A long-term strategy to enhance global crop photosynthesis and yield involves the introduction of cyanobacterial CO₂-concentrating mechanisms (CCMs) into plant chloroplasts. Cyanobacterial CCMs enable relatively rapid CO₂ fixation by elevating intracellular inorganic carbon as bicarbonate, then concentrating it as CO₂ around the enzyme Rubisco in specialized protein micro-compartments called carboxysomes. To date, chloroplastic expression of carboxysomes has been elusive, requiring coordinated expression of almost a dozen proteins. Here we successfully produce simplified carboxysomes, isometric with those of the source organism Cyanobium, within tobacco chloroplasts. We replace the endogenous Rubisco large subunit gene with cyanobacterial Form-1A Rubisco large and small subunit genes, along with genes for two key α-carboxysome structural proteins. This minimal gene set produces carboxysomes, which encapsulate the introduced Rubisco and enable autotrophic growth at elevated CO₂. This result demonstrates the formation of α-carboxysomes from a reduced gene set, informing the step-wise construction of fully functional α-carboxysomes in chloroplasts.
Photosynthetic CO₂ fixation via ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the primary input of carbon into crop biomass. However, Rubisco-mediated CO₂ fixation in C₃ chloroplasts is catalytically slow, competitively inhibited by oxygen and, from an agricultural stand-point, makes inefficient use of water and combined nitrogen. These latter inefficiencies are driven by passive acquisition of CO₂ from the air (leading to water loss via open stomata) and by large investment in Rubisco (up to 50% of leaf protein) to overcome its poor kinetics. A suggested approach to increase CO₂ fixation, minimize water-loss and decrease investment in Rubisco is to translate essential components of the cyanobacterial CO₂-concentrating mechanism (CCM) into C₃ crops.

The cyanobacterial CCM is a single-cell, bipartite system that first generates a high intracellular bicarbonate (HCO₃⁻) pool through action of membrane-bound inorganic carbon (Ci) transporters and CO₂-converting complexes (Fig. 1a). This HCO₃⁻ pool is then utilized by subcellular micro-compartments called carboxysomes, which encapsulate the cell’s complement of Rubisco. The carboxysome’s outer protein shell enables diffusional influx of HCO₃⁻ and RuBP, where the former is converted to CO₂ by a localized carbonic anhydrase (CA). Physiological evidence and mathematical models suggest carboxysomes resist CO₂ efflux, resulting in concentration of CO₂ around Rubisco. Cyanobacterial carboxysomes possess high-catalytic-turnover, but low-CO₂-specificity Rubisco enzymes. When the intracellular HCO₃⁻ pool is elevated, a high CO₂ environment can be generated inside the carboxysome, overcoming this low specificity and enabling rapid CO₂ fixation with reduced inhibition by oxygen.

An engineering strategy to generate a chloroplastic CCM in crop plants (Fig. 1b) relies on transfer of genes encoding HCO₃⁻ transporters, directed to the chloroplast inner-envelope membrane (IEM), to generate an elevated stromal HCO₃⁻ pool, and genes encoding the carboxysome and its Rubisco. Active HCO₃⁻ transporters in the chloroplast IEM alone are expected to improve photosynthesis due to the elevation of CO₂ concentrations around Rubisco. Notably, either a carboxysome-
encapsulated or free cyanobacterial Rubisco in C₃ plant chloroplasts will effectively lead to high CO₂ requirement for growth because cyanobacterial Rubiscos have low affinity and specificity for CO₂. Stromal HCO₃⁻ pools in C₃ plants grown in air approximate 0.5 mM, but the cyanobacterial cytoplasm reaches concentrations between 5 and 20 mM, despite low external C₂, to drive the CCM. In combination, a high stromal HCO₃⁻ pool generated by active HCO₃⁻ transporters and a fully functional carboxysome where CO₂ can be elevated could improve C₃ plant CO₂ fixation and yield up to 60% of CO₂ fixation machinery. Elimination of the native stromal CA and C₁ Rubisco to further improve the accumulation of HCO₃⁻ within the stroma is required to realize an optimal functioning chloroplastic CCM.

Within this proposed engineering strategy, construction of the carboxysome is particularly challenging due to genetic and protein-organizational complexity and requirements for functionality; some carboxysomes require coordinated expression of 13 genes. Carboxysomes are a subset of proteinaceous bacterial microcompartments (BMCs), with specialized CO₂ anabolic function. Two carboxysome types have arisen through convergent evolution: α-carboxysomes encapsulate Form-1A Rubisco in proteobacteria and some unicellular cyanobacteria, and β-carboxysomes encapsulate the plant-like Form-1B Rubisco in the remaining cyanobacteria. Noting that the biogenesis and composition of each carboxysome type is unique, components of the β-type lumen have been successfully expressed in Nicotiana tabacum (hereafter tobacco) chloroplasts. This showed that cyanobacterial Form-1B Rubisco could be successfully expressed and cross-linked with CcmM35 to form large aggregates in the chloroplast. Additionally, transient expression studies showed that carboxysome shell proteins could interact and form structures within chloroplasts. However, these attempts could not generate structural carboxysomes nor encapsulate Rubisco, key requirements to generate CO₂ around Rubisco and for overall CO₂ fixation and yield. While carboxysomes have been heterologously expressed in bacterial systems, there are currently no reports of α- or β-carboxysome biogenesis in eukaryotic systems. This study, we designed simplified α-carboxysomes inspired by those from Cyanobium marinus PCC7001 (hereafter Cyanobium). Cyanobium carboxysomes likely consist of a protein shell primarily made up of CsoS1A, interspersed with proteins CsoS1D and/or CsoS1E (Fig. 1c). Together, these proteins are envisaged to provide a selectively permeable shell, allowing HCO₃⁻ and RuBP into the carboxysome and 3-PGA release but limiting CO₂ efflux. Within the carboxysome, CsoSCA, a CA on the inner shell surface, converts accumulated HCO₃⁻ to CO₂. Rubisco (comprising CbbL and CbbS subunits) is likely anchored to the shell via CsoS2, which arises as two isoforms from one gene in many α-carboxysomal species but only one isoform in Cyanobium. The pentameric vertex proteins (CsoS4A and CsoS4B) complete the icosahedral structure, since their absence can lead to elongated carboxysomes with aberrant assembly design, with the potential for Rubisco encapsulation upon their co-expression. We constructed multigene cassettes for tobacco chloroplast transformation that contained genes for Cyanobium Rubisco large subunit (LSU, cbbL) and small subunit (SSU, cbbS) or these genes in combination with those for α-carboxysome proteins CsoS1A and CsoS2 (Fig. 2). These genetic expression constructs were introduced into the tobacco plastome where they replaced the endogenous Rubisco LSU gene.

Here we report an example of structural carboxysomes, encapsulating a cyanobacterial Form-1A Rubisco, expressed in plant chloroplasts. The primary outcome is the formation of structurally identifiable and purifiable carboxysome structures, formed with Rubisco and just two shell proteins (CsoS1A and CsoS2). This provides a proof-of-concept for the construction of complete and functional carboxysomes within the chloroplast.

