Seminars in Cell and Developmental Biology 155 (2024) 10–22

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

The Calvin-Benson-Bassham cycle in C_4 and Crassulacean acid metabolism species

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ARTICLE INFO

Keywords:
Calvin-Benson-Bassham cycle
C_4
CAM
CO_2 assimilation
Crop improvement
Photosynthesis

ABSTRACT

The Calvin-Benson-Bassham (CBB) cycle is the ancestral CO_2 assimilation pathway and is found in all photosynthetic organisms. Biochemical extensions to the CBB cycle have evolved that allow the resulting pathways to act as CO_2 concentrating mechanisms, either spatially in the case of C_4 photosynthesis or temporally in the case of Crassulacean acid metabolism (CAM). While the biochemical steps in the C_4 and CAM pathways are known, questions remain on their integration and regulation with CBB cycle activity. The application of omic and transgenic technologies is providing a more complete understanding of the biochemistry of C_4 and CAM species and will also provide insight into the CBB cycle in these plants. As the global population increases, new solutions are required to increase crop yields and meet demands for food and other bioproducts. Previous work in C_3 species has shown that increasing carbon assimilation through genetic manipulation of the CBB cycle can increase biomass and yield. There may also be options to improve photosynthesis in species using C_4 photosynthesis and CAM through manipulation of the CBB cycle in these plants. This is an underexplored strategy and requires more basic knowledge of CBB cycle operation in these species to enable approaches for increased productivity.

1. Introduction

All photosynthetic organisms use the Calvin-Benson-Bassham (CBB) cycle to fix atmospheric CO_2 into organic compounds needed for development and growth. The steps in this cycle and the enzymes involved were elucidated starting in the 1950 s by Calvin, Benson, Bassham, and colleagues [1,2]. As the first stable product of the CBB cycle is the 3-carbon compound, 3-phosphoglycerate (3-PGA), this

Abbreviations: Ald, fructose-1,6-bisphosphate aldolase; BS, bundle sheath; CA, carbonic anhydrase; CAM, Crassulacean acid metabolism; CBB, Calvin-Benson-Bassham; CCM, carbon concentrating mechanism; C_i, internal partial pressures; DHAP, dihydroxyacetone phosphate; E-4 P, erythrose-4-phosphate; FB, fructose-1,6-bisphosphatase; Fru-1, 6-BP, fructose 1,6-bisphosphate; Fru-6 P, fructose 6-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; h, hour; HPLC-MS, high performance liquid chromatography-mass spectrometry; LD, light-dark cycle; LL, constant light conditions; LSU, large subunit; OAA, oxaloacetate; MDH, malate dehydrogenase; min, minute; NAD-ME, NAD-malic enzyme; NADP-ME, NADP-malic enzyme; PCK, PEP carboxykinase; 3-PGA, 3-phosphoglycerate; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase; PGK, phosphoglycerate kinase; PNUE, photosynthetic nitrogen-use efficiency; PPDK, pyruvate phosphate dikinase; PRK, phosphoribulokinase; PSII, Photosystem II; PWUE, photosynthetic water-use efficiency; R-5 P, ribose-5-phosphate; RAF1, Rubisco activation factor 1; RBCS, Rubisco small subunit; RCA, Rubisco activase; RNA-Seq, RNA sequencing; RPI, ribose 5-phosphate isomerase; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; SB, sedoheptulose-1,7-bisphosphatase; Sed-1, 7-BP, sedoheptulose 1,7-bisphosphate; Sed-7 P, sedoheptulose 7-phosphate; SSU, small subunit; TK, transketolase; TP, triose phosphate; TPI, triose-P isomerase; TPT, triose phosphate:phosphate translocator; WT, wild type; Xyl-5 P, xylulose 5-phosphate.

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https://doi.org/10.1016/j.semcdb.2023.07.013
Received 7 March 2023; Received in revised form 3 July 2023; Accepted 25 July 2023
Available online 4 August 2023

pathway is also referred to as the C_3 cycle. Plants utilizing only this pathway to assimilate CO_2 are often referred to as C_3 plants and make up over 85% of plant species.

There are 13 reactions in the CBB cycle, which are catalyzed by 11 different enzymes (Fig. 1), and the cycle can be divided into three catalytic phases: carboxylation, reduction, and regeneration [1–3]. The carboxylation and reduction phases form the linear, assimilatory part of the C_3 cycle catalyzed by the enzymes phosphoribulokinase (PRK), ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), phosphoglycerate kinase (PGK), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The reactions catalyzed by PRK and PGK use ATP resulting from the light reactions of photosynthesis while NADPH produced through light harvesting is used by GAPDH. The products resulting from these reactions are triose phosphates (TPs), which can be exported from the chloroplast to the cytosol via the triose phosphate:phosphate translocator (TPT) for sucrose and isoprenoid synthesis, or they can enter the branched, regeneration phase of the cycle (Fig. 1). This regenerative phase results in the formation of the 5-carbon CO_2 acceptor molecule ribulose 1,5-bisphosphate (RuBP), and also produces a number of different carbon compounds which exit the cycle and form the basis of the biosynthetic pathways for starch (fructose 6-phosphate; Fru-6 P), thiamine, nucleotides (ribose 5-phosphate; R-5 P) and shikimate (erythrose 4-phosphate; E-4 P), with the latter being vital for the production of key metabolites including aromatic amino acids and phenylpropanoids (Fig. 1). Although the majority (five-sixths) of the TPs produced in the CBB cycle remains within the cycle to regenerate RuBP, one-sixth of the carbon compounds exit the cycle. The relative amount of carbon going to the different biosynthetic pathways is not fixed, and is likely to change during development, under different environmental conditions, and in response to changes in the rate of photosynthesis. However, a key factor that must remain constant is the retention of five out of every six carbons within the cycle, which prevents the cycle from becoming depleted in the metabolic intermediates it needs to continue to function [1–3].

Rubisco, the enzyme catalyzing the carboxylation of RuBP, has several limitations. It is a relatively inefficient enzyme with a low turnover number, and it also catalyzes the oxygenation of RuBP, with the substrates for the carboxylation and oxygenation reactions competing for the same catalytic site [4]. The oxygenation reaction directs the flow of carbon through the photorespiratory pathway (Fig. 1), and this can result in losses of between 25% and 30% of the carbon fixed [5,6]. Environmental variables, such as high temperature and drought, can result in an increase in the oxygenase activity relative to the carboxylation reaction [7]. Therefore, promoting the Rubisco carboxylation reaction, which would simultaneously minimize the oxygenation reaction, has the potential to increase carbon assimilation significantly and would represent a step change in photosynthesis (up to 100% depending on temperature [8]).

Although all plants use the CBB cycle some species have evolved additional metabolic pathways that capture atmospheric CO_2 using a different carboxylase enzyme that has no oxygenase activity. These pathways act as carbon concentrating mechanisms (CCMs) that limit the oxygenase reaction of Rubisco and photorespiration. Our knowledge of CBB cycle operation, regulation, and metabolic integration in these plants is limited but understanding these processes is critical as it offers opportunities to inform strategies for photosynthetic enhancement and crop improvement.

