Chapter 5
Bioengineering Photosynthesis to Drive Crop Improvement: Engineering Carbon-Concentrating Mechanisms, Kranz Anatomy, Chloroplast Mimics, and New Cellular Compartments

Mouesanao K. Kandjoze and Pallavi Singh

Abstract Global food security is increasingly threatened by climate change and population growth, necessitating enhanced agricultural productivity. Photosynthesis, the foundation of life on Earth, represents a crucial target for bioengineering. This chapter explores innovative strategies for enhancing photosynthetic efficiency, focusing on approaches that optimise natural carbon fixation mechanisms, with a particular emphasis on three key research areas: (1) leveraging biophysical carbon--concentrating mechanisms to integrate functional pyrenoids and carboxysomes into higher plant chloroplasts; (2) introducing CAM, Kranz anatomy, and C_4 photosynthetic traits into staple crops; and (3) engineering chloroplast genomes. Moreover, the chapter highlights breakthroughs in gene editing, cellular reprogramming, and synthetic biology. Emerging tools, such as spatial-omics and synthetic gene circuits, which are transforming conceptual approaches into commercial applications, are also discussed. This exploration of cutting-edge bioengineering approaches offers promising avenues for developing next-generation crops with improved photosynthetic performance, contributing to a more sustainable and food-secure future.

Keywords Photosynthesis · Bioengineering · Chloroplast · Yield · Resilience

Editor's Notes

Crop productivity fundamentally hinges on efficiency of photosynthesis, a natural process powered by solar energy, resulting in fixation of atmospheric CO_2 into chemically available forms. However, photosynthetic efficiency is primarily limited by the sub-par catalytic rate of Rubisco posing a major bottleneck in boosting crop

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productivity. Enhancing photosynthetic efficiency in crop species has therefore been a long-standing goal in agbiotech research.

In this chapter, Kandjoze and Singh discuss the prospects of bioengineering photosynthesis to enhance its efficiency and reduce wasteful CO_2 emissions during photorespiration. To innovate mechanisms for efficient biological fixation of CO_2, the authors propose scholars to pursue three key areas of research: "*(1) leveraging biophysical carbon-concentrating mechanisms (CCMs) to integrate functional pyrenoids and carboxysomes into higher plant chloroplasts, (2) introducing CAM, Kranz anatomy, and C4 photosynthetic traits into staple crops, and (3) engineering chloroplast genomes.*" Appearing as impossible even in recent past, the authors inform how mutational studies, and computational modeling can support re--engineering of functional pyrenoid-based and carboxysome-based CCMs for achieving enhanced photosynthetic efficiency. Bioengineering of thylakoid membranes and stroma, and sequestering Rubisco by way of creating a CO_2 diffusion barrier are ingenious approaches that are being tested currently. The authors also propose integration of several other promising strategies such as introducing CAM, Kranz anatomy, and C4 photosynthetic traits into crops species. The authors present an elegant account on the potential of modern technologies in circumventing bottlenecks in each of these approaches which currently are at the proof-of-concept stage. The chapter is thus a starting point for researchers willing to explore this exciting field.

Actionable Points

1. **Enhancing photosynthetic efficiency:** Discusses current strategies for optimizing photosynthetic carbon fixation through biophysical carbon concentrating mechanisms integrating pyrenoids and carboxysomes.
2. **Engineering approaches of C_4 and CAM photosynthesis:** Explores approaches to introduce anatomical and biochemical traits from C_4 and CAM species into staple crops for improved yield resilience.
3. **Technological advancements:** Highlights experimental innovations and strategies that can pave the way to climate-smart crops.
4. **Future directions:** Provides insights into the technology-readiness levels of different photosynthetic improvement strategies, focusing on a path toward accelerating translational solutions.

5.1 Introduction

The global challenge of food security is intensifying, driven by the combined pressures of climate change and a rapidly growing population. As the global demand for food continues to escalate, traditional agricultural methods are struggling to keep pace. To meet this growing need, enhancing agricultural productivity through innovative approaches has become a priority. At the core of addressing this challenge is

photosynthesis, the fundamental process that sustains life on Earth and drives plant productivity.

5.2 Background

5.2.1 Brief Evolution of Carbon Concentrating Mechanisms

Rubisco is responsible for 90% of inorganic carbon fixation (Erb and Zarzycki 2018). Globally, Rubisco is considered an abundant enzyme, with terrestrial Rubisco estimated to weigh 0.7 gigatons, about 14 times that of global estimates of collagen (Bar-On and Milo 2019). Despite having evolved 3.5 billion years ago, Rubisco is eight to ten times slower than other central metabolic enzymes (Barrett et al. 2021). Evolutionary biologists suggest that Rubisco evolved when atmospheric oxygen levels were low and conferred adaptive advantages due to its carbon-fixing activity (Sage 2004). As O_2 levels rose following the Great Oxygenation Event, Rubisco evolved the capacity to discriminate between CO_2 and O_2, which came at the cost of its catalytic speed (Erb and Zarzycki 2018; Giessen and Silver 2017). Additionally, the chemical similarity between CO_2 and O_2, under O_2-rich atmospheric conditions further impede Rubisco's selectivity for CO_2 (Whitney et al. 2010). Consequently, Rubisco, which has both oxygenase and carboxylase capabilities, still fixes an average of two molecules of O_2 for every five of CO_2 (Giessen and Silver 2017). The promiscuity of Rubisco is not only inefficient, but it also produces toxic 2-phosphoglycolate (2PG), which requires energy to metabolise and leads to the release of CO_2 during photorespiration (Bowes et al. 1971). To overcome this energetically wasteful process, which reduces photosynthetic efficiency by an estimated 30%, photosynthetic organisms have evolved mechanisms to reduce photorespiration by simply producing more Rubisco. In contrast, other systems evolved physical or chemical carbon-concentrating mechanisms (CCMs) in chloroplasts to increase CO_2 concentrations around the site of Rubisco activity (Giessen and Silver 2017; Price et al. 2012; Walsh et al. 2023).

Cyanobacteria, most algae, and most hornwort species developed biophysical CCMs that passively or actively concentrate inorganic bicarbonate (HCO_3^-), convert it to CO_2 via carbonic anhydrases (CA), and release it into a microcompartment that contains Rubisco (Hennacy and Jonikas 2020). Carboxysomes and pyrenoids are the two main types of compartments, both of which evolved physical barriers in the form of a protein shell or starch sheath, respectively, to limit CO_2 leakage (Rae et al. 2017). In cyanobacterial CCMs, the HCO_3^--CO_2 conversion and Rubisco--fixation occurs in the carboxysome matrix (Blikstad et al. 2023). Conversely, algal and hornwort CCM's convert HCO_3^- into CO_2 in specialised thylakoid membrane tubules that traverse thy pyrenoid, prior to releasing the converted CO_2 into the pyrenoid matrix for Rubisco fixation (He et al. 2020).

In contrast, biochemical CCMs, which include C_2, C_4, and crassulacean acid metabolism (CAM), concentrate carbon via organic intermediates such as oxaloacetate (OAA) and malate before delivering it to Rubisco (He et al. 2023). C_4 plants also developed a distinctive Kranz anatomy that further facilitates efficient carbon concentration (Lundgren et al. 2014). Biochemical CCMs have been demonstrated to increase photosynthesis in model species such as *Arabidopsis thaliana* (Arabidopsis) and *Nicotiana tabacum* (tobacco) but also in staple crops including rice and wheat (Sedelnikova et al. 2018).

5.2.2 Agricultural Benefits of CCMs

CCMs offer additional benefits beyond increasing photosynthesis and yield. The more efficient Rubisco activity induced by CCMs reduces the total Rubisco content, thereby improving nitrogen use efficiency (Adler et al. 2022). Furthermore, increased internal CO_2 for Rubisco would decrease stomatal conductance (g_s) and reduce water loss through transpiration in C_3 plants (Adler et al. 2022; Hennacy and Jonikas 2020), thereby enhancing water use efficiency (WUE). Therefore, investing in mechanisms to increase internal carbon concentrations are promising strategies to enhance photosynthetic carbon assimilation, resource use efficiency, and yield.

5.3 Pyrenoid-Based Carbon-Concentrating Mechanisms

Carbon fixation via pyrenoid-based CCMs accounts for about 28–44% of global carbon fixation (Field et al. 1998). *Chlamydomonas reinhardtii* (Chlamydomonas) is the most well-characterised pyrenoid-based CCM and will be used a model to describe how algal CCM systems operate (Adler et al. 2022). Several HCO_3^- transporters located at the plasma, chloroplast, and thylakoid membranes facilitate the accumulation of carbon in the thylakoid lumen (Rottet et al. 2021). HCO_3^- acidifies the lumen, which drives the decarboxylation reaction by carbonic anhydrase 3 (CA3). Finally, released CO_2 diffuses into the pyrenoid matrix for fixation by Rubisco (Barrett et al. 2021; Fei et al. 2022; He et al. 2023).

Pyrenoids have recently attracted research interest as a potential tool for improving photosynthesis in higher plants because of their carbon concentrating efficiency. Pyrenoid-based CCMs have been reported to facilitate an up to 40-fold increase in inorganic carbon (C_i) compared to chloroplasts without pyrenoids (Adler et al. 2022). In addition, the sub-cellular mechanism does not require changes to existing leaf anatomy, such as engineering efforts associated with introduction of C_4 traits (He et al. 2023). Moreover, the genetic components required to express and assemble pyrenoids already exist in eukaryotic genomes (He et al. 2023). Both these factors could streamline the transition of algal carbon-concentrating systems into global crop staples such as wheat or rice.