Results

Generation of transgenic plants. Chloroplasts of the Rhodosphilium rubrum-tobacco (cm₁₆) master line carrying the single-subunit Rubisco (RbcM) were transformed with gene expression cassettes for Cyanobium Rubisco only (CyLS) or CyLS in concert with the carboxysome proteins CsoS1A and CsoS2 (CyLS-S₁S₂; Fig. 2) using biolistic bombardment. Transgene expression cassettes also carried the spectinomycin selection marker gene, adaA, and transformants were recovered through selective tissue culture. Successful plantlets were subsequently grown in soil at 2% (v/v) CO₂ to flowering and seed collection. Southern blot analysis using a DNA probe specific to common sequence in wild-type, cm₁₆, and transgenic plants confirmed the presence of DNA fragments of the anticipated size (Fig. 2). In addition, the complete loss of the R. rubrum rbcM DNA fragment indicated that transformant lines were homoplasmic (Fig. 2).

Protein content in transformed tobacco leaves. To determine whether successful introduction of transgenes led to production of cyanobacterial proteins, we conducted sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of protein in leaf extracts. This confirmed the presence of the LSU and SSU of Cyanobium Rubisco in CyLS plant leaves, along with both carboxysome structural proteins CsoS1A and CsoS2 in CyLS-S₁S₂ leaves (Fig. 3a). Using antibodies specific to the R. rubrum Rubisco (RbcM) in the the cm₁₆ host plants, we confirmed the absence of RbcM from CyLS and CyLS-S₁S₂ leaves. An antibody which detects both the tobacco RbcL and Cyanobium CbbL proteins confirmed that CyLS and CyLS-S₁S₂ lines contained only the Cyanobium Rubisco. We also noted the presence of small quantities of the nuclear-encoded tobacco SSU (RbcS) in CyLS-S₁S₂ leaf material and the successful expression of Cyanobium CbbS in transformants.

Leaf ultrastructure reveals carboxysome formation. To determine whether carboxysome protein expression led to ultrastructural chloroplast changes, we carried out transmission electron microscopy (TEM) of ultrathin leaf sections. Chloroplasts of CyLS plants showed no observable abnormalities, with chloroplasts of these plants typical of those found in wild-type leaves (Fig. 3b). CyLS-S₁S₂ chloroplasts, however, contained multiple electron-dense particles (Fig. 3c, d), similar to Cyanobium α-carboxysomes. Closely packed, localized clusters of geometric structures, approximately 100 nm in diameter, were observed in most chloroplast sections of CyLS-S₁S₂ plants. Presuming that the structures observed in CyLS-S₁S₂ chloroplasts were carboxysomes, a variation of an α-carboxysome purification technique was used to isolate particles from leaf tissue. The same method was also used to isolate carboxysomes.
Fig. 2 Cyanobacterial Form-1A Rubisco and carboxysome gene constructs. a Plastome content at the Rubisco large subunit locus in the cm\textsuperscript{brL} (Rhodospirillum rubrum Rubisco—\textit{rbcM}) master line\textsuperscript{37}, wild-type tobacco (WT) and transformant plants (CyLS and CyLS-S\textsubscript{S1S2}). The recipient line, cm\textsuperscript{brL}, was transformed via homologous recombination with constructs CyLS and CyLS-S\textsubscript{S1S2} (GenBank accession numbers MH051814 and MH051815) containing Cyanobium PCC7001 Rubisco \textit{cbbL} and \textit{cbbS}, together with carboxysome shell gene \textit{csoS1A} (S1A) and \textit{csoS2} sequences, codon optimized for expression in \textit{N. tabacum} chloroplasts. The \textit{Cyanobium} \textit{cbbL} sequence was also codon matched to the tobacco \textit{rbcL} gene where there was amino acid identity.

Transformation vectors also contained the \textit{aadA} selection marker under control of the \textit{NtPpsbA} promoter to allow growth of transformants on spectinomycin. Each transformation construct was flanked by partial plastome \textit{atpB} and \textit{accD} sequence, and transformants were generated using biolistic bombardment. The locations of promoters (P), terminators (T) and intercistronic expression elements (I) are indicated. P\textsubscript{1}, \textit{NtPpsbA}; T\textsubscript{2}, \textit{AtTpetD}; T\textsubscript{3}, \textit{NtPps16}; T\textsubscript{4}, \textit{AtTpsbA}; T\textsubscript{5}, \textit{NtPpsbA} originally from \textit{pcmtrLA37}; T\textsubscript{6}, \textit{NtTrps16}. A DNA probe was constructed by PCR to anneal to the \textit{accD} flanking region in each plastome (black bar), and the corresponding size of Bsp119I (Bsp) digestion fragments are shown for each genotype (in bp).

b DNA blots of total leaf DNA digested with restriction enzyme Bsp119I and probed with the \textit{accD} probe, indicating successful insertion of transgenes and loss of the cm\textsuperscript{brL} genotype. DNA fragment sizes in kbp are shown.

Protein content in isolated carboxysomes. Both the carboxysomes isolated from CyLS-S\textsubscript{S1S2} plants and genuine \textit{Cyanobium} carboxysomes were subjected to SDS-PAGE and immunoblots to determine protein presence and identity. The protein content of plant-derived carboxysomes was consistent with the protein complement of wild-type \textit{Cyanobium} carboxysomes (Fig. 3h).

Since wild-type \textit{Cyanobium} carboxysomes consist of at least nine polypeptides and those of CyLS-S\textsubscript{S1S2} plants only four, there was relatively more of each protein in plant-derived carboxysomes as a proportion of total protein (Fig. 3h). We also noted that the CyLS-S\textsubscript{S1S2} carboxysomes were generally of higher purity than those isolated from \textit{Cyanobium} (Supplementary Fig. 2). Nonetheless, both CyLS-S\textsubscript{S1S2} leaves and their isolated particles contained CbbL, CbbS, \textit{CsoS1A} and \textit{CsoS2} in similar proportion to carboxysomes from \textit{Cyanobium} (Fig. 3a, h). The complete suite of \textit{Cyanobium} carboxysome proteins was absent from those in CyLS-S\textsubscript{S1S2} plants due to the minimized gene set utilized. Despite the presence of small quantities of tobacco Rubisco SSU leaf extracts of CyLS-S\textsubscript{S1S2} plants (Fig. 3a), it was absent from isolated particles, indicating no hybrid \textit{Cyanobium}-tobacco Rubisco formation within the carboxysomes (Fig. 3h).

We identified CsoS2 as a single protein isoform in both \textit{Cyanobium} carboxysomes and CyLS-S\textsubscript{S1S2} particles (Fig. 3a, h). The SDS-PAGE banding pattern of CsoS2 in CyLS-S\textsubscript{S1S2} plant extracts is indicative of potential degradation in the leaf (Fig. 3a), but notably the relatively clean band in isolated carboxysomes suggests that degraded protein is not incorporated into the carboxysome (Fig. 3h).

Using current knowledge of carboxysome protein interactions and the minimal protein set used to generate the structures found in CyLS-S\textsubscript{S1S2} plants, we formulated a structural model of the carboxysomes formed in these plants (Fig. 3i). This model highlights the absence of specific shell components (i.e. proposed facet proteins CsoS1D and CsoS1E and vertex proteins CsoS4A and CsoS4B) and the internal carbonic anhydrase, CsoSCA, compared to the model of the \textit{Cyanobium} carboxysome (Fig. 1c).

