

REVIEW PAPER

The cyanobacterial CCM as a source of genes for improving photosynthetic CO₂ fixation in crop species

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Abstract

Crop yields need to nearly double over the next 35 years to keep pace with projected population growth. Improving photosynthesis, via a range of genetic engineering strategies, has been identified as a promising target for crop improvement with regard to increased photosynthetic yield and better water-use efficiency (WUE). One approach is based on integrating components of the highly efficient CO₂-concentrating mechanism (CCM) present in cyanobacteria (blue-green algae) into the chloroplasts of key C₃ crop plants, particularly wheat and rice. Four progressive phases towards engineering components of the cyanobacterial CCM into C₃ species can be envisaged. The first phase (1a), and simplest, is to consider the transplantation of cyanobacterial bicarbonate transporters to C₃ chloroplasts, by host genomic expression and chloroplast targeting, to raise CO₂ levels in the chloroplast and provide a significant improvement in photosynthetic performance. Mathematical modelling indicates that improvements in photosynthesis as high as 28% could be achieved by introducing both of the single-gene, cyanobacterial bicarbonate transporters, known as BicA and SbtA, into C₃ plant chloroplasts. Part of the first phase (1b) includes the more challenging integration of a functional cyanobacterial carboxysome into the chloroplast by chloroplast genome transformation. The later three phases would be progressively more elaborate, taking longer to engineer other functional components of the cyanobacterial CCM into the chloroplast, and targeting photosynthetic and WUE efficiencies typical of C₄ photosynthesis. These later stages would include the addition of NDH-1-type CO₂ pumps and suppression of carbonic anhydrase and C₃ Rubisco in the chloroplast stroma. We include a score card for assessing the success of physiological modifications gained in phase 1a.

Key words: Bicarbonate pumps, carboxysomes, CO₂-concentrating mechanism, crop improvement, cyanobacteria, photosynthetic efficiency.

Introduction

A number of international agencies have identified food security and food shortages as acute problems that face the global human population by the 2030–2050 period unless a range of measures to ameliorate this looming crisis are adopted. For instance, the United Nations have called for a 50% increase in global food production by 2030, and the World Health Organization has

called for a doubling of food production by 2050 to meet a burgeoning population. The fear is that developing nations will be worst affected by these projected shortages unless action is taken. Productivity gains associated with the ‘Green Revolution’ have essentially plateaued, so a range of biotechnological measures are being considered to meet this challenge, including a major

Abbreviations: C_i, CO₂ concentration in the substomatal cavity; DIC, dissolved inorganic carbon (chiefly CO₂ and HCO₃⁻); HCR, high CO₂-requiring mutant phenotype; WUE, water-use efficiency (kg of water to produce a kg of biomass or grain).

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effort to improve the efficiency of photosynthetic CO₂ fixation so that more food is produced per plant or per hectare (Hibberd *et al.*, 2008; Parry and Hawkesford, 2010; Parry *et al.*, 2011). The theme of this review is to consider approaches relating to the engineering of marked improvements to the efficiency of photosynthetic CO₂ fixation in the chloroplasts of crop plants by integration of components of the cyanobacterial CO₂-concentrating mechanism (CCM). This CCM is a mechanism that operates with high efficiency in cyanobacteria (blue-green algae) to overcome the catalytic deficiencies of Rubisco catalysis.

A key problem that impedes the optimal photosynthetic efficiency of many crop plants revolves around the catalytic limitation of the ubiquitous CO₂-fixing enzyme, known as Rubisco (ribulose biphosphate carboxylase-oxygenase). As the name of the enzyme implies, Rubisco can fix either CO₂ or O₂, and since both substrates are of remarkably similar molecular shape the enzyme is posed with the problem of catalytic difficulty in discriminating between CO₂ and O₂ (Whitney *et al.*, 2011). Although fixing CO₂ is the dominant productive outcome, the inadvertent fixing of O₂ leads to an energetically wasteful cycle known as photorespiration. Some crop improvement approaches are therefore targeting ways to increase the productivity of photosynthetic CO₂ fixation by boosting the steady-state CO₂ concentration around Rubisco in the chloroplasts (the site of CO₂ fixation), thereby reducing photorespiration (Hibberd *et al.*, 2008; Parry *et al.*, 2011). Using components of the cyanobacterial CCM to raise CO₂ levels in the chloroplast is another of these approaches that will be considered here in more detail than in previous reviews (Price *et al.*, 2008, 2011a).

In Earth's evolutionary history the wayward ability of Rubisco to fix O₂ was not always a problem. When Rubisco first evolved in the cyanobacteria some 3.5 billion years ago, it did so in an environment where CO₂ was many fold higher than present levels and O₂ levels were very low (Badger and Price, 2003; Whitney *et al.*, 2011). Under these ancient conditions, Rubisco was CO₂ saturated and seems to have been efficient, possessing a relatively high carboxylation rate, judging from the properties of present-day cyanobacterial Rubisco (Badger *et al.*, 1998). However, due to the onset of oxygenic photosynthesis in cyanobacteria, the levels of O₂ rose steadily until ~1.5 billion years ago where it rose quite dramatically, and CO₂ levels progressively fell to very low levels, creating conditions where atmospheric CO₂ became rate limiting for Rubisco, and fixation of O₂ as an alternative non-productive substrate became significant. In an evolutionary response to changed atmospheric conditions (approaching present-day levels of 0.04% CO₂ and 21% O₂), two general strategies evolved to help Rubisco maximize its net carboxylation rate. One strategy, common to most crop plants, saw the enzyme evolve improvements in catalysis, particularly an increase in enzymatic affinity for CO₂ and an improved ability to discriminate against O₂ (Badger *et al.*, 1998). The downside of this strategy was a >3-fold drop in the carboxylation rate (Tcherkez *et al.*, 2006), requiring more Rubisco enzyme to perform the same amount of CO₂ fixation, and burdening photosynthesis with a rate of photorespiration as high as 30% of the theoretical maximum rate of CO₂ fixation.

The alternative strategy, used by cyanobacteria, many algae, and a subset of land plants, involved the development of active

CCMs to turbo-charge the CO₂ supply to Rubisco, although at a minor metabolic cost (Badger *et al.*, 1998). Among land plants, the strategy led to the development of the efficient, but anatomically and biochemically complex, CCMs that feature in C₄ photosynthesis (Sage, 2004). Paradoxically, very few C₄ species are commercially useful crop plants, although maize, sugarcane, and sorghum are important exceptions. Eukaryotic microalgae, diatoms, and macroalgae have also evolved different types of CCMs (Spalding, 2008; Raven *et al.*, 2011). In cyanobacteria, the strategy led to a highly efficient CCM that can accumulate CO₂ around Rubisco by a factor of 1000-fold above ambient levels (Badger and Price, 2003). Unfortunately, this development seems to have taken place well after the endosymbiotic origins of land plant chloroplasts, thereby precluding the selection of this CCM system by vascular plants. The cyanobacteria and other phytoplankton, chiefly in the oceans, contribute nearly 50% of global annual primary productivity (Field *et al.*, 1998; Liu *et al.*, 1999), thereby underpinning the marine food web. Probably as much as 25% of annual global productivity is achieved by deep ocean cyanobacteria. For example, in oligotrophic oceans located between 40°N and 40°S, photosynthetic CO₂ fixation is dominated by marine cyanobacteria of the *Synechococcus* and *Prochlorococcus* genera, and together these species have been estimated to contribute 30–80% of primary production (Liu *et al.*, 1997). The cyanobacterial CCM arguably contributes to this primary productivity.

Regrettably perhaps, for our most important food crops, whether they are grain crops such as rice, wheat, barley, canola, and soybean, or tuber or vegetable crops, the overwhelming majority are C₃ species, and, therefore, they lack any significant form of CCM at the chloroplast or leaf level. In addition to high rates of photorespiration, another inefficiency in C₃ photosynthesis involves the physical diffusion resistance to CO₂ transfer through the leaf pores (stomata), then across cell walls and cytoplasm, and eventually through to the chloroplasts (Evans and von Caemmerer, 1996; Evans *et al.*, 2009). These diffusive resistances to CO₂ passage result in a drawdown or deficit in the steady-state CO₂ concentration in the chloroplast during photosynthetic CO₂ fixation relative to that in the ambient air. While C₃ plants have adopted strategies to reduce the diffusive resistance to CO₂ by appressing chloroplasts against the intracellular airspaces and maximizing the chloroplast surface area to leaf area ratios, the drawdown effect at the chloroplast remains a significant problem (Evans and von Caemmerer, 1996). This ensuing lower CO₂ concentration in the chloroplast comes at a cost of reduced Rubisco carboxylation and increased oxygenation, thereby enhancing the expenditure of metabolic energy to recycle carbon and nitrogen via photorespiration. This situation is worsened again by increases in temperature due to a drop in the affinity of Rubisco for CO₂ and an increase in the oxygenase reaction, a situation that is quite important in the context of climate change (Kubien and Sage, 2008).

A related downside to C₃ photosynthesis is the need to invest heavily in Rubisco expression to achieve acceptable high rates of photosynthetic CO₂ fixation. For instance, in wheat, up to 25% of leaf nitrogen is invested in Rubisco protein (Evans, 1989), whereas cyanobacteria devote 3–5% to Rubisco. C₃ plants also have to present relatively open stomatal pores to gain sufficient

CO₂ for photosynthesis, but this comes with a water loss penalty of nearly 500 molecules of water per CO₂ fixed, on average. The evolution of the types of CCMs in C₄ plants solved a number of these inefficiencies by actively elevating CO₂ around Rubisco in the bundle sheath chloroplasts, thereby reducing photorespiration, and allowing reduced expenditure in Rubisco and a nearly halving of leaf water loss by being able to operate with more closed stomata (Sage, 2004). Such improvements in resource use have prompted much interest in attempting to introduce components of C₄-type CCMs into important C₃ crops such as rice and other cereal crop plants (Hibberd *et al.*, 2008; Sage and Zhu, 2011).

