0 ± 0	0 ± 0
47.	<i>Cecropia peltata</i> L.	Urticaceae	1.0 ± 2.2	3.7 ± 8.3
48.	<i>Clusia cupulata</i> (Maguire) Maguire	Clusiaceae	169.4 ± 72.7	160.4 ± 26.6
49.	<i>Clusia valerioi</i> Standl.	Clusiaceae	156.9 ± 90.7	109.5 ± 24.2
50.	<i>Garcinia intermedia</i> (Pittier) Hammel	Clusiaceae	20.5 ± 3.8	11.7 ± 5.8
51.	<i>Calophyllum longifolium</i> Willd.	Calophyllaceae	94.3 ± 8.8	86.3 ± 6.2
52.	<i>Jatropha curcas</i> L.	Euphorbiaceae	29.8 ± 1.4	26.1 ± 4.6
53.	<i>Lagerstroemia speciosa</i> (L.) Pers.	Lythraceae	111.2 ± 8.9	97.5 ± 6.5
54.	<i>Eucalyptus camaldulensis</i> Dehnh.	Myrtaceae	54.7 ± 6.1	50.0 ± 5.6
55.	<i>Miconia impetolaris</i> D.Don	Melastomataceae	253.8 ± 31.4	221.4 ± 10.9

Table 1 (Continued)

No.	Species	Family	Titratable acidity (mmol H ⁺ kg ⁻¹ fresh mass)	
			Dusk	Dawn
56.	<i>Protium stevensonii</i> (Standl.) Daly	Burseraceae	55.9 ± 5.2	53.1 ± 5.1
57.	<i>Mangifera indica</i> L.	Anacardiaceae	52.3 ± 4.7	51.1 ± 16.2
58.	<i>Cupania latifolia</i> Kunth	Sapindaceae	19.4 ± 8.1	16.3 ± 2.6
59.	<i>Citrus × limon</i> (L.) Osbeck	Rutaceae	15.3 ± 3.5	16.9 ± 4.3
60.	<i>Luehea seemannii</i> Triana & Planch.	Malvaceae	31.0 ± 3.9	30.6 ± 3.6
61.	<i>Sterculia apetala</i> (Jacq.) H.Karst.	Malvaceae	15.6 ± 0.6	14.8 ± 1.6
62.	<i>Carica papaya</i> L.	Caricaceae	15.4 ± 1.6	15.0 ± 3.5
63.	<i>Coccoloba uvifera</i> (L.) L.	Polygonaceae	5.5 ± 2.3	7.1 ± 3.2
64.	<i>Bougainvillea spectabilis</i> Willd.	Nyctaginaceae	10.5 ± 1.2	8.5 ± 2.3
65.	<i>Isertia haenkeana</i> DC.	Rubiaceae	105.4 ± 5.9	98.6 ± 5.0
66.	<i>Plumeria rubra</i> L.	Apocynaceae	22.4 ± 1.6	23.3 ± 2.3
67.	<i>Nolana reichei</i> M.O.Dillon & Arancio	Solanaceae	2.4 ± 1.6	4.4 ± 3.9
68.	<i>Thunbergia grandiflora</i> Roxb.	Acanthaceae	9.0 ± 2.7	8.4 ± 1.2
69.	<i>Lantana camara</i> L.	Verbenaceae	0.9 ± 2.0	0.5 ± 1.2
70.	<i>Tectona grandis</i> L.f.	Lamiaceae	0 ± 0	0 ± 0

Values are means ± SD ($n = 5$ independent samples).

The survey includes 30 known crassulacean acid metabolism (CAM)-exhibiting species (1–30) and 40 putative C₃ species (31–70). Most plants grew outdoors in the ground at the Tupper Center of the Smithsonian Tropical Research Institute (STRI) in Panama City, Republic of Panama (8°57'45"N, 79°32'36"W), or at STRI's Gamboa facilities (9°07'12"N, 79°42'07"W). Species numbers 13, 15 and 30 grew epiphytically on host trees, and species numbers 4, 18, 23, 24, 29, 31, 32, 54, 56 and 67 grew in pots/soil containers. Most species were studied during the dry season when CAM was probably expressed in species with facultative CAM. Leaf or, in the case of *Epiphyllum* and *Opuntia*, photosynthetic stem tissue was collected on sunny days at dusk and dawn (five samples at each time point per species) and boiled sequentially in 50% (v/v) ethanol and water. For determination of titratable acidity, plant extracts were titrated with 5 or 25 mM NaOH to an end-point of pH 6.5. A one-tailed *t*-test was used to determine whether H⁺ values at dawn were significantly greater than those at dusk at $P < 0.05$.