5.3.1 Understanding Pyrenoid-Based CCM and Strategies for Engineering in Land Plants

5.3.1.1 The Optimal Pyrenoid-Based CCM

Both mutational studies and computational modelling approaches detail the key steps required for functional pyrenoid assembly. Fei et al. (2022) modelled a pyrenoid-based CCM that includes three key modules; first, low-inducible B (LCIB) should be localised to the chloroplast stroma to convert passively imported CO_2 into HCO_3^-; second, a thylakoid membrane channel such as BCT1 (bicarbonate transporter 1) is required to deliver HCO_3^- to luminal CA3 for conversion back into CO_2 near Rubisco; and third, phase-separated Rubisco must be enclosed by a CO_2 diffusion barrier. Notably, the model suggests that a passive CO_2 uptake mechanism at ambient CO_2 levels is possible, while active HCO_3^- uptake is beneficial at low atmospheric CO_2 levels. To achieve this three-module pyrenoid-based CCM, land plants would need to be engineered to condense or phase-separate Rubisco, localise CA-rich thylakoids to the Rubisco condensate, integrate HCO_3^- transporters into the thylakoid membrane, and add a diffusion barrier to limit CO_2 diffusion out of the pyrenoid-based structure (Fig. 5.1).

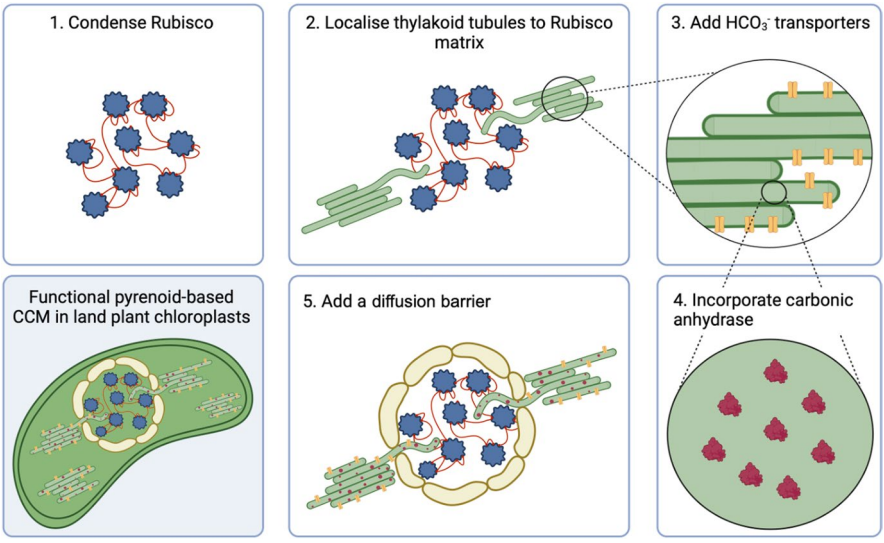

Fig. 5.1 Strategies for engineering a pyrenoid-based CCM in land plants. (1) Rubisco phase separates into a condensate via linker proteins (shown in red) such as EPYC1. (2) Thylakoid membrane tubules are recruited to the matrix core to form pyrenoid tubules, which deliver HCO_3^- to the Rubisco matrix, and supply ATP and NADPH for Rubisco-mediated carbon fixation. (3) HCO_3^- transporters are targeted to the thylakoid membrane to facilitate HCO_3^- accumulation. (4) Targeting carbonic anhydrase to the thylakoid lumen, necessary for HCO_3^- decarboxylation and CO_2 release near the Rubisco matrix. (5) Finally, a diffusion barrier comprised of either starch or stacked thylakoids encloses the pyrenoid to reduce CO_2 leakage. (Created with BioRender.com)

Approach 1: Understanding Pyrenoid Phase Separation and Rubisco Condensation

Understanding phase separation of pyrenoid-associated proteins is a key first step toward pyrenoid-based CCM assembly in higher plants. *Chlamydomonas* phase separation occurs partly due to the interaction between the essential pyrenoid component 1 (EPYC1) linker protein and the small subunit (SSU) of Rubisco (Hennacy and Jonikas 2020). Atkinson et al. (2017) investigated these SSUs by generating *Arabidopsis* mutants lacking a functional SSU and supplemented them with a partial *SSU* sequence encoding the *Chlamydomonas* alpha helix motif, or the complete *Chlamydomonas SSU* sequence. The hybrid Rubisco comprised of the partial *Chlamydomonas* sequence had similar catalytic properties to WT controls, reporting comparable maximum Rubisco carboxylation ($V_{c,max}$) and max photosynthetic electron transport (J_{max}) rates. Atkinson et al. (2020) then introduced the *EPYC1* linker gene in *Arabidopsis* that featured the *Chlamydomonas* Rubisco hybrid, but it failed to phase separate, partly due to EPYC1 being targeted for degradation. This was remedied by adding a native *Arabidopsis* chloroplast signal peptide to GFP-tagged *EPYC1* constructs (Atkinson et al. 2020). Confocal and transmission electron microscopy showed that EPYC1 was able to condense both the partial and mature *Chlamydomonas* Rubisco hybrids to form phase-separated pyrenoid-like compartments in *Arabidopsis*.

The evidence above indicates that modifying the Rubisco *SSU* is a potential avenue through which to initiate pyrenoid assembly in land plants. Rubisco *SSU* sequences are encoded in the nuclear genome rather than the chloroplast genome (He et al. 2023). Modifying nuclear-encoded Rubisco circumvents the challenges associated with editing chloroplast-encoded genes. This allows for a more standardised approach to CCM engineering, which can be translated to various C_3 plants.

Furthermore, research into under-explored algal pyrenoid systems is further increasing our understanding of how pyrenoids assemble. Oh et al. (2023) identified PYCO1 as a key linker protein that mediates *Phaeodactylum tricornutum* red algae pyrenoid phase separation. In *Chlorella sorokiniana*, sequence-independent bioinformatic analysis identified CsLinker proteins as EPYC1 functional analogues (Barrett et al. 2024). Most importantly, Barrett et al. (2024) demonstrated that these CsLinkers bind to highly conserved regions of Rubisco large subunit (LSU) and could phase separate native *Chlamydomonas* and tomato Rubisco in vivo, while tobacco Rubisco phase separated both in vitro and in vivo. This is the first report of native Rubisco phase separation in land plants and presents a critical milestone in pyrenoid-based CCM engineering efforts in C_3 plants.

The present strategies outlined highlight the potential of easily modifiable Rubisco SSUs or conserved LSUs as targets for Rubisco condensation. Additionally, the identification of a diverse set of linker proteins would further expand our repertoire of tools that can be matched to compatible C_3 pyrenoid-based CCMs.

Approach 2: Localising the Thylakoid to the Rubisco Matrix

For functional pyrenoid assembly, thylakoids must traverse the pyrenoid Rubisco matrix. Through cryo-electron transmission microscopy, it has been shown that thylakoid membranes merge as they traverse the starch sheath to form a pyrenoid tubule made up of several smaller, fenestrated tubules (He et al. 2023). The fenestrations connect the thylakoid lumen to the chloroplast stroma and are used to deliver concentrated CO_2 to the Rubisco matrix (Hennacy et al. 2024). Pyrenoid tubules are recruited to the pyrenoid by a starch-sheath-associated protein called Starch Granule Abnormal1 (SAGA1). This is evidenced by *Chlamydomonas* mutations in *SAGA1*, which produced pyrenoids without tubules, or with significantly fewer tubules compared to the WT (Itakura et al. 2019).

Other proteins further implicate the starch sheath in pyrenoid tubule formation. Proximity labelling identified a protein encoded by *Cre09.g394510*, which localised to the starch sheath periphery (Lau et al. 2023). The C-terminal domain of the protein-mediated vesicle fusion and was hypothesised to rearrange the thylakoid grana into a pyrenoid tubule as it traversed the sheath and entered the pyrenoid matrix. Additionally, the *mutation missing thylakoids 1 (MITH1)* in *Chlamydomonas* was associated with tubule-lacking pyrenoids (Hennacy et al. 2024). When expressed together with *SAGA1* in *Arabidopsis* previously engineered to form Rubisco condensates, thylakoid sheets extended into the pyrenoid-like assemblies (Hennacy et al. 2024). Researchers proposed that *MITH1* was required to form an MITH1-SAGA1 interaction that replaced existing Rubisco-EPYC1 interactions, which allowed the thylakoid to traverse the pyrenoid matrix. This was the first time that thylakoid-traversing structures in proto-pyrenoids were observed in planta, bringing this CCM approach one step closer to being fully assembled in land plants.

Approach 3: Expressing HCO_3^- Transporters in Thylakoid Membranes

Biophysical carbon concentration is mediated by several HCO_3^- transporters. Under atmospheric CO_2 levels, C_i passively diffuses across the plasma membrane via the low CO_2-inducible 1 (LCI1) channel (Adler et al. 2022; He et al. 2023). Cytoplasmic CO_2 then diffuses into the chloroplast stroma, where it is rapidly converted to HCO_3^- by the low-inducible B/C (LCIB/LCIC) complex (Rottet et al. 2021). However, at suboptimal CO_2 levels (below 200 ppm CO_2), pyrenoid-based CCMs switch to actively import HCO_3^- into the cytosol and stroma via HLA3 (high light activated 3) and LCIA, respectively (Adler et al. 2022). The stromal HCO_3^- is then shuttled into the thylakoid lumen via BCT1, 2, and 3, where it travels down into the pyrenoid membrane tubule that bisects the pyrenoid (Adler et al. 2022; Fei et al. 2022).