As a parallel alternative, it is now recognized that engineering components of the cyanobacterial CCM into the chloroplasts of C₃ crop species could also achieve improvements in the efficiency of photosynthetic CO₂ fixation similar to that envisaged for the C₄ rice approach (Price *et al.*, 2008, 2011a; Parry *et al.*, 2011). The following sections deal with the basic components of the cyanobacterial CCM, followed by consideration of progressive phases needed to introduce components of the cyanobacterial CCM into C₃ plants.

The features of the cyanobacterial CCM

Basics features of the CCM

Transporting inorganic carbon It is likely that the poor availability of CO₂ in aquatic systems, and the higher availability of HCO₃⁻, are two factors that drove the evolution of the cyanobacterial CCM, especially during periods when atmospheric CO₂ levels became very low and O₂ was high. The evolution of active uptake systems for the major forms of dissolved inorganic carbon (DIC), namely CO₂ and HCO₃⁻, was a key to survival under these conditions. However, the ability to transport CO₂ and HCO₃⁻ actively into the cell, where it is accumulated as a HCO₃⁻ pool, by itself, was not a sufficient advantage until a microcompartment, the carboxysome, had evolved where CO₂ levels could be elevated around Rubisco, to allow enhanced CO₂ fixation (Badger *et al.*, 2002; Price *et al.*, 2008). This final step defines the operation of a CCM, namely that CO₂ has to be elevated around Rubisco to optimize catalysis by operating closer to substrate CO₂ saturation.

Carboxysomes Rubisco in cyanobacteria is encapsulated in unique proteinaceous microcompartments known as carboxysomes which act as the site of CO₂ elevation. The carboxysomes are typically 90–400 nm in diameter, icosahedral in shape (being composed of 20 equilateral triangular sides), and contain most, or all, of the active Rubisco enzyme within the cell. There are two types of carboxysomes (see below), but in either form the carboxysome shells are composed of just 6–8 proteins (Price *et al.*, 1998, 2008; Cannon *et al.*, 2003; Espie and Kimber, 2011). The average unicellular cyanobacterial cells would normally possess 5–15 carboxysomes per cell depending on the species and growth conditions. The critically important supply rate of CO₂, sourced from accumulated cytosolic HCO₃⁻ entering the microcompartment, is catalysed by a carboxysome-located carbonic anhydrase (CA) known as CcaA (Icfa) or

CcmM, or CsoS3, depending on the type of carboxysome (Espie and Kimber, 2011).

Minimizing CO₂ leakage The key to the functional efficiency of any CCM revolves around the ability to minimize the loss of CO₂ from the CO₂ elevation zone or the DIC accumulation zone. In cyanobacteria, this is accomplished by a combination of four key factors (Badger and Price, 2003; Price *et al.*, 2008, 2011a). First, the accumulation of the ionic form of DIC, bicarbonate, which is less membrane permeable than CO₂, acts to reduce leakage. Secondly, CA activity is absent from the cytosol and minimizes leakage due to wasteful conversion to CO₂ and subsequent diffusion back to the external medium. Thirdly, the carboxysome protein shell is proposed to have the special property of retarding CO₂ leakage relative to entry and exit of ionic forms. Finally, thylakoid-located CO₂ pumps play a key role in recycling CO₂ that leaks from the carboxysome back into the HCO₃⁻ pool (Maeda *et al.*, 2002; Price *et al.*, 2008).

Induction of the CCM The cyanobacterial CCM is characterized by a constitutive expression of functional carboxysomes and a constitutive CO₂ pump, and often a basal level of HCO₃⁻ uptake capacity, even when grown at 1–2% CO₂. This uptake capacity is augmented by the induction (de-repression) of high affinity uptake systems for CO₂ and HCO₃⁻ under conditions of DIC starvation. Much is known about the induction process from genetic knockout mutants and transcript analysis, particularly the role of CmpR and CcmR transcription factors in de-repression of gene expression. The details are not particularly relevant to the assessment of the usefulness of cyanobacterial CCM components for crop improvement; however details can be found in the following references: McGinn *et al.* (2003), Wang *et al.* (2004), Nishimura *et al.* (2008), Price *et al.* (2008), Ludwig and Bryant (2011), and Woodger *et al.* (2005, 2007).

DIC transporters

Types of DIC transporters The collective action of the active CO₂ and HCO₃⁻ uptake systems can lead to the accumulation of internal DIC levels well above 20 mM in freshwater model strains such as *Synechococcus* PCC7942, reaching an accumulation ratio of up to 1000-fold. To achieve this, five distinct transport systems for DIC uptake have so far been identified in cyanobacteria (Fig. 1); see Price *et al.* (2008) and Price (2011) for more details and related references. The first two HCO₃⁻ transporters are single-gene systems: SbtA is an inducible, high-affinity Na⁺-dependent HCO₃⁻ transporter (Shibata *et al.*, 2002; Price *et al.*, 2004) that apparently acts as an Na⁺/HCO₃⁻ symporter with a relatively low flux rate. BicA, a low-affinity, high-flux, Na⁺-dependent HCO₃⁻ transporter, belongs to the widespread SulP family and is related to the human SLC26 family of anion transporters (Price *et al.*, 2004; Price and Howitt, 2011); BicA is a probable Na⁺/HCO₃⁻ symporter and is unrelated to SbtA. In addition, BCT1 is a high-affinity HCO₃⁻ transporter (uniporter), inducible under DIC limitation, belonging to the traffic ATPase family, and, unlike BicA and SbtA, it is encoded by four genes (*cmpABCD*) to complete a functional transporter complex.

The two CO₂ pumps are specialized respiration-related NDH-1 complexes that are able to use NADPH as an electron donor to

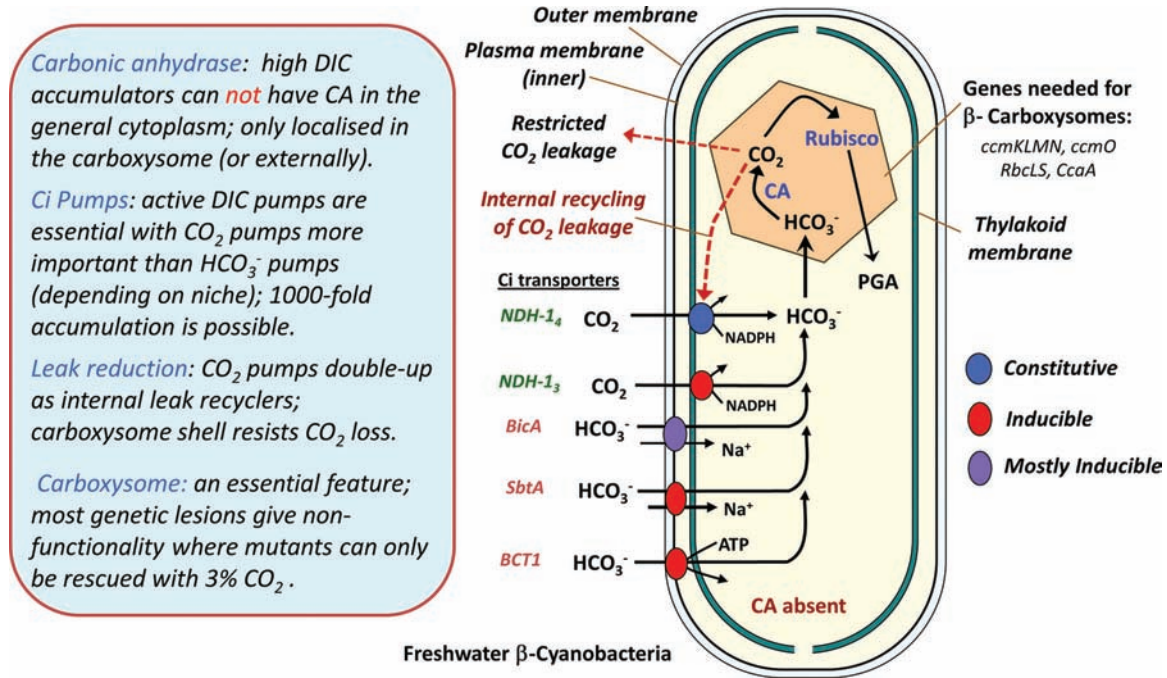


Fig. 1. Basic components of the cyanobacterial CCM of a stylized β -cyanobacterium.

drive conversion of CO₂ to HCO₃⁻ during the uptake step (Price *et al.*, 2002). Each complex is composed of 10 core subunits common to the respiratory NDH-I complex, with 3–4 specialized subunits required for CO₂ uptake. The consensus is that these NDH-I-type CO₂ uptake systems are located on the thylakoid membranes where they use CO₂ diffusing from outside the cell, or arising from leakage from the carboxysomes, as a substrate for directional conversion to HCO₃⁻. NDH-I₄ is a constitutive CO₂ uptake system based on a specialized NADPH dehydrogenase (NDH-I) complex. The second CO₂ pump, NDH-I₃, is inducible under DIC limitation and is of higher uptake affinity than NDH-I₄.