(vacuolar) concentrations of malate are increasing (even if there may be a limited role inside the chloroplast for decarboxylation of malate by NADP-ME for the provision of pyruvate). The key observation of Szecowka *et al.* (2013) was that, when illuminated rosettes of *A. thaliana* were supplied with external ¹³CO₂, the organic acid fraction showed only slow and very limited incorporation of the ¹³C label, implying that much of the daytime carboxylate metabolism was supported by remobilization of vacuolar carboxylates accumulated during the previous night. However, the plants in these experiments were grown under relatively low ('limiting') light conditions, had very high shoot nitrate content (224 mmol kg⁻¹ fresh mass) and rather low concentrations of the major vacuolar carboxylates (citrate, malate and fumarate averaged 1.9, 1.8 and 1.2 mmol kg⁻¹ fresh mass, respectively). These tissue carboxylate concentrations are an order of magnitude lower than those commonly observed in *Arabidopsis* (e.g. Chia *et al.*, 2000; Dyson *et al.*, 2016; Medeiros *et al.*, 2017), and so may have been associated with rather slow rates of *de novo* synthesis and fixation of atmospheric CO₂. It should also be noted that when previously illuminated C₃ tissue is transferred into darkness, this gives rise to the well-known phenomenon of light-enhanced dark respiration (LEDR): this has been shown to be associated with a burst of malate decarboxylation via malic enzyme (Gessler *et al.*, 2009; Gauthier *et al.*, 2010), which again is the opposite of what occurs during the CAM cycle.

While malate (and in some cases fumarate) accumulates during the daytime in C₃ plants, what is the explanation for the

concomitant decline in vacuolar citrate content? Citrate is required in the daytime for conversion to 2-oxoglutarate, in order to provide the carbon skeletons needed for light-dependent nitrogen assimilation and amino acid synthesis. However, TCA cycle activity is strongly downregulated in the light period, so there is only limited *de novo* citrate synthesis via citrate synthase. Instead, labelling evidence suggests that this demand is met by remobilization in the daytime of vacuolar citrate accumulated during the previous night (Gauthier *et al.*, 2010; Tcherkez *et al.*, 2012; Cheung *et al.*, 2014). Subsequent conversion of citrate to 2-oxoglutarate in the light is also not dependent on mitochondrial metabolism, given the existence of cytosolic isoforms of aconitase and isocitrate dehydrogenase (Hanning & Heldt, 1993). The result of this downregulation of TCA cycle activity during the day is an uncoupling of malate and citrate synthesis (Tcherkez *et al.*, 2012), leading to malate accumulation during the daytime and citrate accumulation during the night. In this regard, the nocturnal accumulation of citric acid in some CAM plants may have a similar metabolic basis, although the equivalent labelling experiments with CAM tissues are complex because of the relative pool sizes of metabolites (Tcherkez, 2017).

Malate accumulated during the light period can be remobilized from the vacuole at night and decarboxylated by mitochondrial NAD-malic enzyme (NAD-ME) to contribute to dark respiration (Gessler *et al.*, 2009; Lehmann *et al.*, 2015), but glycolytic breakdown of carbohydrate will also be important in supplying respiratory substrate for entry into the TCA cycle, either via pyruvate kinase (PK) or via PEPC/NAD-ME. Some nocturnal