Research into LCI transporters is shedding light on their role in CO_2 accumulation. LCIA, which was suspected to be an active HCO_3^- transporter, was recently confirmed through a phenotypic rescue assay in *Arabidopsis* (Förster et al. 2023). *Arabidopsis* mutants lacking *bCA5*, which could not grow in ambient CO_2 levels,

grew when rescued with LCIA, indicating that the channel was actively transporting HCO_3^- and facilitating sufficient carbon accumulation to support photoautotrophic growth. However, LCIA expression in WT tobacco did not improve photosynthetic performance (Förster et al. 2023). Similarly, LCIB expression was also able to restore *bCA5*-deficient *Arabidopsis* growth under atmospheric CO_2 levels (Kasili et al. 2023). Additionally, in low CO_2 conditions, LCIB transporters were recruited to the pyrenoid by the *CCM1* regulatory protein and to the surrounding pyrenoid area by starch-sheath-associated isoamylase1 (ISA1) (Toyokawa et al. 2020; Yamano et al. 2022). Furthermore, *Chlamydomonas* LCIB expression in tobacco, along with its target peptide sequence, increased the maximum Rubisco carboxylation rate (Vc_{max}) by 11–13%, fresh shoot biomass by 33% and root biomass by 57% compared to WT tobacco (Nölke et al. 2019). Based on these findings, LCIB is a potential promising target for photosynthetic improvement.

Approach 4: Targeting Functional CA to Pyrenoid Membrane Tubule Lumens

In many pyrenoid-based CCMs, the conversion of HCO_3^- back into CO_2 by CA in the thylakoid lumen is the final stage of carbon concentration in pyrenoids. Several CAs coordinate the accumulation of HCO_3^- in the pyrenoid matrix (Terentyev and Shukshina 2024). Of the various CAs, CA3 localises to the thylakoid lumen and mediates the delivery of CO_2 to the Rubisco matrix (Atkinson et al. 2016). In *Chlamydomonas*, mutations in CA3 impaired pyrenoid functioning and growth in atmospheric CO_2 conditions, despite demonstrating HCO_3^- accumulation in thylakoids and pyrenoid tubules (Mukherjee et al. 2019). CA is therefore crucial for the final step of CO_2 delivery to Rubisco.

In tobacco, although CA3 was correctly targeted to the chloroplasts, it was also largely mis-localised to the cytosol, despite expression with the *Chlamydomonas* transit peptide sequence (Atkinson et al. 2016). However, another study achieved preferential CA3 expression in tobacco thylakoid lumens, with only minor expression in the cytosol (Nölke et al. 2019). CA3 expression was also found together with a 16–17% increase in Vc_{max}, significantly increased fPSII (which indicates photosynthetic capacity), leaf number, and a 28% increase in shoot biomass (Nölke et al. 2019). Enhanced photosynthesis may be due to CA, which has previously been reported to be involved in PSII operation in thylakoids (Terentyev and Shukshina 2024). Therefore, although the findings suggest the potential of algal CA to improve photosynthetic performance, more research is required to determine whether improvement is due to enhanced PSII activity or increased CO_2 accumulation. Nonetheless, the findings confirm the benefits of introducing algal CA into land plants.

Additionally, the role of stromal CA in biophysical CCMs is debated. Some studies suggest stromal CA prevents HCO_3^- from accumulating in the cytosol by causing HCO_3^--CO_2 equilibration, which increases CO_2 leakage (Atkinson et al. 2016; Giessen and Silver 2017). This has motivated the proposal that native stromal CA should be eliminated and replaced with strategically localised CA isoforms.

However, reaction-diffusion modelling demonstrates that a pyrenoid-based CCM can still function with stromal CA, provided the system is otherwise well-optimised (Fei et al. 2022). Their findings emphasise the importance of targeting CA to the thylakoid lumen for efficient CO_2 delivery to the pyreoid matrix but do not support a strict requirement to remove stroml CA. Therefore, engineering efforts are advised to prioritise precise CA localisation rather than fully knocking out native CA—a task yet to be demonstrated in C_3 plants.

Approach 5: Creating a Diffusion Barrier

Experimental data implies that a diffusion barrier surrounding the pyrenoid-based CCM reduces CO_2 leakage (Toyokawa et al. 2020). In *Chlamydomonas*, the barrier is polysaccharide-based, comprising of curved starch granules that enclose the Rubisco condensate (He et al. 2023). Several proteins are also associated with the starch sheath, helping to assemble the individual plates, tether them together, and link the matrix to the sheath (Barrett et al. 2021; He et al. 2023). Of these starch proteins, SAGA1 and SAGA2 are well-characterised proteins that attach the Rubisco condensate to the sheath (Atkinson et al. 2024).

Under atmospheric (0.04% [v/v] CO_2) or very low (0.01% [v/v] CO_2) CO_2 conditions, a starch sheath is essential for pyrenoid-based carbon concentration. In *Chlamydomonas*, mutants lacking *SAGA1* observed increased pyrenoid numbers per chloroplast, thinner and uneven starch sheaths, decreased growth rates under low CO_2, and reduced photosynthetic rates under saturating C_i at both low and high CO_2 levels (Itakura et al. 2019). Additionally, SAGA1 was also demonstrated to recruit stromal HCO_3^- transporters such as HLA3 and LCIA to the surrounding pyrenoid area under CO_2-limiting conditions (Shimamura et al. 2023). The starch sheath may play a role in the proper morphological pyrenoid assembly, which contributes to optimal protein recruitment and functioning, particularly in atmospheric and suboptimal CO_2 conditions (Barrett et al. 2021; Hennacy and Jonikas 2020).

Recently, *Chlamydomonas SAGA 1* and *SAGA2* expression resulted in a partial starch sheath formation around the synthetic proto-pyrenoid in *Arabidopsis* (Atkinson et al. 2024). To form the partial sheath, *SAGA1* was hypothesised to primarily aggregate starch to form plate-like sheaths, while *SAGA2* facilitated the sheath-condensate interactions (Atkinson et al. 2024).

Altogether, starch-based diffusion barriers are essential for pyrenoid function, and its partial engineering into land plants is a promising step toward achieving biophysical carbon concentration in land plants.

Thylakoid-based diffusion barriers have also been observed and modelled to be effective in a pyrenoid-based CCM (Fei et al. 2022; Robison et al. 2025). Experimentally derived data about the gaps between thylakoid membranes and diffusivity were used to model realistic thylakoid geometry and membrane permeability (Fei et al. 2022). Simulation results suggest that tight spacing between thylakoid membranes (Δ_n = 5.6 nm) and low diffusivity ($\approx 10^3$) substantially reduce CO_2 diffusion in the stroma, making them an efficient barrier against CO_2 leakage (Fei et al.

2022). Different thylakoid-based barriers have been observed in other algal species, such as in *Cyanophora paradoxa*, where the thylakoid stacks surround a naked Rubisco condensate, or in *Euglena carterae*, where thylakoid stacks both traverse and surround the Rubisco matrix (He et al. 2023). Additionally, anatomical research into Hornwort species, the only land plants known to have pyrenoid-based CCMs, were recently reported to contain multiple pyrenoids per chloroplast, which are collectively surrounded by thylakoid membrane stacks(Robison et al. 2025). Although the magnitude of CO_2 leakage of thylakoid-enclosed pyrenoids is yet to be experimentally determined, modelled estimates, along with experimental observations, support the feasibility of a pyrenoid-based CCM that encompasses a thylakoid membrane diffusion barrier. It should be noted that research on thylakoid-based pyrenoids are still in its preliminary discovery phase and no attempts at engineering them into higher land plants have been made. Overall, the evidence above confirms the importance of a diffusion barrier in effective pyrenoid-based CCMs.

5.4 Carboxysome-Based Carbon-Concentrating Mechanisms

5.4.1 Introduction to Carboxysomes

Like algae, cyanobacteria and most photosynthesising proteobacteria have also developed a physical component to compensate for the inefficiency of Rubisco, called the carboxysome (Blikstad et al. 2023). Through convergent evolution, two carboxysomes have evolved (Giessen and Silver 2017). α-carboxysomes, expressing Form 1A Rubisco, which is encoded by the *cso* operon, while β-carboxysomes, expressing 1B Rubisco, located on the *ccm* operon (Hennacy and Jonikas 2020). For carboxysome-based carbon concentration, CO_2 passively diffuses across the plasma membrane, while HCO_3^- is pumped into the cytosol, where it diffuses across the selectively permeable protein shell and into the carboxysome (Trettel et al. 2024). Inside the carboxysome, CA converts HCO_3^- back into CO_2 for fixation by Rubisco (Hennacy and Jonikas 2020). Cyanobacteria have been reported to have 10–40 times higher HCO_3^- concentrations in the cytosol compared to stromal levels in C_3 species, making carboxysomes an area of interest for researchers (Long et al. 2018).

5.4.2 Strategies for Carboxysome Engineering

Price et al. (2012) proposed a four-step plan to engineer carboxysomes into higher plants. This plan involves (1) transferring HCO_3^- transporters to chloroplast membranes, (2) removing endogenous stromal CA activity, (3) assembling a functional carboxysome in chloroplast stromal regions, and (4) incorporating an NADH1-CO_2

