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Cyanobacterial CO_2-concentrating mechanism components: function and prospects for plant metabolic engineering

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Global population growth is projected to outpace plant-breeding improvements in major crop yields within decades. To ensure future food security, multiple creative efforts seek to overcome limitations to crop yield. Perhaps the greatest limitation to increased crop yield is photosynthetic inefficiency, particularly in C_3 crop plants. Recently, great strides have been made toward crop improvement by researchers seeking to introduce the cyanobacterial CO_2-concentrating mechanism (CCM) into plant chloroplasts. This strategy recognises the C_3 chloroplast as lacking a CCM, and being a primordial cyanobacterium at its essence. Hence the collection of solute transporters, enzymes, and physical structures that make cyanobacterial CO_2-fixation so efficient are viewed as a natural source of genetic material for C_3 chloroplast improvement. Also we highlight recent outstanding research aimed toward the goal of introducing a cyanobacterial CCM into C_3 chloroplasts and consider future research directions.

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Introduction

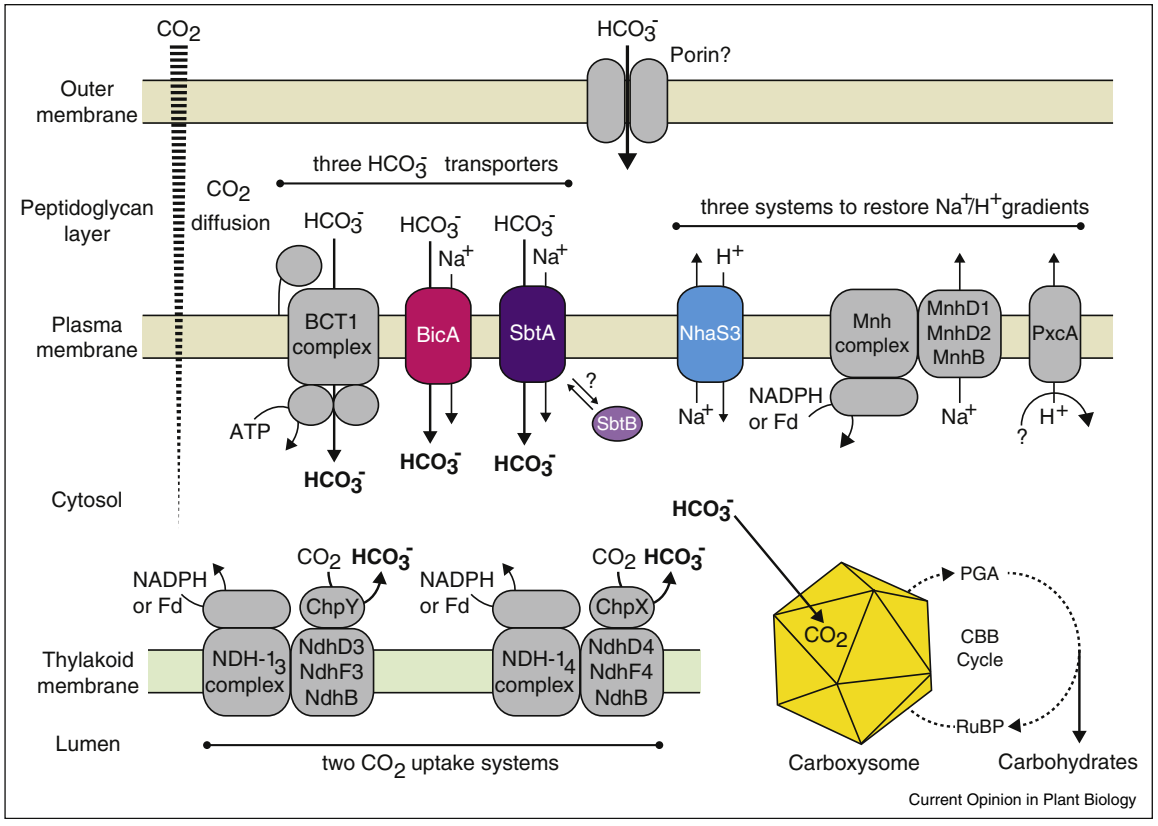
A prime limitation of plant photosynthetic carbon acquisition is the enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), whose slow kinetics and poor discrimination between CO_2 and O_2 as substrates makes it an ideal target for genetic improvement in crop plants [1]. In addition, C_3 chloroplastic CO_2 concentrations typically fall below the $K_M^{CO_2}$ of RuBisCO, often leaving photosynthetic carbon acquisition limited by this critical carbon-fixation step. To overcome RuBisCO's limitations, a number of terrestrial plant species have evolved CO_2-concentrating mechanisms (CCMs), including C_4 photosynthesis and crassulacean-acid metabolism, which elevate CO_2 concentrations near RuBisCO to enhance CO_2-fixation [2]. In aquatic environments, photosynthetic organisms (predominantly microalgae and cyanobacteria) have evolved highly effective CCMs that rely on a range of active and facilitated uptake systems for inorganic carbon (C_i; HCO_3^-, CO_2) to enhance photosynthetic CO_2-fixation [3]. Most of the world's major staple crops perform C_3 photosynthesis, suggesting improvement of net RuBisCO carboxylation rates is a way forward to increased yield potential, urgently needed for future food security [4]. It is expected, based on photosynthetic modelling, that a C_3 chloroplast possessing a cyanobacterial CCM will provide significant improvements in photosynthetic performance and yields [5••,6]. Toward this goal, herein we discuss the current progress, engineering requirements and limitations in current knowledge of cyanobacterial CCMs.

