Engineered Accumulation of Bicarbonate in Plant Chloroplasts: Known Knowns and Known Unknowns

Sarah Rottet1†, Britta Förster2†, Wei Yih Hee1, Loraine M. Rourke1, G. Dean Price1,2* and Benedict M. Long1,2

1Realizing Increased Photosynthetic Efficiency (RIPE), The Australian National University, Canberra, ACT, Australia, 2Australian Research Council Centre of Excellence for Translational Photosynthesis, Research School of Biology, The Australian National University, Canberra, ACT, Australia

Heterologous synthesis of a biophysical CO2-concentrating mechanism (CCM) in plant chloroplasts offers significant potential to improve the photosynthetic efficiency of C3 plants and could translate into substantial increases in crop yield. In organisms utilizing a biophysical CCM, this mechanism efficiently surrounds a high turnover rate Rubisco with elevated CO2 concentrations to maximize carboxylation rates. A critical feature of both native biophysical CCMs and one engineered into a C3 plant chloroplast is functional bicarbonate (HCO3−) transporters and vectorial CO2-to-HCO3− converters. Engineering strategies aim to locate these transporters and conversion systems to the C3 chloroplast, enabling elevation of HCO3− concentrations within the chloroplast stroma. Several CCM components have been identified in proteobacteria, cyanobacteria, and microalgae as likely candidates for this approach, yet their successful functional expression in C3 plant chloroplasts remains elusive. Here, we discuss the challenges in expressing and regulating functional HCO3− transporters, and CO2-to-HCO3− converter candidates in chloroplast membranes as an essential step in engineering a biophysical CCM within plant chloroplasts. We highlight the broad technical and physiological concerns which must be considered in proposed engineering strategies, and present our current status of both knowledge and knowledge-gaps which will affect successful engineering outcomes.

Keywords: CO2-concentrating mechanism, bicarbonate transport, chloroplast envelope, improving photosynthesis, chloroplast engineering

INTRODUCTION

Crop improvement technologies utilizing synthetic biology approaches have been central to a number of recent advances in photosynthetic output (e.g., Kromdijk et al., 2016; Salesse-Smith et al., 2018; Ermakova et al., 2019; South et al., 2019; Batista-Silva et al., 2020; López-Calcagno et al., 2020). These ambitious aims come at an unprecedented time in human history when agricultural productivity must be rapidly boosted in order to feed future populations (Kromdijk and Long, 2016). In a 2008 review, we discussed the potential of utilizing components of the
CO₂-concentrating mechanism (CCM) of cyanobacteria as a means to improve crop photosynthetic CO₂ fixation (Price et al., 2008), with potential to raise rates of carboxylation at ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) while improving nitrogen and water-use efficiencies (Price et al., 2011a; McGrath and Long, 2014; Rae et al., 2017). In the intervening years great steps forward have been made to address this challenge, yet many uncertainties remain on the path to generating a functional chloroplastic CCM.

The CCMs of proteobacteria, cyanobacteria, and microalgae are comprised of bicarbonate (HCO₃⁻) transporters and vectorial CO₂-to-HCO₃⁻ conversion complexes which, in concert, accumulate a high concentration of HCO₃⁻ in prokaryotic cells and microalgal chloroplasts (Figure 1: Kaplan et al., 1980; Badger and Price, 2003; Moroney and Ynalvez, 2007). As a charged species of inorganic carbon (Cᵢ), HCO₃⁻ is not freely diffusible through cell membranes (Tolleter et al., 2017), and allows for the generation of an elevated cellular or stromal HCO₃⁻ pool compared with the external environment (Price and Badger, 1989a). The second chief component of these CCMs are specialized Rubisco compartments called carboxysomes (Rae et al., 2013) and pyrenoids (Figure 1; Moroney and Ynalvez, 2007; Mackinder, 2018; Hennacy and Jonikas, 2020) where co-localized carbonic anhydrase (CA) enzymes dehydrate HCO₃⁻ into CO₂, providing high concentrations of CO₂ as substrate for Rubisco carboxylation.

Collectively, these systems are often termed biophysical CCMs since their function utilizes the active movement of Cᵢ across cellular compartments to release it as CO₂ around Rubisco (Giordano et al., 2005). This is distinct from biochemical CCMs found in C₄ and CAM plants, which generally utilize HCO₃⁻ for the carboxylation of phosphoenolpyruvate into transportable organic acids, prior to spatial or temporal CO₂ re-release and carboxylation by Rubisco.

Modeling has shown that the installation of biophysical CCM HCO₃⁻ transporters in the inner-envelope membrane

**FIGURE 1** | Inorganic carbon uptake components of cyanobacterial, proteobacterial, and microalgal CO₂-concentrating mechanisms (CCMs). Key inorganic carbon transport mechanisms of cyanobacteria (A), proteobacteria (B), and microalgae (C) that facilitate elevated cytoplasmic and stromal HCO₃⁻ concentrations. The HCO₃⁻ pool is utilized to generate localized high concentrations of CO₂ in specialized Rubisco-containing compartments known as carboxysomes (A, B) or pyrenoids (C), supporting high carboxylation rates. In cyanobacteria (A), HCO₃⁻ is potentially supplied to the periplasmic space via an outer-membrane (OM) porin, and is directly transferred across the plasma membrane (PM) by the single-protein Na⁺-dependent transporters bicarbonate transporter A (BicA) and SbtA, or by the ATP-driven complex BCT1. In addition, cytosolic CO₂ acquired by either diffusion, leakage from the carboxysome or spontaneous dehydration of HCO₃⁻ is converted to HCO₃⁻ by the energy-coupled, vectorial CO₂ pumps NHD-1 and NHD-1, in the thylakoid membranes (TM). In proteobacteria (B), DabBA plays a similar role, taking advantage of relatively high rates of CO₂ influx from a low-pH external environment to vectorially generate a cytoplasmic HCO₃⁻ pool (Desmarais et al., 2019). In microalgae (C), HCO₃⁻ is accumulated via a series of transporters located on the PM (LCI1 and presumably ATP-driven HLA3), the chloroplast inner envelope membrane [inner-envelope membrane (IEM); LCI1] and the TM (bestrophins, BST1-3). Thylakoids traverse the Rubisco-containing pyrenoid where the thylakoid lumen-localized carbonic anhydrase (CA) CAH3 is thought to convert HCO₃⁻ supplied to the thylakoid lumen to CO₂. Analogous to the cyanobacterial system, the LGB/C complex constitutes a putative, vectorial CA that may recycle any CO₂ arising in the chloroplast stroma back to HCO₃⁻. Fd, ferredoxin; RuBP, ribulose-1,5-bisphosphate; 3-PGA, 3-phosphoglycerate; SbtB, SbtA regulator protein; and ChpX/ChpY, NDH-1 complex vectorial CO₂-HCO₃⁻ domains. Individual transporter proteins are as listed in Table 1.
ROTTET ET AL. Enhancing Chloroplast Bicarbonate Uptake

Morison et al., 2005; Flamholz and Shih, 2020). This underscores the fact that the chloroplast envelope is a reasonable question to ask is whether HCO$_3^-$ can be elevated in this organelle, and if so, how? There is sufficient HCO$_3^-$ in the mesophyll cytoplasm available for transport into chloroplasts (at least 250μM; Evans and von Caemmerer, 1996). However, a CCM engineering strategy must ensure HCO$_3^-$ can gain passage across both the outer-envelope membrane (OEM) and IEM of the chloroplast. Given that C$_3$ chloroplasts typically access C$_3$ from the external environment (primarily as the more membrane-permeable CO$_2$), chloroplast membranes appear not to have specific HCO$_3^-$ transport mechanisms (Rolland et al., 2012). Nonetheless, a number of oxygenions, such as phosphate, nitrate, and sulfate evidently do diffuse through the OEM (Bölter et al., 1999). Notably, simple diffusion of CO$_2$ through leaf tissue is insufficient to support the supply rates needed for observed rates of CO$_2$ assimilation by plants (Morison et al., 2005), and it is likely that CO$_2$ entry into the chloroplast is also facilitated by CO$_2$-permeable aquaporins (Flexas et al., 2006; Evans et al., 2009; Tollette et al., 2017; Erмакова et al., 2021) and CA-driven distribution of C$_3$ between predominant species (HCO$_3^-$ and CO$_2$; Price et al., 1994). Therefore, the facilitated entry of C$_3$ into C$_3$ chloroplasts is conceptually not counter to contemporary chloroplast function, and on face value would appear beneficial.

In general, solute transport across the chloroplast OEM is considered to be relatively unhindered due to the presence of low-selectivity and large-molecule channel proteins present in this membrane (Bölter et al., 1999; Hemmler et al., 2006; Duy et al., 2007). It is expected that anion passage into the intermembrane space (IMS), and presumably that of HCO$_3^-$, occurs via at least one of the outer envelope protein channels (OEPs; Duy et al., 2007), with OEP21 a potential route for broad anion uptake into the IMS (Figure 2; Hemmler et al., 2006).