Immunogold localization and aberrant carboxysome formation. Immunogold labelling of CyLS-S\textsubscript{S1S2} chloroplast TEM sections, using an antibody specific to the carboxysomal shell protein CsoS1A, showed an association of gold particles with the carboxysome structures (Fig. 4a–c). This confirmed that the structures observed in situ contained CsoS1A. This analysis highlighted the presence of occasional rod-shaped particles in chloroplasts that also reacted positively to CsoS1A antibodies. Approximately 4% of carboxysomes observed in chloroplast sections appeared to be elongated rods (Fig. 4c–f, Table 1). These rod-shaped structures were also present in isolated carboxysome fractions (Fig. 4) at a rate of approximately 16% of purified carboxysome particles (Table 1). They were of variable length but of regular diameter [59 ± 5 (s.d.) nm], with sub-structural particles of ~12 nm in diameter, which we interpreted as Rubisco (Fig. 4f).

\textbf{Cyanobacterial Form-1A Rubisco-dependent plant growth.} Presuming that inclusion of the \textit{Cyanobium} Rubisco in tobacco chloroplasts, either alone or within carboxysomes, should lead to a high CO\textsubscript{2} requirement for growth (Fig. 1), we characterized the
Rubisco and plant CO\textsubscript{2} assimilation characteristics of transformed plants. Analysis of Rubisco catalytic performance in clarified leaf extracts of CyLS and CyLS-S\textsubscript{1S\textsubscript{2}} plants revealed an enzyme with high catalytic turnover rate (\(k_{\text{cat}}\)) and Michaelis–Menten constant for CO\textsubscript{2} (\(K_{C}\)), consistent with values previously reported for the \textit{Cyanobium} enzyme (Table 2 and ref. 18). Both CyLS and CyLS-S\textsubscript{1S\textsubscript{2}} plants demonstrated autotrophic growth at 2% (v/v) CO\textsubscript{2} (Fig. 5). Both transgenic plant types contained similar quantities of Rubisco but ten-fold less than wild-type tobacco (Table 2). For CyLS plants, leaf

Fig. 3 Carboxysomes are synthesized in tobacco chloroplasts from four proteins. a SDS-PAGE and immunoblots of Rubisco and carboxysomal proteins expressed in leaves of the recipient plant line (cm\textsuperscript{+}), wild-type tobacco (WT) and the transformant lines CyLS and CyLS-S\textsubscript{1S\textsubscript{2}}. b Transmission electron micrographs (TEM) of chloroplasts from tobacco expressing \textit{Cyanobium} Rubisco (CyLS plants) and tobacco expressing \textit{Cyanobium} Rubisco along with the shell proteins CsoS\textsubscript{1A} and CsoS\textsubscript{2} (CyLS-S\textsubscript{1S\textsubscript{2}} plants). c, showing aggregations of electron-dense particles of approximately 100 nm. The inset in e at higher magnification (d). Scale bars 500 nm for images b–d. Negatively stained carboxysomes purified from CyLS-S\textsubscript{1S\textsubscript{2}} plants (e) and carboxysomes purified from \textit{Cyanobium} cyanobacterial cells (f). Scale bars for purified carboxysomes 100 nm. g Diameters of carboxysomes from wild-type \textit{Cyanobium} cells (cyan line) and carboxysomes purified from CyLS-S\textsubscript{1S\textsubscript{2}} plants (magenta line) determined using a Nanosight particle analyser. h SDS-PAGE and immunoblots of proteins in particles isolated from CyLS-S\textsubscript{1S\textsubscript{2}} leaves and genuine carboxysomes from cultured \textit{Cyanobium} cells (\textit{Cyanobium} cbx). i A model of the carboxysome structures produced in transgenic CyLS-S\textsubscript{1S\textsubscript{2}} plant chloroplasts indicating the four protein components required to generate the structure. Comparison can be made with the complete wild-type structural model presented in Fig. 1c in which the pentameric vertex proteins (CsoS\textsubscript{4AB}), ancillary shell proteins (CsoS\textsubscript{1D} and CsoS\textsubscript{1E}) and carbonic anhydrase (CsoSCA) are present.
photosynthetic CO₂ response curves were consistent with the quantity and catalytic performance of the Cyanobium Rubisco, confirmed by modelling of photosynthetic rates using the Rubisco catalytic properties of the Cyanobium enzyme (Fig. 5, Table 2). However, the very low rates of CO₂ fixation by both transformants at the CO₂ concentrations provided in gas exchange experiments (Fig. 5a, b), coupled with the high $K_c$ of their Rubisco and its potential progressive deactivation as CO₂ decreases in the gas-exchange chamber, provided potentially misleading information about true photosynthetic performance of these plants. To gather more information regarding the CO₂ assimilation phenotypes of CyLS and CyLS-S1S2 plants, we conducted measurements at high CO₂ using a membrane inlet mass spectrometer (MIMS).

Photosynthetic CO₂ responses of leaf discs by MIMS enabled analysis of photosynthetic assimilation rates up to 20 mbar CO₂ (~2% v/v, Table 2; Fig. 5c), mimicking physiological conditions that are predicted in a fully functional chloroplastic CCM. These conditions indicated a capacity for CyLS plants to reach similar photosynthetic rates to their wild-type counterparts (Table 2; Fig. 5c) despite lower Rubisco content, consistent with the catalytic properties and content of the cyanobacterial Rubisco. MIMS analysis of CyLS-S1S2 plants revealed lower CO₂ assimilation rates at 20 mbar CO₂ (Table 2; Fig. 5c). This was consistently lower than mathematical modelling would indicate, based on Rubisco content and catalysis. CyLS-S1S2 plants also took considerably longer to reach maturity (Fig. 5d–j).

Catalytic parameters of isolated carboxysomes. To investigate the role that Rubisco encapsulation might play in the CO₂ assimilation phenotype of the CyLS-S1S2 plants, carboxysomes were isolated from plant tissue and their functionality compared against the free Rubisco or the CyLS-S1S2 Rubisco (Supplementary Fig. 3). We found with measurements of both CO₂ and RuBP supply to the enzyme that the plant-derived carboxysomes showed a significantly lower $k_{cat}$ than either of its counterparts (Table 2, Supplementary Fig. 3), indicating that a sizable percentage of internalized Rubisco active sites were not capable of the expected rate of catalysis compared to naked Rubisco.

To determine whether the low $k_{cat}$ of the CyLS-S1S2 carboxysomal Rubisco resulted from incorrect formation of L₇S₈ holoenzymes, we assessed the relative stoichiometry of Rubisco isolated from CyLS plants with that found in enriched CyLS-S1S2 and wild-type Cyanobium carboxysomes using western blots (Supplementary Fig. 4). This confirmed that the plant-derived carboxysomes contained Rubisco with stoichiometry not significantly different to that of either the free enzyme from CyLS plants or that of Cyanobium carboxysomes.