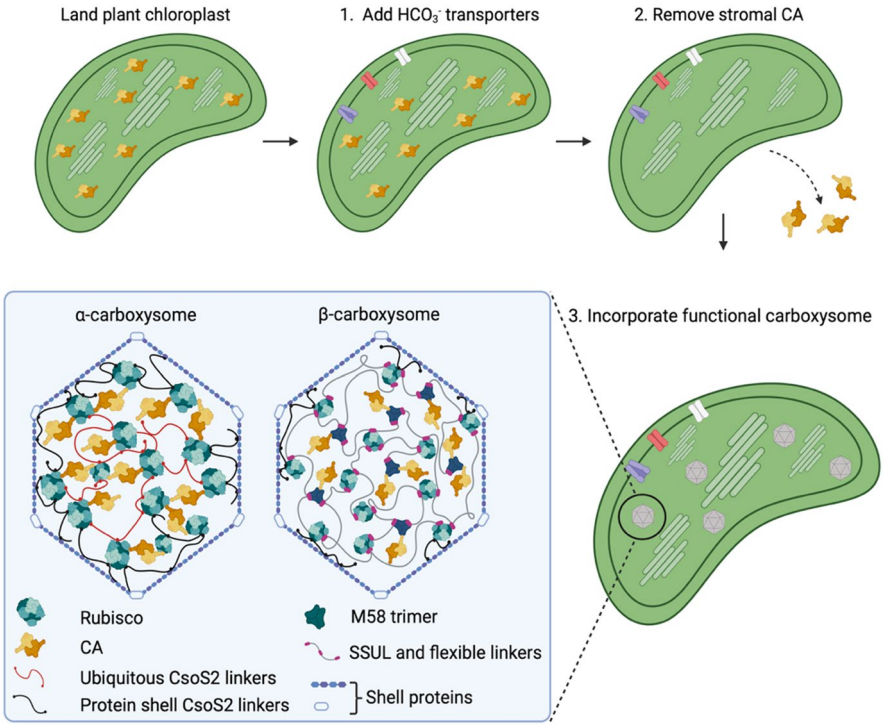

Fig. 5.2 Stepwise construction of a carboxysome-based CCM in land plant chloroplasts. (1) HCO_3^- transporters such as BTC1 (purple), BicA (pink), and SbtA (white) need to express in the inner-envelope membrane of chloroplasts. (2) Stromal CA is removed to allow for efficient HCO_3^- accumulation and to prevent CO_2 leakage. (3) Proteins that comprise the carboxysome shell need to self-assemble to form a carboxysome structure within chloroplasts. Transgenic α- or β-carboxysomes (blue diagram box) need to incorporate the appropriate linkers, condensation modules, and scaffold proteins to enable matrix condensation and protein shell encapsulation. (Created with BioRender.com)

uptake complex within the thylakoid membrane (Fig. 5.2). So far, extensive research has gone into the first three steps, which will be discussed further in this section.

5.4.2.1 Step 1: To Insert Cyanobacterial HCO_3^- Transporters in Chloroplast Membranes

Historically, an early strategy toward carboxysome-based CCMs in land plants involved engineering chloroplasts to express HCO_3^- transporters instead of incorporating whole carboxysomes (Hennacy and Jonikas 2020). This hypothesis is partly supported by reaction-diffusion modelling, which suggests that transforming non-CCM crops with cyanobacterial HCO_3^- transporters could increase daily biomass accumulation by 5–10%, which can have a significant cumulative effect on final

yield (McGrath and Long 2014; Price et al. 2011). Initially, transporters such as bicarbonate transporter A (BicA) and sodium-dependent bicarbonate transporter A (SbtA) were transformed into higher plants, because they were encoded by a single gene (Rae et al. 2017). Single-gene proteins avoid the challenge of targeting multiple domains to the correct cellular location. However, the StbA failed to accumulate carbon in chloroplasts or to increase photosynthetic performance in higher plants, emphasising the need for further research into the mechanisms that regulate HCO_3^- transporter activity (Nguyen et al. 2024; Rae et al. 2017).

Recently, updated reaction-diffusion modelling is challenging the widely cited notion that expressing HCO_3^- transporters in the inner envelope membrane (IEM) of land plant chloroplasts would improve photosynthetic efficiency (Kaste et al. 2024). In this spatially resolved model of land plants with HCO_3^- transporters, Kaste et al. (2024) demonstrate that previous models underestimate lipid membrane permeability, which, when modelled, supports a higher photosynthetic efficiency. However, when the membrane porosity is adjusted to physiologically observed levels, more CO_2 leakage, reduced Rubisco fixation and lower light use efficiency are predicted. This results in energetically wasteful HCO_3^- pumping. The contrasting findings direct researchers and the wider scientific community to be cautious and through when simulating and interpreting modelled data. Additionally, neither simulation model has thus far been experimentally demonstrated.

Inorganic carbon transporter B (ictB) has attracted interest and debate regarding whether it contributes to carbon concentration as an active transporter or passive channel (Koester et al. 2021; Price et al. 2012). *IctB* expression in soybean and maize was associated with modestly increased photosynthesis and yield in greenhouse and field trials, representing a major milestone in the agricultural translation toward carboxysome-based crops (Hay et al. 2017; Koester et al. 2021). However, when expressed in tobacco, *ictB* failed to improve photosynthesis or yield in field trials (Ruiz-Vera et al. 2022). Thus, the role of IctB in photosynthesis and yield appears to be species and environmentally dependent.

There has been recent success in targeting the larger, multi-domain BCT1 to chloroplast membranes in tobacco (Rottet et al. 2024). Rational design and directed evolution were used to optimise *BCT1* expression, localisation, and functionality in *Escherichia coli* prior to transformation in *Arabidopsis*. Although BCT1 domains localised to the IEM, and in the correct orientation, transgenic *Arabidopsis* observed lower CO_2 assimilation rates compared to WT controls. Although the correct localisation and orientation have been achieved, HCO_3^-transporter functionalisation remains to be achieved in planta.

5.4.2.2 Step 2: To Eliminate Endogenous CA from the Chloroplast Stroma

Similar to pyrenoid-based chloroplasts, carboxysome-based CCMs are expected to accumulate carbon more efficiently if native CA is removed from the chloroplast stroma and replaced with carboxysome-associated CAs (Nguyen et al. 2024; Price

et al. 2012). In carboxysome-engineered chloroplasts, the single step of removing CA from the stroma was estimated to increase HCO_3^- to 2–3 mM, which was enough to saturate CO_2 fixation (Price et al. 2012). This was achieved through CRISPR/Cas9 editing in tobacco or by transforming *Arabidopsis* with non-functional variant of CA, which expectedly hindered growth and fertility. Stromal CA was involved in metabolic regulation in C_3 crops. Although it was assumed that relocalised CA would not affect its role in anaplerotic pathway activity, this remains to be demonstrated as no study has concurrently reduced or eliminated stromal CA and expressed encapsulated CA (Nguyen et al. 2024).

5.4.2.3 Step 3: To Assemble a Functional Carboxysome in the Chloroplast Stroma

To assemble a functional carboxysome, cyanobacterial Rubisco must be stoichiometrically co-expressed with CA, scaffold, carboxysome shell, and linker proteins (Chen et al. 2023). In tobacco, a 'minimal carboxysome' was created by replacing endogenous Rubisco with *Cyanobium marinum* PCC7001 (*Cyanobium*) Rubisco, which was introduced together with the shell protein CsoS1A and a CsoS2 linker, which tethers Rubisco to the carboxysome shell (Long et al. 2018). At 2% CO_2, exogenous Rubisco in tobacco had similar catalytic activity to WT *Cyanobium* Rubisco. However, when enclosed in the minimal carboxysome, Rubisco's catalytic rate dropped drastically even at high CO_2 levels. Recently, Chen et al. (2023) demonstrated Rubisco catalytic activity in a carboxysome comprised from nine genes. Despite functionality, transformed tobacco failed to germinate under ambient CO_2 conditions, grew slowly, and had poor net CO_2 assimilation rates at 1% CO_2. Presently, although carboxysomes can be assembled in higher plants and demonstrate functional Rubisco, they can only support autophagic growth under elevated CO_2 (Chen et al. 2023; Long et al. 2018; Nguyen et al. 2023).

Advances in structural biology are paving the way for resolving the mechanisms behind carboxysome assembly, which will be important steps in the engineering of carboxysomal traits into chloroplasts. In α-carboxysomes from two distinct species, cryo-electron transmission resolved that the CsoS2 linker proteins either bind ubiquitously to Rubisco throughout the condensate or preferentially to Rubisco near the carboxysome shell in *Cyanobium* (Ni et al. 2022). Additionally, in cryo-electron microscopy, Blikstad et al. (2023) showed that CA is recruited to the a-carboxysome condensate by interacting directly with Rubisco. In β-carboxysomes, the CcmM protein, termed M58, has an N-terminus gCAL binding site, which organises the scaffold protein into a trimer and binds to the N-terminus of CA (Zang et al. 2021). The M58 trimer also recruits Rubisco via small subunit-like modules, which are strung together by linker proteins. Understanding the interactions between carboxysome enzymes and their associated scaffolds and linkers could optimise carboxysome assembly in planta.

5.5 Biochemical Carbon-Concentrating Mechanisms

In contrast to biophysical CCMs, biochemical CCMs follow a two-step process to metabolically fix and concentrate carbon into organic intermediates, which are then delivered to Rubisco (Rottet et al. 2021). These two processes are achieved either spatially, across two different cells as with C_4 plants, or temporally in CAM plants (Yuan et al. 2020). In C_4 species, the relevant biochemical and anatomical adaptations result in increased photosynthesis, water, and nitrogen use efficiency compared to C_3 species (Zhao et al. 2023). In addition to a tenfold increase in carbon concentration near Rubisco, C_4 photosynthesis also confers adaptive advantages to plants in hot and dry climates (Schlüter and Weber 2016; Simpson et al. 2021). As such, biochemical CCMs present an equally attractive approach for engineering plants to achieve enhanced photosynthetic capacity. This section will discuss the approaches, successes, and challenges currently underway to engineer biochemical CCMs, focusing primarily on engineering C_4 traits into C_3 plants (Fig. 5.3). The C_3 to C_4 transition could equip global crop staples like rice, wheat, and soybean with C_4 characteristics to facilitate faster growth and improved yield. Wherever possible, a perspective on the feasibility of current approaches as well as recommendations for future research will also be discussed.

5.6 Background and Brief Evolution C_4 Photosynthesis

C_4 photosynthesis occurs across two cell types, mesophyll (M) and bundle sheath (BS) (Schlüter and Weber 2016). HCO_3^- produced by CA in M cells is fixed into a four-carbon OAA by phospho*enol*pyruvate carboxylase (PEPC) (Schlüter and Weber 2016). OAA is either converted to malate or aspartate and subsequently shuttled across the BS-M membrane interface and into BS cells by transporters (Singh et al. 2022). C_4 photosynthesis is divided into three subtypes, based on the decarboxylating enzyme (Lin et al. 2020). In the first subtype, NADP-dependent malate dehydrogenase (NADP-ME) converts OAA to malate, which is transported to BS chloroplasts and decarboxylated by NADP-ME (Simpson et al. 2021). The second subtype involves aspartate transport to the BS mitochondria, where it is converted back into malate and decarboxylated by NAD-ME. The third subtype incorporates the NAD-ME pathway alongside a second pathway, in which aspartate is converted back into OAA and decarboxylated by phosphoenolpyruvate carboxykinase (PEPCK). Through these mechanisms, C_4 photosynthesis can dramatically reduce photorespiration, thus enhancing photosynthetic carbon fixation.