The terrestrial nature of C$_3$ plants and their appearance in geological history during a period of relatively high atmospheric CO$_2$ (Flamholz and Shih, 2020) is a possible contributor to the absence of biophysical CCMs from higher plant chloroplasts (Raven et al., 2017). The efficiency of Rubisco carboxylation is hampered by O$_2$, leading to photospiratory expenditure of accumulated CO$_2$ and chemical energy (Busch, 2020). It is assumed that factors selecting for maintenance of relatively high rates of carboxylation, as atmospheric concentrations of CO$_2$ decreased while O$_2$ increased approximately 350 million years ago, may have led to a divergence in mechanistic adaptations between aquatic and terrestrial photosynthetic organisms (Flamholz and Shih, 2020; Long et al., 2021). Thus, cyanobacteria and many eukaryotic algae evolved CCMs to overcome these challenges, while emerging terrestrial C$_3$ plants have maintained a larger investment in Rubisco and evolved to maximize beneficial biochemical contributions from photorespiratory nitrogen and sulfur metabolism (Shi and Bloom, 2021). As a result, terrestrial C$_3$ plant lineages have not evolved with a capability to elevate chloroplastic C$_3$ concentrations like many of their aquatic counterparts. Indeed, there is good argument that biochemical CCM evolution (e.g., C$_3$ photosynthesis) would be favored in terrestrial systems over strategies which accumulate HCO$_3^-$ (Flamholz and Shih, 2020). While horizontal gene transfer may have been involved in the evolution of C$_3$ photosynthesis (Wickell and Li, 2020), there has presumably been very little opportunity or evolutionary pressure for plants to acquire genes from aquatic biophysical CCMs in order to evolve alternative CO$_2$ fixation strategies. In addition, the slower diffusion of C$_3$ species in aquatic environments compared with plant tissue may confine evolutionary trajectories (Raven et al., 2017; Flamholz and Shih, 2020). This underscores the fact that the C$_3$ chloroplast has evolved in a gaseous atmosphere and with alternative solutions to Rubisco promiscuity to its aquatic cousins, highlighting that the concept of an engineered chloroplastic CCM is one in which considerable evolutionary complexity must be considered.

When considering any engineering design for enhanced HCO$_3^-$ uptake into C$_3$ chloroplasts, a reasonable question to ask is whether HCO$_3^-$ can be elevated in this organelle, and if so, how? There is sufficient HCO$_3^-$ in the mesophyll cytoplasm available for transport into chloroplasts (at least 250μM; Evans and von Caemmerer, 1996). However, a CCM engineering strategy must ensure HCO$_3^-$ can gain passage across both the outer-envelope membrane (OEM) and IEM of the chloroplast. Given that C$_3$ chloroplasts typically access C$_3$ from the external environment (primarily as the more membrane-permeable CO$_2$), chloroplast membranes appear not to have specific HCO$_3^-$ transport mechanisms (Rolland et al., 2012). Nonetheless, a number of oxygenions, such as phosphate, nitrate, and sulfate evidently do diffuse through the OEM (Bölter et al., 1999). Notably, simple diffusion of CO$_2$ through leaf tissue is insufficient to support the supply rates needed for observed rates of CO$_2$ assimilation by plants (Morison et al., 2005), and it is likely that CO$_2$ entry into the chloroplast is also facilitated by CO$_2$-permeable aquaporins (Flexas et al., 2006; Evans et al., 2009; Tollette et al., 2017; Erмакова et al., 2021) and CA-driven distribution of C$_3$ between predominant species (HCO$_3^-$ and CO$_2$; Price et al., 1994). Therefore, the facilitated entry of C$_3$ into C$_3$ chloroplasts is conceptually not counter to contemporary chloroplast function, and on face value would appear beneficial.

In general, solute transport across the chloroplast OEM is considered to be relatively unhindered due to the presence of low-selectivity and large-molecule channel proteins present in this membrane (Bölter et al., 1999; Hemmler et al., 2006; Duy et al., 2007). It is expected that anion passage into the intermembrane space (IMS), and presumably that of HCO$_3^-$, occurs via at least one of the outer envelope protein channels (OEPs; Duy et al., 2007), with OEP21 a potential route for broad anion uptake into the IMS (Figure 2; Hemmler et al., 2006).
While inward passage through this specific channel may be hampered by triose-phosphate export in the light (Duy et al., 2007), it and other OEPs offer broad selective import into the IMS. Currently, there is no reason to expect that HCO$_3^-$ cannot access the IMS. Nonetheless, it is worthy of consideration, and additional transport mechanisms or solutions should be considered for the elevation of IMS [HCO$_3^-$] if this becomes a roadblock to the overall strategy. Notably, insertion of an IMS-specific CA would likely generate the requisite HCO$_3^-$ from diffusion of CO$_2$ in this location (depending on the IMS pH) for utilization by an IEM-localized pump, in the unlikely scenario that insufficient HCO$_3^-$ is present here. The ΔpH across the chloroplast IEM has been measured to be up to 1 pH unit (Demmig and Gimmmer, 1983) suggesting that an IMS pH of 7–7.5 is feasible in the light, ensuring that >80% of C$_i$ species would exist as HCO$_3^-$ in the presence of CA.

Assuming sufficient HCO$_3^-$ is available in the IMS from the cytosolic pool, its transport across the IEM into the chloroplasm stroma is predicted to be feasible using either high affinity, low flux transporters [e.g., the cyanobacterial sodium-dependent bicarbonate transporter, SbtA, and the ATP driven bicarbonate transporter, BCT1; Table 1; Figures 1, 2], or low to medium affinity, high flux transporters (e.g., BicA; Table 1; Figures 1, 2). For the most part, the affinity of these HCO$_3^-$ transporter types falls below the proposed cytosolic [HCO$_3^-$] (Table 1), suggesting that sufficient transport is feasible. Either independently, or in concert, modeling suggests that functional forms of these transporter types should provide net import into the stroma and enable increased CO$_2$ supply to Rubisco (Price et al., 2011a).

Once HCO$_3^-$ concentrations in the chloroplast are elevated, it is acknowledged that stromal CA is likely to prevent the desired function of a complete chloroplastic CCM, since its action in converting HCO$_3^-$ to CO$_2$ transforms the C$_i$ pool from one with low membrane permeability to one which can rapidly diffuse away from the site of fixation (Price et al., 2013; McGrath and Long, 2014). This would rob an engineered carboxysome (housing a Rubisco with relatively high K$_m$CO$_2$) of its primary C$_s$ substrate, and ectopic CA is known to lead to a high-CO$_2$-requiring phenotype in cyanobacteria (Price and Badger, 1989a). However, in the development of a simpler CCM with only functional HCO$_3^-$ uptake, stromal CA would provide the rapid, pH-driven development of CO$_2$ needed in the chloroplast to supply additional CO$_2$ to Rubisco. The net effect of such a system is the modest elevation of chloroplastic C$_s$, which leads to enhanced CO$_2$ availability at Rubisco (Price et al., 2011a; McGrath and Long, 2014).