Assuming that the catalytic phenotype of the plant-derived carboxysomes might result from inactivation of CyLS-S1S2 carboxysomal Rubisco, we attempted to compare the catalytic performance of Rubisco in intact carboxysomes with that of the free enzyme after free–thaw treatment commonly used to break the structures and release free Rubisco. While this was extremely successful for Cyanobium carboxysomes, we could not achieve significant rupture of carboxysomes from CyLS-S1S2 plants (Supplementary Fig. 2). Instead, to examine the relative performance of free Rubisco compared to that of carboxysome-encapsulated Rubisco from CyLS-S1S2 leaf extracts, we used the supernatant fraction of crude leaf homogenates after high-speed centrifugation that contained Rubisco but was depleted in carboxysome proteins (Supplementary Fig. 5). This revealed that the free Rubisco from CyLS-S1S2 plants had both a lower $K_{MRuBP}$ and a higher $k_{cat}$ than its encapsulated counterpart (Table 2). Rubisco in broken Cyanobium carboxysome preparations maintained a high $k_{cat}$ but its $K_{MRuBP}$ was lower in the absence of the carboxysome shell (Table 2, Supplementary Fig. 3). As $K_c$ values did not appear to be diagnostic of carboxysome function in our assays, it was not determined for broken Cyanobium carboxysomes (Table 2).

Discussion

The expression of structurally intact carboxysomes within a C₃ plant chloroplast is a critical and complex engineering milestone towards the longer-term goal of attaining a functional chloroplastic CCM in C₃ crop plants83. The structures reported here mimic the gross structure of carboxysomes from Cyanobium but lack specific components expected to be required for full functionality in an operating CCM (viz. the CsoSCA, vertex proteins—CsoS4A and CsoS4B and potential metabolite-pore shell components CsoS1D and CsoS1E; cf. Figs. 1c and 3j). Despite these missing components, we show that a simplified set of proteins (consisting of the major
outer-shell component CsoS1A, the Rubisco linker CsoS2 and Rubisco itself) are capable of self-organizing to produce structural carboxysomes. This finding is an advance on previous attempts to generate either β-carboxysome shells or interlinked Rubiscos in chloroplasts and demonstrates a way forward to functional carboxysome construction in C3 plants.

Simplified carboxysomes in CyLS-S1S2 plants resembled those from Cyanobium but with predictable differences. Occasional elongated carboxysome structures in tobacco chloroplasts are consistent with observed structures resulting from the deletion of csoS4 vertex protein genes in _Halothiobacillus neapolitanus_. A similar phenotype is found in β-carboxysomes where a genetic lesion interrupts expression of the vertex protein homologue CcmL. Vertex protein mutants in both carboxysome types are functionally hindered, probably due to leakiness of the normally gas-tight shell. We therefore conclude that the elongated structures observed in CyLS-S1S2 plants and extracts result from the absence of csoS4A and csoS4B vertex protein genes in the expression cassette. The observed slightly rounded shape of CyLS-S1S2 carboxysomes (Fig. 3e) is possibly attributable to the absence of the CsoS1D protein, which is predicted to be a large metabolite, gated pore in the mature α-carboxysome shell and a likely contributor to shell rigidity.

The generation of carboxysomes with just four proteins represents a pivotal step in our understanding of α-carboxysome biogenesis. Despite the need for additional proteins to produce structurally identical carboxysomes to those of _Cyanobium_, as few as four proteins are required to make an encapsulating body. Thus, relatively simple rules lead to the self-assembly of icosahedral protein bodies, of the correct size, containing Rubisco. This confirms our hypothesis that CsoS1A, CsoS2 and Rubisco alone are required for simple carboxysome formation. More complex requirements for α-carboxysome structure have been assumed in the past. For example, construction of the α-carboxysomes of _H. neapolitanus_ in _Escherichia coli_ utilized an almost complete carboxysome operon of ten genes to achieve structural formation. Our results add further weight to a streamlined approach to carboxysome construction in plants, such as the synthetic domain-fusion approach described for β-carboxysomes, but highlights the existence of already relatively simple gene sets in some cyanobacteria for this purpose.

The relative simplicity of the _Cyanobium_ carboxysome makes it a suitable candidate for expression in chloroplasts. The utility of _Cyanobium_ as a genetic donor is further emphasized by the formation of a single gene product from csoS2 (Fig. 3a, h). CsoS2 plays a critical role in carboxysome formation in
α-cyanobacterial carboxysomes and is a highly disordered protein that contains recognizable repeat domains at the N-terminus and the middle (M) region of the protein and a unique C-terminal region that possibly protrudes through to the carboxysome exterior. In some α-carboxysomes, CsoS2 occurs as two isoforms (CsoS2A and 2B). Cyanobium, however, produces only one form of CsoS2, due to the lack of an internal frameshifting motif in the native gene sequence, which leads to C-terminal truncation during peptide synthesis in homologous sequences. Additionally, Cyanobium carboxysomes require only one CsoS1 shell protein (Fig. 1C,29). The model α-carboxysome from H. neapolitanus has three CsoS1 shell proteins (CsoS1A, B and C), as does its β-carboxysome counterpart from Synechococcus elongatus PCC7942. The α-carboxysome operons of the oceanic α-cyanobacteria are all relatively simple,29 while the Cyanobium Rubisco has kinetic parameters that approach those of the β-cyanobacteria, and outpace those found in its α-cyanobacterial Prochlorococcus relatives. Taken together, our results point to Cyanobium carboxysomes as ideal practical components for use in a chloroplast CCM.

The ability to isolate and analyse purified plant-generated carboxysomes with relative ease is important. The isolation of highly pure carboxysome fractions of α-carboxysomes enables aspects of their structure and functionality to be addressed in vitro. This ensures that analysis can confirm carboxysome function in C3 chloroplasts in the absence of the functional HCO3− transporters required to generate a working chloroplast CCM. This is likely to save considerable time within the chloroplasmatic engineering strategy. Here we have demonstrated the capability to analyse and evaluate aspects of function in our plant-expressed carboxysomes in comparison with those of Cyanobium.

Autotrophic growth of our transformed plants demonstrates the first example of C3 plants reliant on a high-catalytic-rate Form-1A Rubisco. The α-carboxysomal Form-1A Rubisco is phylogenetically distant from the Form-1B isofrom utilized by terrestrial plants, having arisen outside the plant lineage. Limited data are currently available for Form-1A Rubisco catalytic properties, and this report highlights that further research in this area may give rise to alternative sources of Rubisco for C3 photosynthesis augmentation. Furthermore, similar rates of photosynthesis in wild-type and CyLS leaf discs at 20 mbar CO2 (Table 2; Fig. 5c), despite 90% less Rubisco protein in CyLS leaves, highlights the potential to achieve photosynthetic performance with appreciable reduction in nitrogen investment towards Rubisco.

While there was no significant difference in Cyanobium Rubisco content between the two transformant lines, we consistently found a slightly lower content in the leaves of CyLS-S1S2 plants (Table 2) and lower assimilation rates at 20 mbar CO2 in MIMS assays (Fig. 5c). However, the assimilation rates of CyLS-S1S2 plants was lower than predicted by modelling (Fig. 5c), based on the measured Rubisco content in crude leaf homogenates, and maximum catalytic properties of the naked enzyme (Table 2). Importantly, the density of carboxysomes in leaf extracts leads to the separation of two populations of Rubisco in CyLS-S1S2 plants upon centrifugation (viz. insoluble carboxysomal Rubisco and free Rubisco). Thus observed maximum catalytic properties for CyLS-S1S2 Rubisco represents those of the naked enzyme, resulting from either a population of unpacked Rubisco or loss from carboxysomes during homogenization. On the other hand, our Rubisco content measurements from crude homogenates are not determined, n.a. not applicable