C_4 evolution is widely accepted as a notable example of convergent evolution event, having evolved independently over 65 times from the C_3 ancestral state (Sage and Coleman 2001). Understanding how plants arrived at a C_4 system could provide foundational knowledge that could be used to recapitulate the C_3 to C_4 transition. Increased leaf venation and subsequent BS enlargement are believed to have been

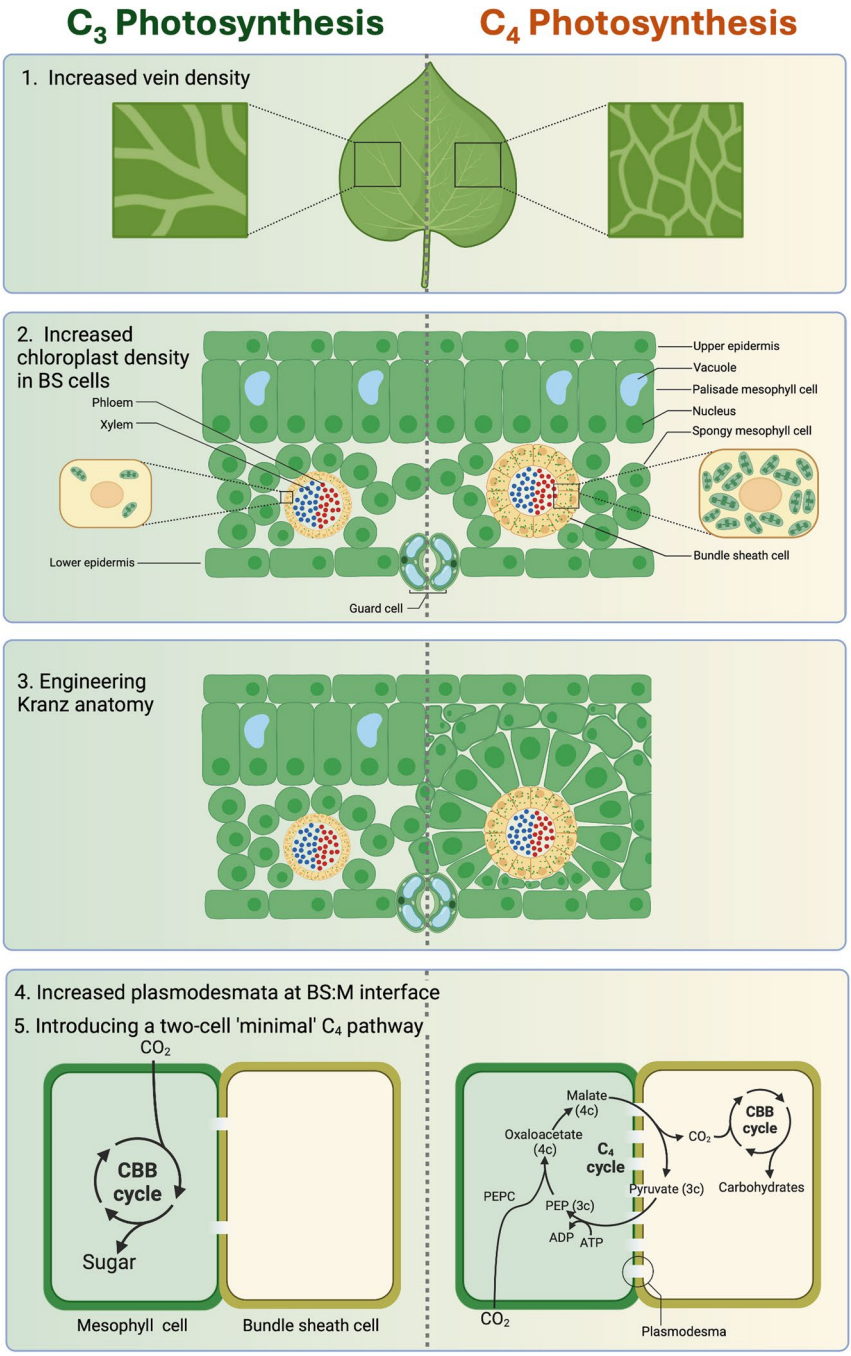

Fig. 5.3 Anatomical and biochemical differences between C_3 and C_4 photosynthesis. To achieve a C_4 anatomy in C_3 species, (1) leaf vein density is increased to improve the supply of water and nutrients to the photosynthesising cells. (2) BS cells increase in size and incorporate more chloroplasts for optimised Calvin-Benson-Bassham cycle operation. (3) M cells are concentrically oriented around the BS cells to supply C_4 intermediates to the BS. (4) Finally, plasmodesmata junctions frequency increases at the BS:M interface, which ensures efficient delivery and regeneration of C_4 intermediates. (Created with BioRender.com)

early steps in the C_3-C_4 transition (Bräutigam and Gowik 2016; Simpson et al. 2021). Increased access to water and nutrients improved the photosynthetic abilities of proximal cells. To keep up with the photosynthetic demand, BS cells, which are closest to the veins, evolved to become larger and house more chloroplasts. Additionally, under limited CO_2, a photorespiratory pump evolved to shuttle photorespiratory glycine to BS cells, where it is decarboxylated for secondary carbon concentration in BS cells (Bräutigam and Gowik 2016). To support increased carbon fixation, the genes that encode the photosynthetic machinery also had to either be upregulated or duplicated. This is evidenced by recent whole-genome duplication of existing C_3 genes which provided additional copies for a C_4 pathway to develop in the background, without compromising the existing C_3 system (Schlüter and Weber 2016).

In addition, whole-genome sequencing efforts are shedding more light on the evolution of C_4 photosynthesis by enabling the end-to-end assembly and annotation of C_4 *Gynandropsis gynandra* (Gynandropsis) and its close C_3 relative *Tarenaya hassleriana* (Tarenaya) (Hoang et al. 2023). The findings led to a model that postulates a whole-genome duplication in both species, which was subsequently lost in the C_3 species. In *Gynandropsis*, genome duplication expanded gene families relating to vein density, heat tolerance, C_4 photosynthesis, and photorespiration. Finally, a recent genome expansion by 50% integrated long terminal repeat retrotransposons (LTR-RT) into promoter genes facilitated the optimisation of C_4 gene expression (Zhao et al. 2023).

High-throughput next-generation sequencing strategies are therefore a valuable tool that evolutionary biologists can use to fill in the phylogenetic gaps. Additionally, research into gene regulatory elements suggest that most of the genes and regulatory elements that facilitated the C_4 pathway were derived from the ancestral C_3 genome and were rewired and recruited for C_4 photosynthesis (Amy Lyu et al. 2023; Singh et al. 2023). The next steps include constructing comprehensive phylogenies and understanding *cis* and *trans* gene regulation that drove C_4 evolution.

5.6.1 Toward a C_3 to C_4 Anatomical Transition

5.6.1.1 Increasing Vein Density

Notable anatomical features, collectively referred to as the Kranz anatomy, complement the biochemical C_4 cycle. This anatomy is defined by a high vein density with chloroplast-rich BS cells surrounding the veins and then M cells surrounding the BS (Bräutigam and Gowik 2016; Lundgren et al. 2014). Increased vein density is widely considered to be an early step toward a C_4 anatomy (Feldman et al. 2017; Walsh et al. 2023). Not only would a higher vein density encourage the correct ratio of BS to M cells, but it would also facilitate the rapid transport of metabolites (Liu and Cheng 2024; Lundgren et al. 2019). Additionally, if appropriate precursor structures exist in a plant, increasing leaf venation could be sufficient to induce a C_3 to

C_4 transition (Lundgren et al. 2019). However, engineering increased venation is not an easy task. The temporal and spatial regulation of venation is a complex process influenced by several known and unknown mechanisms, including auxin and other hormone signalling, protein-protein interactions, and cis-regulatory element signalling, among other factors (Liu and Cheng 2024).

Mutagenic screening of genetically diverse insertion and deletion libraries could yield candidate C_3 lines with increased vein density, thereby circumventing engineering constraints related to venation (Feldman et al. 2014, 2017; Lo et al. 2022). Screening mutant rice libraries identified eight lines with significantly increased venation compared to WT lines Feldman et al. (2014). Phenotypically, there was a strong correlation between vein density and leaf width, which could provide breeders with a quick phenotypic trait marker to accelerate vein density screening. Feldman et al. (2017) then analysed the photosynthetic performance of five of the eight lines and found significantly increased Vc_{max}, J_{max}, net photosynthesis rate, and g_s in mutants, compared to WT lines. Additionally, a mutagenic screening of the same insertion mutant rice line identified a leucine-rich repeat protein as a key regulator of venation (Lo et al. 2022). However, when overexpressed in T_3 rice lines, the initial 12% increase in vein density compared to WT rice dwindled to 4% in later developmental stages. Furthermore, no changes in photosynthetic parameters were reported.

The above findings highlight the need for more detailed transcriptomic and gene-regulatory assays that can help identify candidate genes and molecular mechanisms linked to increased venation in mutant lines. Nonetheless, candidate C_3 mutant lines could provide researchers with the necessary preconditions in which C_4 cycle modifications could be integrated.