It is relevant to consider what effects elevated stromal HCO$_3^-$ might have on chloroplast function beyond the capability of supplying increased CO$_2$ to Rubisco. A role for HCO$_3^-$ as a proton acceptor during water oxidation has been proposed in photosystem II (PSII) function, with HCO$_3^-$ providing stabilizing and protective effects (Shevela et al., 2012). CO$_2$ formation from HCO$_3^-$ at PSII occurs at a rate that correlates with O$_2$ evolution at the donor side (somewhat slower at the acceptor side; Shevela et al., 2020). A simplistic viewpoint therefore is
| C. uptake system | Organism subcellular location | Functional units | Classification | Substrates; Energy | Kinetic properties | References |
|------------------|-------------------------------|------------------|----------------|-------------------|------------------|------------|
| BicA             | Cyanobacteria⁴ plasma membrane | Homodimer        | Sulfate permease (SULP), Solute carrier family (SLC26A) | HCO₃⁻/Na⁺ symport; dependent on Na⁺ gradient | Medium-high flux; low affinity (K₅₀ 7–35 μM HCO₃⁻) | Price et al., 2004; Schrader et al., 2010; Price and Howitt, 2011; Wang et al., 2019 |
| SbtA             | Cyanobacteria⁴ plasma membrane | Possible homotrimer | HCO₃⁻/Na⁺ symport; dependent on Na⁺ gradient | Low flux; high affinity (K₅₀ 2–38 μM HCO₃⁻) | Unknown | Price et al., 2004, 2011a,b; Du et al., 2014, Förster et al., 2021 |
| BCT1 (cmpABCD operon) | Cyanobacteria⁴ plasma membrane | Five subunit complex: CmpA (substrate binding), 2x CmpB (TMD), CmpC (ATPase: substrate binding fusion), and CmpD (ATPase) | ATP-binding cassette (ABC) transporter | HCO₃⁻; ATP hydrolysis | Low flux; high affinity (K₅₀ 10–15 μM HCO₃⁻) | Omata et al., 1999; Koropatkin et al., 2007; Price et al., 2011a |
| LCIA/Nar1.2     | Chlamydomonas⁴ chloroplast envelope | Unknown | Formate-nitrite transporter family | HCO₃⁻; unknown | Unknown | Wang et al., 2015; Atkinson et al., 2016 |
| HLA3            | Chlamydomonas⁴ plasma membrane | Unknown | ABC transporter | HCO₃⁻; ATP hydrolysis | Unknown | Gao et al., 2015; Wang et al., 2015; Atkinson et al., 2016 |
| LCI1            | Chlamydomonas⁴ plasma membrane | Unknown | Anion channel | Cl⁻ (some evidence for CO₂); unknown | Unknown | Wang et al., 2015; Atkinson et al., 2016; Kono and Spalding, 2020 |
| BST-1           | Chlamydomonas⁴ thylakoid membrane | BST-1 pentamer | Bestrophin-like proteins, Anion/Cl⁻ channel family | HCO₃⁻; unknown | Unknown | Mukherjee et al., 2019 |
| BST-2           | Chlamydomonas⁴ thylakoid membrane | BST-2 tetrad | Bestrophin-like proteins, Anion/Cl⁻ channel family | HCO₃⁻; unknown | Unknown | Price et al., 2011a; Laughlin et al., 2020; Schuller et al., 2020 |
| BST-3           | Chlamydomonas⁴ thylakoid membrane | BST-3 tetrad | Bestrophin-like proteins, Anion/Cl⁻ channel family | HCO₃⁻; unknown | Unknown | Price et al., 2011a; Laughlin et al., 2020; Schuller et al., 2020 |
| NHD-1₃         | Cyanobacteria⁴ thylakoid membrane | 21 subunit complex: CupS, CphY (CupA, type II β-CA), NdhD3, and F3 (specialized for CO₂ hydration) | Specialized respiratory NDH-1-type complex, energy-coupled vectorial CA | CO₂; photosynthetic electron transport/ redox-coupled H⁺ pumping, reduced Fd-dependent | Low flux; high affinity (K₅₀ 1–2 μM CO₂) | Maeda et al., 2002; Price et al., 2011a; Laughlin et al., 2020; Schuller et al., 2020 |
| NHD-1₄         | ndhA,B,C,D3,E,F3,G-Q,S/V chpV/cupB | 20 subunit complex: ChpX (CupB, type II β-CA), NdhD4, and F4 (specialized for CO₂ hydration) | Specialized respiratory NDH-1-type complex, energy-coupled vectorial CA | CO₂; photosynthetic electron transport/ redox-coupled H⁺ pumping, reduced Fd-dependent | High flux; medium affinity (K₅₀ 10–15 μM CO₂) | Maeda et al., 2002; Price et al., 2011a; Laughlin et al., 2020; Schuller et al., 2020 |
| DAB2; dabA2, dabB2 | Halothiobacillus neapolitanus plasma membrane | Heterodimer: DabB2 (type II β-CA homolog), DabB2 (H⁺ pumping protein homolog) | Energy-coupled vectorial CA | CO₂; cation gradient-coupled | Unknown | Desmarais et al., 2011 |
| LOIB/C; lciB, lciC | Chlamydomonas⁴ Chloroplast stroma, pyrenoid periphery | Heterodimer: LciB-LciC (β-CA subtype) | Vectorial? CA | CO₂; unknown | Unknown | Duanmu et al., 2009; Wang et al., 2015; Jin et al., 2016 |

CA, Carbonic anhydrase; Fd, ferredoxin; PQ, plastoquinone; K₅₀, substrate concentration supporting half-maximum C transport activity; TMD, transmembrane domain.

¹Identified and characterized in several species incl. Synechococcus elongatus PCC7942, Synechocystis sp. PCC6803, Synechococcus sp. PCC7002, and Thermosynechococcus elongatus.

²Identified in Chlamydomonas reinhardtii.

³Energyization is to some extent speculative based on structural homology.
that greater quantities of stromal HCO$_3^-$ may support PSII function rather than having any negative effects, as appears to be the case for cyanobacteria and microalgae. This PSII property highlights potential conversion of HCO$_3^-$ to CO$_2$ in an engineered chloroplastic CCM, however, and longer-term goals would be to generate systems which recycle stromal CO$_2$ back to HCO$_3^-$, whether it is generated through PSII action, anaplerotic reactions, or indeed leakage from an engineered carboxysome or pyrenoid (Price et al., 2013). Nonetheless, CO$_2$ losses via these processes are likely to be minimal within an engineering scheme utilizing a HCO$_3^-$ transporter and a carboxysome, benefiting only marginally from the addition of vectorial CO$_2$-to-HCO$_3^-$ conversion complexes (McGrath and Long, 2014).

**WHICH C$_i$ UPTAKE SYSTEMS COULD FACILITATE CHLOROPLASTIC HCO$_3^-$ ACCUMULATION?**

Inorganic carbon acquisition is an essential step in driving a biophysical CCM and for maximizing its efficiency. Acquisition of the predominant C$_i$ species (CO$_2$ and HCO$_3^-$) contributes to the accumulation of an intracellular/chloroplastic HCO$_3^-$ pool well above external C$_i$ levels, with up to 1,000-fold increases observed in cyanobacteria (Price, 2011). This can only be achieved by active C$_i$ uptake against a concentration gradient, requiring energy, as opposed to passive diffusional uptake through protein channels such as CO$_2$ aquaporins (Uehlein et al., 2012; Li et al., 2015). Active C$_i$ uptake systems can be divided into two categories, energy-coupled CAs (also known as vectorial CO$_2$ pumps or CO$_2$-to-HCO$_3^-$ conversion systems) and active HCO$_3^-$ transporters. A number of C$_i$ transport systems have been identified through genetic screens of high CO$_2$ requiring mutants in the microalga *Chlamydomonas* (Spalding, 2008; Fang et al., 2012), several cyanobacteria (Price and Badger, 1989b; Badger and Price, 2003; Price et al., 2008) and, recently non-photosynthetic, CO$_2$-fixing γ-proteobacteria (Scott et al., 2018; Desmarais et al., 2019), summarized in Table 1 and Sui et al. (2020).

**Cyanobacterial C$_i$ Uptake Systems**

In cyanobacteria, five C$_i$ uptake systems have been verified, subsets of which are present in all species (Figure 1A; Table 1). These transport systems differ in subcellular localization, substrate affinity, flux rates, energization and regulation of gene expression, and transport activity (Price, 2011). These properties somewhat determine their suitability for function in a proposed chloroplastic CCM. Dependent on the species, some C$_i$ uptake systems are constitutively expressed, but in most cases, their expression is controlled by a combination of limiting C$_i$ and light (Badger and Andrews, 1982; Kaplan et al., 1987; McGinn et al., 2003; Price et al., 2011b).

Intracellular CO$_2$-to-HCO$_3^-$ conversion in cyanobacteria is facilitated by two specialized, thylakoid-located NAD(P)H dehydrogenase (NDH1) complexes related to the respiratory complex-I from mitochondria: the low C$_i$-inducible, high affinity NDH-1$_{3}$, and the constitutively, slightly lower affinity NDH-1$_{4}$ complexes (Maeda et al., 2002; Ohkawa et al., 2002). The CO$_2$ hydration subunits ChpY (CupA) and ChpX (CupB) of NDH-1$_{3}$ and NDH-1$_{4}$ respectively, convert cytoplasmic CO$_2$ to HCO$_3^-$, energized by reduced ferredoxin or NADPH that are generated by photosynthetic electron transport, and hence light-dependent (Ogawa et al., 1985; Maeda et al., 2002; Price et al., 2008; Bättichikova et al., 2011). Recently, catalytic properties of the cryo-EM structure of the NDH-1$_{3}$ complex have been analyzed applying quantum chemical density modeling to the cryo-EM structure, which has shed light onto putative regulatory mechanisms. CO$_2$ hydration by NDH-1$_{3}$ (and by analogy NDH-1$_{4}$) is energetically linked to plastoquinone oxido-reduction coupled to proton-pumping, which controls the opening and closing of the putative CO$_2$ diffusion channel and lateral removal of H$^+$ generated in the CO$_2$ hydration reaction catalyzed by the ChpY (CupA) subunit. This mechanism ensures that the backward reaction, and unfavorable CO$_2$ release, is prevented (Badger and Price, 2003; Schuller et al., 2020). In plant chloroplasts, we expect such systems would require thylakoid localization for correct function.