### Table 2. Rubisco catalytic properties, leaf Rubisco content and photosynthetic performance

| Rubisco source          | $k_{cat}$ (s−1) | $K_{M,RUBP}$ (μM) | $K_{C_3S2}$ (μM) | $K_{C_3S2}O_2$ (μM) | $S_{CO}$ (M M−1) | Rubisco (μmol m−2 s−1) at 20 mbar CO₂ | Assimilation rate (μmol CO₂ m−2 s−1) | CO₂ compensation factor (E, μbar) |
|-------------------------|----------------|-------------------|-----------------|-------------------|-----------------|--------------------------------------|-----------------------------------|----------------------------------|
| Tobacco                 | 3.1 ± 0.3      | 19 ± 3            | 9.7 ± 0.1       | 290 ± 3           | 18.3            | 80 ± 2.6                             | 21.9 ± 0.7                        | 27.6 ± 0.5                      | 55 ± 1                           |
| CyLS plants             | 9.8 ± 0.2      | 38 ± 1            | 158 ± 8         | 4724 ± 249        | 275 ± 8         | [8234 ± 240]                         | (n = 4)                           | (n = 3)                          | (n = 3)                          |
| CyLS-S1S2 plants        | Free Rubisco   | 9.4 ± 0.4         | 36 ± 1          | 169 ± 14          | 5063 ± 416      | 285 ± 13                             | n.d.                              | n.d.                             | n.d.                             |
| Isolated carboxysomes   | 4.9 ± 0.3      | 59 ± 3            | n.d.            | n.d.              | 248 ± 21        | [7430 ± 629]                         | n.d.                              | n.d.                             | n.d.                             |
| Cyanobium cells         |               |                   |                 |                   | n.d.            | n.d.                                 | n.d.                              | n.d.                             | n.d.                             |
| Broken carboxysomes     |               |                   |                 |                   | n.d.            | n.d.                                 | n.d.                              | n.d.                             | n.d.                             |

Leaf samples of wild-type tobacco plants, CyLS and CyLS-S1S2 plants, grown at 2% CO₂ (v/v), were extracted and prepared for Rubisco catalytic analysis as described in Methods. Tobacco $k_{cat}$ and $K_{C_3S2}$ values are from Sharwood et al.12 and $K_{M,RUBP}$ is from Whitney et al.13. Free Rubisco from CyLS-S1S2 plants was obtained after high-speed centrifugation of crude leaf homogenates to pellet insoluble carboxysomes. Carboxysomes from both CyLS-S1S2 plants and Cyanobium cells were isolated as described in Methods and those of Cyanobium were broken to release free Rubisco by freeze-thaw treatment. Rubisco specificity values for CO₂ ($S_{CO}$) were determined as described in Methods and are in vitro estimates from wild-type tobacco and CyLS plant Rubiscos. Data are means ± s.e.m. from 4 replicate measurements. Rubisco active site content of CyLS-S1S2 leaves was consistently lower than for CyLS leaves, but this was not statistically significant (P = 0.068; two-tailed, homoscedastic Student’s T test). Assimilation rates at 20 mbar CO₂ were determined for independent leaf discs via membrane inlet mass spectrometry analysis. Values in brackets are in μmol CO₂ M−2 s−1 and bar bar bar bar for $S_{CO}$ n.d. not determined, n.a. not applicable

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limiting carboxysome function in cyanobacteria. For example, mutations leading to carboxysome shell protein or CA loss (but maintenance of Rubisco encapsulation) result in phenotypes that can be overcome by growth at high CO2. Additionally, cyanobacterial Rubisco in aberrant carboxysomes is quickly activated at high CO2. We also found that both CyLS and CyLS-S1S2 plants achieved maximum photosynthetic rates more rapidly than wild-type tobacco (Supplementary Fig. 6), indicating rapid activation of Cyanobium Rubisco in tobacco chloroplasts. Thus, we reasoned that Rubisco activation status was both unlikely to provide an explanation for CyLS-S1S2 plant CO2 assimilation rate nor provide meaningful results in this case. Instead, we determined whether the catalytic properties of the Rubisco in plant-derived carboxysomes were impaired relative to the naked enzyme or that found in Cyanobium carboxysomes (Table 2). This revealed that Rubisco in plant-derived carboxysomes, lacking many of the protein factors found in wild-type carboxysomes, had a lower $k_{cat}$ than either their naked counterpart or that found in Cyanobium carboxysomes (Table 2). In addition, a lower $k_{cat}$ was predicted from modelling observed CO2 assimilation in MIMS assays (Fig. 5). A similar observation was made by Occhialini and co-workers where they successfully measured CO2 assimilation rate in transformed plants. Data are presented as means ($n=3-6$) ± s.e.m. Fitted lines (WT, black; CyLS, cyan; CyLS-S1S2, yellow) were calculated as the minimum of modelled Rubisco- and electron transport-limited rates of CO2 assimilation according to Farquhar et al. using the Rubisco catalytic parameters presented in Table 2, and a $J_{max}$ of 140 μmol m$^{-2}$ s$^{-1}$, at 25 °C. The model predicts Rubisco active site concentrations of 1.8 ± 0.4 (s.e.m.) and 1.7 ± 0.4 (s.e.m.) μmol sites m$^{-2}$ for CyLS and CyLS-S1S2 leaves, respectively, within the range determined from leaf tissue (Table 2). Equations are outlined in Methods. CO2 assimilation rates of leaf discs from each plant line from plants grown at 2% (v/v) CO2 in membrane inlet mass spectrometer (MIMS) assays, carried out as described in Methods. Solid lines (WT, magenta; CyLS, cyan; CyLS-S1S2, yellow) are averaged data from $n=3$ independent leaf discs (±s.d., shaded areas). The dashed lines for CyLS and CyLS-S1S2 are modelled assimilation rates using the same parameters as a, b. Using the model to estimate $k_{cat}$ for the Rubisco in CyLS-S1S2 leaf discs gives an estimate of 0.46 s$^{-1}$ ± 0.01 (s.e.m.) based on the measured Rubisco content in Table 2. Growth measured as plant height post germination (±s.e.m., $n=4-6$) for wild-type, CyLS and CyLS-S1S2 plants grown at 2% (v/v) CO2. Growth phenotypes at 30 days after germination (e–g) and at maturity (h–j) of wild-type (e, h), CyLS (f, i) and CyLS-S1S2 (g, j) plants grown in soil at 2% (v/v) CO2 in 20 cm pots. Note the delayed germination and time to reach maturity in both transformant lines. Scale bars 5 cm.
co-expressed the cyanobacterial Form-1B Rubisco from *S. elongatus* with its cognate carboxyosome-binding protein CcmM35 in tobacco chloroplasts. In that instance, a decrease in \( K_{\text{cat}} \) was also observed, although they attributed this to an association with tobacco Rubisco SSUs. We did not find any tobacco SSU associated with our purified CyLS-S_{S2} carboxysomes (Fig. 3h). However, when we attempted to isolate free Rubisco from CyLS-S_{S2} carboxysomes using the freeze–thaw technique we were not successful (Supplemental Fig. 2). We speculate that this is due to incomplete composition of our minimal carboxysomes leading to different physical characteristics and changed Rubisco kinetics. This implies that our minimal carboxysomes lack a factor, or factors, required to ensure correct carboxysome structure and Rubisco catalytic performance. Future step-wise addition of other carboxysomal proteins should enable an increase in catalytic performance to levels observed for the wild-type *Cyanobium* carboxysomes.