5.6.1.2 Increasing Chloroplast Density in BS Cells

One of the four characteristics of the Kranz anatomy deemed essential for the proper functioning of a C_4 system is that the cells where the Calvin-Benson-Bassham cycle occurs (predominantly in the BS cells) should be chloroplast rich (Lundgren et al. 2014). Therefore, engineering the C_4 anatomy in C_3 species involves increasing chloroplast density in cells surrounding leaf veins. *GOLDEN2-LIKE* (GLK) genes are transcription factors that are known to promote chloroplast development and have emerged as promising targets for chloroplast enrichment in BS cells (Susila et al. 2023). When overexpressed under either under constitutive and cell-specific maize promoters, *GLK2*, which is preferentially expressed in BS cells, significantly increased chloroplast size and/or number in a tissue specific manner (Wang et al. 2017; Yeh et al. 2021). When looking at yield data, maize-specific promoters outperformed constitutive promoters, evidenced by an 80% increase in growth and 70% increase in grain yield in a double *GLK1/2* overexpression rice line (Yeh et al. 2021). Identifying candidate C_4 genes and combining them with appropriate promoter sequences are a feasible strategy that is realising the goal of improving photosynthesis in C_3 crops.

Mutagenic screening, sequencing, and gene editing are key driving forces in understanding BS development and manipulation. Transgenic *Arabidopsis* lines that expressed luciferase or chloroplast-tagged GFP under a C_4 BS-specific promoter were chemically mutagenized (Döring et al. 2019). Screening via confocal microscopy lines revealed nine M_2 lines with increased or diminished reporter signalling. In lines with increased reporter signalling, BS cell counts were higher and more radially arranged compared to the reference line. Mutants showing increased reporter signalling were sequenced and mapped to identify candidate genes, which were then investigated further using CRISPR/Cas9-knockout assays (Bano and Westhoff 2024). Knocking out the mutated *At2g25970* gene was shown to increase the GFP reporter signal and as well as leaf surface area. WT *At2g25970* is likely a multifunctional gene that negatively regulates the cell cycle. Although the identified KH domain gene may not be the most ideal candidate for enhancing BS proliferation in the C_3 plants given its pleiotropic functions, the study indicates that reporter-based forward genetics could elucidate BS regulation.

Through modifications of CRISPR/Cas9 technology, Lee et al. (2021) demonstrated a proof of concept for multiple gene regulation in a tissue-specific manner using a dead Cas9 (dCas9). The team generated transgenic rice lines that overexpressed a native chloroplast biosynthesis-gene *OsCGA1*. Transgenic lines were reported to have significantly increased BS chloroplast numbers. By fusing Cas9 to activation modules, dCas9 acquired a transcriptional activation function. In addition, a series of guide RNAs (gRNAs), which were complementary to different promoter regions, targeted the dCas9 module to upregulate both endogenous and exogenous CGA1. As the dCas9 and transgenic *OsCGA1* constructs were both placed under a C_4 *Flaveria trinervia* promoter, which is highly active in BS cells, both *CGA1* genes were expressed exclusively in BS cells. These findings introduce the possibility of regulating multiple Kranz anatomy or C_4 cycle genes using a streamlined system using gRNAs.

5.6.2 The Road to Kranz: Engineering Enhanced Photosynthetic Leaf Anatomy

The Kranz anatomy formation is delineated by three steps: (1) the innermost leaf cells differentiate into procambium, which then develop into vasculature systems; (2) M and BS cells are radially arranged in a 'wreath-like' structure, around the vasculature; and (3) sheath and mesophyll cells acquire increased photosynthetic capacity (Sedelnikova et al. 2018). Engineering the first two steps presents an incredible anatomical feat that requires correct spatial and temporal regulation (Sedelnikova et al. 2018). Additionally, the complex interactions of the gene-regulatory pathways involved are yet to be elucidated (Sedelnikova et al. 2018).

Despite these challenges, progress is evident. Wang et al. (2017) observed features of a Kranz-like structure in rice via BS-specific GLK2 overexpression, which

included enlarged mitochondria in sheath cells and anatomical ratios comparable to a proto-Kranz structure. However, no improvements in photosynthesis or yield were found when compared to WT controls, suggesting that the anatomical changes were insufficient to confer photosynthetic advantages.

Singh and Reeves (2020) highlight the relevance of epigenetics and gene regulatory networks that regulate leaf development and anatomy by expanding on research done by van Rooijen et al. (2019). A post-transcriptional repressor NAC052 was found to regulate leaf development and arrest it before it progresses into a Kranz-like anatomy in *Arabidopsis*. However, when ectopically expressed, NAC052 assumed transcriptional activator function, which was noted by increased BS cells among other changes. Further studies into gene regulatory networks and *trans* elements in C_4 species are therefore crucial in unravelling the intricacies that define C_4 regulation (Singh and Reeves 2020). Given the complexity of leaf development and Kranz anatomy, characterising the multitude of genes and regulatory elements will take years (Sedelnikova et al. 2018). Consequently, modifying C_3 leaf anatomy will require more fundamental research and understanding before being considered as a feasible photosynthetic improvement strategy.

5.6.3 Intercellular Communication Through Plasmodesmata

For a dual-cell photosynthetic system to be efficient, robust intercellular transport of metabolites is crucial. Intercellular junctions called plasmodesmata (PD) facilitate this flux and are more frequent and closely clustered in C_4 species compared to C_3 counterparts (Danila et al. 2016; Simpson et al. 2021). It is estimated that if the C_3 to C_4 biochemical transition is established, current C_3 plasmodesmata structures would need to achieve a flux 12 times that of current C_4 rates (Danila et al. 2016). Therefore, optimising the BS-M plasmodesmata frequency is necessary to support the biochemical changes that could accompany the C_3 to C_4 transition.

Electron and confocal microscopy, as well as immunological assays, are common techniques used to visualise and quantify plasmodesmata frequency (Danila et al. 2016; Liu and Cheng 2024). Using these approaches, Schreier et al. (2024) found that C_4 *Gynandropsis* had an eightfold increase in PD frequency at the M-BS junction compared to C_3 *Tarenaya* and *Arabidopsis*. This M-BS PD frequency is partly mediated by photosynthetic metabolite formation during the early developmental stages following de-etiolation. These findings may explain why increased plasmodesmata frequency between mestome and BS cells was observed upon *GLK2* overexpression in BS cells discussed above (Wang et al. 2017). Increased photosynthetic activity and metabolite synthesis may necessitate intercellular connections between sheath cells in the form of increased PD frequency.

Previous work with understanding plasmodesmata clustering and pore size has been hindered by the inaccessibility to penetrate the cell walls and challenges in isolating the associated proteins that localise to plasmodesmata. Recent advancements such as Plant-Enzyme-Assisted (PEA) CLARITY renders clear plant tissue

for high-resolution confocal or electron microscopy, allowing for more accurate PD visualisation and quantification (Liu and Cheng 2024; Tsang et al. 2024). Other approaches include GF tagging of known PD proteins for quantification using confocal microscopy, together with mRNA sequencing and gene co-expression network analysis (Tsang et al. 2024). By applying these techniques at different developmental stages of rice and millet development, a spatiotemporal atlas of PD density was created, along with the identification of up to 57 genes that mediate PD frequency (Tsang et al. 2024). With these advances, researchers can look forward to more streamlined quantification of PD-associated proteins and can investigate the identified candidate genes for functional analysis.

5.6.4 Toward a C_3 to C_4 Biochemical Transition

Recapitulating the key biochemical events that facilitated C_4 evolution one at a time will help fill in the gaps in our understanding of C_4 photosynthesis. For example, the re-localisation of CA from the mesophyll chloroplast to the mesophyll cytosol is considered an important step that facilitated and sustained the development of a C_4 biochemical pathway (Singh et al. 2022). This was successfully achieved in rice by genetically mutating or truncating the chloroplast transit peptide sequence that targets CA to chloroplasts (Jethva et al. 2024). However, cytosolic CA showed no differences in terms of yield, growth rates, photosynthetic rates, or CO_2 concentrations near Rubisco. Despite this, successful re-localisation indicates that the current C_3 genome can be edited to direct its evolution toward a C_4 system.

Advances are being made to develop a functional 'minimal' C_4 cycle, which consists of genes from the NADP-ME subtype. Initially, C_4 genes were inserted individually and then crossed and verified repeatedly to achieve trait stacking and was a time-consuming process (Lin et al. 2020). The advent of Golden Gate cloning has fast-tracked this process, allowing a suite of C_4 genes to be integrated into host genomes in one transformation event (Ermakova et al. 2021). In addition, incorporating cell-specific promoters in the constructs also enable tissue-specific gene expression, which partly circumvents localisation challenges (Ermakova et al. 2021). Both traditional and Golden Gate trait stacking were combined with $^{13}CO_2$ labelling to measure carbon flux through the modified pathway and demonstrated a partially functional C_4 pathway (Ermakova et al. 2021).

Similarly, single-celled omics is quickly becoming a key player in uncovering C_4 photosynthetic regulation. Single-celled indexed assay for transpose accessible chromatin sequencing (sciATACS-Seq) of different cell types from four different C_4 grasses successfully produced a C_4 cell-specific gene regulatory map (Mendieta et al. 2024). This map suggests that cell-specific regulatory networks are a necessary regulatory element for a functional C_4 pathway. Additionally, the rapid sequence diversification of central regulatory elements across C_4 species is a strong indicator of ongoing evolution (Zhang and Zhang 2022). More specifically, transposable elements (TEs), which modulate proximally located genes, are found to be

instrumental components of accessible chromatin regions (ACRs) in rice (Zhang and Zhang 2022). TE-derived ACRs were found to be highly genetically variable, enabling the selection of advantageous agronomic traits. These traits, in turn, drove the selective fixation of the TE-derived ACRs during rice domestication. The C_3 to C_4 transition will therefore need to identify strongly conserved *cis* regulatory elements as well as consider suitable methods to recapitulate key regulators in a cell-specific manner.