Diversity of the DIC transporters There are two broad CCM phylogenies in cyanobacteria, known as α - or β -cyanobacteria, based on Rubisco and carboxysome types (Tabita, 1999; Badger *et al.*, 2002; Espie and Kimber, 2011); each displays differences in the range of DIC uptake systems employed (Badger *et al.*, 2006; Price *et al.*, 2008; Rae *et al.*, 2011). β -Cyanobacteria, prevalent in freshwater and coastal marine areas, utilize form-1b Rubisco and *ccm*-type carboxysomes, whereas α -cyanobacteria utilize form-1a Rubisco and *cso*-type carboxysomes. The abundant, and therefore globally productive, α -cyanobacteria usually occur in the deep ocean areas of the world, at typical depths of 100–150 m where the light intensity is as low as 1% of the surface radiance. Generally, β -cyanobacteria have a greater diversity of DIC uptake systems, while α -cyanobacteria have the fewest (Badger *et al.*, 2006; Rae *et al.*, 2011). However, α -cyanobacteria, particularly those of the *Cyanobium* clade, can also be found in freshwater lakes, apparently as a result of the horizontal transfer of transporter genes from β -cyanobacteria (Rae *et al.*, 2011).

Carboxysomal function

Types and composition Although β - and α -carboxysomes appear have arisen by parallel evolution, each performs the same basic functional role of acting as a microcompartment where CO₂ can be elevated around Rubisco. Both types are obligate for cell survival at low and moderate CO₂ levels, and both structures appear to be icosahedral in shape, and thus possessing 20 identical facets each fashioned as an equilateral triangle. The icosahedral shape has passing resemblance to some viral capsids, and parts of the structure may be able to self-assemble to some extent. See the following reviews for more detail: Badger and Price (2003), Badger *et al.* (2006), Price *et al.* (2008, 2011a), and Espie and Kimber (2011). There are a large number of carboxysome mutations known that result in non-functional carboxysomes, and therefore generate high CO₂-requiring (HCR) phenotypes. These mutants require in excess of 2% CO₂ to survive (Friedberg *et al.*, 1989; Price and Badger, 1989b; Ludwig *et al.*, 2000; Woodger *et al.*, 2003). These mutants also feature an abnormally large accumulated internal DIC pool (up to 2-fold more than wild-type levels) in the cell because they are unable to perform the final essential step of converting HCO₃⁻ to CO₂ inside the polyhedral structures to support CO₂ fixation by Rubisco. Both carboxysome structures have potential application towards engineering a functional CCM in C₃ plants.

The carboxysome shell is composed largely of a number of related small proteins which have been termed bacterial microcompartment proteins (CcmK family and CcmL in β -carboxysomes; CsoS1 and CsoS4 families in α -carboxysomes); in addition, the carboxysomes also contain a number of unrelated proteins which perform various functions in each structure (Badger and Price, 2003; Price *et al.*, 2008;

Espie and Kimber, 2011; Yeates *et al.*, 2011). Both types of carboxysomes contain an essential and specific CA for catalytic conversion of HCO_3^- to CO_2 in the vicinity of Rubisco. For β -carboxysomes, the CA function is carried out by a shell-associated protein known as CcaA (IcfA), or, in some strains that are missing CcaA, by a full-length version of CcmM, that contains an active N-terminal γ -CA domain that is generally inactive in most strains (Fukuzawa *et al.*, 1992; Yu *et al.*, 1992; So *et al.*, 2002; Pena *et al.*, 2010). In α -carboxysomes, the shell protein CsoS3 (CsoSCA) is a functional CA with a β -CA-like protein fold, although lacking sequence homology with β forms (So *et al.*, 2004; Sawaya *et al.*, 2006). In a typical β -carboxysome, such as that present in the model cyanobacterium *Synechococcus* PCC7942, the carboxysome is composed of 10 proteins (including form-1B Rubisco, *rbcLS*) with Rubisco tightly packed inside the structure through the action of the 58 kDa and 35 kDa forms of the Rubisco-organizing proteins of CcmM (Long *et al.*, 2007, 2010). The CcmK2 protein crystallizes as a flattened hexameric tile that is proposed to tessellate and form the outer coat of the carboxysome (Kerfeld *et al.*, 2010). CcmK2 (formerly known as CcmK1 or CcmK) and CcmO are the most abundant proteins of the outer shell and both are essential for CCM function, while CcmK3 and CcmK4 are low abundance proteins that help refine the function of the shell (Rae *et al.*, 2012). Another rare protein, CcmL, crystallizes as a flattened pentamer, and is believed to complete the outer layer of the shell by filling the twelve 5-fold vertices of the structure (Tanaka *et al.*, 2008). The inner layer of the carboxysome shell is composed of the 58 kDa form of CcmM (Rubisco-organizing protein) bound to RbcL by multiple RbcS-like domains and complexed to CcaA and CcmN (Long *et al.*, 2007, 2011; Cot *et al.*, 2008); this complex is also known as the hydration complex. The organization of *Synechococcus* PCC7942 carboxysome genes features a five-gene operon of core carboxysome genes, *ccmK2-LMNO* situated upstream of the dicistronic *rbcLS* operon. The essential *ccaA* (*icfA*) gene is located elsewhere on the genome, as is the dicistronic *ccmK3-K4* operon which plays a peripheral but important role in functionality of the carboxysome.

The structure of a typical α -carboxysome, such as that from the sulphur bacterium *Halothiobacillus neapolitanus* C2, differs somewhat from that of the β -carboxysome in that they seem to lack any identified analogues that act as Rubisco-organizing proteins. In contrast, the details of the shell seem to be quite similar to those to the outer shell of β -carboxysomes, where CsoS1 hexameric forms tessellate to form the bulk of the shell and the CsoS4 forms close the structures at the vertices. Of the larger CsoS2 and CsoS3 forms, CsoS3 is bound to the shell as the essential CA, but the precise function of CsoS2 shell protein is still unresolved. The α -carboxysome genes are commonly organized in a single operon. A significant leap in assembling carboxysomes in a heterologous expression host was recently achieved when the *H. neapolitanus* carboxysome operon was successfully expressed in *Escherichia coli* (Bonacci *et al.*, 2012). Including the two form-1A Rubisco genes (*cbhLS*) a total of 10 proteins were expressed, resulting in active Rubisco and normal carboxysome ultrastructure. This is an important milestone that may help pave the way for introduction of a functional carboxysome in C_3 chloroplasts (phase 1b; see below).

Further details about the gene products involved in forming the two carboxysome types can be found in previous reviews (Price *et al.*, 2008; Yeates *et al.*, 2008; Espie and Kimber, 2011; Kinney *et al.*, 2011).

Correct localization of CA is essential for DIC accumulation

The CA enzyme rapidly catalyses the reversible hydration and dehydration of CO_2 and HCO_3^- , respectively; otherwise this is normally a slow reaction with the half-time at pH 8 (at 25 °C) of >15 s (Walker *et al.*, 1980). As such, appropriate localization of CA within specific compartments is therefore critical to the functionality of cyanobacterial CCMs, or indeed any CCM. It is the absence of CA in the cytosol, coupled with the directional CO_2 uptake systems that convert CO_2 to HCO_3^- at the thylakoid membrane, which allows the cell to accumulate HCO_3^- and keep it out of rapid chemical equilibrium with CO_2 . This is effective in minimizing outward diffusion of CO_2 to the external medium, owing to the slow dehydration of HCO_3^- in the absence of CA. Thus it follows that incorrect placement of CA has a marked negative effect on CCM efficiency. This was demonstrated by an earlier experiment where human CA was expressed in the cytoplasm of a model cyanobacterium, *Synechococcus elongatus* PCC7942 (Fig. 2). As ectopic expression of HCA was progressively increased, the eventual result was dissipation of the accumulated HCO_3^- pool due to the CA-mediated equilibration between CO_2 and HCO_3^- , which in turn led to increased CO_2 diffusion out of the cell (Price and Badger, 1989a). This is very different from C_3 chloroplasts where CA is highly abundant in the stroma in order to maximize diffusion of CO_2 across the envelope and throughout the chloroplast (Badger and Price, 1994).

Pathways for engineering a cyanobacterial CCM into C_3 chloroplasts

Based on what we know about the cyanobacterial CCM components and the physiology of chloroplasts in C_3 plants, a pathway for engineering aspects of the cyanobacterial CCM into C_3 plant chloroplasts can be proposed (Figs 3–5).

- Phase 1a. Transferring active HCO_3^- pumps to the chloroplast envelope.
- Phase 1b. Building a functional cyanobacterial carboxysome in the chloroplast stroma.
- Phase 2. Combining the traits from phase 1a and 1b.
- Phase 3. Eliminating CA from the stroma.
- Phase 4. Building a functional NDH-1 CO_2 uptake complex in the thylakoid membranes.