We also found that both CyLS-S_{S2} and *Cyanobium* carboxysome Rubisco had an elevated \( K_{MRubP} \) (Table 2, Supplementary Fig. 3), indicative of resistance to substrate influx in both carboxysomes. This suggests that the reduced Rubisco fluxes are not only due to carboxysomal CO2 diffusion resistance but also due to the relatively intact and that the absence of the vertex proteins does not necessarily result in substantial changes in permeability to RuBP. The observed increase in \( K_{MRubP} \) is unlikely to explain the CyLS-S_{S2} plant CO2 assimilation phenotype alone, although we cannot rule out the possibility that this diffusion resistance effect might be magnified in the observed clusters of carboxysomes inside chloroplasts (Figs. 3 and 4). This clustering is reminiscent of carboxysomes in β-cyanobacterial mutants lacking the ability to properly distribute their carboxysomes. We hypothesize that an even distribution of carboxysomes within the stroma is preferable to prevent localized concentration gradients that could limit substrate access to carboxysomes.

We could not detect a predicted resistance to CO2 flux across the carboxysome shell as assessed by no observable change in \( K_{C} \) for intact carboxysomes compared with the free enzyme (Table 2). We also found that the CA inhibitor AZ had no effect on carboxysomal Rubisco kinetics in our assays (Supplementary Fig. 3). We propose that this results from an inability to reproducibly create carboxysomes. The relatively intact and the absence of the vertex proteins does not necessarily result in substantial changes in permeability to RuBP. The observed increase in \( K_{MRubP} \) is unlikely to explain the CyLS-S_{S2} plant CO2 assimilation phenotype alone, although we cannot rule out the possibility that this diffusion resistance effect might be magnified in the observed clusters of carboxysomes inside chloroplasts (Figs. 3 and 4). This clustering is reminiscent of carboxysomes in β-cyanobacterial mutants lacking the ability to properly distribute their carboxysomes. We hypothesize that an even distribution of carboxysomes within the stroma is preferable to prevent localized concentration gradients that could limit substrate access to carboxysomes.

**Methods**

**Tobacco chloroplast transformation.** Chloroplasts of the *R. rubrum*Rubisco tobacco master line were transformed with CyLS and CyLS-S_{S2} constructs through biolistic bombardment according to Maliga and Tungsuz-Chuang using 2.5 mg of tungsten particles coated with freshly prepared plasmid DNA (10 μg). Each leaf was bombarded with 0.5 mg DNA-coated tungsten particles. This enabled recombination of the genes of interest in place of the *R. rubrum* Rubisco LSU (*rbcm*) gene locus in the plastid genome and selection using a spectinomycin-resistance marker gene (aadA) downstream of the genes of interest, under the control of the tobacco *psbA* promoter (Fig. 2). Successful explants were cultured on regeneration medium (Murashige and Skoog medium supplemented with 3% (w/v) sucrose, 1 mg mL\(^{-1}\) *L*-tryptophan, 1 mg mL\(^{-1}\) thiamine-HCl, 100 mg L\(^{-1}\) 6-benzylationopurine (BA), 1 mg L\(^{-1}\) t-naphthaleneacetic acid [NAA], 1 mg L\(^{-1}\) thiamine-HCl 100 mg L\(^{-1}\) myo-inositol and solidified with 0.6% (w/v) agar) in controlled temperature cabinets (Thermoline, Wetherill Park, NSW, Australia) supplied with 2% (v/v) CO2. Plantlets were assessed with western immunoblot and PCR to identify successful transformants. Homoplasmic transformants were obtained by subculturing the plantlets in regeneration medium. Homoplasmic plantlets were transferred to rooting medium (regeneration medium lacking BAP, NAA, thiamine and myo-inositol) and then to soil to grow to maturity for seed harvest.

**Plant growth analysis and gas exchange.** For growth and gas-exchange experiments, plants were grown from seed germinated in Green Wizard Premium Potting Mix (Scotts Australia, Bella Vista, NSW, Australia) in 20 cm pots supplemented with Osmocote Exact* (ICL Australia & New Zealand, Bella Vista, Australia) at a rate of 4 g L\(^{-1}\) potting mix. Plants were grown in a growth room located at the Research School of Biology Control Environment Facility.
Gas exchange and modelling. Assimilation rates (A; μmol m⁻² s⁻¹) at 25 °C over the range of chloroplastic CO₂ partial pressure (Cₚ;μbar) were examined during gas exchange experiments using the portable flow-through LI-6400 gas-exchange system (LI-COR, Nebraska, USA). Data were modelled according to Farquhar et al. and von Caemmerer as the minimum of the following equations

\[
A = \frac{\mu C_i - 0.5O_2}{C_i + K_{\text{O}_2}} - R_d \\
A = \frac{\mu C_i - 0.5O_2}{C_i + K_{\text{O}_2}}f(\mu C_i + 0.5O_2 - R_d)
\]

where μ = 0.033 M bar⁻¹, Rubisco leaf content parameters (μ; mol active sites m⁻²) and in vivo SₚO values listed in Table 2. An ambient O₂ partial pressure (O₂) of 200 μbar, and a non-photorespiratory CO₂ release (R_d) of 1 μmol m⁻² s⁻¹ was used. For Eq. 2, an electron transport rate (J) of 140 μmol m⁻² s⁻¹ was used. In these simulations, it was assumed that Cₕ = Cᵢ the intercellular CO₂ partial pressure measured by gas exchange.

Southern blot analysis. DNA (0.5 µg) was digested with FastDigest Bsp119I (Thermo Fisher Scientific, Waltham, MA, USA). Purified DNA was electrophoresed in 0.9% (w/v) agarose by gel electrophoresis at 90 V for 2 h. The gel was prepared with 0.5× TBE and 1× SYBR Safe (Thermo Fisher Scientific). DNA fragments were visualized using a UV transilluminator. DNA was then ultraviolet (UV) cross-linked to the membrane using the Spectrolinker XL-1000 UV Crosslinker (Spectronics Corporation, Westbury, NY, USA) in the Optimal Crosslink mode. The membrane was prehybridized for 2 h in 0.5 M NaCl with 4% (w/v) blocking reagent (GE Healthcare Life Sciences) in a hybridization oven at 55 °C. The probe was prepared using the AlkPhos Direct Labelling System (GE Healthcare Life Sciences) and a 483 bp PCR product generated from the accD gene in the 3′ flanking region of the plastome insertion locus (Fig. 2) (Primer accD accD reverse: 5′-AAAGGGGCGCTTCTTTACAT-3′, accD forward: 5′-TGCATTAAAC TGGCGCCCA-3′). The probe was hybridized to the membrane overnight at 55 °C. The following day, two 10 min primary washes were performed at 55 °C in the hybridization oven. The wash buffer contained 2 M urea, 0.1% SDS (w/v), 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1 μM MgCl₂ and 0.2% (v/v) blocking reagent (GE Healthcare Life Sciences). Two 5 min secondary washes were performed at room temperature in 50 mM Tris with 2 mM MgCl₂. The blot was then placed for 1 h in the dark in 1 mM AttoPhos Fluorescent Substrate (Promega, Alexandria, NSW, Australia) and imaged with a ChemiDoc MP Imaging System (Bio-Rad, Gladesville, NSW, Australia) using Epi-blue illumination and a 530/20 filter.