Despite these advancements, the road toward C_3 to C_4 photosynthesis bioengineering comes with its challenges. It has been evolutionarily shown that the C_4 elements already exist in C_3 genomes (Singh et al. 2023). It has also been demonstrated that in part, rewiring these elements to mimic a C_4 pathway is possible. However, it seems that C_4 ancestral genes are primarily involved in stress responses in C_3 genes (Chen et al. 2022; Singh et al. 2022). Developing mechanisms that will coopt current C_3 isoforms to perform photosynthetic functions in addition to their current stress-related functions remains a challenge. Another stumbling block is that C_4 gene expression is significantly elevated compared to C_3 pathway enzymes (Furbank et al. 2023). RNAi knockdown studies suggest that reducing any of the C_4 enzymatic pool by up to 50% contributes to a maximal 21% reduction in photosynthesis (Furbank et al. 2023). This means that the full C_4 enzyme levels are not required to achieve a functional system in C_3 species. However, current C_3 to C_4 expression levels do not meet the threshold for functional C_4 photosynthesis (Ermakova et al. 2020). Furthermore, although concurrent gene regulation of two genes was recently demonstrated, this technology would need to be developed further to regulate the five 'minimal' C_4 enzymes for optimal gene expression and modulation. Overall, engineering the anatomical components of the biochemical C_4 transition in C_3 crops is forging ahead in a non-sequential order with successes and challenges being experienced simultaneously.

5.7 CAM Photosynthesis

High temperatures and arid conditions created a unique selection pressure for CAM species, which evolved the ability to fix CO_2 at night (Perron et al. 2024). CAM photosynthesis emerged through alterations in the circadian regulation of ancestral C_3 photosynthesis genes and in stomatal opening and closing patterns (Perron et al. 2024). Due to nocturnal stomatal opening, CAM plants have a sixfold increase in WUE compared to C_3 crops (Yang et al. 2015). CAM-engineered C_3 crops present a unique opportunity to grow crops using less water while also expanding the use of marginal land for crop farming, both of which are pertinent in meeting rising food demands amidst dwindling fresh water supplies (Yuan et al. 2020).

CAM photosynthesis is broadly divided into four phases. The first phase begins at night, when CO_2 diffuses into leaves through open stomata and is fixed into OAA by PEPC (Perron et al. 2024). The four-carbon compound is then converted to malate and is stored in the vacuole as malic acid until daybreak (Yuan et al. 2020).

Phase two is initiated in the early morning hours and is a transitional phase during which carbon is fixed by both PEPC and Rubisco (Burgos et al. 2022). Malic acid is decarboxylated back into PEP by malate dehydrogenase (MDH) during phase three, which releases CO_2 for fixation by rubisco (Guan et al. 2020). The stomata remain closed during this phase. Finally, during phase four, stomatal reopening is partly triggered by the depletion of low malate levels toward the end of the day, which restarts the cycle (Tan and Chen 2023).

CAM photosynthesis is another example of convergent evolution and is found in at least 400 genera (Yuan et al. 2020). Facultative CAM species have evolved to operate the C_3 pathway when conditions are optimal but switch to CAM photosynthesis under abiotic stress (Winter and Holtum 2024). Harnessing this facultative CAM system in C_3 would confer crops with the dual benefits of high yield through C_3 photosynthesis and CAM-mediated drought tolerance (Yuan et al. 2020). Yang et al. (2024) proposed a simplified strategy to engineer facultative CAM in C_3 species (Fig. 5.4). The first step proposed reprogramming the timing of gene expression

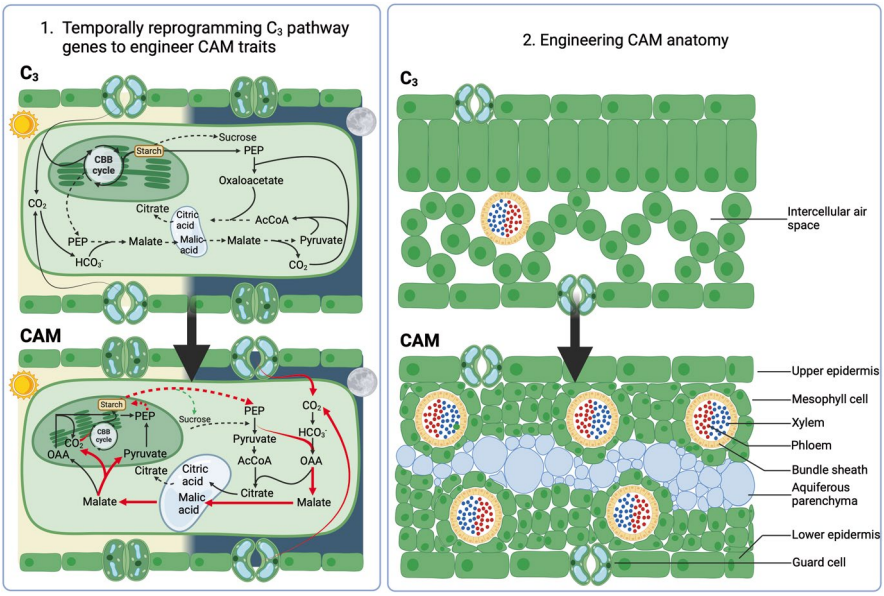

Fig. 5.4 Metabolic and anatomical modifications that enable C_3-CAM transition. (1) Transitioning from C_3 to CAM photosynthesis involves modifying the circadian expression of C_3 genes. This includes engineering stomatal opening at night to facilitate CO_2 uptake. Additional nocturnal reprogramming upregulates starch digestion for glycolysis, PEP conversion to malate, and malic acid storage in the enlarged vacuole. During the day, malate decarboxylation is prioritised to supply CO_2 to the CBB cycle for starch production. Starch accumulates throughout the day, partly due to the downregulation of starch hydrolysis into sucrose. (2) CAM anatomy features densely packed mesophyll cells and specialised water storage cells called aquiferous parenchymal cells, the latter of which ensures diurnal photosynthesis when stomata are closed. Dotted arrows denote a multi-step process. Upregulated metabolic pathways are shown in red, and downregulated pathways are illustrated with green arrows. (Created with BioRender.com)

for genes common to both C_3 and CAM pathways. Secondly, C_3 leaf anatomy must be modified to a CAM-like anatomy, which would sustain the temporal shift in metabolic activity.

5.7.1 *Reprogramming C_3 Circadian Regulation to Induce CAM*

Reshuffling the circadian cycle of the genes that mediate carboxylation, decarboxylation, and stomatal activity is the foremost factor for engineering CAM photosynthesis. A comparative genomics study identified 54 CAM genes that showed altered diurnal cycle patterning (Yuan et al. 2020). A key challenge will be to determine the minimal number of genes that need to be temporally rewired to achieve CAM-like photosynthesis in C_3 species. Among these genes and regulatory changes, the malate pathway regulation is believed to be crucial in inducing CAM biochemistry (Burgos et al. 2022). Stomatal closure is regulated by high C_i through negative feedback (Schiller and Bräutigam 2021). It will therefore be important to increase malate levels to a high enough concentration such that decarboxylation increases CO_2 release and C_i, thus triggering stomatal closure (Burgos et al. 2022). Presently, circadian reprogramming of any of the CAM metabolic pathways has not been demonstrated in model or C_3 species.

Recent advances in omics studies are uncovering the mechanisms of CAM photosynthesis and regulation. Following 6 days of salinity treatment, the facultative CAM species *Mesembryanthemum crystallinum* (ice plant) upregulated V-type H^+-ATPase expression, which pumps H^+ into the vacuole and mediates salt and drought tolerance (Guan et al. 2020). Additionally, significant changes in abscisic acid (ABA) regulation initially induced drought-mediated stomatal closure, which was followed by the nocturnal upregulation of ABA inhibitors. Other omics assays are continuing to shed light of CAM induction, particularly with stomatal regulation, which also undergo a C_3-CAM transition when under salt stress (Kong et al. 2020). Although CAM engineering in C_3 is still in the preliminary research stages, advancements in single-cell omics are rapidly filling the gaps in our understanding of temporally regulated CAM.

5.7.2 *Engineering CAM Anatomy*

Increased succulence is a characteristic feature of most CAM species. Succulence is mediated by an extensive parenchymal network (Yuan et al. 2020). CAM plants have also densely packaged mesophyll cells, which reduce both intercellular air spaces (IAS) and the mesophyll surface area that is exposed to IAS (Yuan et al. 2018). These anatomical features reduce the conductance of CO_2, which allows for

efficient CAM CO_2 fixation (Yuan et al. 2020). Overexpressing succulence-associated genes, such as *xyloglucan endotransglucosylase/hydrolase (XTH)* and *cell expansion basic helix-loop-helix (CEB1)* has shown moderate success in reducing IAS, increasing cellular packing, higher salt tolerance, and increased WUE (Yuan et al. 2020). Additionally, C_3 vacuoles need to expand to accommodate large malate pool that supports CAM photosynthesis (Burgos et al. 2022). Modulating V-type H^+-ATPase mentioned earlier alongside circadian modification may facilitate vacuole enlargement. Further research is needed to fully understand the CAM anatomy before optimisation into C_3 species can proceed.

Insights into C_4 species that feature CAM photosynthesis demonstrate the plasticity of CAM photosynthesis. *Portulaca oleracea*, a C_4 species known to operate CAM under stress conditions, was initially thought to operate both systems in different cells through unknown mechanisms (Guralnick et al. 2002). However, recent spatial transcriptomics study has shown that, under drought conditions, C_4 and CAM pathways both operate in the same BS and M cells, but at different times (Moreno-Villena et al. 2022). C_4 gene homologues evolved to be repurposed for CAM, which are upregulated at night to avoid futile cycling between the two pathways. Additionally, malate produced during nocturnally active CAM is integrated into the C_4 pathway during the day. Furthermore, the success of C_4 + CAM operation is partly due to mild succulence, combined with sufficient vein density to support a high BS:M ratio (Moreno-Villena et al. 2022). This finding demonstrates that CAM is a flexible system that integrates seamlessly with C_4 photosynthesis, provided the appropriate temporal rearrangements are made. Spatial transcriptomics is resolving long-unsolved questions while expanding the possibilities regarding integrating different systems for improved photosynthesis and climate resilience in crops.