Phase 1a engineering of cyanobacterial CCM components into the chloroplasts of C_3 model plants can be loosely described as adding functional forms of the single-gene HCO_3^- transporters, SbtA and BicA, into the chloroplast envelope for the purpose of reducing the drawdown of CO_2 in the chloroplast. In addition to this, a parallel approach (phase

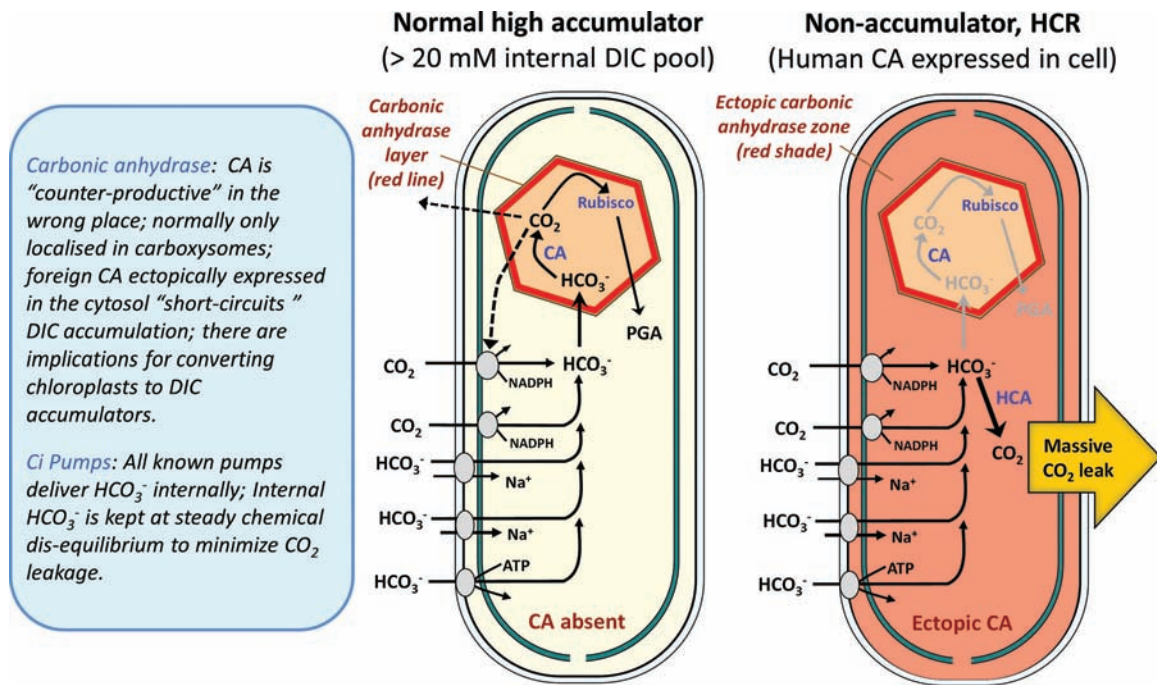


Fig. 2. Carbonic anhydrase (CA) zones in cyanobacteria and the role that CA location has in determining if cells are to be DIC accumulators or non-accumulators.

1b) can also be pursued through adding functional cyanobacterial carboxysomes to the chloroplast. Phase 2 would involve combining phases 1a and 1b, for assessing the combined benefits of both HCO₃⁻ pumps and carboxysomes, whereas phases 3 and 4 consider the progressive engineering of a more advanced form of the cyanobacterial CCM into plant chloroplasts. Elimination of CA from the stroma in phase 3 would enhance stromal DIC levels and improve carboxysome function, while the complex engineering of a CO₂ uptake NDH-1 complex in phase 4 would reduce CO₂ leakage.

Phase 1a

The initial engineering approach would aim to transfer single-gene HCO₃⁻ transporters, such as SbtA and BicA, into the chloroplast envelope (Fig. 3a) with the objective of not achieving significant net accumulation of DIC in the chloroplast, but to reduce the deficit (drawdown) in the steady-state CO₂ concentration between the leaf intracellular level and that in the chloroplast. This scenario has been considered in some detail, and theoretical modelling has been used to track the theoretical benefits of this approach (Price *et al.*, 2011a). Inevitably this approach will entail a degree of fine-tuning to obtain optimal and fully functional bicarbonate transport capacity. Phase 1a is considered in more detail below.

Phase 1b

This phase assumes that building a carboxysome in the chloroplast, using cyanobacterial Rubisco, can result in the enhancement

of Rubisco fixation without addition of chloroplast envelope pumps and the accumulation of significant stromal DIC levels. Most of the model strains of cyanobacteria used for physiological analysis are actually prone to being superaccumulators of DIC under optimal conditions, with achieved maximal DIC pools of 20–40 mM (Badger and Andrews, 1982; Price and Badger, 1989b; Woodger *et al.*, 2005). These levels of DIC accumulation would be challenging to achieve in the chloroplast. However, the observation that cyanobacterial CO₂ fixation is near substrate saturation with as little as 6.4 mM accumulated DIC (Price and Badger, 1989a), and recent data and modelling showing that 5 mM DIC may be sufficient for saturation (M.R. Badger, L.F. Whitehead, B.M. Long, and G.D. Price, unpublished results), suggests that carboxysomes provide a steady-state CO₂ level which is higher than the chemical equilibrium at pH 8. The mechanism for this is unknown, but this opens up the possibility of having functional carboxysomes working effectively at relatively low stromal HCO₃⁻ levels (Fig. 3b). Calculations indicate that between 0.48 mM and 0.76 mM HCO₃⁻ (for a stromal C_i of ~190 μbar, at 25 °C and a stromal pH of 8.0–8.2) is present in the chloroplast under steady-state photosynthetic conditions (Evans and von Caemmerer, 1996). This would be enough to provide 15–20% saturation of carboxysome-enclosed Rubisco with relatively low rates of Rubisco oxygenase activity. The supplementation of the existing C₃ CO₂ fixation rate with carboxysomal CO₂ fixation should be readily detectable through a gas exchange analysis, and more so when phases 1a and 1b are combined. This would at least allow the assessment of whether introduced carboxysomes are correctly assembled and functional within the chloroplast, and may give a small increase in net CO₂ fixation. The real impact of this advance would be seen in phases 3 and 4.

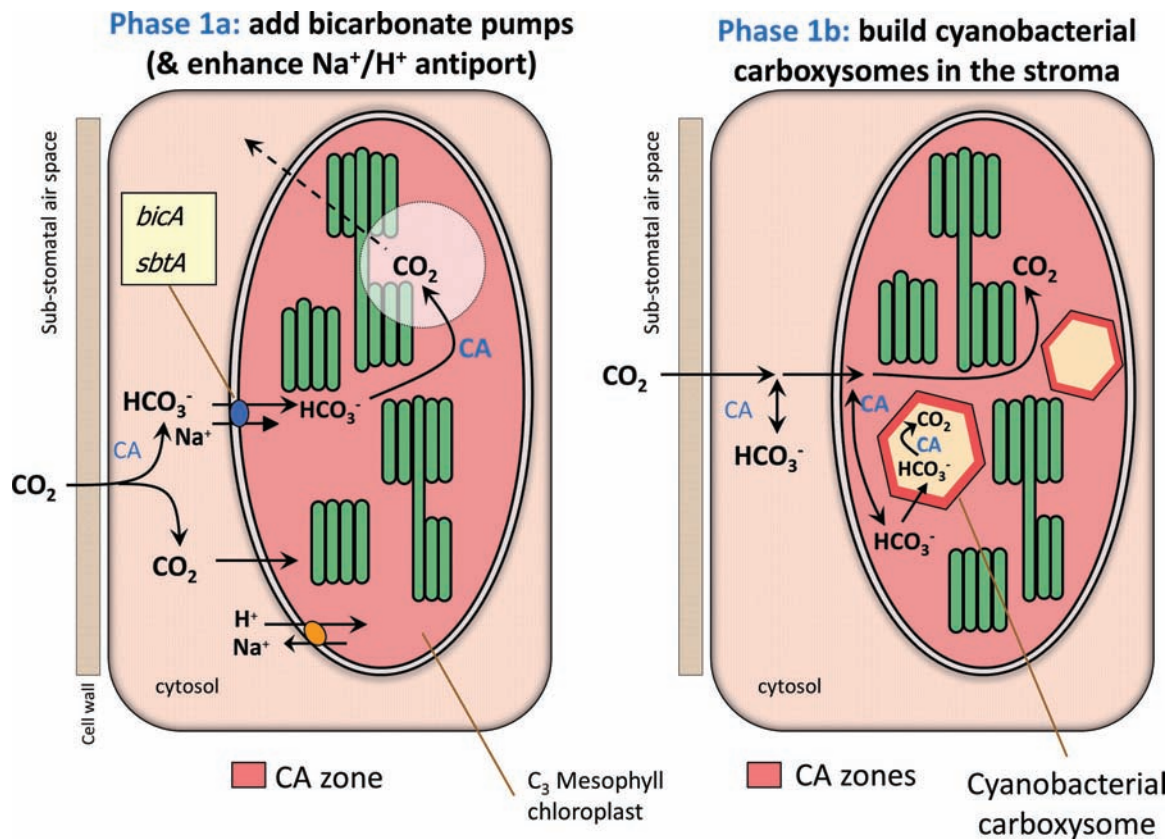


Fig. 3. Phase 1a and 1b engineering—addition of bicarbonate transporters and carboxysomes.

The fundamentals of carboxysome assembly and function have advanced in recent years, aided by advances in determining the crystal structures of some key components of the shell (Yeates *et al.*, 2008) and identification of proteins required as key Rubisco- and shell-organizing proteins (Long *et al.*, 2007, 2010). We have reached a point where engineering the assembly of a carboxysome in the chloroplast via chloroplast genome transformation seems to be feasible, especially with the recent breakthrough for the expression of a structurally correct α -carboxysome in *E. coli* (Bonacci *et al.*, 2012). Inevitably, optimization would be required to achieve expression in the chloroplast, as correct assembly of cyanobacterial Rubisco in higher plants so far appears to be problematic (Kanevski *et al.*, 1999), and optimization of a synthetic carboxysome operon would still be required to balance the expression of 9–10 proteins. For proof-of-concept, expression in tobacco chloroplasts using chloroplast genome transformation seems to be the simplest approach to test for correct protein folding and correct functional assembly of carboxysomes. Chloroplast transformation in rice and wheat would be the preferred way to transfer carboxysomes to these key crop plants, but if such techniques are not yet reliable, each carboxysome component would need to be expressed with a chloroplast transit protein from host genome integrations. New techniques for expressing whole bacterial operons from the host genome by viral-type vectors are becoming available (Mozes-Koch *et al.*, 2012) and may become effective alternatives.