Direct transfer of HCO$_3^-$ from the outside into the cytoplasm is facilitated by three types of plasma membrane-located HCO$_3^-$ transporters (Figure 1). The high affinity transporters, BCT1 and SbtA, were shown to be newly synthesized upon activation of HCO$_3^-$ uptake, while constitutively expressed BicA was induced without further de novo protein synthesis (Sültemeyer et al., 1998; McGinn et al., 2003). The heteromeric BCT1 complex (encoded by the *cmpABCD* operon; Table 1) is a high affinity-low flux HCO$_3^-$ transporter (Omata et al., 1999) of the ATP binding cassette (ABC) transporter superfamily, strongly suggesting ATP is used for energization. However, ATPase activity has not yet been demonstrated. BCT1 is composed of the membrane-anchored, substrate-binding protein CmpA, the homodimeric, membrane integral CmpB domain, and the cytoplasmic ATPase subunits CmpC and CmpD. CmpC appears to be a fusion protein which contains both the ATPase moiety and a putative regulatory substrate-binding domain homologous to CmpA. CmpA requires Ca$^{2+}$ as co-ligand for binding of HCO$_3^-$, yet it is unclear whether Ca$^{2+}$ plays a role in HCO$_3^-$ transport (Koropatkin et al., 2007). The complexity of the proposed subunit localization of BCT1 for chloroplast envelope expression (one subunit in the IMS, one in the IEM, and two in the stroma; see below) provides further plant engineering challenges in addition to correct transporter function.

Both, BicA and SbtA (Table 1) are HCO$_3^-$/Na$^+$ symporters that require a cell-inward directed Na$^+$ gradient for HCO$_3^-$ uptake (Shibata et al., 2002; Price et al., 2004), and as single protein transporters are attractive considerations for chloroplast engineering. BicA, a medium affinity-high flux transporter of the SLC26A solute carrier superfamily, is thought to function as a homodimer (Compton et al., 2011; Price and Howitt, 2014; Wang et al., 2019). The high affinity-low flux SbtA transporter, constitutes its own Na$^+$-dependent HCO$_3^-$ transporter superfamily, and is likely to be active as a trimer (Du et al., 2014; Fang et al., 2021; Förster et al., 2021). These requirements for
Na\textsuperscript+ for HCO\textsubscript{3}\textsuperscript{−} uptake highlight the potential for excessive influx of Na\textsuperscript+ in a chloroplast-based CCM which we discuss below.

**Non-photosynthetic Bacterial C\textsubscript{4} Uptake Systems**

The DAB proteins (encoded by the *dab1* and *dab2* operons) first identified in *Halothiobacillus neapolitanus* are distributed throughout prokaryotic phyla and have been proposed to function as energy-coupled CAs accumulating HCO\textsubscript{3}\textsuperscript{−} in the cytoplasm (Desmarais et al., 2019). A heterodimeric functional unit consists of the cytoplasmic exposed β-CA-like DabA protein coupled to the membrane-integral cation antiporter-like membrane subunit DabB (Figure 1B). Vectorial CO\textsubscript{2} hydration by DabA has been hypothesized to be driven by a cation (H\textsuperscript{+} or Na\textsuperscript{+}) gradient but has not yet been proven experimentally (Laughlin et al., 2020). From an engineering standpoint, DAB proteins may represent a viable alternative to NDH\textsubscript{1/4} complexes as candidates for CO\textsubscript{2} uptake/recapture in chloroplasts as introduction of only two proteins is required for DABs compared to 20–21 different proteins for NDH\textsubscript{1/4} (Price et al., 2019). However, the suitability of DABs to function in chloroplasts will be uncertain until mechanisms of energization/regulation are resolved. In addition, we need to consider that DABs or any vectorial CA will only be effective in the final engineering stages once the endogenous stromal CA has been successfully removed (Price et al., 2011a).

**Microalgal C\textsubscript{4} Uptake Systems**

In *Chlamydomonas*, HCO\textsubscript{3}\textsuperscript{−} transporter genes induced under low C\textsubscript{4} include plasma membrane-located HLA3 and LCI1 (Figure 2; Kono and Spalding, 2020), the chloroplast envelope-located LCIA (Nar1.2; Wang et al., 2011; Atkinson et al., 2016; Kono and Spalding, 2020), thylakoid membrane-integral bestrophin-like proteins BST1, BST2, and BST3 (Mukherjee et al., 2019), and the chloroplast-located CIA8 (Machingura et al., 2017). In addition, stromal LCIB/C complex and the thylakoid luminal carbonic anhydrase CAH3 have been implied in CO\textsubscript{2} recapture (reviewed in Mackinder, 2018; Mallikarjuna et al., 2020). Importantly, neither substrate affinities, net accumulation capacity, and energization nor regulatory mechanisms of individual transporters are sufficiently understood to evaluate their suitability for expression in C\textsubscript{4} chloroplasts at this time (Table 1). It is highly likely though that HLA3 (Figure 1C), as a member of the ABC and transporter family, is energized by ATP hydrolysis (Wang et al., 2015), and heterologous expression of HLA3 or LCIA in *Xenopus* oocytes showed some HCO\textsubscript{3}\textsuperscript{−} uptake activity but were not characterized further (Atkinson et al., 2016).

**WHAT ARE THE ENERGETIC AND FUNCTIONAL REQUIREMENTS OF C\textsubscript{4} UPTAKE SYSTEMS?**

One major challenge for heterologous expression of C\textsubscript{4} uptake systems is the regulation of protein function, which encompasses both primary energization and fine-tuning of activity to match dynamic photosynthetic CO\textsubscript{2} assimilation capacity of plant leaves (Price et al., 2013; Rae et al., 2017; Mackinder, 2018). Irrespective of the organism, C\textsubscript{4} uptake appears to be controlled at the level of gene expression as well as protein function. While our current knowledge allows us to control expression of transgenes quite effectively, control of protein function in a non-native environment is still vastly empirical and, without greater understanding, far from attaining control by rational design.

Regulation of transporter function appears to be as little understood as energization. Most knowledge has been gathered for the cyanobacterial C\textsubscript{4} uptake systems (Table 1). In cyanobacteria, as in chloroplasts, elevated HCO\textsubscript{3}\textsuperscript{−} concentration is only beneficial for photosynthetic carbon gain in the light. For maximum efficiency, C\textsubscript{4} uptake activity needs to be in tune with day/night cycles and changes in light intensity. In cyanobacteria, CO\textsubscript{2} uptake and HCO\textsubscript{3}\textsuperscript{−} transport are activated within seconds in the light, with CO\textsubscript{2} uptake preceding HCO\textsubscript{3}\textsuperscript{−} uptake (Badger and Andrews, 1982; Price et al., 2008, 2011b), and both SbtA and BicA are inactivated within seconds in the dark (Price et al., 2013; Förster et al., 2021). A link between light-activation/dark-inactivation of C\textsubscript{4} uptake and the state of photosynthetic electron transport and/or to a redox signal has been suggested by Kaplan et al. (1987), the identity of the light signal, signal transduction pathways and sensory/response mechanisms of the C\textsubscript{4} uptake proteins are still elusive. Furthermore, protein phosphorylation may play a role in post-translational modulation of HCO\textsubscript{3}\textsuperscript{−} transporter activity (Sültemeyer et al., 1998), and it is uncertain whether the native cyanobacterial regulatory kinases/phosphatases could function correctly in plastids when co-expressed with their transporter targets. This level of regulation dependency needs to be addressed to ensure replication of cyanobacterial-like control of C\textsubscript{4} uptake mechanisms in a C\textsubscript{3} system.

**Light/Dark Control of C\textsubscript{4} Uptake**

There is some evidence for redox-regulation of CO\textsubscript{2} uptake by the NDH-1 complexes in cyanobacteria. NDH\textsubscript{1/4} function is directly linked to the trans-thylakoid proton motive force and cyclic electron transfer at photosystem I through interaction with ferredoxin and plastoquinone intermediates of the photosynthetic electron transport chain (ETC; Schuller et al., 2020). Light-driven changes in photosynthetic electron transport cause instantaneous changes of the redox state of the ETC which modulates CO\textsubscript{2} fixation via changes in NADPH production, ATP synthesis, and the redox-sensitive activation state of the Calvin-Benson-Bassham (CBB) cycle enzymes. In cyanobacteria, oxidizing conditions activate the small, inhibitory CP12 protein and ferredoxin-thioredoxin redox signaling cascades which inhibit the CBB cycle enzymes (via thiol-oxidation of cysteines; McFarlane et al., 2019), thus coordinating CO\textsubscript{2} uptake and carboxylation. Given that the ETC and the ferredoxin-thioredoxin-CP12 regulatory system are highly conserved and present in all plant chloroplasts, regulatory features may already be present in chloroplasts if large, multi-gene NDH-1 complexes could be heterologously expressed. However, it is unlikely that
this modus of redox-regulation applies to plasma membrane-located HCO$_3^-$ transporters, which are spatially separated from the ETC and have not been detected among proteins targeted by thioredoxin (Lindahl and Florencio, 2003).