5.8 Engineering Chloroplast Genomes and New Cellular Compartments

5.8.1 Chloroplast Genome Engineering

Concurrently with nuclear photosynthetic improvement strategies, chloroplast genome editing is an active area of exploration and could serve as a foundational step toward engineering chloroplast mimics. Their small size, maternal transmission, and lack of epigenetic silencing make chloroplast genomes, or plastomes, an attractive system to introduce suites of transgenes that can be expressed at high levels (Bock 2022). Plastomes are characterised by multiple operon-mediated gene expression and several steps of post-transcriptional mRNA processing (Zhang et al. 2023). This operon style of gene expression can easily be replicated through

synthetic operons introduced by Golden Gate cloning. Additionally, integrating the operons with the appropriate post-transcriptional machinery is also important for successful plastid gene expression. For example, intercistronic elements (IEEs) are essential recognition sites which mediate the cleavage of polysictronic mRNA transcripts into individual transcripts and are associated with mRNA stability and translational efficiency (Bock 2022). In transplastomic tobacco, IEE incorporation into synthetic operons carrying carotenoid-related enzymes were shown to redirect the carotenoid pathway by increasing zeaxanthin production relative to astaxanthin and other carotenoids in tobacco (Tanwar et al. 2023).

Additionally, more efficient transformation strategies are encouraging the progression from modifying endogenous plastomes to synthetically engineering entirely new ones. Homologous recombination has been the standard plastid transformation protocol for the last 30 years and often involves applying sustained selective pressure across several rounds of positive selection to ensure the polyploidal plastomes all acquire the transgene (Jakubiec et al. 2021; Occhialini and Lenaghan 2023). Episomal DNA, which can replicate independently from plastid or nuclear DNA, circumvents the challenge of homoplastic integration (Occhialini and Lenaghan 2023). These independent replication vectors were either incorporated into the geminivirus replication system in tobacco or into a chloroplast specific origin of replication into potatoes (Occhialini and Lenaghan 2023). The resulting 'minichromosomes' or 'mini-synthetic plastome' replicate independently and are transmitted to subsequent generations (Jakubiec et al. 2021; Occhialini et al. 2023). It is easy to envisage the evolution of plastome engineering from individual gene transformations toward the bespoke design and integration of whole, independently replicating and heritable plastid genomes that deliver the photosynthetic code directly to the source.

5.9 New Cellular Compartments

As new technologies or advancements in current methods develop, so does the discovery of potentially groundbreaking science. Recently, reports of the nitrogen-fixing Candidatus *Atelocyanobacterium thalassa* (UCYN-A) symbiont and its algal host *Braarudosphaera bigelowii* (Braarudosphaera) suggest that UCYN-A is likely in the early stages of evolving into an organelle, aptly termed a nitroplast (Coale et al. 2024). By combining proteomics and high-resolution 3D X-ray technology, nitroplasts were observed to have undergone a genome contraction and recruited proteins expressed by nuclear *Braarudosphaera*. Additionally, the UCYN-A to *Braarudosphaera* size ratio is also consistent with those found in endosymbionts that evolved to become organelles (Cornejo-Castillo et al. 2024). Together, the speculative nitroplast provides a novel model system through which to study the

evolution of an exceptionally rare event, perhaps with the hope of eventually replicating primary endosymbiosis. Although still in its infancy, possibilities of engineering a nitrogen-fixing organelle into land plants would undoubtedly revolutionise current agricultural practices, not to mention the benefits to plant productivity and yield.

5.10 Conclusions and Future Perspectives

Advancing photosynthesis remains a cornerstone of the global agricultural strategy to combat the challenges posed by climate change and an increasing global population. Technological advancements are accelerating the transition from concept development toward the commercial deployment of CCM-engineered crops. This process is structured into Technology Readiness Levels (TRLs), which outline the pathway from fundamental research and conceptual development to the full commercialisation of crops with enhanced photosynthetic efficiency (Fig. 5.5) (CAPITALISE 2024).

Researchers have achieved several key milestones which advance the progression of different photosynthetic improvement strategies toward commercially viable CCM-engineered crops. Partial pyrenoid assembly in planta places this strategy on the border between concept development and proof-of-concept testing (TRL 2–3). Meanwhile, an assembled and lowly functional carboxysome in tobacco together with modestly successful field trials advance carboxysome-based CCMs to TRL 4. Similarly, moderate success with C_3 BS expansion in improving photosynthesis and yield in rice also infer proof of principle and a translational readiness of level four. In contrast to other strategies, CAM photosynthesis research is preferentially focused on understanding how it works, thus positioning it at TRL 2–3. Technical advancements are also enabling potentially revolutionary concepts for photosynthetic improvement through synthetic chloroplast-like compartments and the discovery of entirely new organelles.

Progression along the TRLs is often hindered by common bottlenecks, including gaps in understanding of regulatory elements, achieving correct protein localisation, and overcoming challenges in anatomical reprogramming. However, emerging techniques like spatial transcriptomics and spatial proteomics, directed evolution, and synthetic gene circuit engineering are unlocking new pathways to decipher the molecular mechanisms underpinning various CCMs. These breakthroughs hold transformative potential to revolutionise agricultural productivity by increasing yield, enhancing WUE, improving climate adaptability, and reducing fertilizer dependency, thereby providing innovative solutions for crop improvement. Ultimately, enhancing photosynthesis will be central to ensuring sustainable, resilient agricultural systems in the face of environmental pressures.FundingMKK and PS were supported by the UKRI-Future Leaders Fellowship (MR/Y016882/1), *REVOLUTON*, awarded to PS. The authors declare that they have no competing interests.

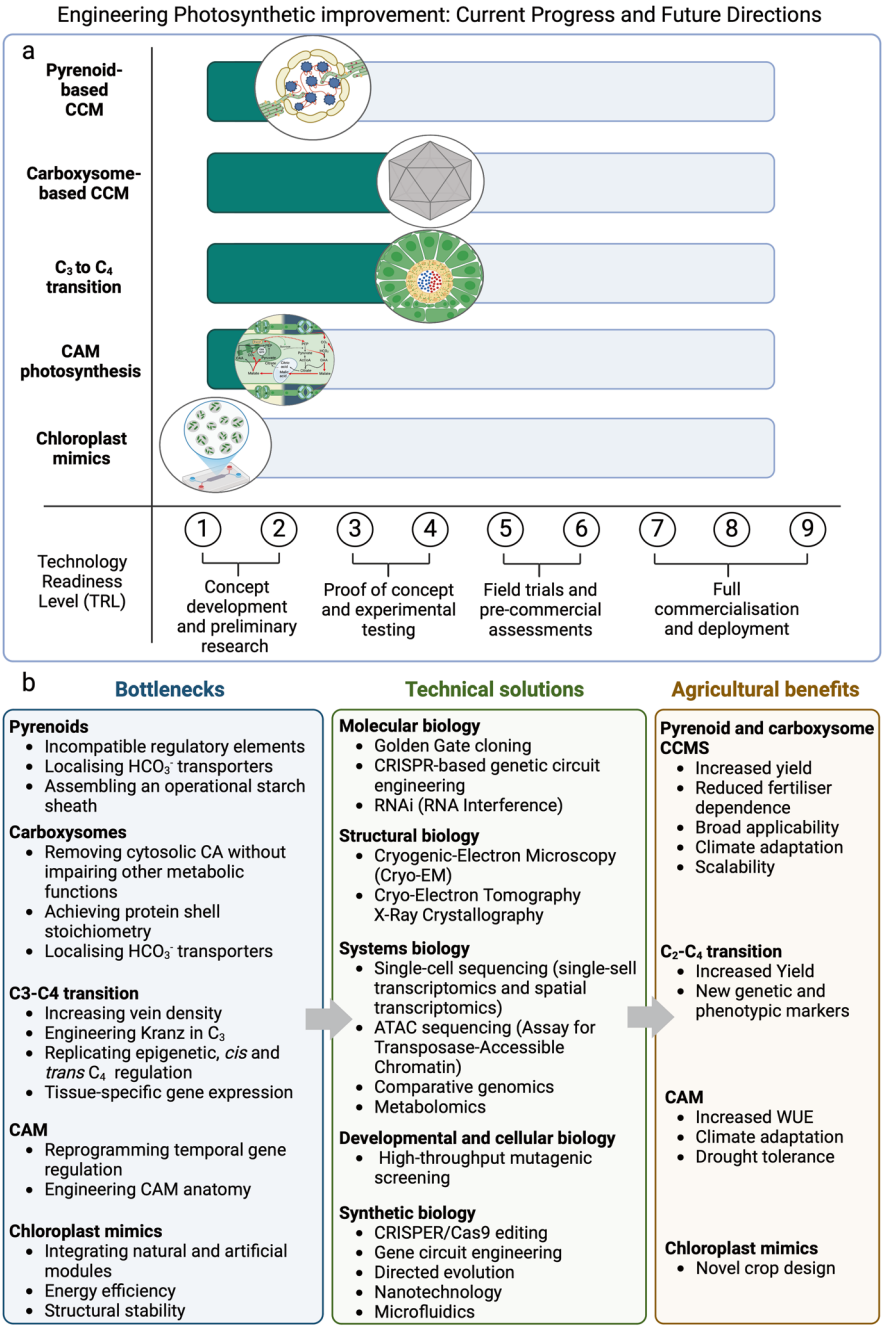

Fig. 5.5 Mapping photosynthetic pathways for crop improvement. (**a**) Technology readiness level (TRL) of photosynthetic improvement strategies. The timeline positions pyrenoid-based CCMs and CAM photosynthesis at TRL 2–3, carboxysome-based CCMs at TRL 4, and chloroplast mimics at TRL 1. (**b**) Identified bottlenecks of current improvement strategies, proposed technical solutions to accelerate these strategies, and an outline of the potential agricultural benefits. (Created with BioRender.com)

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