Carboxysome purification. Carboxysomes were purified from culture-grown Cyanothrix cells and CyLS-S₃ plants essentially as described by So et al. for α-carboxysomes, with some minor modifications. For the purification of carboxysomes from the cyanobacterium Cyanothrix, cells were grown in 1 L BG-11 freshwater medium, sparged with air enriched with 2% (v/v) CO₂. Cells were collected by centrifugation (6000 x g, 10 min) and resuspended in 25 mL TEMB buffer (5 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10 mM MgCl₂, 20 mM NaHCO₃) containing 0.55 M mannitol and 60 kU lysozyme (Merck-Millipore, Bayswater, VIC, Australia). Cells were incubated at 37 °C in the dark for 2–16 h with gentle shaking to enable cell wall degradation and then collected by centrifugation as above. Cells were placed on ice and resuspended in 10 mL ice-cold TEMB for 15 min prior to three passages through an EmulsiFlex-B15 cell disruptor (Avestin, Ottawa, ON, Canada) at a homogenizing pressure of ~15,000 psi. IGEPA C6-30 (Sigma-Aldrich, Castle Hill, NSW, Australia) was added to a final concentration of 1% (v/v), and broken cells were mixed with ice and a rotating shaker at 4 °C for 1 h. Cell debris and unbroken cells were removed by centrifugation at 3000 x g, 1 min, and the supernatant centrifuged at 40,000 x g for 20 min to generate a crude carboxysome pellet. The pellet was washed again in 20 mL TEMB containing 1% (v/v) IGEPA C6-30. The final pellet was resuspended in 1.5 mL TEMB and centrifuged by straining at 3000 x g, 1 min prior to loading onto a ~10–60% (v/v) linear sucrose gradient in TEMB. Gradients were centrifuged at 105,000 x g for 60 min and the milky-white band towards the bottom of the gradient was collected, diluted in 35 mL TEMB and recentrifuged at 100,000 x g for 60 min. The final carboxysome pellet was resuspended in 500 μL TEMB prior to analysis for protein content, particle size analysis, and Rubisco assay.

Purification of carboxysomes from CyLS-S₃ plants was carried out in an analogous manner except that 10 g (fresh weight) leaves were extracted in 100 mL TEMB (containing plant protease inhibitor cocktail; Sigma-Aldrich) using an Omni MS homogenizer (Kennesaw, GA, USA) on ice with three 15 s pulses. IGEPA CA-630 was added to a final concentration of 1% (v/v) and extracts were mixed with gentle inversion for 60 min at 4 °C. Extracts were then filtered through a single layer of Miracloth (Merck-Millipore, Bayswater, VIC, Australia) prior to removal of heavy leaf debris and starch at 3000 x g for 1 min. The supernatant was subjected to centrifugation at 40,000 x g for 1 min and subsequent isolation procedures carried out on the resulting pellet in an identical manner to those used for wild-type Cyanobium carboxysomes.

Carboxysomes isolated from both CyLS-S₃ leaves and Cyanothrix were subjected to particle size analysis using a Nanosight NS300 apparatus (Malvern Instruments, Malvern, UK) essentially according to the manufacturer’s instructions. Samples were diluted 1:10,000 in reverse-osmosis purified, de-ionized, filtered water and delivered to the instrument via syringe at a pump speed of 50 units. Particles were illuminated with a blue laser at 405 nm and the instrument operated at 25 °C. A series of five 1 min videos were collected for each sample and subsequently analysed. Video data from multiple, independent carboxysome extractions were analysed and data combined to determine a final particle diameter, reported as the estimated mode ± s.e.m.

For analysis of carboxysome catalytic performance, highly enriched carboxysome fractions obtained immediately prior to application onto sucrose gradients were used for both Cyanothrix and CyLS-S₃ plants. This ensured a high concentration of material with low probability of carboxysome shell breakage sometimes observed after removal from sucrose gradients and enabled same-day Rubisco analysis.

Protein analysis. For protein analysis of whole-leaf extracts, leaves were extracted in extraction buffer (50 mM EPPS, 20 mM NaHCO₃, 10 mM MgCl₂, 1% (w/v) PVPP, 5 mM DTT) using a plastic pestle in a 1.5 mL microfuge tube. Protein samples were denatured by adding 4% Laemmli Sample Buffer (Bio-Rad) and heating at 95 °C for 10 min, and insoluble debris was removed by centrifugation at 20,000 x g for 5 min. A volume of supernatant equivalent to 5.6 mm² leaf area was loaded into 4–20% Stain-free polyacrylamide gels (Bio-Rad). Proteins were separated at 180 V for 35 min in denaturing buffer (1% (w/v) SDS; 25 mM Tris, 30 mM Tris, 100 mM NaCl, 0.1% (w/v) Tween 20). For carboxysomes, bands were visualized using 5% (w/v) skim-milk powder. Blocked membranes were probed and detected with alkaline phosphatase-conjugated antibody against tobacco Rubisco. In the case of CyLS-S₃ leaves, cytosolic and chloroplast Rubisco contents were compared on SDS-PAGE gels, although CsaS1A contains no tryptophan residues and cannot be visualized using Stain-free gels. As a result, images of Stain-free gels containing these proteins show only CBB for 12 kDa. Conversely, CsaS1A is visualized using Coomassie blue stain, whereas CBB gels stain poorly. These opposing characteristics could be used effectively to identify each protein in the absence of western blots. For western blot analysis, separated proteins on Stain-free gels were transferred to Immobilon-P polyvinylidene fluoride membranes using a Trans-Blot apparatus (Bio-Rad) and bands were visualized using the Attophos Substrate Kit (Promega). The tobacco Rubisco antibody cross-reacts with the Cyanobium large subunit (CBB) but not RubcM.