Currently without experimental evidence, other putative redox-sensitive regulatory mechanisms for cyanobacterial C$_i$ uptake, such as eliciting signaling molecules such as Ca$^{2+}$ (Torrecilla et al., 2004; Dominguez et al., 2015), light-stimulated changes in membrane potential (Murvanidze and Glogolev, 1982), and Ca$^{2+}$ sensory phosphorylation relays triggered by light-dark transitions (Mata-Cabana et al., 2012) are speculative. However, regardless of the regulatory mechanism, the main concern remains whether an analogous regulatory system exists in the chloroplast and whether it can interact appropriately with the introduced foreign proteins, or, whether such systems need to be transplanted into chloroplasts alongside C$_i$ uptake systems. Importantly, Ca$^{2+}$ plays a major regulatory role for photosynthesis and related metabolism in chloroplasts and light-dark transitions elicit specific Ca$^{2+}$ responses (Pottosin and Shabala, 2016). Therefore, chloroplasts harbor an extensive Ca$^{2+}$ signaling infrastructure and are part of the whole plant signaling network which includes crosstalk between chloroplastic and cytoplasmic Ca$^{2+}$ signaling responses to environmental stimuli (Navazio et al., 2020). How the incorporation of additional systems, which could have Ca$^{2+}$ dependencies, might impact on overall inter- and intra-cellular signaling is yet to be seen.

So far, evidence for control of HCO$_3^-$ uptake involving interaction of the transporter with regulatory proteins and/or additional co-factors has only emerged for SbtA. Heterologous co-expression of SbtA and its cognate P$_H$-like SbtB proteins in _E. coli_ abolished SbtA-mediated HCO$_3^-$ uptake constitutively and formed SbtA:SbtB containing protein complexes (Du et al., 2014). This suggests activity of SbtA can be modulated through binding its respective SbtB (Fang et al., 2021). Effects on SbtA activity have not been observed in low C$_i$-acclimated, SbtB-deficient cyanobacterial mutants (Förster et al., 2021), although initial C$_i$ acclimation and growth appeared to be compromised in _Synechocystis_ sp. PCC6803 (Selim et al., 2018). However, so far, in _vivvo_ evidence suggest that certain SbtA and SbtB pairs interact in response to adenylate ratios and adenylate energy charge sensed through SbtB (Kaczmarski et al., 2019; Förster et al., 2021), and even though the _in vivo_ role of the SbtA:SbtB interaction is not clear yet, co-expression of SbtA and SbtB may be necessary for appropriate functional control in chloroplasts.

**Implications for pH Balance, Ion Homeostasis, and Energetic Requirements**

While single gene HCO$_3^-$ transporters such as the SbtA HCO$_3^-$/Na$^+$ symporters are prime candidates for chloroplast expression (Du et al., 2014), accumulation of HCO$_3^-$ and Na$^+$ in the stroma in the dark could theoretically lead to pH imbalances and high concentrations of Na$^+$ impairing chloroplast biochemistry (Price et al., 2008; Mueller et al., 2014; Myo et al., 2020). Cellular pH is tightly regulated to ensure near optimal conditions for biochemical reactions to occur. The cytoplasmic pH in _Arabidopsis_ is maintained at about 7.3 (Shen et al., 2013), whereas the chloroplast stroma has been reported to vary between pH 7.2 in the dark to about pH 8 in the light (Höhner et al., 2016). All membrane systems in plant cells possess numerous transport systems (comprised of cation/H$^+$ and anion/H$^+$ exchangers) that maintain pH homeostasis in different subcellular compartments, and transmembrane H$^+$ gradients as a proton motive energy source. In the light, the capacity for pH-regulation and buffering in chloroplasts is likely to accommodate the alkalization caused by continued HCO$_3^-$ import into the chloroplast. Bicarbonate accumulation in the chloroplast _via_ a single transporter type is unlikely to exceed the pool sizes of up to 50 mM measured in CCM-induced and actively photosynthesizing cyanobacteria (Kaplan et al., 1980; Woodger et al., 2005). Moreover, the pH disturbance associated with short-term (~ 5 min) exposure of leaves to high CO$_2$, which elevated stromal HCO$_3^-$ up to 90 mM in the dark and 120 mM in the light, was counteracted rapidly within seconds (Hauser et al., 1995). However, it is uncertain whether pH buffering is as effective if continued HCO$_3^-$ uptake in the dark were to accumulate substantial HCO$_3^-$ pools without consumption by Rubisco. Consideration must therefore be given to this uncertainty in CCM engineering strategies.

The second potentially confounding issue with expression of the SbtA and BicA transporters on the chloroplast envelope is the influx of Na$^+$. Assuming a stoichiometry of 1:1 for Na$^+$ and HCO$_3^-$ co-transport, these transporters could increase chloroplast [Na$^+$] by at least as much as the [HCO$_3^-$] mentioned above. In contrast to halophytes which tolerate higher chloroplastic Na$^+$ concentrations, photosynthesis in glycophyles (including many C$_3$ crop plants) becomes impaired by subtle elevation of stromal Na$^+$ from 0.21 to 0.38 mM in _Arabidopsis_ (Mueller et al., 2014). The NHD1 Na$^+$/H$^+$ antiporter on the chloroplast envelope is active in Na$^+$ extrusion (_Figure 2_), maintaining a positive Na$^+$ gradient for other Na$^+$-dependent carriers on the chloroplast envelope, regulating stromal pH, and contributing to salt tolerance (Höhner et al., 2016; Tsujii et al., 2020). This suggests that, in particular, light/dark regulated Na$^+$ extrusion and Na$^+$/HCO$_3^-$ symport need to be synchronized. Thus, boosting Na$^+$ export systems on the chloroplast envelope may be required to restore ion/pH balance, which could involve overexpression of the endogenous NHD1 or expression of foreign Na$^+$/H$^+$ antiporters such as cyanobacterial NhaS proteins (Price et al., 2013).

Unfortunately, regulation of Na$^+$ fluxes between different compartments of plant cells and the characteristics of Na$^+$ carriers are not well understood, therefore making it difficult to predict how active HCO$_3^-$ uptake might influence Na$^+$ fluxes. In addition to the potential over-accumulation of stromal [Na$^+$], it is not clear whether the cytoplasmic [Na$^+$] and the magnitude of the Na$^+$ gradient across the chloroplast envelope will be sufficient for optimal energization of SbtA or BicA and in varying environments. Estimates of cytoplasmic [Na$^+$] range between 3 and 30 mM (Karley et al., 2000; Tester and Davenport, 2003), which exceeds the $K_{S_3}$ (Na$^+$ concentration supporting half-maximum HCO$_3^-$ uptake rates) of 1–2 mM Na$^+$ for SbtA and BicA (Price et al., 2004; Du et al., 2014). Stromal Na$^+$ concentrations have been reported between 0.2 and 7 mM.
Therefore, dependent on the plant species and/or environmental conditions, cytoplasmic Na\(^+\) is in the lower concentration range, and the differential between cytoplasm and stroma, could impose limits on HCO\(_3^-\) uptake rates depending on substrate availability. However, plants under field conditions experience relatively higher salinity in most agricultural soils than in controlled growth environments, which means we can expect their cells to operate at slightly elevated cytoplasmic Na\(^+\) levels (Tester and Davenport, 2003), which renders Na\(^+\) limitation fairly unlikely.

Based on homology to ABC transporters, the cyanobacterial BCT1 and the *Chlamydomonas* HLA3 (Figures 1A,C) are thought to be energized by ATP hydrolysis, but the ATP required per HCO\(_3^-\) transported has not been determined. Modeled ATP requirements for SbtA and BicA activity, which consume ATP indirectly as costs for proton transport to maintain the Na\(^+\) gradient, project 0.5 and 0.25 ATP, respectively, per HCO\(_3^-\) transported (Price et al., 2011a). Particularly at low external C\(_3\), suppression of photosynthesis by active HCO\(_3^-\) uptake is more ATP cost-effective than typical C\(_3\) photosynthesis, and ATP demand for transporter function should be readily covered by photophosphorylation in the chloroplast. The modeling did not consider additional ATP requirements for synthesis and maintenance of C\(_3\) uptake complexes though, since protein accumulation and turnover rates are unknown in both native organisms and chloroplasts, which is a modest pressure onto ATP production compared to the overall daily expenditure in living cells. Recent modeling of proposed pyrenoid-based systems also highlights ATP costs to chloroplastic CCMs; however, these can be limited depending on the engineering strategy (Fei et al., 2021).

**HOW CAN WE GET C\(_3\) UPTAKE SYSTEMS INTO THE CHLOROPLAST?**

The expression of transgenes from the nuclear genome of terrestrial plants is the favored means to introduce a CCM into crop plants due to current difficulties associated with successful insertion of exogenous genes into the chloroplast genomes of some major crops (Hanson et al., 2013). Nonetheless, many proof-of-concept approaches utilize plastome expression to assess CCM components (Lin et al., 2014b; Pengelly et al., 2014; Long et al., 2018). We focus here on strategies relating to the import of nuclear-encoded proteins into chloroplastic membranes and stroma where broader application to the majority of globally important crops is feasible. This approach introduces many complicating challenges when considering the transfer of systems from a cyanobacterium where proteins are targeted to the membrane from the inside, whereas in chloroplast proteins would come from the outside.