PEPC activity is needed to provide OAA to support net synthesis of (1) any citrate that is removed from the TCA cycle for accumulation in the vacuole (Fig. 1b), and (2) aspartate, which together with malate is one of the major radiolabelled products following dark $^{14}\text{CO}_2$ feeding in tobacco leaves (Kunitake *et al.*, 1959). Indeed, based on the natural enrichment of ^{13}C in aspartyl residues in protein extracted from tobacco leaves, Melzer & O'Leary (1987) estimated that more than half of cellular aspartate is synthesized by direct fixation of atmospheric CO_2 (as HCO_3^-) by PEPC via a C_4 -type pathway, rather than by refixation of CO_2 originally assimilated by Rubisco. Based on this observation, Bräutigam *et al.* (2017) have proposed that, if at least one isoform of PEPC is active at night, the C_4 -like isotope distribution in aspartate could be generated by nocturnal fixation of external CO_2 by PEPC, and that this may represent a plausible precursor in C_3 plants of CAM-like dark fixation. However, it should be borne in mind that, because of dark respiration, the overall CO_2 diffusion gradient at night in C_3 plants is outwardly directed and the stomata are largely or partially closed at this time, so the rate of uptake of external CO_2 molecules would be limited. As a more likely alternative, the distribution of 'heavy' ^{13}C atoms in aspartate could be readily explained by PEPC-mediated fixation of atmospheric CO_2 during the daytime, when PEPC will be active in the light-dependent synthesis of malate (see earlier): this could either occur by immediate transamination of the OAA formed to aspartate, or more indirectly via accumulation of malate in the vacuole during the light period, followed by its remobilization and decarboxylation by NAD-ME in the subsequent dark period, and refixation of this 'heavy' CO_2 via PEPC to form aspartate. The pyruvate resulting from NAD-ME activity at night could then be used to produce acetyl-CoA to support vacuolar citrate accumulation, as shown in Fig. 1(b), and indeed NAD-ME loss-of-function mutants of *A. thaliana* have been shown to possess reduced citrate contents (Tronconi *et al.*, 2008).

Thus, although at least one isoform of PEPC may be active at night in C_3 plants, and this allows radiolabel from externally supplied $^{14}\text{CO}_2$ in feeding experiments to appear in malate, the main function of malate at night in C_3 plants is as a substrate for other reactions (citrate synthesis and respiration) and as a metabolic intermediate (in the TCA cycle). By contrast, in CAM plants in the dark there is much greater and more sustained incorporation of label into malate (Kunitake *et al.*, 1959), reflecting its function as the metabolic end-product of nocturnal CO_2 fixation that accumulates overnight in the vacuole until being remobilized at the start of the light period in the next phase of the CAM cycle.

The discreteness of the ability to perform CAM

Schiller & Bräutigam (2021) suggest that the continuous spectrum of phenotypes observable between C_3 and CAM supports their proposal that CAM photosynthesis merely requires the upregulation of fluxes already present in C_3 plants. In contrast to the comparison of C_3 and C_4 photosynthesis, the authors suggest that CAM cannot be regarded as a binary character-state because 'it can be plastic with plant age and external conditions such as water availability and salinity'. However, an alternative view is that the

ability to perform net dark CO_2 fixation represents a discrete metabolic innovation, which has only arisen in a limited number of plant lineages, even if the phenotypic expression of CAM is strongly influenced by developmental and environmental factors. In obligate CAM plants, the assimilatory tissues show an ontogenetic progression from C_3 to CAM during development (Winter *et al.*, 2011; Winter, 2019), but even at maturity most CAM species retain the ability to acquire atmospheric CO_2 directly via the C_3 pathway – notably during the early morning (phase II) and late afternoon (phase IV) – when environmental conditions are favourable. The entire spectrum from C_3 to CAM gas exchange is exhibited particularly clearly in facultative CAM plants as they transition from C_3 to CAM in response to water deficit stress and/or high soil salinity (e.g. Winter & Holtum, 2014). It is also notable that, while performing C_3 photosynthesis before the ontogenetic or environmentally induced transition to CAM, the young leaves of *Kalanchoë* (Amagasa, 1982) and *Mesembryanthemum* (Winter *et al.*, 1978) show small daytime increases in malate content, before switching to the night-time malate accumulation characteristic of CAM. But this phenotypic continuum does not mean that CAM is a purely quantitative trait: the ability to show net dark CO_2 fixation and nocturnal acidification is not present in C_3 plants (cf. Table 1), and so can be regarded as a discrete metabolic innovation only found in lineages that have evolved CAM.