Cyanobacterial CCMs include energised bicarbonate transporters, CO_2-uptake complexes [7], and ancillary and regulatory proteins [8] which elevate the cytoplasmic C_i pool, primarily as less membrane-permeable bicarbonate ion (Figure 1). Cytoplasmic bicarbonate is transferred across a selectively permeable protein shell into the carboxysome, a RuBisCO micro-compartment [9,10], where it is dehydrated to CO_2 and incorporated into 3-phosphoglycerate (Figure 1) [9,10]. This arrangement favours cyanobacterial RuBisCOs, which have high enzyme fluxes (high V_{CO_2}) compared with higher plant RuBisCOs, but at the cost of substrate affinity (high $K_M^{CO_2}$) [11]. Elevated CO_2 within the carboxysome enables high kinetic turnover, minimizing photorespiration.

Prior work from our laboratory has outlined a hypothetical C_3 chloroplastic CCM incorporating cyanobacterial components [6,12]. A first-order analysis reveals an engineering trajectory (further outlined in Figure 2):

1. Introduction of active bicarbonate transporters and potential ancillary systems.
2. Introduction of carboxysomes and carboxysomal RuBisCO.

Figure 1

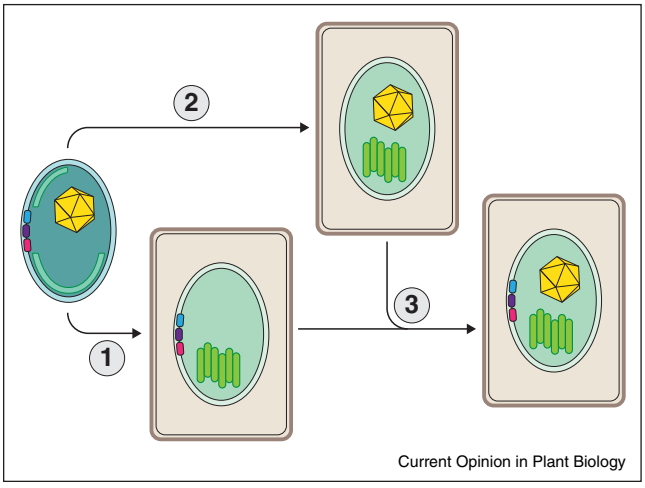


The CCM of cyanobacteria. Protein components discussed in this review are highlighted, whilst those outside the expected chloroplast engineering strategy are shown in grey. The cyanobacterial CCM utilises three known bicarbonate transporters: BicA (magenta), SbtA (purple), and the BCT1 complex (an ATP-binding cassette transporter comprising Cmp A, B, C and D subunits). Further, two thylakoid-bound CO_2 uptake complexes recover cytoplasmic CO_2: NDH-I_3, and NDH-I_4. These are supported by at least three ion exchangers which recover the chemical gradients energising active Ci-uptake: Na^+/H^+ antiporter NhaS3 (blue); and PxcA and Mnh complexes exporting H^+. The potential role of the companion protein SbtB (light purple) in SbtA activation is indicated. The accumulated bicarbonate pool is utilised within the carboxysome (yellow), where bicarbonate is dehydrated to CO_2 and fixed into organic carbon by RuBisCO in the Calvin–Benson–Bassham cycle (CBB). Low rate, diffusive movement of CO_2 into the cell is indicated on the left of the figure. CO_2 uptake complexes also play a role in scavenging of CO_2 lost from carboxysomes. Locations of the proteins and structures within the cell are indicated in Figure 2.

3. Genetic deletion of stromal CA and endogenous RuBisCO.