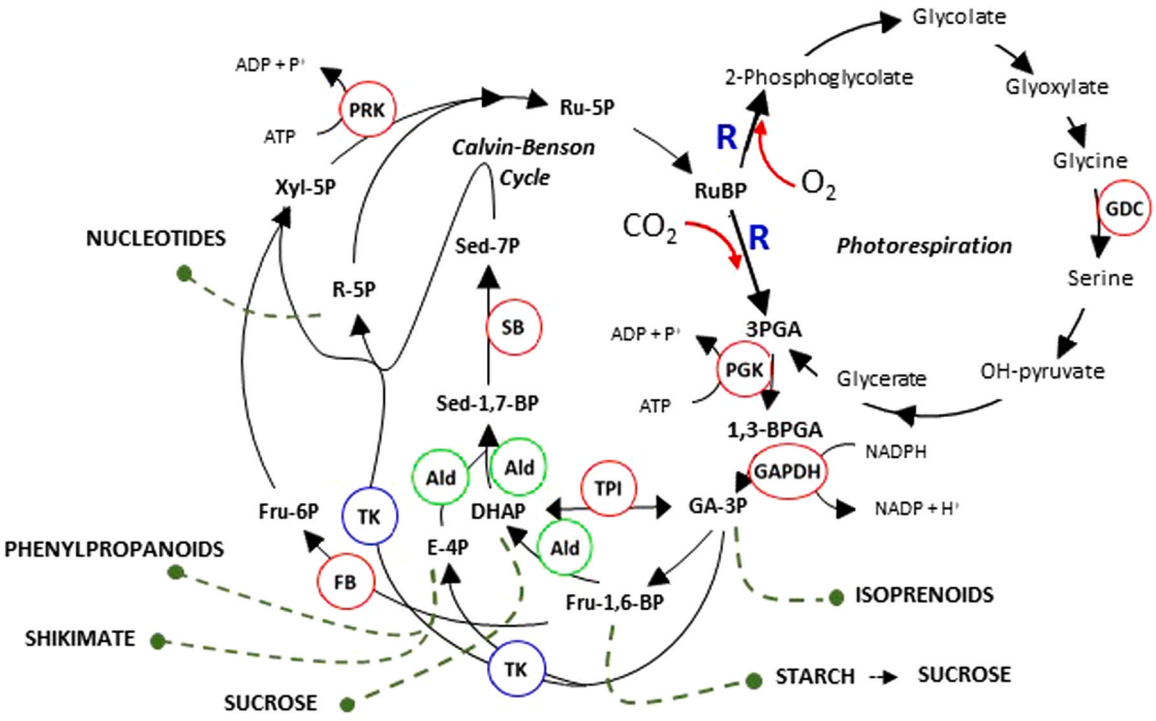

Fig. 1. Schematic representation of the Calvin-Benson-Bassham (CBB) Cycle. Fructose-1,6-bisphosphatase (FB: EC.3.1.3.11), fructose-1,6-bisphosphate aldolase (Ald: EC.4.1.2.13), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glycine decarboxylase (GDC), triosephosphate isomerase (TPI: EC 5.3.1.1), phosphoglycerate kinase (PGK: EC.2.7.2.3), phosphoribulokinase (PRK: EC.2.7.1.19), ribulose-1,5-bisphosphate carboxylase/oxygenase (R: EC.4.1.1.39), sedoheptulose-1,7-bisphosphatase (SB: EC.3.1.3.37), transketolase (TK: EC.2.2.1.1), 1,3-bisphosphoglyceric acid (1,3-BPGA), dihydroxyacetone phosphate (DHAP), erythrose-4-phosphate (E-4 P), fructose 1,6-bisphosphate (Fru-1,6-BP), fructose 6-phosphate (Fru-6 P), glyceraldehyde 3-phosphate (GA-3 P), 3-phosphoglycerate (3-PGA), ribose-5-phosphate (R-5 P), ribulose 5-phosphate (Ru-5 P), ribulose bisphosphate (RuBP), sedoheptulose 7-phosphate (Sed-7 P), sedoheptulose 1,7-bisphosphate (Sed-1,7-BP), xylulose 5-phosphate (Xyl-5 P) (see Simkin [127]).

2. Angiosperm carbon concentrating mechanisms favour carboxylation, minimise photorespiration, and have evolved in diverse lineages

Within angiosperms, two distinct metabolic adaptations of photosynthetic CO_2 fixation have evolved that limit the oxygenation of RuBP and loss of fixed carbon through photorespiration (Fig. 2). The adaptations are known as C_4 photosynthesis, where the first stable compound synthesized is a 4-carbon acid, oxaloacetate (OAA), and Crassulacean acid metabolism (CAM), which also generates OAA as the initial product of primary atmospheric CO_2 fixation [9]. Both pathways are metabolic additions to the CBB cycle and increase the concentration of CO_2 around Rubisco, thereby reducing the oxygenation reaction and flux through the photorespiratory pathway.

The C_4 and CAM pathways concentrate CO_2 around Rubisco through either a spatial separation of primary and secondary CO_2 fixation across two distinct, but connected, photosynthetic cell types within the leaf (C_4; Fig. 2), or a temporal separation of primary and secondary CO_2 fixation within each photosynthetic mesophyll cell (CAM; Fig. 2). Both systems catalyze primary atmospheric CO_2 fixation using phospho*enol*-pyruvate (PEP) carboxylase (PEPC), a cytosolic enzyme that does not recognize oxygen and has a higher affinity for CO_2 (specifically HCO_3^-) than Rubisco, making it a superior carboxylase [10].

2.1. C_4 photosynthesis

A C_4 photosynthetic syndrome has evolved more than 60 independent times in the angiosperms, in both monocot and eudicot taxa [11]. While species operating a C_4 pathway in a single cell type are known [12], most C_4 species demonstrate Kranz anatomy in which leaf mesophyll cells surround bundle sheath (BS) cells, which surround the vascular tissue. In Kranz C_4 species, carbonic anhydrase (CA) converts CO_2 entering the cytosol of leaf mesophyll cells to HCO_3^-, which is then used by PEPC to produce the 4-carbon OAA from PEP in the light period, and malate and/or aspartate move through plasmodesmata connections to BS cells where they are decarboxylated (Fig. 2). This decarboxylase activity results in high CO_2 concentrations in the vicinity of Rubisco, which is found only in BS chloroplasts in C_4 plants [13], and the CO_2 is fixed by the CBB cycle [14]. The 3-carbon compound resulting from the decarboxylation reaction is used to regenerate PEP in the mesophyll cells through the activity of pyruvate phosphate dikinase (PPDK; Fig. 2). Under standard growth conditions, the production of carbohydrates is split between the two leaf cell types in C_4 species with starch synthesized predominantly in the BS and mesophyll cells being the primary site of sucrose production although the BS also has capacity for sucrose synthesis in many C_4 species (Fig. 3) [15–17].

Historically, C_4 species were categorized into one of three subtypes based on the decarboxylase with the highest activity in their leaf tissue [14]. In NADP-malic enzyme (NADP-ME) species, decarboxylation occurs in the BS chloroplasts whereas in plants using NAD-malic enzyme (NAD-ME) as the major decarboxylase, CO_2 is released in the BS mitochondria. In the third subtype, PEP carboxykinase (PCK), CO_2 is released in the BS cytosol through the activity of PCK while an NAD-ME isoform is also active in BS mitochondria. Currently, it is recognised that for most C_4 species, more than one type of C_4 acid moves into the BS where more than one of the above decarboxylases are active [18,19]. Due to the high affinity of PEPC for HCO_3^-, the sequestration of Rubisco to BS chloroplasts, and Kranz anatomy where BS cell connections to intracellular airspaces are limited, the concentration of CO_2 around Rubisco is high. This combination of biochemistry and anatomy results in a C_4 CO_2 pump that limits Rubisco oxygenase activity and photorespiration and allows C_4 plants to outcompete C_3 species in hot, dry, and/or high light