An additional rationale for treating CAM as a distinct functional trait has come from ecophysiological surveys of carbon-isotope ratios ($\delta^{13}\text{C}$ values), which show a strongly bimodal distribution in several large families containing both C_3 species and obligate CAM plants (Winter & Holtum, 2002; Winter *et al.*, 2015). This has facilitated character-mapping onto phylogenies as a means of investigating photosynthetic pathway evolution in families such as Bromeliaceae (Crayn *et al.*, 2004), Orchidaceae (Silvera *et al.*, 2009; Bone *et al.*, 2015; Gamisch *et al.*, 2021), Euphorbiaceae (Horn *et al.*, 2014) and Asparagaceae (subfamily Agavoideae: Heyduk *et al.*, 2016). Even if some substructure is discernible in the frequency histograms of $\delta^{13}\text{C}$ values within the CAM cluster (Winter *et al.*, 2015; Messerschmid *et al.*, 2021), the pronounced minimum in the bimodal frequency distributions consistently seen around -20‰ (observed in at least seven families so far) is concordant with CAM being a discrete evolutionary innovation, with C_3 –CAM intermediate states not being favoured.

In facultative CAM species, by contrast, in which the full spectrum of phenotypes may be seen over the plant's life cycle, a complete range of intermediate $\delta^{13}\text{C}$ values can be found. For example, the well-studied facultative CAM plant *Mesembryanthemum crystallinum* shows C_3 -type $\delta^{13}\text{C}$ values as low as -27‰ in its natural habitat soon after germination in the rainy season, and these gradually shift to values as high as -15‰ as the dry season progresses, indicative of an almost complete switch to CAM photosynthesis (Winter *et al.*, 1978). This pattern appears to be common among annual plants of the family Aizoaceae, with the result that a bimodal distribution of $\delta^{13}\text{C}$ values is not observed in the corresponding frequency histograms for such families (Messerschmid *et al.*, 2021). Nevertheless, it is the genetically encoded ability to induce net dark CO_2 fixation in response to water deficits that distinguishes these facultative CAM plants from C_3 species. A

more inclusive approach, as pointed out by Winter *et al.* (2015), will eventually incorporate more systematic surveys of species displaying only weakly expressed CAM, but these cannot be recognized on the basis of their $\delta^{13}\text{C}$ values alone, and so require physiological measurements on living material under conditions when CAM activity may be expressed (e.g. Hancock *et al.*, 2019; Torres-Morales *et al.*, 2020). Dawn–dusk measurements of tissue titratable acidity provide a highly sensitive method for detecting low-level CAM activity, which in gas-exchange studies is often discernible only as a slight reduction in the rate of net CO_2 loss during the dark period in comparison with well-watered plants.

Thus, the fact that dark CO_2 fixation may contribute anywhere between 0 and 100% of total 24 h carbon gain is not incompatible with CAM being treated as a categorical trait. Certain plant lineages possess the ability to engage the CAM cycle, whereas others (the majority) do not.

Conclusions

The evidence from comparative biochemistry and genomics suggests that synthesis of malate via PEP carboxylase, and accumulation of the resulting carboxylate anion in the cell vacuole for ion homeostasis and turgor generation are ubiquitous features of central metabolism in the green plant lineage. In the heterotrophic tissues of C_3 plants such as roots, PEPC activity has traditionally been associated with the ‘dark’ fixation of CO_2 into malate (Lips & Beevers, 1966), but in photosynthetic tissues the accumulation of malate typically occurs during the daytime in association with light-dependent assimilation of nitrate (e.g. Strack *et al.*, 1986). The key metabolic innovation underpinning CAM photosynthesis is arguably the switch from daytime to night-time synthesis of malate in the assimilatory tissue, coupled to the glycolytic breakdown of storage carbohydrate (Borland *et al.*, 2016; Tay *et al.*, 2021). This results in the stoichiometric fixation of CO_2 , with malic acid accumulating as the end-product in the vacuole, before its remobilization and decarboxylation in the following daytime. Even when expressed at relatively low levels, this cycle would appear to involve a fundamental reprogramming of day–night carbon metabolism not easily conceptualized as part of a seamless continuum between C_3 and CAM. Realization of the full water-saving potential of the CAM pathway, as seen in obligate CAM plants, would undoubtedly involve optimization of other evolutionary modifications, but these can be viewed as contingent upon (rather than necessary precursors for) the reconfigured day–night cycle of malic acid metabolism. For example, the distinctive period of stomatal closure during phase III of the CAM cycle is a direct consequence of the wholesale remobilization and decarboxylation of the malic acid accumulated during the previous night. And the characteristic succulence of the assimilatory tissue, attributable to highly vacuolated cells with relatively thin cell walls, is important in maximizing the acid- and water-storage capacity of the cells (Lüttge *et al.*, 1982; Smith *et al.*, 1996; Edwards, 2019; Töpfer *et al.*, 2020; Heyduk, 2021).

