REVIEW PAPER

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

G. Dean Price*, Jasper J.L. Pengelly, Britta Forster, Jiahui Du, Spencer M. Whitney, Susanne von Caemmerer, Murray R. Badger, Susan M. Howitt and John R. Evans

Molecular Plant Physiology Cluster, Research School of Biology, Australian National University, Canberra, Australian Capital Territory 0200, Australia

* To whom correspondence should be addressed. E-mail: dean.price@anu.edu.au

Received 5 July 2012; Revised 20 August 2012; Accepted 22 August 2012

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
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 suppressing chloroplasts against the intracellular spaces 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 photosynthate.
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

Transferring 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 fixation. 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⁺/HCO₃⁻ symporter with a relatively low flux rate. BicA, a low-affinity, high-flux, Na⁺/HCO₃⁻ symporter, 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 (unipporter), 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
drive conversion of CO$_2$ to HCO$_3^-$ 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 CO$_2$ uptake. The consensus is that these NDH-I-type CO$_2$ uptake systems are located on the thylakoid membranes where they use CO$_2$ diffusing from outside the cell, or arising from leakage from the carboxysomes, as a substrate for directional conversion to HCO$_3^-$.

**NDH-I$_4$** is a constitutive CO$_2$ uptake system based on a specialized NADPH dehydrogenase (NDH-I) complex. The second CO$_2$ pump, NDH-I$_3$, is inducible under DIC limitation and is of higher uptake affinity than NDH-I$_4$.

**Diversity of the DIC transporters**

There are two broad CCM phylogenies in cyanobacteria, known as $\alpha$- or $\beta$-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). $\beta$-Cyanobacteria, prevalent in freshwater and coastal marine areas, utilize form-1b Rubisco and ccm- type carboxysomes, whereas $\alpha$-cyanobacteria utilize form-1a Rubisco and cso-type carboxysomes. The abundant, and therefore globally productive, $\alpha$-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, $\beta$-cyanobacteria have a greater diversity of DIC uptake systems, while $\alpha$-cyanobacteria have the fewest (Badger et al., 2006; Rae et al., 2011). However, $\alpha$-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 $\beta$-cyanobacteria (Rae et al., 2011).

Carboxysomal function

**Types and composition** Although $\beta$- and $\alpha$-carboxysomes appear have arisen by parallel evolution, each performs the same basic functional role of acting as a microcompartment where CO$_2$ can be elevated around Rubisco. Both types are obligate for cell survival at low and moderate CO$_2$ 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 CO$_2$-requiring (HCR) phenotypes. These mutants require in excess of 2% CO$_2$ 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 HCO$_3^-$ to CO$_2$ inside the polyhedral structures to support CO$_2$ fixation by Rubisco. Both carboxysome structures have potential application towards engineering a functional CCM in C$_3$ plants.

The carboxysome shell is composed largely of a number of related small proteins which have been termed bacterial micro-compartment proteins (CcmK family and CcmL in $\beta$-carboxysomes; CsoS1 and CsoS4 families in $\alpha$-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 $\beta$-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 $\gamma$-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 $\alpha$-carboxysomes, the shell protein CsoS3 (CsoSCA) is a functional CA with a $\beta$-CA-like protein fold, although lacking sequence homology with $\beta$ forms (So et al., 2004; Sawaya et al., 2006). In a typical $\beta$-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 $\alpha$-carboxysome, such as that from the sulphur bacterium *Halothiobacillus neapolitanus* C2, differs somewhat from that of the $\beta$-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 $\beta$-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 $\alpha$-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 (*cbbLS*) 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...
Phase 1a

The initial engineering approach would aim to transfer single-gene HCO$_3^-$ 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 CO$_2$ 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 CO$_2$ 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 CO$_2$ 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 HCO$_3^-$ levels (Fig. 3b). Calculations indicate that between 0.48 mM and 0.76 mM HCO$_3^-$ (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 C$_3$ CO$_2$ fixation rate with carboxysomal CO$_2$ 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 CO$_2$ fixation. The real impact of this advance would be seen in phases 3 and 4.

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 HCO$_3^-$ 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 CO$_2$ uptake NDH-1 complex in phase 4 would reduce CO$_2$ leakage.
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 transgensics 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
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-1 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-1 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 PxC (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
be best achieved by expressing one or two cyanobacterial HCO$_3^-$ transporters on the inner envelope of the C$_3$ chloroplast (Fig. 3a). Single-subunit HCO$_3^-$ 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.