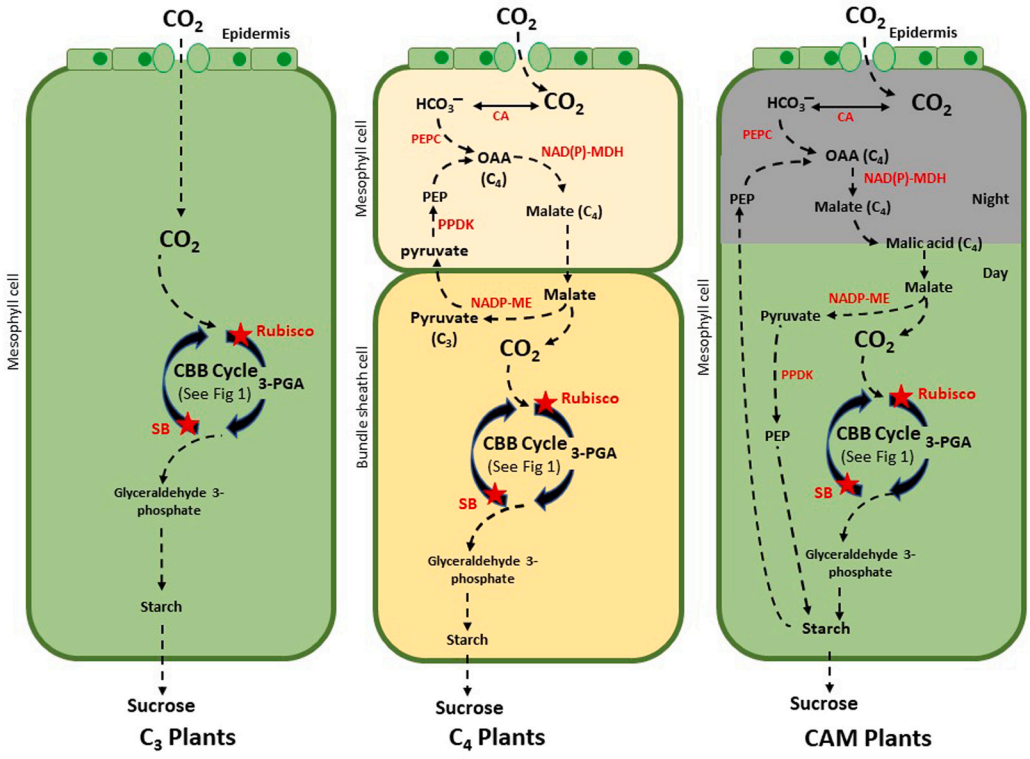

Fig. 2. Schematic representation of the CO_2 fluxes in C_3, C_4, and Crassulacean acid metabolism leaves. C_3 plants convert atmospheric CO_2 into 3-phosphoglycerate (3-PGA) via ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Fig. 1) Sedoheptulose-1,7-bisphosphatase (SB) catalyzes a step in the regeneration phase of the Calvin-Benson-Bassham (CBB) cycle. In the leaves of C_4 plants, CO_2 is converted into the 4-carbon oxaloacetate (OAA) by the enzyme phospho*enol*pyruvate (PEP) carboxylase (PEPC). C_4 plants are categorized into three subtypes, based on the C_4 acid decarboxylation enzyme (see text for detail). The NADP-malic enzyme (NADP-ME) type is shown in the figure (see also Fig. 3). The CO_2 released through the decarboxylation event in the bundle sheath is fixed by Rubisco (Fig. 1). Crassulacean acid metabolism (CAM) plants fix CO_2 at night (grey area), with PEPC converting CO_2 into OAA, which is stored as the 4-carbon intermediate malic acid. During the day (green area), malate is decarboxylated and the CO_2 released is fixed by Rubisco (Fig. 1). CAM plants are represented by two subtypes, 1, the NAD(P)-ME type, and 2, the PEP carboxykinase type. Carbonic anhydrase (CA), NAD(P)-malate dehydrogenase (NAD(P)-MDH); pyruvate phosphate dikinase (PPDK). Stars show locations of enzymes targeted for manipulation to increase photosynthesis (see Tables 1–2). Adapted from Yamori et al. [168] and Ainsworth et al. [169].

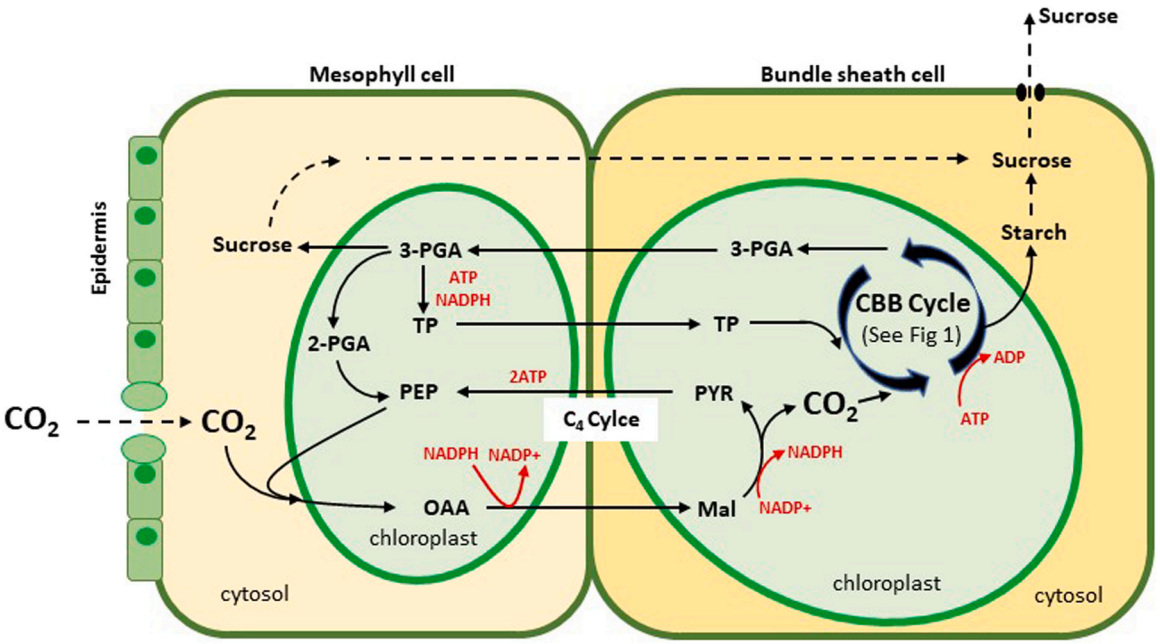

Fig. 3. Schematic representation of NADP-malic enzyme-type C_4 photosynthesis. Atmospheric CO_2 and phospho*enol*pyruvate (PEP) are converted into the 4-carbon oxaloacetate (OAA) in the mesophyll cell cytosol. In the mesophyll cell chloroplast, OAA is converted into malate (Mal), which is then translocated to a bundle sheath cell and decarboxylated in the chloroplast. The CO_2 released enters the Calvin-Benson-Bassham (CBB) cycle and the 3-carbon pyruvate (PYR) diffuses into a mesophyll cell where it is regenerated into PEP. 3-phosphoglycerate (3-PGA) made in the CBB cycle is shuttled to a mesophyll chloroplast from the bundle sheath chloroplast where it can be either reduced to triose phosphate (TP) and returned to the CBB cycle in the bundle sheath chloroplast or converted to PEP via phosphoglycerate mutase and enolase. Sucrose synthesis occurs mainly in the mesophyll cell cytosol while starch is made in bundle sheath chloroplasts. Adapted from von Caemmerer and Furbank [68].

environments.

2.2. Crassulacean acid metabolism

In CAM species (Fig. 2), primary CO_2 fixation by PEPC occurs in the cytosol of each chloroplast-containing mesophyll cell and happens in the dark period [20]. Stomata open at night and PEPC fixes both atmospheric and internal respiratory CO_2 with PEP, forming OAA initially. OAA is rapidly converted to malate by malate dehydrogenase (MDH). Malate is stored in the mesophyll vacuole as malic acid throughout the night and reaches high concentrations by dawn. This nocturnal primary atmospheric CO_2 fixation during CAM has been defined as phase I [21].

Phases II, III and IV of CAM occur in the light period [21]. Phase II represents a brief period of 1 to 2 h of atmospheric CO_2 fixation after sunrise when stomata open and PEPC and Rubisco are active simultaneously. Phase III follows and represents a period of stomatal closure in the light period when CO_2 from malate decarboxylation is refixed by Rubisco. Phase IV defines a period that can occur when all malate has been consumed and stomata reopen, allowing direct atmospheric CO_2 fixation by Rubisco later in the light period [21].

In terms of the timing of the biochemical steps of the CAM pathway (Fig. 2), once the sun has risen, malic acid leaves the vacuole and is decarboxylated by either NAD(P)-ME or the combination of MDH, working in the opposite direction to the night, and PCK [20]. The mechanism of malate decarboxylation in the light depends on the specific CAM species, with the adaptation being found in at least 37 plant families and having evolved independently multiple times in several families [22]. In addition, the extent of CAM expression is dependent on the species and, in some species, dependent on the environmental conditions. CAM species have therefore been categorized as displaying either constitutive/obligate CAM or facultative/inducible CAM [23]. Obligate CAM species develop CAM even when well watered and continue to rely on CAM as their main mode of primary CO_2 fixation throughout their lives. Facultative CAM species use C_3 when well watered and induce CAM in response to drought stress. A range of other environmental signals has also been reported to induce CAM in specific species, including high light intensity and a decrease in day length. Importantly, facultative CAM species are able to return to C_3 when rewatered [23].

The CO_2 released through malate decarboxylation in the light period results in high internal partial pressures of CO_2 (C_i) within the leaf air spaces due to diffusion of CO_2 from the mesophyll cells. This rise in C_i is believed to promote stomatal closure and efficient secondary fixation of CO_2 to sugars via the CBB cycle (phase III of CAM). It has been hypothesized that CAM species close their stomatal pores for much of the hot and dry light period in response to this build up of internal CO_2 from malate decarboxylation [24].