Successful transport of HCO\(_3^-\) into C\(_3\) plant chloroplasts requires that a transporter will be pumping solute across the chloroplast IEM, into the chloroplast stroma. This sounds simple in principle but implies several assumptions about the transporter are true. Firstly, that it is successfully expressed and targeted to the chloroplast. Secondly, correct direction of the imported protein to the chloroplast IEM occurs. Thirdly, the protein must fold and orient itself in the appropriate manner such that its intended direction of transport is inward to the stroma. Finally, any processes which ensure activation and energization of the transporter must be met (discussed above). Correct targeting of HCO\(_3^-\) transporters to the chloroplast IEM has been the subject of several reports in recent years (Atkinson et al., 2016; Rolland et al., 2016, 2017; Uehara et al., 2016, 2020; Nölke et al., 2019), however, correct localization, orientation, and activation of these proteins to ensure favorable function remain an engineering challenge.

**Foreign Protein Expression and Targeting**

The initial step of expressing foreign genes in transgenic plants is a common point of failure due to a myriad of factors relating to gene positional effects (Pérez-González and Caro, 2019) and silencing (Jackson et al., 2014), codon usage (Nakamura and Sugita, 2009), promoter and terminator combinations (Beyene et al., 2011; de Felippes et al., 2020), and potential degradation of the precursor protein (Lee et al., 2009; Shen et al., 2017; Hristou et al., 2020). This usually requires the analysis of relatively large numbers of plant transformation events and somewhat laborious testing of gene expression cassettes (often in transient expression systems) to ensure appropriate levels of protein expression can be achieved. We do not provide further discussion on this point but highlight that fine-tuning this aspect of CCM engineering in C\(_3\) plants is not trivial and can heavily impact on the trajectories of engineering approaches.

Once expressed, nuclear encoded proteins targeted to the chloroplasts are translocated as pre-proteins within the cytosol where chaperones, such as Hsp70, Hsp90, and the 14–3–3 protein complex are involved throughout the translocation process (Figure 3; May and Soll, 2000; Schwenkert et al., 2011). Proteins translated in the cytosol and destined for the chloroplast either remain unfolded with the help of chaperones (Jarvis, 2008), or can be imported to the chloroplast in a fully-formed state (Ganesan et al., 2018), prior to translocation across the chloroplast envelope. These chaperones are crucial to prevent the premature folding of large proteins and aggregation and/or degradation of pre-proteins (Wojcik and Kriechbaumer, 2021).

Upon reaching the chloroplast, pre-proteins enter through the TIC-TOC pathway and are then directed within the chloroplast to their final destination (e.g., IEM, OEC, stroma, thylakoid membrane, or lumen; Figure 3; Oh and Hwang, 2015; Lee et al., 2017; Xu et al., 2020). Noticeably, post-import insertion into the IEM could involve additional processing by the Cpn60/Cpn10 chaperonin complex within the stroma, prior to insertion into the IEM through a membrane bound translocase (SEC2; Li and Schnell, 2006; Li et al., 2017). These various processes are facilitated by the pre-protein chloroplast transit peptide (cTP) which possesses binding sites for chaperones and is crucial for targeting nuclear-encoded proteins into the chloroplast (Ivey et al., 2000; Rial et al., 2000; Lee and Hwang, 2018). Therefore, the types of chaperones that would mediate foreign pre-protein chloroplast import would depend on the cTP used. There is
of membrane proteins (Day and Theg, 2018), and the emergence of cTPs to enable protein trafficking through the TIC-TOC complex and to the correct membrane (Figure 3; Knopp et al., 2020; Ramundo et al., 2020).

Correct targeting to the chloroplast membranes is further complicated by the presence of additional organelles in plant cells, and dual targeting between chloroplasts and mitochondria is commonly observed (Peeters and Small, 2001; Sharma et al., 2018). This complexity of organelle targeting (Bruce, 2000; Wojcik and Kriechbaumer, 2021) requires specific choice of cTP in proposed photosynthetic engineering strategies, and we suggest that the direction of foreign proteins to the appropriate cellular compartment is unlikely to be a one-size-fits-all solution (Rolland et al., 2017). There are also likely to be protein cargo-specific requirements which determine the choice of cTP for each heterologous membrane protein directed to the chloroplast IEM, thus identifying the need to test and tailor genetic constructs on an individual basis. This strategy is also required to optimize promotor/terminator requirements and is highly relevant in systems where protein stoichiometry (such as for multi-protein complex transporters such as BCT1) may be essential for function.

Successful incorporation of multi-component membrane transporter complexes such as BCT1 (Figures 1, 2) will require subunits which lie not only in the IEM, but also in the IMS and the stroma of the chloroplast. Targeting to the IMS has not been well investigated, with few examples in the literature investigating the subject (Kouranov et al., 1999; Vojta et al., 2007). At least two pathways to this location are thought to exist, one where proteins mature in the IMS (e.g., the TIC complex subunit Tic22; Kouranov et al., 1999), and one where proteins transit through the stroma and are then re-inserted into the IMS (e.g., MGD1; Vojta et al., 2007). Which may be the most appropriate pathway and whether foreign proteins can utilize either approach is yet to be described. In contrast, targeting to the stroma has been thoroughly studied and might therefore be the easiest to achieve (reviewed in Li and Chiu, 2010). One aspect worth mentioning is the stromal processing peptidase (SPP) which is known to cleave cTPs from several nuclear-encoded proteins imported into the stroma (Figure 3; Richter and Lamppa, 1998, 2002). The complete removal of cTPs is highly desirable in chloroplast engineering, as N-terminal additions to foreign proteins can impede their function. However, successful cTP cleavage may be prevented by cargo protein secondary and tertiary structure. With difficult cargoes, cTPs may need to be extended beyond the cleavage site with a flexible linker which will ultimately leave a scar that might also impede protein function. Notably, however, some novel cTPs have been designed to reduce the proteolytic scars while enhancing targeting of difficult protein cargoes. These engineered cTPs, such as RC2 and PCI (Shen et al., 2017; Yao et al., 2020) include about 20 residues from its native mature cargo (a spacer to allow translocating factors better access to the cTP) which are followed by a second SPP cleavage site (to allow removal of the additional 20 residues used as spacer). Another approach that has been specifically used for the HCO\textsuperscript{−} transporters SbtA and BicA included a TEV protease cleavage...
site after the cTP to enable removal by a heterologously expressed TEV protease (Uehara et al., 2016, 2020).

As mentioned above, the NDH complex may depend on plastoquinone for energization, and if we were to use such a complex for CO₂ recapture, the chloroplast thylakoid membrane would be the destination of choice (Figure 2; Long et al., 2016; Price et al., 2019; Hennacy and Jonikas, 2020). While the chloroplast twin arginine translocation, and secretory pathways directly mostly soluble proteins to the thylakoid lumen, it is the chloroplast signal recognition pathway (SRP) that targets membrane proteins to the thylakoid membrane (Figure 3; Smeekens et al., 1985; Schnell, 1998; Aldridge et al., 2009; Ouyang et al., 2020; Xu et al., 2020). Note that dual targeting between thylakoid and IEM was encountered when foreign transporters were targeted to the IEM (Pengelly et al., 2014; Rolland et al., 2017). A study on two closely related Arabidopsis proteins, SCY1 (thylakoids) and SCY2 (IEM), shed light on the sorting mechanism between IEM and thylakoids. In brief, the N-terminal region of SCY2 alone was not sufficient for exclusive targeting to the IEM. Instead, two internal transmembrane domains (TMDs) were required to achieve unambiguous localization to the IEM with no leakage toward the thylakoid membrane (Singhal and Fernandez, 2017). This study demonstrated that targeting is cargo-dependent. Hence, a more complex engineering of cargo TMDs might be required to successfully target foreign HCO₃⁻ transporters within the chloroplast (Rolland et al., 2017).

Control of Membrane Protein Orientation

Due to the inverted targeting strategy proposed for cyanobacterial transporters, there is potential for nuclear-encoded membrane proteins to be incorrectly oriented in the chloroplast IEM, even if targeting is successful. Most of the work done to understand membrane protein orientation (i.e., TMD topology) has been carried out in bacteria, establishing the positive-inside rule (Lys and Arg rich loops orient in the cytoplasm; von Heijne, 1986) and the charge-balanced rule (Dowhan et al., 1999). However, little is known about topology determinants in C₃ plant chloroplast membranes. Membrane lipid composition is known to influence the orientation of membrane proteins in the OEM (Schleiff et al., 2001). However, since the lipid composition of the C₃ chloroplast OEM differs from the IEM (Block et al., 2007), it is difficult to draw parallels between their orientation determinants. Interestingly, specific TMDs also appear to affect membrane protein orientation (Viana et al., 2010; Okawa et al., 2014). While changing lipid composition to control orientation is unrealistic in plants (but was achieved in bacteria; Dowhan et al., 2019), rational design of TMDs, and interconnecting loops (Rapp et al., 2007) from HCO₃⁻ uptake systems might be an option. As shown for the secretory pathway in plant endoplasmic reticulum, membrane protein signal peptides may also play a role in the orientation of some proteins (Wojcik and Kriechbaumer, 2021). Hence, it is reasonable to assume that correct targeting and orientation of membrane proteins in the chloroplast IEM are dependent on both the cargo protein and its targeting sequence (Rolland et al., 2016; Uehara et al., 2016, 2020). As a result, broad screening of targeting peptides for candidate cyanobacterial membrane protein cargos is likely required, both on a case-by-case basis and possibly between heterologous hosts. Membrane protein orientation must therefore be considered when addressing CCM component expression in plant systems and will affect predicted outcomes of functional HCO₃⁻ uptake assessment in transformed plants.