C$_3$ plants suffer from a standing CO$_2$ diffusion gradient, or drawdown, that exists between the substomatal cavity of the leaf ($C_i$) and the steady-state level of CO$_2$ 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 CO$_2$ drawdown but not necessarily elevate it significantly above $C_i$. Any attempt to raise the CO$_2$ level above $C_i$ would probably result in wasteful CO$_2$ 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 CO$_2$ fixation rates at a constant substomatal CO$_2$ level of 250 µbar is theoretically feasible (Figs 7a, 7b). This modelling is based on previous approaches used to consider the theoretical addition of a CO$_2$ pump of the single-cell C$_4$ 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 CO$_2$ 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 CO$_2$ drawdown is effectively reduced enabling an indicative assimilation rate >28% higher than in wild-type C$_3$ 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 CO$_2$ compensation point by 11–26 µbar (Fig. 9), providing scope for net CO$_2$ fixation at very low $C_i$ levels (closed stomata). Fortunately, measurement of CO$_2$ compensation points by gas exchange analysis provides a robust means of verifying successful incorporation of HCO$_3^-$ 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 CO$_2$ into the leaf, a typical C$_3$ stomata loses ~250–500 molecules of water to the atmosphere for every CO$_2$ fixed. The modelling shows that added HCO$_3^-$ transporters provide the largest benefit at low $C_i$ values, thus the stomata of a plant with HCO$_3^-$ pump enhancement could afford to operate
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 C3 photosynthesis at low C\textsubscript{i} levels where net CO\textsubscript{2} fixation is highly influenced by photorespiration. It was estimated that transport might require a \(\frac{1}{4}\) ATP per HCO\textsubscript{3}\textsuperscript{-} transported by BicA and \(\frac{1}{2}\) ATP per HCO\textsubscript{3}\textsuperscript{-} for SbtA. Combined with the offset to photorespiration, it was estimated that introduction of HCO\textsubscript{3}\textsuperscript{-} transporters reduces the ATP cost at low C\textsubscript{i} below that normally experienced during C\textsubscript{3} photosynthesis, and increases marginally above the C\textsubscript{3} requirement at higher C\textsubscript{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\textsubscript{2} around Rubisco.

Expression of cyanobacterial bicarbonate transporters, BicA and SbtA, in tobacco chloroplasts through chloroplast genome...
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. Constructs for expression of these two transporters would obviously need to take these differences into account.

**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; Ci minus Cₘₚ) 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.’

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

**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.
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 (Kₘ₅ 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 hydropathy 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 Δ4 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 Δ4 knockout (giving rise to the Δ5 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 sensors/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.

**Acknowledgements**

The authors acknowledge financial assistance from Australian Research Council Discovery grants (GDP and MRB) and a SuperScience Fellowship (JP, SVC, GDP, and MRB). Some of the ideas presented in this review were developed with the assistance of an Australian Centre for International Agricultural Research (ACIAR) sponsored workshop held in Canberra, September 2009.

**References**


Friedberg D, Kaplan A, Ariel R, Kessel M, Seijffers J. 1989. The 5’-flanking region of the gene encoding the large subunit of ribulose-1,5-bisphoshate carboxylase/oxygenase is crucial for growth of the cyanobacterium *Synechococcus* sp. strain PCC7942 at the level of CO2 in air. *Journal of Bacteriology* 171, 6069–6076.


Long BM, Rae BD, Badger MR, Price GD. 2011. Over-expression of the beta-carboxysomal CcmM protein in *Synechococcus* PCC7942...
reveals a tight co-regulation of carboxysomal carbonic anhydrase (CcoA) and M58 content. Photosynthesis Research 109, 33–45.


Ludwig M, Bryant DA. 2011. Transcription profiling of the model cyanobacterium Synechococcus sp. strain PCC 7002 by NextGen (SOLID™) sequencing of cDNA. Frontiers in Microbiology 2, 41.

Ludwig M, Sulstemeier D, Price GD. 2000. Isolation of ccmKLMM genes from the marine cyanobacterium, Synechococcus sp PCC7002 (Cyanobacteria), and evidence that CcmM is essential for carboxysome assembly. Journal of Phycolgy 36, 1109–1118.


Price GD, Badger MR. 1989b. Isolation and characterization of high CO2-requiring-mutants of the cyanobacterium Synechococcus PCC7942. Two phenotypes that accumulate inorganic carbon but are apparently unable to generate CO₂ within the carboxysome. Plant Physiology 91, 514–525.