Phase 2

It is envisaged that phases 1a and 1b could be combined by crossing of tobacco transgenics if BicA/SbtA additions are initially as host genome integrations and a carboxysomal line is achieved by chloroplast genome transformation (Fig. 4). If the drawdown in the chloroplast can be eliminated, then between 0.63 mM and 1.0 mM HCO₃⁻ should be present in the stroma (for C_{chlo} of ~250 μbar , at 25°C and a stromal pH of 8.0–8.2), giving a better opportunity for carboxysomal CO₂ fixation to contribute to net photosynthesis in the chloroplast. In addition, the proposed ability of the carboxysomes to limit O₂ entry from the thylakoids may provide a second beneficial effect by reducing photorespiration. Achieving phase 2 modifications would allow an assessment of the value of combining introduction of bicarbonate transporters and carboxysomes on photosynthetic efficiency.

Phase 3

On the pathway towards implementing a full cyanobacterial CCM into the chloroplast it would be necessary to eliminate CA from the chloroplast stroma (Fig. 5a). Without stromal CA present, the accumulated HCO₃⁻ concentration should reach 2–3 mM, which would be more than half the concentration to saturate CO₂ fixation in the transferred carboxysomes. In addition, it would reduce CO₂ leakage from transported HCO₃⁻. Gene knockouts of chloroplast CA would be the most effective

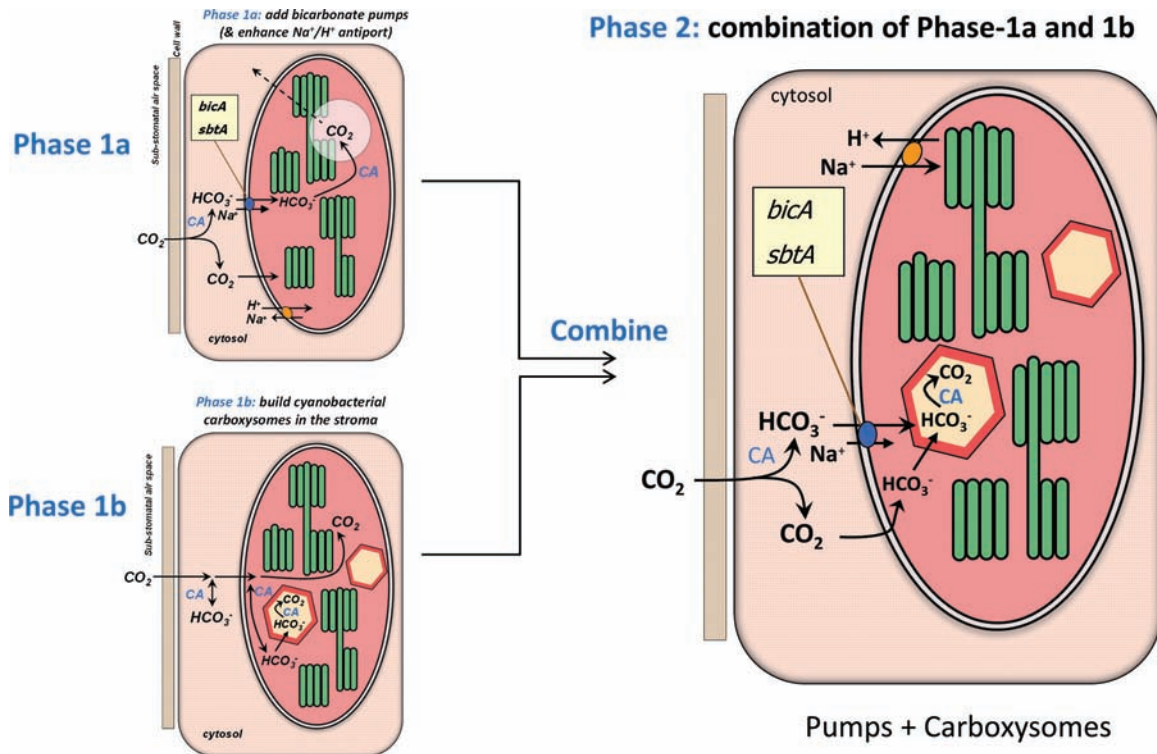


Fig. 4. Phase 2 engineering—combination of 1a and 1b.

approach, although in some species such as hexaploid wheat this would be more difficult because of gene copy number. The use of iRNA (interfering RNA) could also be an effective tool as this has been 99% successful in reducing chloroplastic CA levels in tobacco (Majeau *et al.*, 1994; Price *et al.*, 1994). Of additional benefit in phase 3 would be the elimination of the host C₃ Rubisco, either by targeted deletion of the chloroplast *rbcL* gene or by iRNA depression of the SSU gene family, so that CO₂ fixation would then be entirely dependent on carboxysomal Rubisco of cyanobacterial origin. This would be instructive in assessing how well the basic cyanobacterial CCM works in the chloroplast and would provide data for further experimental and modelling refinements.

Phase 4

Over the longer term, once phase 3 alterations are completed, it may be possible to consider the more complex addition of an NDH-1-based CO₂ pump (Fig. 5b), recognizing that this would be extremely challenging and may require the availability of cereal chloroplast genome transformation technologies. The purpose would be to add CO₂ as another actively acquired DIC species and to reduce CO₂ leakage. Since the NDH-1₄ complex is located on the thylakoid membrane in cyanobacteria, with the extrinsic part facing the stroma, this would also be the obvious location in the chloroplast. A full complex would probably require the expression of 14 genes, although some core parts of the chloroplast–NDH-1 complex are already present and play a role in cyclic electron flow under heat stress conditions (Wang *et al.*, 2006; Peng *et al.*, 2011). There is no certainty that core components of the existing complex would be able to bind the CO₂

pump-specific components (NdhF4, D4, and ChpX) and function correctly. For proof-of-concept, a full NDH-1₄ operon would be first expressed in the tobacco chloroplast genome as an artificial operon of all 14 genes assembled by commercial DNA synthesis, allowing adjustments for codon bias and translational efficiency. Phase 4 engineering would aim at achieving DIC accumulation of up to 5 mM in the stroma, thereby approaching the net photosynthetic efficiencies typical of C₄ plants. It has been claimed that photorespiration plays a role in nitrogen assimilation in C₃ plants such that any reduction in photorespiration might inhibit the assimilation of nitrate into organic nitrogen compounds (Bloom *et al.*, 2010). If this is the case, some enhancement of nitrogen assimilatory capacity might be required for phase 3 onwards.

High rates of active CO₂ uptake in cyanobacterial cells have been shown to require an active H⁺ extrusion pump, known as PxcA (formerly known as CotA; related to CemA from *Chlamydomonas*) for the purposes of pH balancing (Sonoda *et al.*, 1998). Depending on the steady-state balance between active HCO₃⁻ and CO₂ uptake achieved within the chloroplast it might also be necessary to introduce *pxcA* for correct pH balancing. Another issue is to consider ways of making the chloroplast envelope less conductive to CO₂ loss as a means to improve the efficiency of the transplanted CCM. Targeted reductions in aquaporin activity have already been shown to reduce leaf mesophyll conductance in tobacco (Flexas *et al.*, 2006), and may provide a partial solution.

A more detailed look at phase 1A

As previously indicated (Price *et al.*, 2011a), the objective of reducing the CO₂ drawdown effect in C₃ chloroplasts appears to