Leaf Rubisco content and kinetic analysis. Leaf disc samples (0.5 cm²), taken at the site of gas exchange immediately after measurement, were used for Rubisco content and kinetic analysis. Radiolabelled [¹⁴C]carboxyarabinitol-1-P (CABP) was used to measure leaf Rubisco content as described. In the case of CyLS-S₃ plants, crude leaf extracts, clarified only by a low-speed centrifugation step (3000 x g, 1 min), were used in the determination of Rubisco content to avoid excess losses of the insoluble carboxysomes. The same method used to measure leaf Rubisco content was applied to both leaf extracts containing isolated Rubisco and enriched carboxysomes used in activity assays. Radiolabelled [¹⁴C]CO₂ fixation assays were carried out using leaf discs for kinetic analyses (measured relative to Rubisco content for CO₂ at both ambient O₂ (K₅ₐ₅(ambient)) and under nitrogen (K₅ₐ₅(NO₂)) using 30–440 μM [¹⁴C]CO₂ at 25 °C, pH 8.04 as described by Sharwood et al. Samples containing Rubisco were typically activated for 5 min prior to assay. Catalytic turnover rates (kₑ₅, s⁻¹) were determined by dividing the Vₐ₅₅₁₅ by Rubisco content. Determined by 1 µg α-carboxysome pellets, extraction of Similar assays were performed using 10–800 μM RuBP at 20 mM MgCl₂, ambient O₂ and 20 mM NaHCO₃ to determine K₅ₐ₅. In the case of CyLS-S₃ plants, analysis of Rubisco
kinetics was carried out on what we deemed to be free Rubisco after centrifugation of leaf extracts at 20,000 × g, 1 min, or carboxysomal Rubisco after enrichment of carboxysomes. To generate broken Cyanobium carboxysomes with released Rubisco, enriched carboxysome samples were centrifuged (20,000 × g, 10 min) and the supernatant discarded. Pellets were then frozen at −20°C for at least 30 min and then quickly resuspended in TEMB using a pipette to achieve breakage. The response of CyLS Rubisco, CyLS-S1S2 carboxysomes and Cyanobium carboxysomes to the CA inhibitor AZ was carried out under normal assay conditions except that 500 µM AZ was included in both activation and assay buffers, allowing incubation of samples with AZ for 5 min at 25°C prior to assay. The same concentration of the more membrane permeable analogue EZ has been shown to reduce CoCScA activity to 15% of maximum in preparations of affinity-purified, recombinant CoCScA enzyme from H. neapolitanus carboxysomes, expressed in E. coli.16 In vitro ScCo for Cyanobium Rubisco was determined for protein extracted from CyLS plants using 5–10 cm2 leaf material. Protein was extracted in 50 mM EPPS, pH 8.6, 5 mM MgCl2 containing plant protease inhibitor cocktail (Sigma–Aldrich) and initially purified using ion-exchange chromatography on 1 mL High Q cartridge columns (Bio-Rad). Rubisco was eluted using 2 mL elution buffer (50 mM triethanolamine pH 8.3, 30 mM Mg acetate) using an ÄKTA Pure chromatography system (GE Healthcare). Fractions containing Rubisco were pooled and concentrated by centrifugation through 30 kDa molecular weight cutoff filters. Purified Rubisco was used to catalyse the production of radiolabelled and 3-PGA from wild-type tobacco, CyLS and CyLS-S1S2 plants were determined according to the methods described by Maxwell et al.17 using a purpose-built cuvette attached to a Micromass membrane inlet mass spectrometer. Discs were taken from third leaf of the same plants used for attached-leaf gas exchange (grown at 2% (v/v) CO2, 25°C, 300 µmol m−2 s−1). In vitro ScCo for Cyanobium Rubisco was determined for protein extracted from CyLS plants using 5–10 cm2 leaf material. Protein was extracted in 50 mM EPPS, pH 8.6, 5 mM MgCl2 containing plant protease inhibitor cocktail (Sigma–Aldrich) and initially purified using ion-exchange chromatography on 1 mL High Q cartridge columns (Bio-Rad). Rubisco was eluted using 2 mL elution buffer (50 mM triethanolamine pH 8.3, 30 mM Mg acetate) using an ÄKTA Pure chromatography system (GE Healthcare). Fractions containing Rubisco were pooled and concentrated by centrifugation through 30 kDa molecular weight cutoff filters. Purified Rubisco was used to catalyse the production of radiolabelled and 3-PGA from wild-type tobacco, CyLS and CyLS-S1S2 plants were determined according to the methods described by Maxwell et al.17 using a purpose-built cuvette attached to a Micromass membrane inlet mass spectrometer. Discs were taken from third leaf of the same plants used for attached-leaf gas exchange (grown at 2% (v/v) CO2, 25°C, 300 µmol m−2 s−1).
29. Roberts, E. W., Cai, F., Kerfeld, C. A., Cannon, G. C. & Heinhorst, S. Isolation and characterization of the Prochlorococcus carboxysome reveal the presence of the outer shell protein CsoS1D. J. Bacteriol. 194, 787–795 (2012).
30. Klein, M. G. et al. Identification and structural analysis of a novel carboxysome shell protein with implications for metabolite transport. J. Mol. Biol. 392, 319–333 (2009).
31. Dou, Z. et al. CO2 fixation kinetics of Halothiobacillus neapolitanus mutant carboxysomes lacking a carboxy anhydrase suggest the shell acts as a two diffusion barrier for CO2. J. Biol. Chem. 283, 10377–10384 (2008).
32. Baker, S. H., Williams, D. S., Aldrich, H. C., Gambrell, A. C. & Shively, J. M. Identification and localization of the carboxysome peptide CsoS3 and its corresponding gene in Thiobacillus neapolitanus. Arch. Microbiol. 173, 278–280 (2003).
33. Cai, F. et al. Advances in understanding carboxysome assembly in Prochlorococcus and Synechococcus implicate CsoS2 as a critical component. Life 5, 1141–1171 (2015).
34. Cai, F. et al. The pentamer vertex proteins are necessary for the icoshedral carboxysome shell to function as a CO2 leakage barrier. PLoS ONE 4, e7521 (2009).
35. Sutter, M. et al. Visualization of bacterial microcompartment facet assembly using high-speed atomic force microscopy. Nano Lett. 16, 1590–1595 (2016).
36. Tsai, Y. et al. Structural analysis of CsoS1A and the protein shell of the Halothiobacillus neapolitanus carboxysome. PLoS Biol. 5, e144 (2007).
37. Price, G. D. & Badger, M. R. Isolation and characterization of high CO2-requiring mutants of the cyanobacterium Synechococcus PCC7942. J. Mol. Biol. 98, 503–517 (1975).
38. Shively, J. M., Ball, F. L. & Kline, B. W. Electron microscopy of the carboxysomes (polyhedral bodies) of Thiobacillus neapolitanus. J. Bacteriol. 116, 1405–1411 (1973).
39. Ruuska, S. et al. The interplay between limiting processes in C3 photosynthesis studied by rapid-response gas exchange using transgenic tobacco imparted in plastidic photosynthesis. Aust. J. Plant Physiol. 29, 357–373 (2002).
40. Whitney, S. M. & Andrews, T. J. Plastome-encoded bacterial ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) supports photosynthesis and growth in tobacco. Proc. Natl. Acad. Sci. USA 98, 14738–14743 (2001).
41. Shively, J. M., Ghanourou, O., Kapralov, M. V., Gunn, L. H. & Whitney, S. M. Temperature responses of Rubisco from Pinusaceae grasses provide opportunities for improving C3 photosynthesis. Nat. Plants 2, 16186 (2016).
42. Price, G. D., Coleman, J. R. & Badger, M. R. Association of carboxy anhydrase activity with carboxysomes isolated from the cyanobacterium Synechocystis PCC7002. Plant Physiol. 100, 784–793 (1992).
43. Schwarz, R., Reinhold, L. & Kaplan, A. Low activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase in carboxysome-defective Synechococcus mutants. Plant Physiol. 108, 183–190 (1995).
44. Occhialini, A., Lin, M. T., Andralojc, P. J., Hanson, M. R. & Parry, M. A. J. Transgenic tobacco plants with improved cyanobacterial Rubisco expression separated by gel electrophoresis. J. Mol. Biol. 232, 5881 (2000).
45. Price, G. D., Campbell, J. M. & Espie, G. S. Characterization of a mutant lacking carboxysomal carboxy anhydrase from the cyanobacterium Synechocystis PCC6803. Plant Physiol. 124, 456–467 (2002).

Acknowledgements
We thank L. M. Bourke and L. Wey for technical assistance; E. Martin Avila for advice on tobacco seed and tobacco Rubisco antibody. We also thank M. Groisman for critical comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript. Figures 1c and 3i were drawn by Erin I. Walsh under comments on the manuscript.
Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-06044-0.

Competing interests: The authors declare no competing interests.

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