The high CO_2 concentration that is established in CAM photosynthetic tissues in phase III is widely assumed to favour the carboxylase activity of Rubisco over the oxygenation reaction. However, the extent to which photorespiration is suppressed in CAM species during phase III requires further detailed studies across a diverse range of independent CAM origins. This is important because stomatal closure in the light period also prevents oxygen, generated in chloroplasts through water splitting, escaping from the leaf. Thus, oxygen concentrations inside the leaf rise simultaneously with C_i. Data have been reported for the concentration of CO_2 and O_2 in the leaves of CAM species during phase III, and the results revealed that the concentration of O_2 inside CAM tissues varied from 21% to 80% [25,26]. In turn, the ratio between the partial pressure of O_2 and CO_2 inside leaves of CAM species varied from 285 in *Hoya carnosa* to 81 in *Sedum praealtum* [26]. These ratios support the conclusion that CO_2 will be enriched over O_2 behind closed stomata in the light in phase III as the equivalent ratio for ambient air is 525 and that for the air in a C_3 leaf is 840. Thus, the highest ratio reported for a CAM species (285 in *H. carnosa*) was three times better, in terms of favouring Rubisco carboxylation over oxygenation, relative to the air in a C_3 leaf. Lüttge [25] used these published values for the concentrations of CO_2 and O_2 inside CAM tissues in phase III to calculate theoretical

values for the ratio between the reaction rate of Rubisco with O_2 and CO_2. These calculations were performed using two possible equations and a range of published kinetic constants for Rubisco. The resulting calculated values led Lüttge [25] to conclude that the level of photorespiration in CAM leaves or stems behind closed stomata in the light period may be either very similar to that in C_3 leaves, at best half that in C_3 leaves, or that photorespiration may be suppressed very strongly in phase III of CAM.

2.3. Increased photosynthetic and nitrogen- and water-use efficiencies result from C_4 and Crassulacean acid metabolism carbon concentrating mechanisms

In plants that use C_4 photosynthesis, the concentration of CO_2 in the vicinity of Rubisco within the chloroplast, where the CBB cycle functions, is increased, thereby favouring carboxylation over oxygenation. This facet results in an increase in the amount of CO_2 being fixed per photon of sunlight absorbed by C_4 plants when compared to C_3 species [27,28]. Moreover, increasing the CO_2 concentration around Rubisco allows it to operate at close to its maximum catalytic rate, which leads to C_4 species being able to function optimally with lower amounts of Rubisco than C_3 plants [29]. Given that Rubisco accounts for a high proportion of total protein, and thus nitrogen, in C_3 leaves, the lower requirement for Rubisco in C_4 plants leads to increased photosynthetic nitrogen-use efficiency (PNUE; reviewed in Long, [29]). It is less clear that CAM plants achieve higher PNUE relative to C_3 species, but this may, at least in part, be due to a lack of appropriate studies in CAM species [30–32]. Overall, this means that CO_2 assimilation rates are greater per unit leaf nitrogen in the leaves of C_4 plants compared to those of C_3 plants [33]. In addition, C_4 biochemistry allows C_4 plants to achieve rates of CO_2 fixation sufficient to satisfy the C_4 pump at intercellular CO_2 partial pressures much lower than those found in C_3 leaves [34]. Thus, C_4 plants can maintain high rates of photosynthesis with lower stomatal conductance relative to a C_3 plant achieving the same rate, which in turn means that the transpirational loss of water during atmospheric CO_2 uptake for photosynthesis is lower in C_4 species than in C_3 plants [20,35]. Consequently, C_4 species demonstrate higher photosynthetic water-use efficiency (PWUE) than C_3 plants, and CAM plants have substantially greater PWUE than both C_3 and C_4 species because they open their stomata during the cooler and more humid dark period and close them for much of the hot and dry light period [20,36]. Under optimal conditions, these advantages allow C_4 plants to fix more carbon per unit of light, per unit of nitrogen, and per unit of water transpired, when compared to C_3 plants [20,30,33]. While these relationships are often less well characterized for CAM species, CAM plants do fix more CO_2 per unit of water transpired relative to C_3 and C_4 plants, and may display superior fixation of carbon per unit of light and per unit of nitrogen, although more data are required for CAM species for these latter two use efficiencies.

3. Biochemistry of the Calvin-Benson-Bassham cycle in C_4 photosynthesis and Crassulacean acid metabolism

While the enzymes catalyzing the steps of the CBB cycle are the same in all organisms, their properties, concentrations, and/or cellular locations differ between photosynthetic types and notably between plants using the same photosynthetic pathway.

3.1. C_4 photosynthesis: Calvin-Benson-Bassham cycle reactions are shared between mesophyll and bundle-sheath cells

In early work involving the isolation of mesophyll and BS cells and/or chloroplasts from plants representing all three C_4 subtypes, the enzymes catalyzing the reduction phase of the CBB cycle, PGK and GAPDH, as well as triose-P isomerase (TPI), were found in both cell types, while Rubisco, PRK, and chloroplast fructose-1,6-bisphosphatase (FB) showed BS specificity (Fig. 1) [37–39]. While these results should be considered with the knowledge that most enzymes of C_4 photosynthesis are encoded by gene families [40], they have been supported by more recent proteomic studies that identified CBB cycle-associated isoforms in maize through mass spectrometry-based technologies [41,42]. Analyses of the maize leaf proteome data also indicated preferential localization of sedoheptulose-1,7-bisphosphatase (SB), fructose-1,6-bisphosphate aldolase (Ald), and transketolase (TK) (Fig. 1) to the BS [41,42].

Rubisco has been the most examined and best characterized of all the CBB cycle enzymes within datasets for C_4 species [43–46]. As described above, the C_4 CCM concentrates CO_2 around Rubisco in BS chloroplasts, which results in C_4 plant leaves containing less Rubisco than those of C_3 species [47]. Consistent with the activity of a CCM, C_4 plant Rubisco isoforms demonstrate lower CO_2 specificities compared to those of C_3 species, but with the trade-off of greater k_{cat} values ([48,49] and references therein). Studies in which C_3 plant Rubisco small (SSU) or large (LSU) subunits were replaced with those of C_4 plants have indicated elements controlling Rubisco kinetics are located on both subunits of the enzymes in both C_3 and C_4 species (Section 5.1.1) [50–52].

Comparisons of the amount or activity of other CBB cycle enzymes between C_3 and C_4 species are limited. Whole leaf extracts of several NADP-ME-, NAD-ME-, and PCK-type C_4 grasses showed PRK activity, which is predominantly in the BS (see above), is similar to that of wheat whole leaf extracts [39]. Whole leaf TPI activity, which is split between mesophyll and BS cells (see above), in these same C_4 grasses was comparable to or greater than that of wheat leaf extracts [39]. By contrast, the activity of chloroplast GAPDH, which is also split between the two cell types (see above), in species representing the three C_4 subtypes was lower than that of wheat [53]. As for studies reporting CBB cycle enzyme location, the presence of multiple enzyme isoforms needs to be considered when interpreting the results of these whole leaf enzyme activity studies.

Chloroplast dimorphism is typically seen between mesophyll and BS cells of NADP-ME-type C_4 species although the extent of the differences between the plastids is variable among species and likely dependent on development and environmental factors [54–63]. Under standard growth conditions, when dimorphic chloroplasts are present, the BS organelles exhibit few grana stacks and significant reduction in Photosystem II (PSII) activity [64], which contribute to maintaining low O_2 levels around Rubisco due to the absence of PSII H_2O-splitting complexes. However, limited linear electron flow and only about half the NADPH requirement of the CBB cycle being met through the NADP-ME decarboxylation reaction in the BS chloroplasts [65] would inhibit RuBP regeneration. This photochemical imbalance is mitigated through a 3-PGA/TP shuttle with approximately half the 3-PGA generated by Rubisco transported into mesophyll chloroplasts (Fig. 3) [14], which are characterised by thylakoid grana with PSII activity [64]. In mesophyll chloroplasts, 3-PGA is converted to TPs, of which at least two-thirds [66] must be translocated to BS cells for entry into the CBB cycle and regeneration of RuBP as well as starch synthesis (Fig. 3). The TPs remaining in the mesophyll are used predominantly for sucrose synthesis (Fig. 3) [17] while some BS-generated 3-PGA may also be converted to PEP in the mesophyll, thereby supporting PEPC activity (Fig. 3) [67].

Although BS chloroplasts of NAD-ME- and PCK-type C_4 species contain grana stacks and PSII activity [64], as noted above, the enzymes of the CBB cycle reduction phase are also found in both mesophyll and BS cells of these plants, indicating they also contain a functional 3-PGA/TP shuttle [14,39,68]. The separation of the CBB cycle reactions between mesophyll and BS cells along with the likelihood of mixed decarboxylation pathways operating in all C_4 species allow flexibility in balancing the photochemical load and carbohydrate synthesis between the two cell types in response to environmental changes and/or during leaf and plant development (reviewed in [17,18,69–71]). However, details on the flux through the individual C_4 acid transfer cycles in a single species, including its regulation, involvement of a 3-PGA/TP

shuttle, and mesophyll/BS partitioning of sucrose and starch synthesis, under different growth conditions and stages of development are limited.