Mathematical modelling shows that substantial increases in substrate-saturated CO_2 assimilation rate (A_{sat}) and yield can be achieved (A_{sat} increase up to 60%; 36–60% yield increase) by incorporation of a cyanobacterial CCM in C_3 chloroplasts [5••]. At minimum, photosynthetic improvements can be made by incorporation of functional cyanobacterial bicarbonate transporters in the chloroplast inner membrane. However, maximum improvements will only be achieved by incorporation of further components of a complete CCM (Figures 1 and 2; Table 1) and genetic deletion of stromal carbonic anhydrase (CA) [5••,6]. The importance of ancillary systems involved in aiding C_i uptake by maintenance of electrochemical gradients (Figure 1) is currently either unknown or unclear [6] and will not be discussed further. The bicarbonate transporters, BicA and SbtA (Figure 1), have been suggested as primary candidates for chloroplast engineering since they likely function as homomeric complexes, thus eliminating the need to express and assemble different protein subunits in correct stoichiometry. In contrast, engineering of multi-component C_i uptake systems (Figure 1) such as BCT1, NDH-I_3 and NDH-I_4 (NADPH dependent, CO_2 uptake complexes) will only marginally increase photosynthesis [5••], but add the complication of coordinated expression of both membrane-associated and cytosolic proteins. While the construction of a fully functional carboxysome in the chloroplast stroma is equally challenging, early steps have recently been made [13,14]. We discuss here some of the recent advances, remaining questions and constraints toward carboxysome formation in C_3 chloroplasts.

Figure 2



Engineering a C_3 chloroplastic CCM. Mathematical models suggest that the ideal engineering trajectory occurs in three general stages [5••,6]. **(1)** Active bicarbonate transporters [BicA (magenta), SbtA (purple)] from cyanobacteria are introduced into the chloroplast inner membrane within a C_3 plant cell (brown); subsequently, ancillary Na^+/H^+ pumps [Na^+/H^+ antiporter NhaS3 (blue)]. **(2)** Carboxysomes (yellow) with their cognate RuBisCO are introduced. **(3)** Stromal carbonic anhydrase (CA) enzymes, as well as the endogenous RuBisCO, are eliminated and carboxysomes and transporters are combined in the one chloroplast. Genetic removal of the endogenous chloroplastic RuBisCO LSU gene can be carried out during incorporation of carboxysomal RuBisCO via recombination [14]. The genetic excision or down-regulation of stromal CA has little effect on photosynthetic performance in WT plants [69] and can therefore be carried out at any time. However, the existence of stromal CA will short-cut a chloroplastic CCM by dissipating the stromal bicarbonate pool as CO_2 [5••] and must at least be a final step in the process before maximal advantage of the CCM can be achieved. Further steps are likely required for fine-tuning of the system.