PERSPECTIVES AND CONCLUSION

Application of synthetic biology approaches to elevate HCO₃⁻ concentrations in C₃ plant chloroplasts, as a means to enhance Rubisco carboxylation, is an ongoing engineering endeavor among plant biologists. It is, however, a complex task which needs to be considered within a broad framework of molecular and physiological complexity. Efforts to heterologously express candidate HCO₃⁻ transporters and CO₂-to-HCO₃⁻ converting complexes in C₃ plants must therefore be contemplated within this context. Therefore, it is critically important that researchers addressing this challenge gather evidence of correct targeting, orientation and processing of protein transporters in plant systems. Functionality should be addressed where possible, and techniques which provide evidence of successful HCO₃⁻ import (e.g., Tolleter et al., 2017) and elevated leaf-level carboxylation should accompany reports of plant growth and productivity to ensure that predicted physiological outcomes correlate with enhanced growth. In addition to this, greater detail is required on the functional characterization of existing HCO₃⁻ uptake systems in their native systems (Table 1), while an understanding of the broader natural variation in HCO₃⁻ uptake systems (e.g., Scott et al., 2018; Desmarais et al., 2019) should be accumulated to provide greater options for engineering purposes.

AUTHOR CONTRIBUTIONS

BF generated the table. BML generated the figures. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a subaward from the University of Illinois as part of the research project Realizing Increased Photosynthetic Efficiency (RIPE) that is funded by the Bill & Melinda Gates Foundation, Foundation for Food and Agriculture Research, and the UK Government's Department for International Development under grant number OPP1172157. We also acknowledge funding support from the Australian Research Council Centre of Excellence for Translational Photosynthesis (CE140100015).

ACKNOWLEDGMENTS

The authors thank Suyan Yee and Nghiem Nguyen for proofreading the final manuscript.
Hristou, A., Grimmer, J., and Baginsky, S. (2020). The secret life of chloroplast precursor proteins in the cytosol. Mol. Plant 13, 1111–1113. doi: 10.1016/j.molp.2020.07.004

Ivey, R. A., Subramanian, C., and Bruce, B. D. (2000). Identification of a Hsp70 recognition domain within the rubisco small subunit transit peptide. Plant Physiol. 122, 1289–1299. doi: 10.1104/pp.122.4.1289

Jackson, M. A., Sternes, P. R., Madge, S. R., Graham, M. W., and Birch, R. G. (2014). Design rules for efficient transient expression in plants. Plant Biotechnol. J. 12, 925–933. doi: 10.1111/pbi.12197

Jarvis, P. (2008). Targeting of nucleus-encoded proteins to chloroplasts in plants. New Phytol. 179, 257–285. doi: 10.1111/j.1469-8137.2008.02542.x

Jin, S., Sun, J., Wunder, T., Tang, D., Cousins, A. B., Sze, S. K., et al. (2016). Structural insights into the LCI3B protein family reveals a new group of β-carbonic anhydrases. Proc. Natl. Acad. Sci. U. S. A. 113, 14716–14721. doi: 10.1073/pnas.1612941113

Kaczmarski, J. A., Hong, N. S., Mukherjee, B., Wey, L. T., Rourke, L., Förster, B., et al. (2019). Structural basis for the allosteric regulation of the StbA bicarbonate transporter by the P_i-like protein, StbB, from Cyanobium sp. PCC7001. Biochemistry 58, 5030–5039. doi: 10.1021/acs.biochem.9b00880

Kaplan, A., Badger, M. R., and Berry, J. A. (1980). Photosynthesis and the intracellular inorganic carbon pool in the bluegreen alga Anacystis variabilis: response to external CO2 concentration. Planta 149, 219–226. doi: 10.1007/BF00384557

Kaplan, A., Zenvirth, D., Marcus, Y., Omata, T., and Ogawa, T. (1987). Energization and activation of inorganic carbon uptake by light in cyanobacteria. Plant Physiol. 84, 210–219. doi: 10.1104/pp.84.2.210

Karley, A. J., Leigh, R. A., and Sanders, D. (2000). Where do all the ions go? The cellular basis of differential ion accumulation in leaf cells. Trends Plant Sci. 5, 465–470. doi: 10.1016/s1360-1380(00)01758-1

Knopp, M., Garg, S. G., Handrich, M., and Gould, S. B. (2020). Major changes in plastid protein import and the origin of the Chloroplastida. Science 2130896. doi: 10.1126/science.2130896

Kono, A., and Spalding, M. H. (2020). CI21, a Chlamydomonas reinhardtii plasma membrane protein, functions in active CO2 uptake under low CO2. Plant J. 102, 1127–1141. doi: 10.1111/tpj.14761

Koropatkin, N. M., Koppenaal, D. W., Palcusi, H. B., and Smith, T. J. (2007). The structure of a cyanobacterial bicarbonate transport protein, CmpA. J. Biol. Chem. 282, 2606–2614. doi: 10.1074/jbc.M61022220

Kouranov, A., Wang, H., and Schnell, D. J. (1999). Tic22 is targeted to the intermembrane space of chloroplasts by a novel pathway. J. Biol. Chem. 274, 25181–25186. doi: 10.1074/jbc.274.35.25181

Kromdijk, J., Glowacka, K., Leonelli, L., Gablil, S. T., Iwai, M., Niyogi, K. K., et al. (2016). Improving photosynthesis and crop productivity by increasing recovery from photoprotection. Science 354, 857–861. doi: 10.1126/science.aai8878

Kromdijk, J., and Long, S. P. (2016). One crop breeding cycle from starvation? How engineering crop photosynthesis for rising CO2 can recover from photoprotection. Trends Plant Sci. 21, 53–63. doi: 10.1016/j.tplants.2015.08.003

Laughlin, T. G., Savage, D. F., and Davies, K. M. (2020). Recent advances on Kromdijk, J., and Long, S. P. (2016). Thiol-based redox modulation of a cyanobacterial eukaryotic-type serine/threonine kinase required for oxidative stress tolerance. Antioxid. Redox Signal. 17, 521–533. doi: 10.1089/ars.2011.4483

May, T., and Soll, J. (2000). 14-3-3 proteins form a guidance complex with chloroplast translocase. Proc. R. Soc. B 267, 1851–1856. doi: 10.1098/rspb.2000.12578

Malingra, M. C., Baja-Hirschel, J., Laborde, S. M., Schwartzenburg, J. B., Mukherjee, B., Mukherjee, A., et al. (2017). Identification and characterization of a solute carrier, CIA4, involved in organic carbon acclimation in Chlamydomonas reinhardtii. J. Exp. Bot. 68, 3879–3889. doi: 10.1038/jxb.201618

Mackinder, C. L. M. (2018). The Chlamydomonas CO2-concentrating mechanism and its potential for engineering photosynthesis in plants. New Phytol. 217, 54–61. doi: 10.1111/nph.14749

Maeda, S. I., Badger, M. R., and Price, G. D. (2002). Novel genes products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic CO2 hydration in the cyanobacterium, Synechococcus sp. PCC7942. Mol. Microbiol. 43, 425–435. doi: 10.1046/j.1365-2958.2002.02753.x

Mallickarjuna, K., Narendra, K., Ragalaitha, R., and Rao, B. J. (2020). Elucidation and genetic intervention of CO2 concentration mechanism in Chlamydomonas reinhardtii for increased plant primary productivity. J. Biosci. 45, 1–18. doi: 10.1007/s12038-020-00080-z

Mata-Cabana, A., Garcia-Dominguez, M., Florencio, F. J., and Lindahl, M. (2012). Thiol-based redox modulation of a cyanobacterial eukaryotic-type serine/threonine kinase required for oxidative stress tolerance. Antioxid. Redox Signal. 17, 521–533. doi: 10.1089/ars.2011.4483

McFarlane, C. R., Shah, N. R., Kabasakal, B. V., Echeverria, B., Cotton, C. A. R., Bubeck, D., et al. (2019). Structural basis of light-induced redox regulation in the Calvin–Benson cycle in cyanobacteria. Proc. Natl. Acad. Sci. U. S. A. 116, 20984–20990. doi: 10.1073/pnas.1905672116