3.2. Crassulacean acid metabolism: the temporal integration of two carboxylases

Although a relatively limited number of studies have explored the properties of the enzymes of the CBB cycle in CAM species, and/or studied the operation of the CBB cycle as a whole in the photosynthetic tissues of CAM plants, some insights have been gleaned from studies of in vitro enzyme activities in extracts from *Kalanchoë* leaves and instantaneous online carbon isotope discrimination. Early work established that several enzymes within the CBB cycle are activated by light in a redox-dependent manner, and thus share this characteristic in common with their counterparts in C_4 and C_3 species [72,73]. Specifically, Gupta and Anderson [72] reported that PRK, NADP-GAPDH and SB were light-activated in *K. blossfeldiana* cv. Tetra Vulcan, but FB was not, whereas Hutcheson and Buchanan [73] demonstrated that FB and SB from leaves of *K. daigremontiana* were subject to light-activation by reduced thioredoxin-*f*.

Additional understanding of the CBB cycle in a CAM species (pineapple; *Ananas comosus*) was provided by applying high performance liquid chromatography-mass spectrometry (HPLC-MS) with mixed-mode stationary phases to identify and quantify the notoriously difficult CBB cycle intermediates. The concentrations of Fru-6 P, glucose 6-phosphate, dihydroxyacetone phosphate (DHAP), 3-PGA, PEP, and fructose 1,6-bisphosphate (Fru-1,6-BP) were all measured at levels comparable to those in the C_3 model species *Arabidopsis thaliana*, plus R-5 P, xylose 5-phosphate (Xyl-5 P), and E-4 P were below the level of detection, which was also the case for *A. thaliana* [74]. However, *A. thaliana* and three other C_3 species (barley, peppermint, and pea) all had detectable levels of RuBP at the level of < 5 μmol m^{-2}, whereas RuBP was below the level of detection in pineapple [74]. Despite the absence of a large body of literature studying the characteristics of the CBB cycle enzymes and associated metabolite levels in a diverse range of CAM species, these studies do support the general conclusion that the CBB cycle in CAM species is operating behind closed stomata in the light period in much the same way as it is known to be operating in C_3 species behind open stomata.

Most work on CAM species in relation to the CBB cycle has focused on Rubisco, which like the C_4 homologues have lower CO_2 specificity and greater k_{cat} values relative to the Rubiscos of C_3 species [48], as well as the interplay between PEPC and Rubisco activities throughout the light period. In particular, studies in species of *Kalanchoë* that perform constitutive/obligate CAM, and bromeliads such as the facultative CAM species *Guzmania monostachia,* demonstrated that Rubisco activation during the light period is delayed relative to the norm for C_3 species, where Rubisco is activated rapidly at dawn [75,76]. Findings demonstrated that Rubisco activation state remains low during malate decarboxylation in phase III of CAM, but then Rubisco activase (RCA) protein abundance and Rubisco activation state rise in anticipation of phase IV of CAM when stomatal pores can re-open later in the light period and atmospheric CO_2 is assimilated directly via the CBB cycle [76]. Furthermore, online carbon isotope discrimination data for the leaves of *K. daigremontiana* sampled at different time points across the light period revealed that during phase II of CAM, a short period of stomatal opening and atmospheric CO_2 fixation for 1–2 h after dawn, the instantaneous carbon isotope discrimination transitioned from a signal indicative of PEPC fixing most of the CO_2 (−6‰ to −7‰) to a Rubisco signal (−16‰) [76,77]. Likewise, in the bromeliad *Tillandsia utriculata*, discrimination transitioned from PEPC in early phase II to Rubisco late in phase II [78] and a similar progression was measured in *Clusia rosea*, which displays constitutive CAM. By contrast the facultative CAM species *C. minor* had an instantaneous discrimination signal indicative of Rubisco dominating in both phase II and phase IV in the light period [79]. In phase IV, when stomata re-open for the final few hours of the light period, instantaneous discrimination in *K. daigremontiana* declined from − 13‰ to − 11‰, indicating a transition from Rubisco to PEPC as dusk was approached [76]. Equivalent data for phase IV in *T. utriculata* also showed that the instantaneous discrimination transitioned from a Rubisco signal early in phase IV to a more PEPC-influenced signal late in phase IV [78]. More recently, experiments using short periods of darkness applied during phase IV of CAM with the orchid *Phalaenopsis* cv. Sacramento revealed that PEPC was the major carboxylase in the last few hours of phase IV as CO_2 fixation did not decline in response to short 5-min blackouts applied at 2 h and 0.5 h before dark [80]. By contrast, *K. blossfeldiana* cv. Saja leaves displayed a sharp decline in CO_2 fixation in response to 5-min blackouts applied throughout phase IV, indicating that Rubisco remained the major carboxylase right through to dusk [80]. However, it is important to note that the 24 h pattern of CO_2 exchange for *Phalaenopsis* cv. Sacramento in this study did not show the classic decline in CO_2 fixation to zero or below zero at dawn and dusk that mark the start of phase II and end of phase IV in other CAM species that display the four phases of gas exchange. Instead, phase IV in *Phalaenopsis* cv. Sacramento appeared to be a prolonged start to phase I, with the rate of CO_2 assimilation continuing without a dip through the light to dark transition, and phase II also had the characteristics of a continuation of phase I into the early hours of the light period, with no dip in CO_2 fixation at the dark to light transition [80]. Hence, the physiology of *Phalaenopsis* cv. Sacramento reported by van Tongerlo et al. [80] was abnormal at dawn and dusk relative to all previous reports of CAM gas exchange patterns in other species; consequently, the response to short periods of darkness applied in phase IV may not be broadly applicable across diverse CAM species.

Such data from both *Kalanchoë* species, the orchid *Phalaenopsis* cv. Sacramento, and the bromeliads *G. monostachia* and *T. utriculata*, which represent distantly related, independent origins of the CAM adaptation, emphasize that the circadian clock most likely regulates this fine balancing between PEPC and Rubisco activity at different times across the light period in a wide diversity of CAM origins. An important role for the clock is likely because PEPC and Rubisco are clock controlled via PEPC kinase and RCA, respectively. However, it is also clear that the specific timing of the transitioning and balancing between PEPC and Rubisco differs between CAM species, which in turn emphasizes the need to study the carboxylase interplay over the light period in a much greater diversity of CAM plants. Furthermore, far greater attention should be paid to studying these aspects of CAM under natural dawn and dusk transitions in light, temperature and humidity, as many lab experiments use square wave light/ dark cycles that do not reflect the gradual transitions that occur in the field.

Addressing the role of the circadian clock in balancing the two carboxylases during CAM directly, Davies and Griffiths [81] studied the facultative CAM species, *Mesembryanthemum crystallinum* (common iceplant) under constant light (LL) and temperature conditions. LL conditions are a key experimental method to establish whether or not the endogenous circadian clock is regulating the observed temporal changes. Again, instantaneous carbon isotope discrimination measurements on CAM-induced leaves measured under circadian free-running LL conditions demonstrated that there was a transition from a Rubisco-mediated C_3 discrimination signal in the early hours of the subjective night, to a C_4 discrimination due to PEPC late in the subjective dark [81]. This study also demonstrated that in vitro Rubisco activation state reached a 24 h minimum at the start of the dark period under light-dark (LD) cycles, but then rose for much of the dark period, perhaps pre-empting dawn. The activation state also reached a minimum shortly after subjective dawn under LL conditions, coincident with the timing of maximal malate decarboxylation [81]. Under LL, Rubisco activation state reached its peak level in the subjective dark period, overlapping with the timing of the Rubisco-dominated isotope discrimination signal in the first hours of the subjective dark, and prior to the transition to a PEPC-dominated C_4 discrimination signal later in

the subjective dark. Furthermore, immunoblot analysis showed that two isoforms of RCA were detected in C_3 leaves of *M. crystallinum* when measured every 24 h across 3 days of LL conditions, whereas the larger RCA isoform was barely detectable in the CAM-induced leaves under LL. These findings, spanning multiple independent origins of CAM across monocots and eudicots, emphasize that there is a delicate interplay between the regulation and balancing of the two competing carboxylases in CAM leaves, with circadian control important for the optimal timing of peak PEPC activity in the dark, and Rubisco in the light.