Challenges of expressing functional bicarbonate transporters in chloroplasts

A milestone toward the generation of a chloroplastic CCM was the successful expression of the cyanobacterial bicarbonate transporter BicA in chloroplasts of *Nicotiana tabacum* via plastome transformation [15•]. However, predominant localization of BicA to the thylakoids, and lack of function, highlights the challenges in correct targeting and activation of foreign transporters in chloroplasts. Indeed, BicA expressed in *Escherichia coli* [16••] or in *Xenopus* oocytes (Förster, B., and Price, G. D., unpublished) is also inactive. It is therefore paramount to understand the activation mechanism in order to use BicA effectively in the chloroplast envelope. While plastome-encoding alleviates the need for organellar targeting peptides, the fact that most C_3 crop plants are recalcitrant to plastid transformation makes expression from the nucleus a promising alternative.

Table 1

Protein components of α-carboxysomes and β-carboxysomes and RuBisCO ancillary proteins.[a]

Component	Carboxysome type	
	α	β
RuBisCO	Form 1A	From 1B
RuBisCO chaperones/ activases	acRAF, CbbQ/O, CbbX	RbcX, Rca
Structural proteins	CsoS2 (A/B)	CcmM (long/short), CcmN
Shell proteins	CsoS1A–E	CcmK (1–4), CcmO, CcmP
Shell vertex proteins	CsoS4A/B	CcmL
Carbonic anhydrase	CsoSCA (CsoS3)	CcmM (N-term), CcaA

[a] Location of most RuBisCO ancillary proteins within the carboxysome is not yet known, although CbbQ and CbbO have recently been identified as components of the *H. neapolitanus* carboxysome shell [38].

To be functional in higher plants, nuclear-encoded cyanobacterial bicarbonate transporters BicA and SbtA need to be: first, efficiently targeted to chloroplasts; second, inserted in the correct membrane; third, in an orientation allowing transport of bicarbonate into chloroplasts; and fourth, active and regulated.

Chloroplast targeting and orientation in the envelope

Chloroplast protein import is well understood, and generally relies on a cleavable chloroplast transit peptide (cTP) [17]. Efficient import of foreign proteins is more difficult and until recently data only existed for soluble proteins, in which case both a cTP and part of the mature protein are required (a combination called a transit peptide, TP) [18,19]. No 'universal' TP able to deliver any cargo to the chloroplast is known, and instead TPs are likely to be cargo-specific. In fact, a recent study showed that TPs used for stromal cargos were too short to target large transmembrane proteins such as BicA and SbtA, to the inner envelope membrane [20]. However, longer TPs were sufficient for chloroplast targeting, without the need for extra transmembrane domains [20]. In a separate study, BicA and SbtA were targeted to chloroplasts by fusion to another transmembrane protein which could be cleaved off post-import [21].

Chloroplasts contain three membranous compartments: the outer-envelope and inner-envelope membranes (OEM and IEM), and the thylakoids. The OEM is a porous membrane and, to present knowledge, only one of its proteins contains an identifiable cTP [22]. The IEM is the selective membrane in which most of the transporters regulating solute fluxes are located [23,24], and is the proposed membrane in which BicA and SbtA need to be localized in order to effect bicarbonate transport [12].

Overexpression of proteins in the IEM or OEM result in differently shaped membrane out-foldings (stromules), suggesting it is possible to identify the membrane containing the protein of interest [25].

The thylakoids are fused at focal points with the IEM, such that the IEM-thylakoids form a continuous membrane [26], and protein movement between the two membranes is not well understood. However, the membrane domain of proteins with a single trans-membrane-domain (TMD) contains sufficient information to target it to the IEM or the thylakoid [27]. The case of foreign proteins with multiple TMDs such as BicA and SbtA, which have evolved to be localized in the plasma membrane in cyanobacteria [7] is likely to be more complex [20].