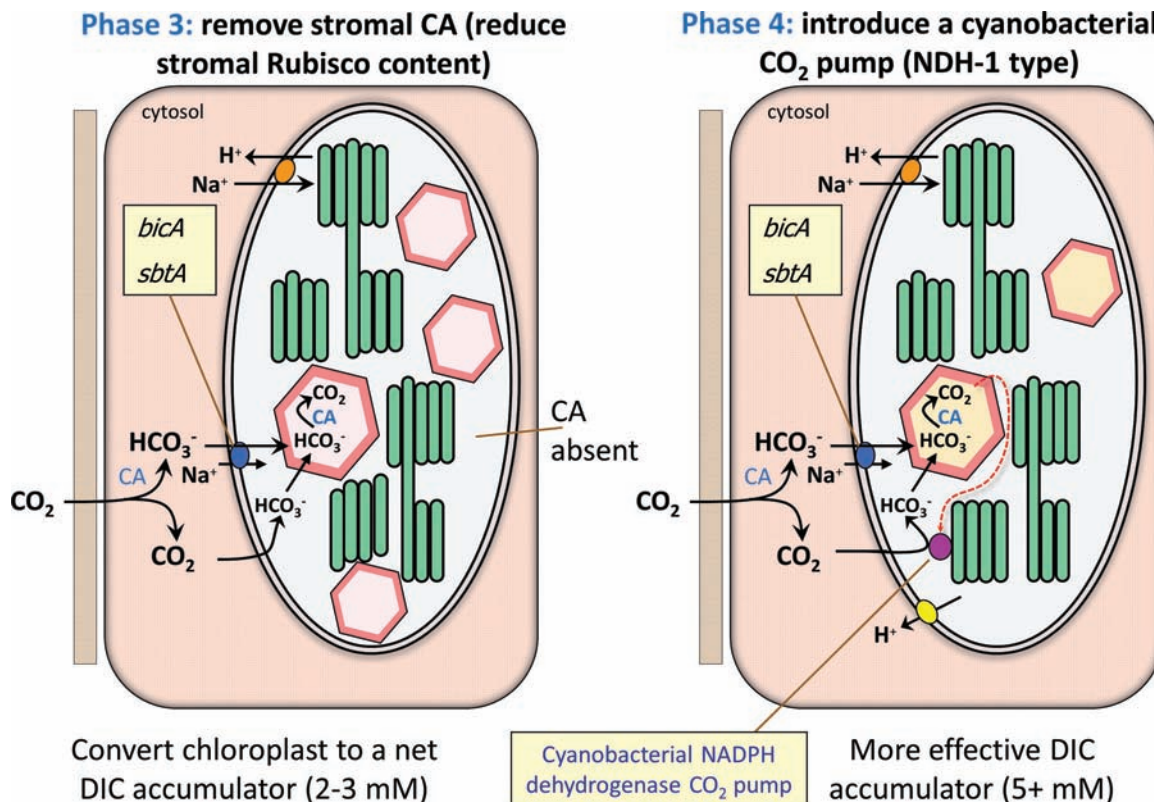


Fig. 5. Phase 3 and 4—progressive addition of CCM components to C₃ chloroplasts and suppression of endogenous CA and Rubisco levels in the chloroplast stroma.

be best achieved by expressing one or two cyanobacterial HCO₃⁻ transporters on the inner envelope of the C₃ chloroplast (Fig. 3a). Single-subunit HCO₃⁻ transporters such as BicA and SbtA are the most appropriate initial candidates because of simplified plant transformation requirements. Interestingly, the modelling indicates that installation of both transporters is an advantage as the kinetic properties can be blended to give a better outcome; namely BicA for a low-affinity, high-flux rate, and SbtA for a high-affinity (low-flux rate) uptake. To achieve proof-of-concept, the expression of these transporters from the tobacco chloroplast genome is desirable, but extension to crop plants is likely to require host genome transformation techniques.

C₃ plants suffer from a standing CO₂ diffusion gradient, or drawdown, that exists between the substomatal cavity of the leaf (C_i) and the steady-state level of CO₂ in chloroplast stroma; the magnitude of this gradient is ~40% below C_i at high irradiance (Evans and von Caemmerer, 1996); see Fig. 6. The objective of phase 1 engineering is to diminish the size of this CO₂ drawdown but not necessarily elevate it significantly above C_i. Any attempt to raise the CO₂ level above C_i would probably result in wasteful CO₂ leakage because the chloroplast stroma contains high CA levels. However, this remains to be tested. Our modelling of phase 1 installation of BicA and/or SbtA transporters into the chloroplast inner envelope (Price *et al.*, 2011a) indicates that up to a 28% improvement in photosynthetic CO₂ fixation rates at a constant substomatal CO₂ level of 250 μbar is theoretically feasible (Figs 7a, 78). This modelling is based on previous approaches used to consider the theoretical addition of

a CO₂ pump of the single-cell C₄ type (von Caemmerer, 2003; von Caemmerer and Furbank, 2003). As such, the magnitude of improvements depends on the kinetic properties of the transporters and the conductance of the chloroplast envelope to CO₂ diffusion (von Caemmerer, 2003). The modelling indicated that addition of the high-affinity SbtA transporter is more effective at reducing the compensation point (the point where net assimilation is zero) than addition of the BicA transporter because of its lower K_m. Introduction of both transporters can be even more effective (Figs 7a, 8), particularly at higher C_i levels, where CO₂ drawdown is effectively reduced enabling an indicative assimilation rate >28% higher than in wild-type C₃ plants (at a C_i of 200–250 μbar; Fig. 8). It is also notable that addition of one or both pumps can reduce the CO₂ compensation point by 11–26 μbar (Fig. 9), providing scope for net CO₂ fixation at very low C_i levels (closed stomata). Fortunately, measurement of CO₂ compensation points by gas exchange analysis provides a robust means of verifying successful incorporation of HCO₃⁻ pumps.

While not specifically modelled, it was noted that enhancing leaf photosynthesis also has the potential to improve leaf water-use efficiency (WUE) depending on the way stomata respond, or can be engineered to respond. The problem relates to the fact that in an effort to gain CO₂ into the leaf, a typical C₃ stomata loses ~250–500 molecules of water to the atmosphere for every CO₂ fixed. The modelling shows that added HCO₃⁻ transporters provide the largest benefit at low C_i values, thus the stomata of a plant with HCO₃⁻ pump enhancement could afford to operate

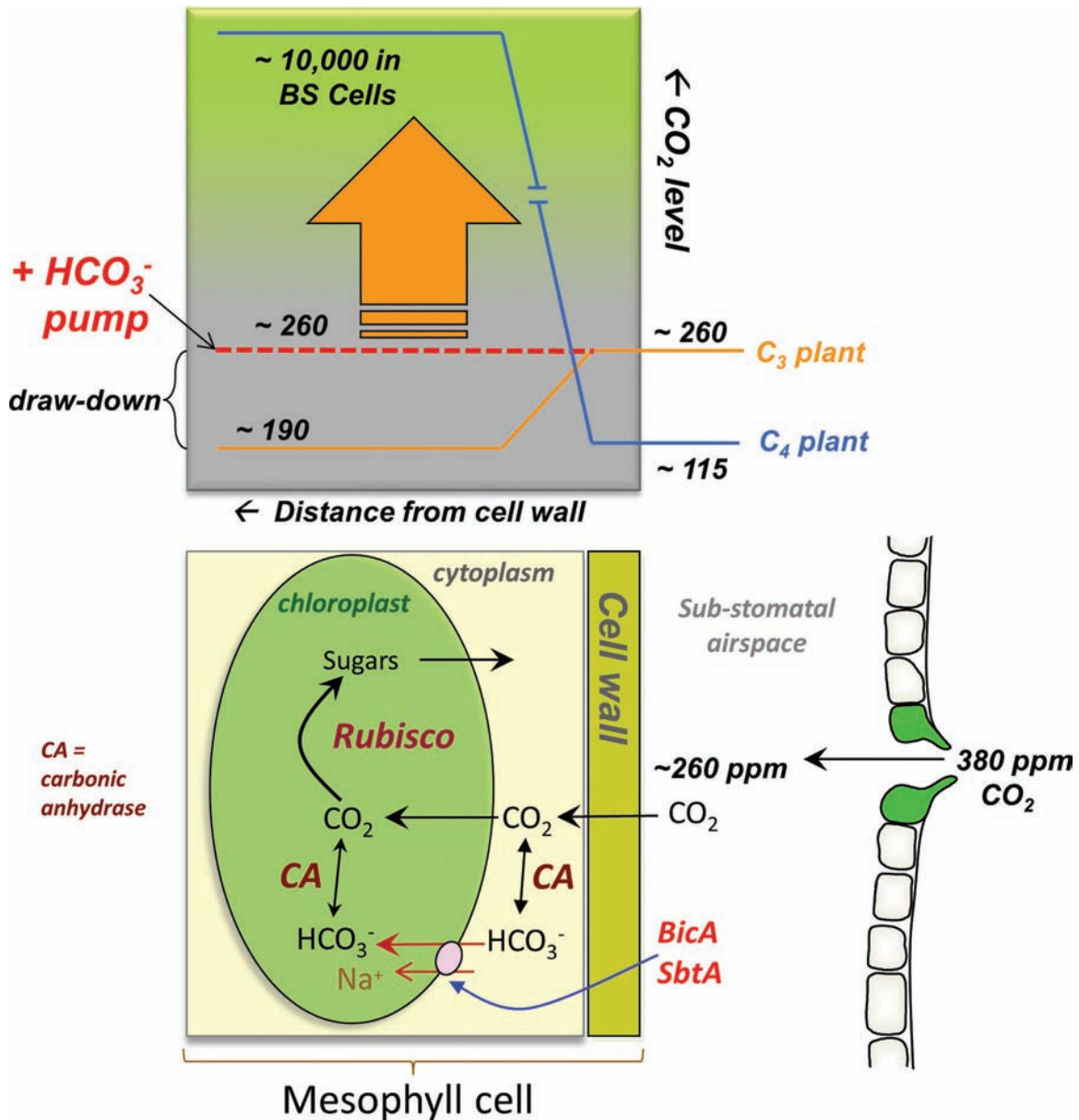


Fig. 6. Schematic showing the existence of a downhill diffusion gradient for CO_2 (drawdown) from the ambient air (380 ppm CO_2) to the chloroplast stroma (typically ~ 190 ppm CO_2). The CO_2 gradient from the substomatal cavity (C_i ; ~ 260 ppm CO_2) to the chloroplast stroma is the first target of phase 1a addition of cyanobacterial bicarbonate transporters. Later stage engineering would aim to raise the CO_2 concentration around Rubisco (carboxysome located) to levels typically encountered in the chloroplasts of C_4 bundles sheath (BS) cells.

at a smaller stomatal aperture whilst providing the same rate of assimilation, thereby resulting in less loss of water from the leaf. The SbtA transporter could be capable of improving WUE under dry air conditions more effectively than BicA, and addition of both transporters has additive benefits.