4. Insights into Calvin-Benson-Basham cycle operation in C_4 photosynthesis and Crassulacean acid metabolism from omics

In this era when high-throughput omics approaches have been leveraged to delve more deeply and globally into a wide diversity of biological questions, it is not surprising that advances are being made possible in the fields of C_4 and CAM research through applying genome-, transcriptome-, proteome- and metabolome-wide approaches. Early insights into genome content, organization, and evolution were achieved through the publication of the sorghum and maize genomes for C_4 [82,83], and the genomes of two orchid (Orchidaceae) species, *Phalaenopsis equestris* and *Dendrobium officinale*, and *Ananas comosus* for independent origins of CAM in monocots, plus *K. fedtschenkoi* for CAM in a eudicot [84–86]. Since these initial breakthroughs, complete genome sequences have continued to be published for species representing independent origins of both C_4 and CAM [87]. Further novel insights into each pathway have also been gained from several detailed large-scale transcriptome, proteome, and/or metabolome datasets generated from photosynthetic tissues of a range of C_4 and CAM species to answer specific questions on C_4 photosynthesis and CAM [88–96]. The availability of such 'big data' for a wide diversity of C_4 and CAM species offers unprecedented opportunities to elucidate the complete genetic blueprint for each pathway, and for a range of independent evolutionary origins. In contrast to most previous work, it is now possible to globally explore the regulation and functioning of the genes, transcripts, proteins, and metabolites of the CBB cycle in a wide diversity of C_4 and CAM species. As C_4 and CAM species use the CBB cycle for secondary CO_2 fixation to provide the building blocks supporting wider metabolism, growth, and development, it is clear that understanding the regulation and operation of the CBB cycle and its integration with the C_4 cycle and CAM are vitally important for achieving a complete understanding of both pathways. Such work is now possible through analyses of existing published datasets.

4.1. C_4 photosynthesis

In addition to solidifying mesophyll and/or BS cell locations of CBB enzymes (Section 3.1) [41,42], proteome analyses have also given insight into CBB enzyme expression patterns during the differentiation of mesophyll and BS chloroplasts in maize [55]. A developmental gradient exists in grass leaves with cells at the leaf base containing proplastids and those near the tip fully differentiated mesophyll and BS chloroplasts [97]. Using maize leaf and BS-strand sections along this developmental gradient for proteome analysis, Majeran et al. [55] showed the differential enrichment of CBB cycle enzymes seen in mesophyll and BS chloroplasts at the leaf tip begins at about 4 cm from the leaf base, at the source and sink transition zone. Plastids at the base of the leaf were found to lack CBB cycle enzymes although mesophyll and BS chloroplasts could be distinguished in electron micrographs of sections between 2 and 3 cm from the leaf base due to differences in grana stacking (Section 3.1) [55]. Interestingly, the abundance of C_4 cycle enzymes (Section 2.1; Figs. 2, 3) also begins to increase in leaf cells located about 4 cm from the base, indicating the activities of the enzymes in this cycle are closely linked with those of the CBB cycle [55]. While the above work has increased our knowledge of CBB cycle operation during leaf development in a NADP-ME-type C_4 species, corresponding information for NAD-ME- and PCK-type species is lacking. A systems biology study reported Rubisco protein amounts did not differ between mature and immature phytomers of *Setaria italica*, another NADP-ME-type C_4 species [98]; however, no information was reported for other CBB enzymes. Consequently, it is also not known if C_4 species of the same decarboxylation subtype exhibit similar developmental patterns.

Variation in CBB cycle operation among different C_4 species of the NADP-ME subtype has been detected at the level of CBB cycle intermediates in recent metabolite profiling studies, with the diversity attributed to differing levels of 3-PGA/TP shuttle activity in the four species (Section 3.1; [65,99] and Clapero et al. [100], this special issue). Principal component analyses in these studies also showed that RuBP was the driver separating the C_3 and C_4 species examined; C_4 taxa have lower RuBP levels, enabled by the presence of a CCM [65,99,100]. Metabolite profiles of C_3, C_3-C_4 (species showing leaf anatomy and/or photosynthetic biochemistry intermediate to C_3 and C_4 plants), C_4-like (species lacking optimization of C_4 anatomy and/or biochemistry), and C_4 species of *Flaveria* have shown the C_4 species have higher levels of 3-PGA relative to the non-C_4 congeners [101]. Few differences in the levels of CBB cycle intermediates were found between the C_3 and C_3-C_4 intermediate *Flaveria* species, whereas RuBP, Fru-6 P; Fru-1,6-BP, sedoheptulose 7-phosphate (Sed-7 P), and sedoheptulose 1,7-bisphosphate (Sed-1,7-BP) (Fig. 1) levels showed an overall trend toward lower levels in the C_4-like species relative to the C_3-C_4 species and lower again in the true C_4 species [101]. These modifications are consistent with the integration of the CBB cycle in the progressive evolution of a full C_4 syndrome [102].

At the transcript level, RNA-Seq datasets have been generated from whole leaf tissue of closely related members of the Cleomaceae, *Flaveria*, Paniceae, and *Moricandia* for comparative transcriptomic studies [103–106]. Not surprisingly, relative to the C_3 comparators, a decreased abundance of transcripts encoding CBB cycle enzymes was found in all the C_4 species [104–106]. Bräutigam et al. [105] suggested this down-regulation of expression should be considered in strategies to engineer a C_4 pathway into C_3 crop plants as it, along with reduced expression of proteins in the photorespiratory pathway and protein translation, may be important for achieving the high PNUE demonstrated by C_4 plants (Section 2.3). The genus *Moricandia* contains C_3 and C_3-C_4 photosynthetic intermediate species but no true C_4 species [107]. A decreasing trend in the abundance of transcripts coding for most of the CBB cycle enzymes was found for two C_3-C_4 intermediate *Moricandia* species relative to the C_3 congener *M. moricandioides*, and differences between the intermediate species were apparent [103]. This decrease in transcripts coding for CBB cycle enzymes in *Moricandia* C_3-C_4 intermediates, however, was not paralleled in the C_3-C_4 intermediate *F. ramosissima*, where only transcripts encoding Rubisco were decreased relative to the levels of the *Flaveria* C_3 congener [104]. In addition, an increase in transcripts encoding components of the C_4 cycle accompanied the decrease in transcripts coding for the CBB cycle enzymes in *Flaveria* C_3-C_4 and C_4 species but not in *Moricandia* photosynthetic intermediates [103].

Predictably, transcript profiles along leaf developmental gradients from the NADP-ME-type C_4 grasses maize, sugarcane, and the non-model *Antaenanthia lanata*; the NAD-ME-type C_4 eudicot *Gynandropsis gynandra*; and C_3 and proto-Kranz (incipient C_3-C_4 photosynthetic intermediate) comparators showed investment in transcripts encoding CBB cycle enzymes was high in mature regions of the leaf but lower in C_4 than in C_3 or proto-Kranz species [71,108–112]. In the case of maize for which corresponding proteome information is available [55], a high correlation was seen between CBB cycle enzyme transcript and protein profiles across the gradient [71,109]. Interestingly, *G. gynandra* exhibited correlated delays in the accumulation of mRNAs encoding CBB cycle enzymes and leaf cell differentiation relative to its C_3 congener *Tarenaya hassleriana* [110]. A co-expression study using leaf developmental transcriptomes of sorghum, *Setaria viridis*, maize, and rice found

Ald, FB, and SB are important in regulating carbon flux through the CBB cycle and it was suggested these enzymes should be considered in crop improvement strategies [113]. Further analysis of these developmental datasets would likely add to our understanding of how the components of the CBB and C_4 cycles become integrated to produce a full C_4 syndrome.

Information on the mesophyll and BS cell compartmentation of transcripts encoding CBB enzymes has also been provided for diverse C_4 species, including maize, *S. viridis*, *G. gynandra*, and *Panicum virgatum* [108,109,114–116]. This cell-specific information has highlighted some differences and similarities within and between decarboxylation subtypes of C_4 species. A high correlation was reported between transcriptomes from laser-capture microdissected maize mesophyll and BS cells and previous mesophyll and BS cell proteome datasets, with transcripts encoding CBB cycle enzymes enriched in BS cells [109,116]. Consistent with enzyme activity data (Section 3.1) and maize proteome and transcriptome studies [41,42,109,116], transcripts encoding the CBB cycle-associated GAPDH, PGK, and TPI were more abundant in mesophyll cells than in BS cells of the two NAD-ME-type species *G. gynandra* and *P. virgatum*, while mRNAs coding for other CBB cycle enzymes preferentially accumulated in the BS cells [108,114]. Compartmentation of transcripts encoding CBB cycle enzymes between mesophyll and BS cells from *S. viridis* was overall very similar to that of maize [115]; however, two exceptions indicate differences exist in CBB cycle operation and regulation in these two NADP-ME-type species. Transcripts encoding PGK are more abundant in maize mesophyll cells, as is the cognate protein [41,42], whereas in *S. viridis*, BS cells preferentially accumulate mRNA coding for PGK [115]. It was suggested that this difference may be due to posttranscriptional or posttranslational mechanisms controlling PGK expression in the mesophyll of *S. viridis* [115]. Posttranscriptional or translational regulation was suggested to explain the difference in accumulation of transcripts encoding ribose 5-phosphate isomerase (RPI) in the two species. In *S. viridis*, mRNA coding for RPI is enriched in BS cells [115], which agrees with the compartmentation of the maize RPI protein [41,42]; however, in maize, transcripts encoding RPI show preferential accumulation in the mesophyll [109,115].