BicA and SbtA likely follow the positive-inside rule governing their orientation in the plasma membrane [28,29], but it is unknown how foreign proteins insert into the plastid IEM. Therefore it is critical to assess protein orientation *in vivo*. As described recently, a self-assembling split GFP-fluorophore may be used to unravel the orientation of transporters in the IEM [30,31,32,33].

Regulation of BicA and SbtA transport activity

Rapid regulation in response to C_i availability and light is a common feature of all C_i transporters [7]. However, the underlying post-translational regulatory mechanisms in cyanobacteria are poorly understood. Insights from heterologous expression in *E. coli* suggest that SbtA may be directly regulated by a companion protein SbtB [16••]. A mechanism for SbtA–SbtB interaction has been proposed based on similarities of an unpublished SbtB crystal structure from *Anabaena* (PDB: 3DFE), with cyanobacterial PII proteins that regulate nitrogen metabolism [(16, and references therein)]. By analogy to the regulation of the *E. coli* ammonia channel AmtB by its PII protein counterpart GlnK [34], it is conceivable that small-molecule co-factors may mediate a reversible binding of SbtB to SbtA. Thus, we view SbtA as being manageable once expressed in the chloroplast envelope.

Although we presently have no experimental evidence for SbtA or BicA function in higher plants, it is promising that several algal bicarbonate transporters localize to equivalent sub-cellular compartments both in *Chlamydomonas* and higher plants [35•]. Two of those transporters (LCIA and HLA3) facilitate bicarbonate uptake into *Xenopus* oocytes, albeit exerting no measurable phenotype *in planta*, re-iterating the necessity to understand regulation of transporters and the need for highly sensitive assays for bicarbonate transporter activity in plant cells.

Carboxysomes: expression and engineering in C_3 chloroplasts — current advances

Carboxysomes are protein mega-complexes containing the cellular RuBisCO and CA enzymes in model species [9,10] (and elsewhere in this issue, [70]). Formation of these bodies in cyanobacteria and proteobacteria relies on the stoichiometric expression of as few as eight, or as many as 13 genes (Figure 3). Two architecturally distinct types of carboxysomes are evident; α-carboxysomes possess bacterial RuBisCO form-1A, whereas β-carboxysomes encapsulate higher-plant RuBisCO form-1B [36]. Both types utilise many thousands of homologous proteins in tessellated arrays forming an icosahedral outer shell structure [37]. The outer shell is selectively permeable, allowing transit of RuBisCO substrates and the bicarbonate ion, but limited CO_2 and O_2 diffusion (Figure 1; Table 1) [38].

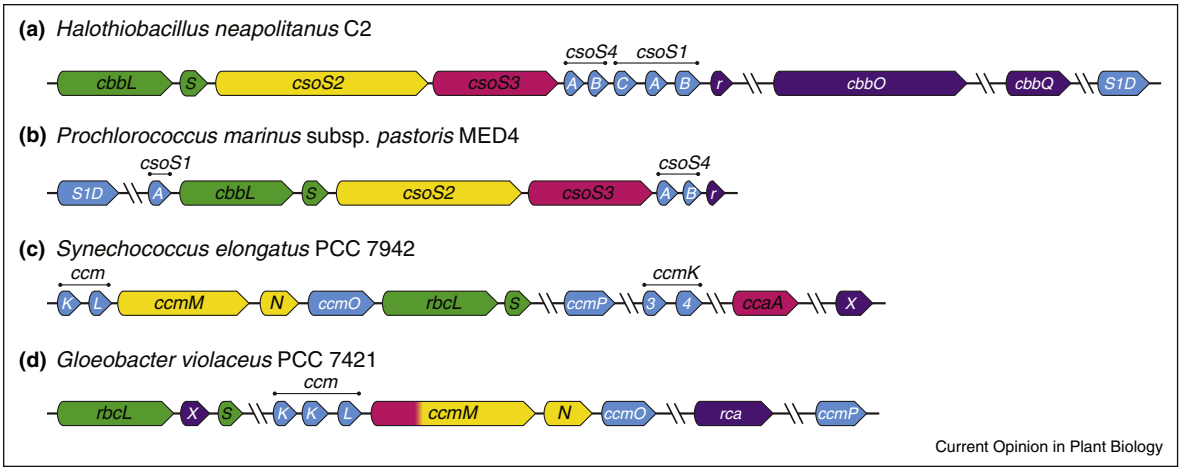
Complex requirements for RuBisCO folding and activation