Another notable prediction of the modelling indicates the potential for phase 1a to cost less energy than normal C_3 photosynthesis at low C_i levels where net CO_2 fixation is highly influenced by photorespiration. It was estimated that transport might require a $\frac{1}{4}$ ATP per HCO_3^- transported by BicA and $\frac{1}{2}$ ATP per HCO_3^- for SbtA. Combined with the offset to photorespiration, it was estimated that introduction of HCO_3^- transporters reduces the ATP cost at low C_i below that normally experienced

during C_3 photosynthesis, and increases marginally above the C_3 requirement at higher C_i (Price et al., 2011a).

Some milestones for establishing progress on phase 1A

Table 1 details some of the milestones needed to prove that SbtA and/or BicA have been successfully integrated into the chloroplast envelope, thereby functioning in raising the stromal DIC level and resulting in a concomitant increase in CO_2 around Rubisco.

Expression of cyanobacterial bicarbonate transporters, BicA and SbtA, in tobacco chloroplasts through chloroplast genome

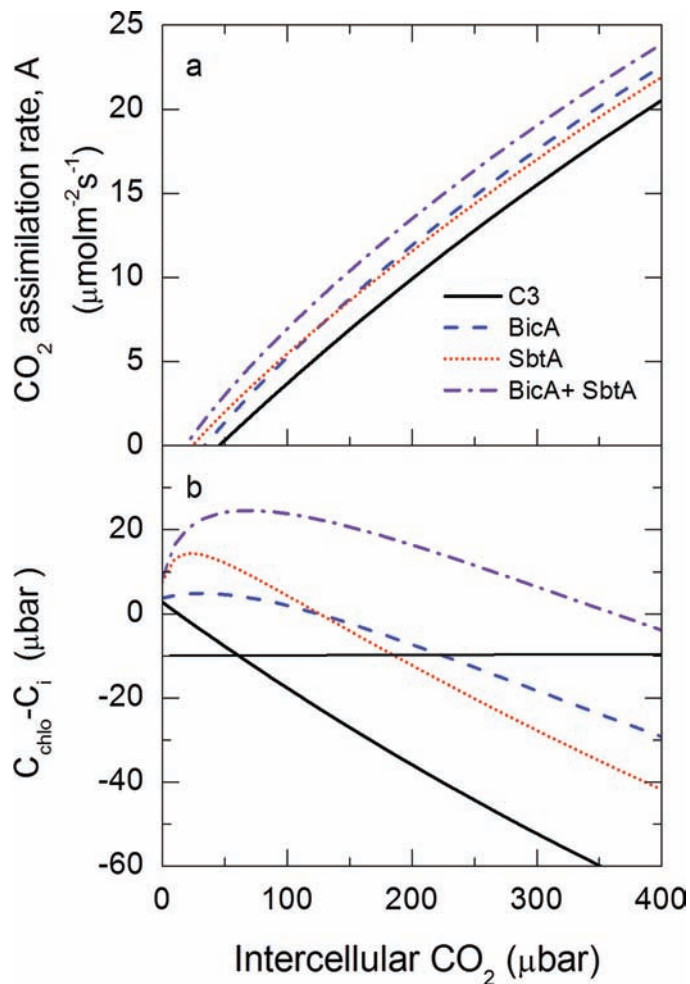


Fig. 7. Modelled data, replotted from Price *et al.* (2011a), showing the theoretical increase in the rate of CO₂ assimilation (a) and the reduction in the magnitude of the substomatal to stromal drawdown (b; C_i minus C_{chlo}) for plants expressing BicA or SbtA, or both. For details of the model and parameters used see Price GD, Badger MR, von Caemmerer S. 2011. The prospect of using cyanobacterial bicarbonate transporters to improve leaf photosynthesis in C₃ crop plants. *Plant Physiology* **155**, 20–26. www.plantphysiol.org 'Copyright American Society of Plant Biologists.'

transformation is likely to be more successful than their being targeted from the host genome; however, this needs to be established by thorough experimentation. Until such time that chloroplast genome transformation becomes available for cereal crops, attention will need to centre on the best ways to achieve expression of SbtA and BicA in a model C₃ plant by host genome expression. The general approach would be based on using known envelope-located proteins from, for example, *Arabidopsis* (AT_CHLORO; Ferro *et al.*, 2010) as gene fusion partners for *sbtA* and *bicA*. Topology mapping has shown that SbtA and BicA orientation in the membrane is different (Shelden *et al.*, 2010; Price *et al.*, 2011b), with BicA having both the C- and N-termini inside the cell, while SbtA has both termini outside the cell.

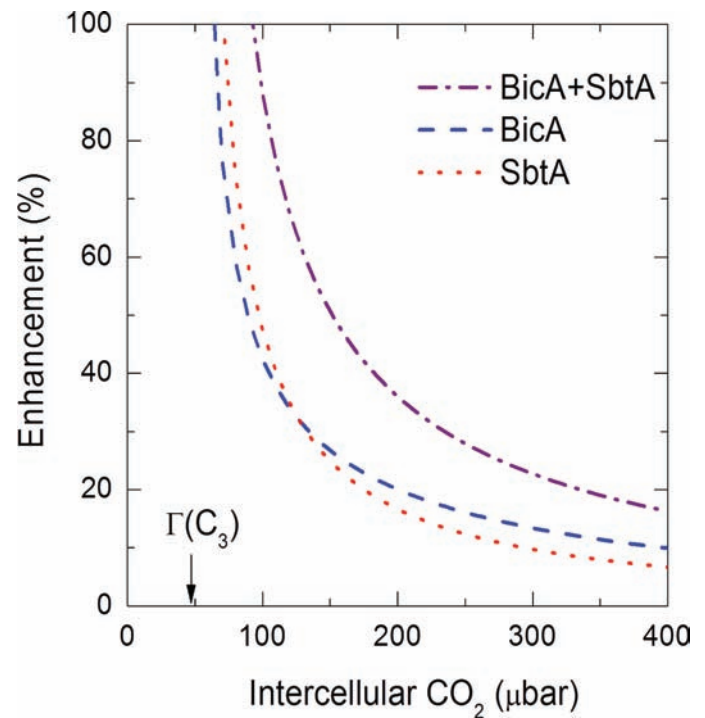


Fig. 8. The modelled percentage improvement in assimilation rate due to addition of bicarbonate transporters as relative to the base C₃ rate (assuming constant C_i values for each scenario). Data calculated from the data in Fig 7a. Γ refers to the position of the CO₂ compensation point for C₃.

Constructs for expression of these two transporters would obviously need to take these differences into account.

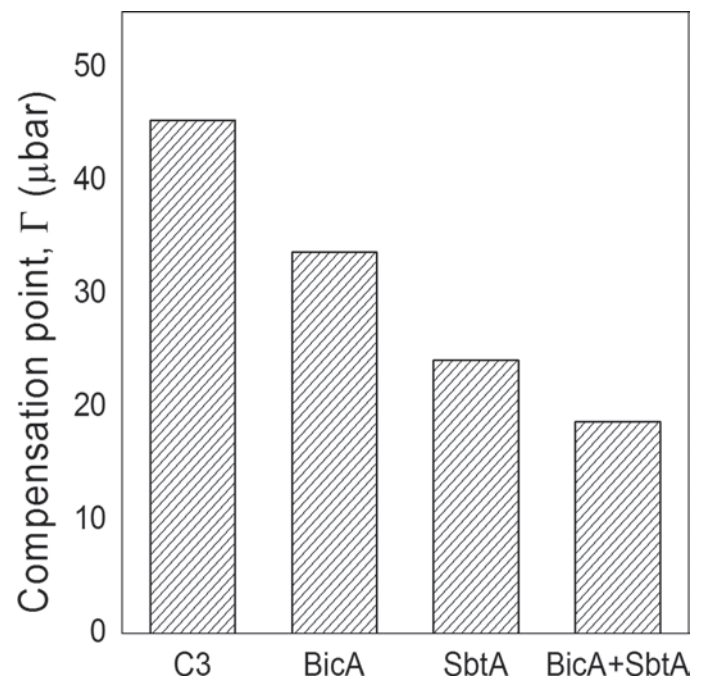


Fig. 9. The modelled reduction in the leaf CO₂ compensation point (Γ) due to addition of bicarbonate transporters, as compared with the base C₃ rate. Data calculated from the data in Fig 7a.