4.2. Crassulacean acid metabolism

Despite the extensive nature of the published quantitative RNA-Seq transcriptome analysis datasets for a wide diversity of CAM species that represent both obligate/constitutive CAM and facultative/inducible CAM, and that span many independent origins, few insights into the regulation of transcripts encoding the individual enzymes of the CBB cycle have been highlighted to date. In some cases, a decline in the transcript abundance of genes encoding the small subunit of Rubisco (*RBCS*) has been documented (e.g., [91,93]). Furthermore, in *Portulaca oleracea*, a species with C_4 photosynthesis that induces weak CAM (low, but detectable, net atmospheric CO_2 fixation to malate in the dark) in response to drought-stress, both *RBCS* transcripts, and transcripts for most of the enzymes of the photorespiratory pathway, were down-regulated in response to drought and thus coincident with increased CAM [91]. However, detailed insights into the transcript-level regulation of all genes encoding CBB cycle enzymes in CAM species are hiding in plain sight in the published RNA-Seq datasets. Re-analysis of these datasets with a specific focus on the genes encoding CBB cycle enzymes will yield new understanding that can guide more detailed studies into the regulation of associated CBB cycle enzymes and their regulators, and the associated metabolite levels of the CBB cycle intermediates and levels of flux through the pathway during phase III of CAM.

Within our own quantitative RNA-Seq datasets for *K. fedtschenkoi* leaves, comparing the 12-h-light/12-h-dark temporal profile of all transcripts detected from leaf pair 1 (the youngest visible leaves at the shoot apical meristem, C_3) and leaf pair 6 (fully expanded leaves that use full CAM defined by only performing phase I and phase III), a number of intriguing observations can be made regarding the regulation of transcripts encoding proteins associated with the CBB cycle. The most abundant *RBCS* transcript shifts its daily temporal peak by 12 hours from the end of the dark/beginning of the light period in leaf pair 1 C_3 to the end of the light/dusk in leaf pair 6 full CAM (Boxall, Dever, Kneřová and Hartwell, unpublished). The transcript levels of *PGK* display a very similar pattern to *RBCS*, both in terms of differential regulation at different diel times between leaf pair 1 C_3 and leaf pair 6 full CAM, and the 12-h shift in the timing of the daily peak transcript level; *PGK* transcripts also peak at the end of the light in leaf pair 6 full CAM. In addition, *RCA* displays a dramatic, up to 8-fold induction in leaf pair 6 full CAM relative to leaf pair 1 C_3, with the transcript level rising sharply throughout the dark period, reaching a peak 2 h before dawn. Genes encoding various chloroplast *GAPDH* subunits, two *ALD* transcripts, a ribose 5-phosphate epimerase transcript, *PRK*, *FB*, and transcripts for two *SB* isogenes were all induced in leaf pair 6 CAM to differing extents, and at a range of times across the 24 h cycle. By contrast, transcript levels for *TPI*, one of two isogenes encoding TK, and RPI were all repressed in leaf pair 6 CAM relative to leaf pair 1 C_3 (Boxall, Dever, Kneřová and Hartwell, unpublished). Although one of the major changes coincident with leaf development in *K. fedtschenkoi* is the transition from C_3 photosynthesis in leaf pair 1 to full CAM in leaf pair 6, it is important to note that this developmental range also spans from leaf pair 1 being sink leaves to leaf pair 6 being source leaves, and leaf pair 1 are unable to generate clonal plantlets from the notches in their margins when detached from the plant, whereas leaf pair 6 have gained the competence to develop leaf margin adventitious plantlets. Thus, the detected changes in the regulation of transcript levels for genes encoding CBB cycle enzymes between leaf pair 1 and leaf pair 6 may also be associated with these developmental changes in the biology of the leaves either in concert with a role in relation to CAM, or distinct from the development of full CAM in leaf pair 6.

These findings contrast with the current assumption that the CBB cycle is universally down-regulated following CAM development and/or induction due to the increased efficiency of Rubisco following the establishment of the CAM CCM. Furthermore, these results highlight the value of exploring the regulation of CBB cycle gene transcript levels in the transcriptome datasets for the many other diverse CAM species that are already published and awaiting re-analysis. Such studies can help to identify key genes coding for CBB cycle enzymes in CAM species that merit more detailed investigation through the generation of transgenic mutant lines using techniques such as RNAi and gene editing [36,117,118].

5. Engineering improvement

Overcoming stagnating crop productivity for current and future food, fodder, biofuel, and bioproduct demand is a major goal for plant scientists [119]. Identification of targets and development of strategies to improve CO_2 assimilation in C_4 plants are complicated by the separation of the CO_2 assimilation reactions between two cell types, including flux through the PGA/TP shuttle and differing electron transport capacity between mesophyll and BS cells, as well as the presence of mixed decarboxylation pathways. Nevertheless, several recent reviews have highlighted targets and strategies to improve both C_4 and C_3 crop performance [68,120–127]; however, little or no attention has been directed at enhancing CAM. Below are summarized transgenic experiments in C_3 and C_4 species that have given insights into the regulation of the CBB cycle (Fig. 2) that may lead to crop improvement.

5.1. Current knowledge gleaned from transgenic studies in C_3 and C_4 plants

5.1.1. Rubisco

As the primary carboxylase in C_3 plants, Rubisco is limiting for CO_2 assimilation and CBB cycle function in these species [128]; however, antisense experiments in *Flaveria bidentis* indicated Rubisco is also a major determinant limiting CO_2 assimilation at saturating light levels in this C_4 eudicot species, with photosynthetic rate decreasing in transformants as Rubisco content and activity decreased (Fig. 2;Table 1) [129,130].

Further evidence of the importance of Rubisco in the photosynthetic flux of C_4 plants has come from the overexpression of genes encoding maize Rubisco SSU, LSU, and Rubisco activation factor 1 (RAF1) in maize (Table 1) [132]. These LSSS-RAF1 transformants showed increases in Rubisco content, CO_2 assimilation, growth, and biomass production relative to wild type (WT) maize. Moreover, while most C_4 plants demonstrate decreases in CO_2 assimilation under chilling conditions [133], the LSSS-RAF1 transformants outperformed WT maize in photosynthetic and growth characteristics as well as recovery following the chilling period [131]. The LSSS-RAF1 transformants also demonstrated better recovery, e.g., higher CO_2 assimilation and biomass, following drought stress than WT maize although no benefits in photosynthesis or growth relative to the control plants were observed during the stress treatment [134]. These results support earlier work that implicated Rubisco content and/or activity as limiting photosynthetic capacity in several C_4 species under chilling stress [133,135–141] and water limitation [142,143].

Either partial or complete replacement of rice Rubisco SSU with sorghum homologues led to a Rubisco with C_4-like kinetics, including a higher k_{cat} and K_m for CO_2 and a reduced specificity for CO_2 (Table 1) [50,52]. No change in CO_2 assimilation at the various CO_2 concentrations tested was apparent in the plants containing a mixture of rice and sorghum SSU isoforms [50] and, except for a reduction in non-productive tillers in the transformants, there were no obvious growth differences relative to WT rice. By contrast, transformants containing only sorghum SSU demonstrated higher CO_2 assimilation rates than WT rice and equal biomass production when CO_2 concentrations were higher than ambient levels [52]. The above results indicate manipulation of Rubisco has the potential to increase CO_2 assimilation, growth, and yield under non-stress and stress conditions in both C_3 and C_4 crops.

5.1.2. Sedoheptulose-1,7-bisphosphatase

Knockdown experiments in a number of C_3 plants, including tobacco (*Nicotiana tobacum*), have shown that small reductions in SB (Fig. 1) activity (>9%) result in a >12% reduction in carbon assimilation (A_{max}), growth rates, and biomass yields [144–147]. These data demonstrate that SB exerts strong regulation over CBB cycle flux, identifying it as a good target for crop improvement strategies.