McGinn, P. J., Price, G. D., Maleszka, R., and Badger, M. R. (2003). Inorganic carbon limitation and light control the expression of transcripts related to the CO2-concentrating mechanism in the cyanobacterium Synechocystis sp. strain PCC6803. Plant Physiol. 132, 218–229. doi: 10.1104/pp.199349

McGrath, J. M., and Long, S. P. (2014). Can the cyanobacterial carbon–concentrating mechanism increase photosynthesis in crop species? A theoretical analysis. Plant Physiol. 164, 2247–2261. doi: 10.1104/pp.113.232611

Moreno, J. V., and Navález, R. A. (2007). Proposed carbon dioxide concentrating mechanism in Chlamydomonas reinhardtii. Eukaryot. Cell 6, 1251–1259. doi: 10.1128/ECC.00064-07

Mueller, M., Kunz, H.-H., Schroeder, J. I., Kemp, G., Young, H. S., and Neuhaus, H. E. (2014). Decreased capacity for sodium export out of Arabidopsis chloroplasts impairs salt tolerance, photosynthesis and plant performance. Plant J. 78, 646–658. doi: 10.1111/tjp.12501

Mukherjee, A., Lau, C. S., Walker, C. E., Rai, A. K., Prejean, C. I., Yates, G., et al. (2019). Thylakoid localized bestrophin-like proteins are essential for.
the CO$_2$ concentrating mechanism of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U. S. A.* 116, 16915–16920. doi: 10.1073/pnas.1907061116

Murvanidze, G. V., and Glagolev, A. N. (1982). Electrical nature of the taxis signal in cyanobacteria. *J. Bacteriol.* 150, 239–244. doi: 10.1128/JB.150.1.239-244.1982

Niole, G., Barsoum, M., Houdelet, M., Arcalis, E., Kreuzaler, F., Fischer, R., et al. (2019). The integration of algal carbon concentration mechanism components into tobacco chloroplasts increases photosynthetic efficiency and biomass. *Biotechnol. J.* 14, e1800170. doi: 10.1002/biot.201800170

Ogawa, T., Miyano, A., and Inoue, Y. (1985). Photosystem-I-driven inorganic carbon transport in the cyanobacterium, *Anacystis nidulans*. *BBA Bioenerg.* 808, 77–84. doi: 10.1016/0005-2728(85)90029-5

Ouyang, M., Li, X., Zhang, J., Feng, P., Pu, H., Kong, L., et al. (2020). Liquid-liquid phase transitions enable xerophytic growth in crop plants. *Proc. Natl. Acad. Sci. U. S. A.* 117, 32739–32749. doi: 10.1073/pnas.2002018110

Price, G. D., and Howitt, S. M. (2011). Membrane topology of the cyanobacterial bicarbonate transporter, BicA and Sbta, and identification of potential regulatory loops. *Mol. Membr. Biol.* 28, 265–275. doi: 10.1080/09687688.2011.930049

Price, G. D., Badger, M. R., and Förster, B. (2019). DABs accumulate bicarbonate. *Nat. Metab.* 4, 2029–2030. doi: 10.1038/s41536-019-01629-9

Price, G. D., Pengelly, I. J. I., Förster, B., Du, J., Whitney, S. M., von Caemmerer, S., et al. (2013). The cyanobacterial CCM as a source of genes for improving photosynthetic CO$_2$ fixation in crop species. *J. Exp. Bot.* 64, 753–768. doi: 10.1093/jxb/ers257

Price, G. D., Shelden, M. C., and Howitt, S. M. (2011b). Membrane topology of the cyanobacterial bicarbonate transporter, Sbta, and identification of potential regulatory loops. *Mol. Membr. Biol.* 28, 265–275. doi: 10.1080/09687688.2011.930049

Price, G. D., von Caemmerer, S., Evans, J. R., Yu, J. W., Lloyd, J., Oja, V., et al. (1994). Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO$_2$ assimilation. *Planta* 193, 331–340. doi: 10.1007/BF00201810

Price, G. D., Woodger, F. J., Badger, M. R., Howitt, S. M., and Tucker, L. (2004). Identification of a SulI-type bicarbonate transporter in marine cyanobacteria. *Proc. Natl. Acad. Sci. U. S. A.* 101, 18228–18233. doi: 10.1073/pnas.0405211101

Rae, B. D., Long, B. M., Badger, M. R., and Price, G. D. (2013). Functions, compositions, and evolution of the two types of carboxysomes: polyhedral microcompartment that facilitate CO$_2$ fixation in cyanobacteria and some proteobacteria. *Microbiol. Mol. Biol. Rev.* 77, 357–379. doi: 10.1128/MMBR.00061-12

Rae, B. D., Badger, M. R., Förster, B., Nguyen, N. D., Velantis, C. N., Atkinson, N., et al. (2017). Progress and challenges of engineering a biophysical CO$_2$-concentrating mechanism into higher plants. *J. Exp. Bot.* 68, 3717–3737. doi: 10.1093/jxb/erx133
Schuler, J. M., Saura, P., Thiemann, J., Schuller, S. K., Gamiz-Hernandez, A. P., Kurisu, G., et al. (2020). Redox-coupled proton pumping drives carbon concentration in the photosynthetic complex I. Nat. Commun. 11:494. doi: 10.1038/s41467-020-11524-4

Schwenkert, S., Soll, J., and Böltzer, B. (2011). Protein import into chloroplasts—how chaperones feature into the game. Biochim. Biophys. Acta. 1808, 901–911. doi: 10.1016/j.bbamem.2010.07.021

Scott, K. M., Leonard, J. M., Boden, R., Chaput, D., Dennison, C., Haller, E., et al. (2018). Diversity in CO₂-concentrating mechanisms among chemolithoautotrophs from the genera Hydrogenovibrio, Thiomicroinsertus, and Thiomicrospira, ubiquitous in sulfidic habitats worldwide. Appl. Environ. Microbiol. 85, e02096–e02018. doi: 10.1128/AEM.02096-18

Selim, K. A., Haase, F., Hartmann, D. M., Hagemann, M., and Forchhammer, K. (2018). Psi-like signaling protein SbB links cAMP sensing with cyanobacterial inorganic carbon response. Proc. Natl. Acad. Sci. U. S. A. 115, E4861–E4869. doi:10.1073/pnas.1803790115

Sharma, M., Bennweitz, B., and Klüsgen, R. B. (2018). Rather rule than exception? How to evaluate the relevance of dual protein targeting to mitochondria and chloroplasts. Photosynth. Res. 138, 335–343. doi:10.1007/s11120-018-0543-7

Shelden, M. C., Howitt, S. M., and Price, G. D. (2010). Membrane topology of the cyanobacterial bicarbonate transportor, BicA, a member of the SulP (SLC26A) family. Mol. Membr. Biol. 27, 12–23. doi: 10.3109/0968768903400120

Shen, J., Zeng, Y., Zhuang, X., Sun, L., Yao, X., Pirpml, P., et al. (2013). Organelle pH in the Arabidopsis endomembrane system. Mol. Plant. 6, 1419–1437. doi: 10.1016/j.molp.2013.04.006

Shen, B. R., Zhu, C. H., Yao, Z., Cui, L. L., Zhang, J. I., Yang, C. W., et al. (2017). An optimized transit peptide for effective targeting of diverse foreign proteins into chloroplasts in rice. Sci. Rep. 7:46231. doi:10.1038/srep46231

Shevela, D., Do, H. N., Fantuzzi, A., Rutherford, A. W., and Messinger, J. (2020). Bicarbonate-mediated CO₂ formation on both sides of photosystem II. Biochemistry 59, 2442–2449. doi:10.1021/acs.biochem.0c00208

Shevela, D., Eaton-Rye, J. J., Shen, J. R., and Govindjee, (2012). Photosystem II and the unique role of bicarbonate: a historical perspective. Biochim. Biophys. Acta. 1817, 1134–1151. doi:10.1016/j.bbabi.2012.04.003

Shi, X., and Bloom, A. (2021). PhotosRespiration: the futile cycle? Plan. Theory 10,908. doi:10.1111/ptp.12628

Shibata, M., Katoh, H., Sonoda, M., Ohkawa, H., Shimoyama, M., Fukuzawa, H., et al. (2002). Genes essential to sodium-dependent bicarbonate transport in cyanobacteria: function and phylogenetic analysis. J. Biol. Chem. 277, 18658–18664. doi: 10.1074/jbc.M112468200

Singhal, R., and Fernandez, D. E. (2017). Sorting of SEC translocase SCY components to different membranes in chloroplasts. J. Exp. Bot. 68, 5029–5043. doi:10.1093/jxb/erx318

Smeeckens, S., De Groot, M., Van Binsbergen, J., and Weisbeek, P. (1985). Sequence of the precursor of the chloroplast thylakoid lumen protein plastocyanin. Nature 317, 456–458. doi:10.1038/317456a0

South, P. F., Cavanagh, A. P., Liu, H. W., and Ort, D. R. (2019). Synthetic glycolate metabolism pathways stimulate crop growth and productivity in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Copyright © 2021 Rottet, Förster, Hee, Roucke, Price and Long. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.