Table 1. A checklist of evidence required to achieve proof-of-concept for phase 1a (active expression of BicA and/or SbtA)

1. **Gene selection:** expression targets must be proven HCO₃⁻ transporters (or Na⁺/H⁺ antiporters) and the functional transporter should be a single-gene product. BicA and SbtA fit this criterion, IctB does not.
2. **Construct design:** expression constructs require proven delivery attributes such as the type of chloroplast transit peptide, and potentially fusion with the first one or two membrane-spanning domains of a known chloroplast inner envelope protein. It is highly advantageous to know the in-membrane topology of each transporter.
3. **Transgene expression:** verification that transformed plants produce transgene mRNA (PCR-based or Southern) and that transgenes are stably maintained in the progeny.
4. **Whole-membrane-located expression:** isolation of whole-membrane fractions from the leaves and verification that the HCO₃⁻ transporter is expressed and folded correctly (using immunodetection). Assess multiple transgenic lines for best expression lines.
5. **Chloroplast located:** isolate intact chloroplasts and verify that the HCO₃⁻ transporter is present in the whole-membrane-enriched fraction (using immunodetection).
6. **Envelope located:** fractionate chloroplast membranes and verify that the HCO₃⁻ transporter is located in the inner chloroplast envelope versus thylakoid membranes (using immunodetection and use of envelope- and thylakoid-specific antibodies).
7. **Transporter activity:** verify that each HCO₃⁻ transporter is correctly activated and capable of taking up HCO₃⁻ in isolated chloroplasts under illumination (silicone oil centrifugation filtration).
8. **Reduced compensation point:** verify that the leaf CO₂ compensation point is reduced by a statistically significant amount (leaf gas exchange measurements over a range of O₂ levels).
9. **Photosynthetic enhancement:** verify that the leaf photosynthesis rates are elevated by a statistically significant amount particularly in the 200 μbar stomatal subcavity range.
10. **Changed isotopic signature:** verify that the leaf dry matter and on-line ¹³C discrimination is consistent with a minor level of active C_i elevation in the chloroplast (reduced discrimination).
11. **Growth/yield enhancement:** verify that elevated leaf photosynthesis rates translate into enhanced growth rate and/or enhanced water-use efficiency (WUE) for vegetative matter and grain yield.
12. **Field growth enhancement:** verify that elevated leaf photosynthesis rates translate into enhanced growth rate and WUE in field trials. Must be a stable trait and stably inherited.

Factors that could affect expression and activity of bicarbonate transporters in the chloroplast

Substrates and energization of bicarbonate pumps

As discussed previously (Price *et al.*, 2011b), HCO₃⁻ levels in the cytoplasm of a typical C₃ cell appear sufficient to supply both types of HCO₃⁻ transporters with substrate at levels above the affinity of each transporter. It is estimated that at least 250 μM HCO₃⁻ is present in the cytosol of a leaf cell in ambient air (Evans and von Caemmerer, 1996), which is well above the substrate affinities ($K_{0.5}$ HCO₃⁻) of SbtA (low flux rate) and BicA (high flux rate) for HCO₃⁻, namely 5–15 μM and 90–170 μM, respectively (Shibata *et al.*, 2002; Price *et al.*, 2004).

Likewise there are good prospects that chloroplasts possess and maintain an inwardly directed Na⁺ gradient sufficient to energize both transporters. Both SbtA and BicA require ~1 mM Na⁺ for half-maximal activity in the form of an inwardly directed Na⁺ gradient (Shibata *et al.*, 2002; Price *et al.*, 2004), whereas the leaf cytosol possesses 1–3 mM Na⁺ (Karley *et al.*, 2000). In addition, proteomic analyses have revealed that the *Arabidopsis* chloroplast envelope possesses several potential Na⁺-coupled transporters and Na⁺/H⁺ antiporters that are homologous to cyanobacterial forms (Rolland *et al.*, 2003). Indeed, it was recently discovered by transcriptomic comparison of C₄ and C₃ *Cleome* species (Brautigam *et al.*, 2011) that C₄ *Cleome gynandra* has an Na⁺/H⁺ antiporter gene (AT1G49810) that is highly up-regulated along with an Na⁺/pyruvate transporter (Furumoto *et al.*, 2011). The AT1G49810 gene would be an ideal candidate

for overexpression in *Arabidopsis* plants expressing BicA and SbtA with the objective of enhancing the regeneration of the Na⁺ gradient across the envelope. The NhaS3 Na⁺/H⁺ antiporter that is up-regulated as part of the cyanobacterial BicA operon in response to DIC limitation (Woodger *et al.*, 2007) is also a valid candidate. Any perturbations in pH regulation, as a result of the addition of BicA/SbtA with enhanced Na⁺/H⁺ antiporter activity, are likely to be minor, since elevating steady-state HCO₃⁻ levels by up to 15% in the chloroplast, or even by as much as 25-fold relative to air-exposed leaves, has been shown to have a minimal effect on plastid pH owing to high stromal buffering capacity (Wagner *et al.*, 1990).

Bicarbonate transporter activation/inactivation

DIC uptake by a cyanobacterium represents the largest nutrient flux encountered by a cyanobacterial cell in the light, and as such is likely to have the largest effect on maintenance of pH and membrane potential charge. It is perhaps not surprising that cyanobacterial DIC transporters are inactive in darkness, presumably to prevent any waste of metabolic energy due to futile cycling. Following illumination, CO₂ uptake is fully activated within a few seconds, whereas full activation of HCO₃⁻ uptake takes up to 90 s; both systems are inactivated by darkness in <15 s (B. Forster and G.D. Price, unpublished). We do not know precisely how activation/inactivation of HCO₃⁻ transporters occurs, but some lines of evidence indicate that post-translational or allosteric modifications are involved, possibly involving activation due to a protein kinase-mediated phosphorylation of transporters and deactivation by a specific protein phosphatase. Phosphorylation

via protein phosphokinases of the Ser/Thr class has already been implicated in the rapid induction of latent HCO_3^- uptake capacity in *Synechococcus* PCC7942 and PCC7002 species subjected to severe DIC limitation (Sültemeyer *et al.*, 1998).

There are about eight protein phosphatases and five protein kinases of this broad Ser/Thr class that have not been well characterized in *Synechococcus* PCC7942, and certainly not in regard to activation/inactivation of HCO_3^- transporters. Any kinases or phosphatases involved would probably target conserved serine or threonine residues on putative regulatory domains on the cytoplasmic faces of SbtA and BicA. Identification of these cytoplasmic domains was a main reason for determining the membrane topology structure of BicA and SbtA transporters (Shelden *et al.*, 2010; Price *et al.*, 2011b). Research aimed at testing conserved serine or threonine residues as possible sites for activation of DIC transporters by phosphorylation is ongoing (B. Forster and G.D. Price, unpublished). Any phosphorylated serine or threonine (or tyrosine) residues that might be involved in the activation process can be mimicked by conversion to a glutamate residue. Such modified BicA and SbtA transporters would be useful for proof-of-concept work in tobacco, but long term, the genes involved in activation/inactivation would need to be added to the chloroplasts of crop species to stop futile uptake in the dark.

The enigma of IctB

There has been a considerable amount of confusion concerning IctB and its perceived role in crop improvement. Originally the gene product was reported as a likely HCO_3^- transporter, based on physiological data where no defined knockout was obtained and where high CO_2 performance of the mutant showed only a partial recovery to wild-type levels (Bonfil *et al.*, 1998). The original mutant in *Synechococcus* PCC7942 arose due to a single-sided genomic insertion that duplicated *ictB* and possibly removed a small part of the C-terminal end of IctB. The IctB protein is certainly a membrane protein based on its strong hydrophathy profile and presence of 12 predicted membrane-spanning domains. It is also a highly conserved protein present in every cyanobacterial genome so far sequenced, which is a property typical of core essential genes. Of the five known DIC uptake systems in cyanobacteria, all have been deleted and fully segregated as single and multiples deletions, so none is considered a core gene that is essential for function. So far, no segregated defined gene knockout of *ictB* has been achieved in cyanobacteria in two species by three separate labs.

From subsequent work, based on analysis of a $\Delta 4$ knockout of four known DIC uptake systems in *Synechocystis* PCC6803, it was concluded that IctB could not possibly be a bicarbonate transporter (Shibata *et al.*, 2002). This work overlooked the constitutively expressed *bicA* gene in this species because it was not known then (Price *et al.*, 2004). However, a *bicA* knockout combined with the $\Delta 4$ knockout (giving rise to the $\Delta 5$ knockout) has since been analysed as a high CO_2 -requiring phenotype and a physiology featuring no residual CO_2 or HCO_3^- uptake (Xu *et al.*, 2008), confirming that IctB is not a HCO_3^- transporter.

Despite a lack of support for IctB being a HCO_3^- transporter, transgenic plants overexpressing *ictB* produced an intriguing

5–9 ppm reduction in the CO_2 compensation point in tobacco and *Arabidopsis* and a higher relative growth rate under 30% humidity than wild-type *Arabidopsis* (Lieman-Hurwitz *et al.*, 2003). While the data could be interpreted as IctB being a HCO_3^- transporter in plants, other explanations should be explored. For instance, one possibility is that overexpression of a strongly hydrophobic membrane protein (such as IctB) causes a build-up of unfolded membrane protein in the endoplasmic reticulum (ER), producing a classic ER shock response. This would induce membrane-bound ER stress sensor/transducers (AtbZIP60 and AtbZIP28 leucine zipper transcription factors in *Arabidopsis*) that are activated by ER intramembrane proteolysis systems leading to expression of the unfolded protein response (UPR) genes, which in turn show cross-talk with pathogen and heat stress responses (Urade, 2009). Could overexpression of *ictB* lead to the up-regulation of drought resistance transcription factors such as DREB as part of the UPR (Agarwal *et al.*, 2006), thus giving resistance to water stress (Pellegrineschi *et al.*, 2004) and potentially changes in CO_2 transfer resistance in the plant leaf? If so, overexpression of any strong membrane protein from a strong promoter could lead to a build-up of unfolded membrane protein in the ER, leading to induction of UPR. Certainly this, and other possibilities, need further attention.

Conclusion

The cyanobacterial CCM is very effective at raising the CO_2 levels around Rubisco and, given the similarities between the cyanobacteria cell and the chloroplast, the prospects for achieving the phase 1 goals of integrating bicarbonate transporters and functional carboxysomes are promising. The modelled benefits of achieving phase 1a engineering, of adding bicarbonate transporters into crop plant chloroplasts, are quite significant. However, as with other engineering approaches for crop improvement, the ability to bring about success in the required time frame will require a concerted effort, and the resources to support those efforts.

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