Deciphering the first critical steps on the evolutionary path to net dark CO_2 fixation and malic acid accumulation will certainly help to guide efforts directed at the bioengineering of the CAM trait. Schiller & Bräutigam (2021) have proposed that, considering the

widespread occurrence and the plasticity of the CAM trait, there are ‘therefore likely no general barriers to trait engineering’ and that ‘[c]ircadian and diurnal rhythms in CAM plants are likely similar to those in C_3 plants’, but we believe that both of these opinions are difficult to reconcile with the available evidence. Despite its dispersed taxonomic occurrence and phenotypic plasticity, CAM activity has only been detected so far in 37 out of a total of 450 families of vascular plants (Winter & Smith, 1996b; Winter *et al.*, 2021). Acquisition of this trait by such a limited number of lineages suggests that it does not represent a facile evolutionary step requiring simply enhancement of flux pathways already present in C_3 plants. Also, the switch from diurnal to nocturnal malate synthesis in CAM plants would seem, *prima facie*, to require significant modification of the way in which central metabolism is integrated with the circadian clock. Indeed, the ability of CAM plants to show a persistent free-running endogenous rhythm of dark CO_2 fixation provided early evidence that this core component of CAM is directly linked to the central circadian oscillator (Wilkins, 1959). This rhythm of dark CO_2 fixation is now known to be attributable to the periodic activity of a specific, highly expressed isoform of PEP carboxylase, which becomes post-translationally activated at night by a dedicated protein kinase whose expression is under endogenous circadian control (Hartwell *et al.*, 1996; Boxall *et al.*, 2020). Evidence of a tight interconnection between the circadian oscillator and dark CO_2 fixation is also shown by the observation that suppression of PEPC expression by RNA interference results in altered expression of certain core circadian clock genes (Boxall *et al.*, 2020). On the other hand, overexpression of the CAM isoform of PEPC in a C_3 background (*Arabidopsis* or tobacco), while leading to increased malate content and daytime accumulation, does not itself result in a switch to night-time acidification (Lim *et al.*, 2019; Liu *et al.*, 2021).

Many facets of the gene regulatory networks involved in the core CAM cycle remain to be explored. Much attention has focused so far on the regulation of PEPC, the key enzyme involved in the initial carboxylation reaction. This is encoded by a multigene family even in C_3 plants, so it is plausible that one isoform of PEPC was coopted in evolution for the key function of night-time CO_2 fixation in CAM plants. But less work has been performed to date on regulation of the decarboxylation process, which will be equally critical to coordination of the CAM cycle. The α and β subunits of plant NAD-ME, the principal decarboxylase in many CAM species, are each encoded by a single gene, and their expression is linked to the central circadian clock (Dever *et al.*, 2015; Yang *et al.*, 2017; Francisco *et al.*, 2021). A fundamental reprogramming is thus needed to ensure that NAD-ME is active during the day in the CAM cycle, rather than during the night as in C_3 plants. Similarly, coordinate regulation of the key transport proteins at the vacuolar membrane with the phases of malic acid synthesis and decarboxylation will be essential to maintain cytoplasmic homeostasis and minimize futile cycling, but details of these mechanisms remain to be fully elucidated (Smith *et al.*, 1996; Hafke *et al.*, 2003; Ceusters *et al.*, 2021). Further exploration of the gene regulatory networks in closely related C_3 and CAM species, and especially those implicated in the C_3 –CAM

transition in facultative species (e.g. Brillhaus *et al.*, 2016; Heyduk *et al.*, 2019; Wai *et al.*, 2019), will undoubtedly help in identifying core elements of the circadian circuitry needed to bioengineer the dark CO₂ fixation pathway into C₃ plants.



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Author contributions


KW designed the research, collected the plant material, analysed the data and wrote the initial draft. JACS revised the manuscript and designed Fig. 1. Both authors discussed, reviewed and approved the final manuscript.

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Data availability

All data required to draw the conclusions in the paper are present in the main text.

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