Additional to the challenge of successful carboxysome construction in the chloroplast stroma is the expression of a fully functional, foreign, carboxysomal RuBisCO [11]. Attempts at ectopic expression of RuBisCO proteins have been hampered by the complex chaperone requirements of this enzyme [11]. Depending on the species of origin, numerous RuBisCO chaperones are utilised (Table 1) [39,40•,41•]. The extent to which these chaperone requirements can be satisfied by the C_3 chloroplast remains a topic of extensive research, as is the chaperone requirement of *bona fide* C_3 RuBisCO enzymes [42]. While numerous barriers prevent the effective expression of even closely related RuBisCO enzymes in C_3 chloroplasts, recent advances suggest cognate chaperones are required [43•]. Further studies have also elaborated the roles of α-carboxysome-specific RuBisCO chaperones and activases acRAF, CbbQ-CbbO, and CbbX (Table 1) [40•,41•,44,45]. On the other hand, β-carboxysomal RuBisCO requires the Rca activase (Table 1) [46], but not RbcX when co-expressed with RuBisCO in *N. tabacum* [14].

Structural and ancillary proteins

Gross differences in structure exist among carboxysomes (Table 1). Both types utilise a CA enzyme, however β-carboxysomes may use either of two genes for this purpose [47]. The main body of RuBisCO enzymes is arranged differently in α-carboxysomes and β-carboxysomes [recently reviewed in depth, 9]. RuBisCO is attached to the shell via CsoS2 proteins in α-carboxysomes [48,49••], whereas in β-carboxysomes RuBisCO enzymes are scaffolded to one another, and to CA enzymes, by CcmM proteins [50,51]; the shell is attached to this body via another structural protein CcmN [52]. Lin *et al.* [13] successfully co-expressed CcmM and RuBisCO from *Synechococcus elongatus* PCC 7942 in chloroplasts of *N. tabacum*, resulting in apparent macromolecular complexes, in some ways reminiscent of structures upon which β-carboxysomal models are based [53,54,55•]. However, similar to transgenic plants expressing form II RuBisCO in chloroplasts [56], those generated by Lin

Figure 3



Genetic operons and associated genes encoding model carboxysomes, and carboxysomes suited to expression in chloroplastic CCMs. In all panels: green arrows are the RuBisCO large and small subunit genes; purple are known or putative RuBisCO activases and chaperones; magenta are carboxysomal carbonic anhydrases (CA); blue are outer shell proteins including vertex proteins; and yellow are structural proteins (Table 1). **(a)** α-Carboxysomes from the gammaproteobacterium *Halothiobacillus neapolitanus* C2 are the best understood of the α-type, and utilise the acRAF chaperone r [44], and CbbQ/O activase [41•]. **(b)** A comparatively simple α-carboxysome operon from *Prochlorococcus marinus*, utilising the acRAF (r) chaperone only, although its absolute requirement is unknown. Other cyanobacterial α-carboxysomes appears to also utilise a CbbX activase [58]. **(c)** Genetic components encoding the best understood β-carboxysome from *Synechococcus elongatus* PCC 7942, utilising the putative chaperone RbcX (X), and the additional carboxysomal CA CcaA. D. A comparatively simple β-carboxysome operon from *Gloeobacter violaceus* PCC 7421, utilising the RuBisCO activase Rca, and the CcmM protein both as a structural component and its N-terminal CA [47].

et al. [13] could not grow without CO_2 supplementation, highlighting the important fact that we do not expect cyanobacterial RuBisCO alone, or in the carboxysome, to operate effectively in the absence of bicarbonate transport or stromal CA removal [5••,6].