Several studies have overexpressed the target enzyme SB, in an effort to increase plant biomass (Fig. 2; Table 2). In the model plant *A. thaliana* , transgenic plants showed a 42% increase in plant biomass and 39–53% increase in seed yield compared to WT [148]. In tobacco, constitutive overexpression of SB resulted in a 40% increase in shoot biomass under controlled greenhouse conditions [149,150]. More recently, the overexpression of SB in wheat was shown to result in an increase in biomass yield and more importantly, a 30–40% increase in seed yield [151] and in tomato, photosynthesis increased by 20–25%, increasing vegetative biomass by 30%, resulting in early onset flowering [152]. These results demonstrate that the manipulation of this CBB cycle enzyme can result in biomass/yield increases in model crops, a cereal crop, and a fruiting crop, suggesting it is a viable route for the manipulation of carbon assimilation in plants (see [122,127] for review). However, a contrasting result was observed when SB was overexpressed in the C_4 plant *Setaria viridis* (Fig. 2; Table 2).

Transgenic plants with 1.5- to 3.2-times increase in SB levels showed no correlation between SB content and saturating rates of CO_2 assimilation [155]. This result suggests that alternate limiting steps may exist in C_4 plants compared to C_3 species. It should be noted that targeting multiple steps in the CBB cycle in C_3 plants can result in a step change in assimilation increase [122,127]. In C_4 plants, it may be necessary to target multiple steps to illicit the same response observed in C_3 plants, while also considering that limiting steps in CBB cycle function in C_4 plants may be different to those in C_3 plants.

5.2. Other targets and opportunities to enhance photosynthesis

For C_4 species, non-CBB cycle targets have been suggested or predicted to improve C_4 photosynthesis and increase yield under non-stress and stress conditions, including chloroplast electron transport, transpirational water loss, bundle-sheath leakiness, C_4 cycle enzyme activity, and transport/diffusion of metabolites between mesophyll and BS cells [58,68,120,124,156,157]. While enhancing CAM has not been a research focus, potential non-CBB targets for engineering CAM in C_3 plants to improve photosynthesis have been identified [20,158–163] and include genes encoding proteins involved in the C_3-to-CAM transition, stomatal regulation, and PWUE. In addition, the genetic variation in wild relatives of modern-day C_4 and CAM crop species is largely an untapped resource and offers potential novel strategies to overcome limitations in productivity [124,158,160,164–166].

Table 1
The impact of manipulating ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubsico) expression on CO_2 assimilation, biomass, and yield in C_3 and C_4 plants. Nuclear located transgenes introduced under the control of photosynthetic tissue specific promoters, or a constitutive promoter are specified. Growth conditions are indicated: Controlled Environment * , Greenhouse * *. SSU, small subunit; LSU, large subunit; RAF1, Rubisco activation factor 1.

Plant Type	Plant	Transgene (s) Expressed			Functional Description	Biomass and Yield	Ref
C_4	*Flaveria bidentis*	Anti-sense Rubisco SSU	-	-	Constitutive expression. Decrease in CO_2 assimilation.	Decrease in plant growth rates and biomass in plants with < 20% wild type activity.	[129, 130] *, * *
	Maize	Maize SSU	maize LSU	maize RAF1	Constitutive expression. Significant increase in CO_2 assimilation.	Increase in plant growth and biomass.	[131] *, * *
C_3	Tobacco	*F. pringlei* or *F.* floridana or *F. bidentis* LSU	-	-	Plastome transformation. Decrease in CO_2 assimilation.	Decrease in growth in air.	[51] *
	Rice	Sorghum SSU	-	-	Tissue-specific expression. Partial replacement of rice SSU. No change in CO_2 assimilation. C_4 Rubisco kinetic traits.	Decreases in non-productive tillers.	[50] *
	Rice	Sorghum SSU	-	-	CRISPR/Cas9. Complete replacement of rice SSU. Increases in CO_2 assimilation at ambient CO_2. C_4 Rubisco kinetic traits.	Decreases in total dry weight at ambient CO_2.	[52] *, * *

Table 2
The impact of the overexpression of sedoheptulose 1,7-bisphosphatase (SB) on plant growth in C_3 and C_4 plants. Transgenes were either under the control of photosynthetic tissue specific promoters or a constitutive promoter. Growth conditions are indicated: Controlled environment * , Greenhouse * *, Field experiments * ** .

Plant Type	Plant	Functional Description	Biomass and Yield	Ref
C_3	Arabidopsis	Tissue specific expression. 37–85% increase in SB activity, 37% increase in CO_2 assimilation.	42% increase in dry weight and a 53% increase in seed yield.	[148]*
	Tobacco	Constitutive expression. 90–110% average increase in SB activity, Increase in photosynthetic rates. Increases in sucrose and starch content.	30–34% increase in dry weight.	[149, 150] * *
	Tomato	Constitutive expression. 55–139% increase in SB activity. An approximately 25% increase in CO_2 assimilation. Increases in sucrose and starch content.	Up to 39% increase in dry weight in best lines.	[153] * *
	Wheat	Constitutive expression. Up to 90% increase in SB activities in some lines. Increase in CO_2 assimilation.	Up to 40% increase in grain yield.	[151] * *
	Rice	Constitutive expression. Up to 200% increase in SB activities in some lines. Increased CO_2 assimilation rates under elevated temperature.	Higher growth rates under elevated temperature.	[154] * *
C_4	*Setaria viridis*	Bundle-sheath cell-preferential promoter. Transgenic plants with 1.5- to 3.2-times higher SB content. No correlation between SB content and saturating rates of CO_2 assimilation.	No increase in development or biomass was reported.	[155] *

6. Conclusions and future prospects

It has been well over half a century since the reactions making up the CBB cycle and the C_4 and CAM photosynthetic pathways were elucidated [1,11,167]. Nevertheless, there are significant gaps in our knowledge of how the ancestral C_3 biochemistry became integrated and operates in C_4 and CAM syndromes and these limitations restrict our abilities to improve plant photosynthetic performance, biomass, and yield.

Experiments to increase our understanding of CBB cycle operation more fully in CAM plants include instantaneous online discrimination measurements to ascertain the temporal interplay between the two carboxylases across the 24-h light/dark cycle. These studies should include a diverse range of CAM types representing independent CAM origins as well as facultative CAM species under well-watered and drought-stressed conditions. Similar species surveys are also needed to fill the knowledge gap regarding PNUE in CAM species. For C_4 plants, resolution of the presence and operation of mixed decarboxylation pathways and/or a 3-PGA/TP shuttle in individual species will contribute to attempts to enhance C_4 photosynthetic efficiency. The future work needs to include a focus on the integration of the CBB cycle with these pathways in species from diverse C_4 monocot and eudicot lineages. For both C_4 and CAM species, the kinetics and regulation of CBB cycle enzymes reflect significant gaps in our knowledge.

The abundance and regulation of transcripts encoding CBB cycle enzymes have not been a focus of most transcriptome studies of C_4 and CAM photosynthetic tissues; consequently, the level of information reported in the scientific literature for these transcripts is variable. In addition, metabolite profiles and proteome datasets have been generated for only a very limited number of C_4 and CAM species. These information gaps highlight opportunities to not only re-examine and further mine existing datasets, but to also expand omics technologies to additional CAM types and C_4 decarboxylation subtypes from diverse evolutionary lineages, including more taxa with closely related congeners that use different photosynthetic pathways and wild relatives of crop plants. The resulting knowledge will increase our molecular toolbox, aiding attempts to bioengineer improvements in photosynthetic efficiency and crop yields.

A multi-target approach will likely be needed to enhance photosynthesis in crop plants. Generating bespoke C_4, CAM, and C_3 crops for growth conditions and environments of the future to meet global food and fodder, fiber, and fuel demands will undoubtedly involve manipulating the expression of a combination of enzymes and pathways using both modern breeding strategies and targeted genetic manipulation.

Role of funding sources

The funding sources had no role in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Declaration of Competing Interest

As corresponding author of the manuscript entitled, "**The Calvin-Benson-Bassham cycle in C_4 and Crassulacean acid metabolism species**", I submit this declaration of interest on behalf of all the authors: Martha Ludwig, James Hartwell, Christine A Raines, and Andrew J Simkin. For all the authors, there are no declarations of interest.

Acknowledgements

ML was supported by the Australian Research Council, (DP180102747). JH was supported by the Biotechnology and Biological Sciences Research Council, UK (BBSRC grant no. BB/F009313/1), the US Department of Energy (DOE) Office of Science, Genomic Science Program under Award Number DE-SC0008834, and a Newton Advanced Fellowship funded by the Royal Society, UK (grant no. NA140007 awarded to Prof. Luciano Freschi and JH). The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of DOE. CAR was supported by the European Union's Horizon 2020 research and innovation programme (No. 862201) project CAPITALISE. AJS was supported by the Innovate UK Growing Kent and Medway Program, UK (Ref 107139).

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