Complex carboxysome genetics

Carboxysomes with the smallest genetic footprint are the most appealing for C_3 chloroplast transformation (Figure 3). With lower genetic load, and minimal potential for recombination between homologous shell genes, we expect that simple carboxysomes might be better able to self-assemble in chloroplasts. While α-carboxysomes and β-carboxysomes appear to assemble from their components with remarkable ease, complex expression of the major organising proteins of both types is troubling. Two forms of CcmM are present in β-carboxysomes, with a short form arising from an internal ribosome-entry site within *ccmM* [50]. Two isoforms of the α-carboxysomal protein CsoS2 arise from the gene via translational frameshifting in many organisms [49••]. Both forms of CcmM are essential to the functioning of the β-carboxysome [54], hence it must be ascertained whether these can be accurately produced in C_3 chloroplasts. On the other hand, it appears that only the long-form of CsoS2 is truly essential in α-carboxysomes [49••].

To achieve similar goals, Gonzalez-Esquer *et al.* [57••] generated carboxysome-like bodies from a single carboxysomal fusion-protein, CcmC (containing shell, CA, and RuBisCO-binding domains). Further work is required to align the physiology with the observed structure, but we recognise this innovative work to have significantly advanced the field.

Ideal carboxysomes from genomic data

Candidate operons encoding the minimal gene requirements for both α-carboxysomes and β-carboxysomes formation exist (Figure 3): *Gloeobacter violaceus* PCC 7421 has the smallest known β-carboxysome gene content, encoding just five shell proteins, and utilising CcmM as its CA enzyme [47]. Candidate α-carboxysome operons are evident in the high-light adapted *Prochlorococcus marinus* clade [58], possessing the simplest known α-carboxysome operons, and also likely to use a single form of CsoS2 [49••,58]. Typically, we recognise α-carboxysomes as most desirable in terms of gene content, self-assembly in transgenic hosts, and simplicity of gene expression.

Because C_3 carboxysome models are sensitive to RuBisCO kinetics [5••], we must take into account the varied kinetics observed in extant RuBisCO enzymes. Essentially, a carboxysomal RuBisCO should operate near its maximum rate of catalysis. Therefore, β-carboxysomal RuBisCO enzymes, being catalytically superior to α-carboxysomal homologues [59,60,61,62•,63,64,65] might be preferred. Interestingly, the *Cyanobium* genus possess a high affinity CCM and fast α-carboxysomal

RuBisCO [62•,66], overcoming this shortfall, hence we view *Cyanobium* carboxysomes as bearing many desirable traits for chloroplast engineering. Nonetheless, a dearth of kinetic data for RuBisCOs from α-carboxysomes suggests that an ideal α-carboxysomal RuBisCO candidate for a chloroplastic CCM is yet to be identified.

Conclusions

Recent advances toward a chloroplastic CCM [13,14,15•,35•] are vital first steps, but highlight shortcomings in achieving this goal. These developments emphasise the need to examine targeting and regulation (in the case of C_i transporters), and issues of protein folding and activity (in the case of carboxysomes). Indeed, our efforts focus on engineering C_3 chloroplasts with simple cyanobacterial systems, such as the single-subunit C_i transporters BicA and SbtA in the hope that regulation is achievable. This extends to genetically simple carboxysomes, which are apparent from both types (Figure 3). The difficult nature of β-carboxysome purification [67] makes them problematic in confirming their functional presence in transgenic plants. Whereas, α-carboxysomes are readily expressed in, and purified from, tractable bacterial and cyanobacterial systems [48,58,68]. Despite the catalytic superiority of β-carboxysomal RuBisCOs, we identify a lack of data for α-carboxysomal RuBisCOs and work in this field is needed. In what is a rapidly developing field of research, our expectations for progress toward a chloroplastic CCM utilising cyanobacterial components are optimistic.

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