Journal of Experimental Botany Advance Access published October 1, 2012

Journal of Experimental Botany
doi:10.1093/jxb/ers257

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

The cyanobacterial CCM as a source of genes for improving photosynthetic CO_2 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 CO_2-concentrating mechanism (CCM) present in cyanobacteria (blue-green algae) into the chloroplasts of key C_3 crop plants, particularly wheat and rice. Four progressive phases towards engineering components of the cyanobacterial CCM into C_3 species can be envisaged. The first phase (1a), and simplest, is to consider the transplantation of cyanobacterial bicarbonate transporters to C_3 chloroplasts, by host genomic expression and chloroplast targeting, to raise CO_2 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 C_3 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 C_4 photosynthesis. These later stages would include the addition of NDH-1-type CO_2 pumps and suppression of carbonic anhydrase and C_3 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, CO_2-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, CO_2 concentration in the substomatal cavity; DIC, dissolved inorganic carbon (chiefly CO_2 and HCO_3^-); HCR, high CO_2-requiring mutant phenotype; WUE, water-use efficiency (kg of water to produce a kg of biomass or grain).

effort to improve the efficiency of photosynthetic CO_2 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 CO_2 fixation in the chloroplasts of crop plants by integration of components of the cyanobacterial CO_2-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 CO_2-fixing enzyme, known as Rubisco (ribulose bisphosphate carboxylase-oxygenase). As the name of the enzyme implies, Rubisco can fix either CO_2 or O_2, and since both substrates are of remarkably similar molecular shape the enzyme is posed with the problem of catalytic difficulty in discriminating between CO_2 and O_2 (Whitney *et al*., 2011). Although fixing CO_2 is the dominant productive outcome, the inadvertent fixing of O_2 leads to an energetically wasteful cycle known as photorespiration. Some crop improvement approaches are therefore targeting ways to increase the productivity of photosynthetic CO_2 fixation by boosting the steady-state CO_2 concentration around Rubisco in the chloroplasts (the site of CO_2 fixation), thereby reducing photorespiration (Hibberd *et al*., 2008; Parry *et al*., 2011). Using components of the cyanobacterial CCM to raise CO_2 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, 2011*a*).

In Earth's evolutionary history the wayward ability of Rubisco to fix O_2 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 CO_2 was many fold higher than present levels and O_2 levels were very low (Badger and Price, 2003; Whitney *et al*., 2011). Under these ancient conditions, Rubisco was CO_2 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 O_2 rose steadily until ~1.5 billion years ago where it rose quite dramatically, and CO_2 levels progressively fell to very low levels, creating conditions where atmospheric CO_2 became rate limiting for Rubisco, and fixation of O_2 as an alternative non-productive substrate became significant. In an evolutionary response to changed atmospheric conditions (approaching present-day levels of 0.04% CO_2 and 21% O_2), 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 CO_2 and an improved ability to discriminate against O_2 (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 CO_2 fixation, and burdening photosynthesis with a rate of photorespiration as high as 30% of the theoretical maximum rate of CO_2 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 CO_2 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 C_4 photosynthesis (Sage, 2004). Paradoxically, very few C_4 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 CO_2 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 CO_2 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 C_3 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 C_3 photosynthesis involves the physical diffusion resistance to CO_2 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 CO_2 passage result in a drawdown or deficit in the steady-state CO_2 concentration in the chloroplast during photosynthetic CO_2 fixation relative to that in the ambient air. While C_3 plants have adopted strategies to reduce the diffusive resistance to CO_2 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 CO_2 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 CO_2 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 C_3 photosynthesis is the need to invest heavily in Rubisco expression to achieve acceptable high rates of photosynthetic CO_2 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. C_3 plants also have to present relatively open stomatal pores to gain sufficient

CO_2 for photosynthesis, but this comes with a water loss penalty of nearly 500 molecules of water per CO_2 fixed, on average. The evolution of the types of CCMs in C_4 plants solved a number of these inefficiencies by actively elevating CO_2 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 C_4-type CCMs into important C_3 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 C_3 crop species could also achieve improvements in the efficiency of photosynthetic CO_2 fixation similar to that envisaged for the C_4 rice approach (Price *et al*., 2008, 2011*a*; 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 C_3 plants.

The features of the cyanobacterial CCM

Basics features of the CCM

Transporting inorganic carbon It is likely that the poor availability of CO_2 in aquatic systems, and the higher availability of HCO_3^-, are two factors that drove the evolution of the cyanobacterial CCM, especially during periods when atmospheric CO_2 levels became very low and O_2 was high. The evolution of active uptake systems for the major forms of dissolved inorganic carbon (DIC), namely CO_2 and HCO_3^-, was a key to survival under these conditions. However, the ability to transport CO_2 and HCO_3^- actively into the cell, where it is accumulated as a HCO_3^- pool, by itself, was not a sufficient advantage until a microcompartment, the carboxysome, had evolved where CO_2 levels could be elevated around Rubisco, to allow enhanced CO_2 fixation (Badger *et al*., 2002; Price *et al*., 2008). This final step defines the operation of a CCM, namely that CO_2 has to be elevated around Rubisco to optimize catalysis by operating closer to substrate CO_2 saturation.

Carboxysomes Rubisco in cyanobacteria is encapsulated in unique proteinaceous microcompartments known as carboxysomes which act as the site of CO_2 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 CO_2, sourced from accumulated cytosolic HCO_3^- 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 CO_2 leakage The key to the functional efficiency of any CCM revolves around the ability to minimize the loss of CO_2 from the CO_2 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, 2011*a*). First, the accumulation of the ionic form of DIC, bicarbonate, which is less membrane permeable than CO_2, acts to reduce leakage. Secondly, CA activity is absent from the cytosol and minimizes leakage due to wasteful conversion to CO_2 and subsequent diffusion back to the external medium. Thirdly, the carboxysome protein shell is proposed to have the special property of retarding CO_2 leakage relative to entry and exit of ionic forms. Finally, thylakoid-located CO_2 pumps play a key role in recycling CO_2 that leaks from the carboxysome back into the HCO_3^- 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 CO_2 pump, and often a basal level of HCO_3^- uptake capacity, even when grown at 1–2% CO_2. This uptake capacity is augmented by the induction (de-repression) of high affinity uptake systems for CO_2 and HCO_3^- 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 CO_2 and HCO_3^- 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 HCO_3^- transporters are single-gene systems: SbtA is an inducible, high-affinity Na^+-dependent HCO_3^- transporter (Shibata *et al*., 2002; Price *et al*., 2004) that apparently acts as an Na^+/HCO_3^- symporter with a relatively low flux rate. BicA, a low-affinity, high-flux, Na^+-dependent HCO_3^- 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^+/HCO_3^- symporter and is unrelated to SbtA. In addition, BCT1 is a high-affinity HCO_3^- 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 CO_2 pumps are specialized respiration-related NDH-1 complexes that are able to use NADPH as an electron donor to

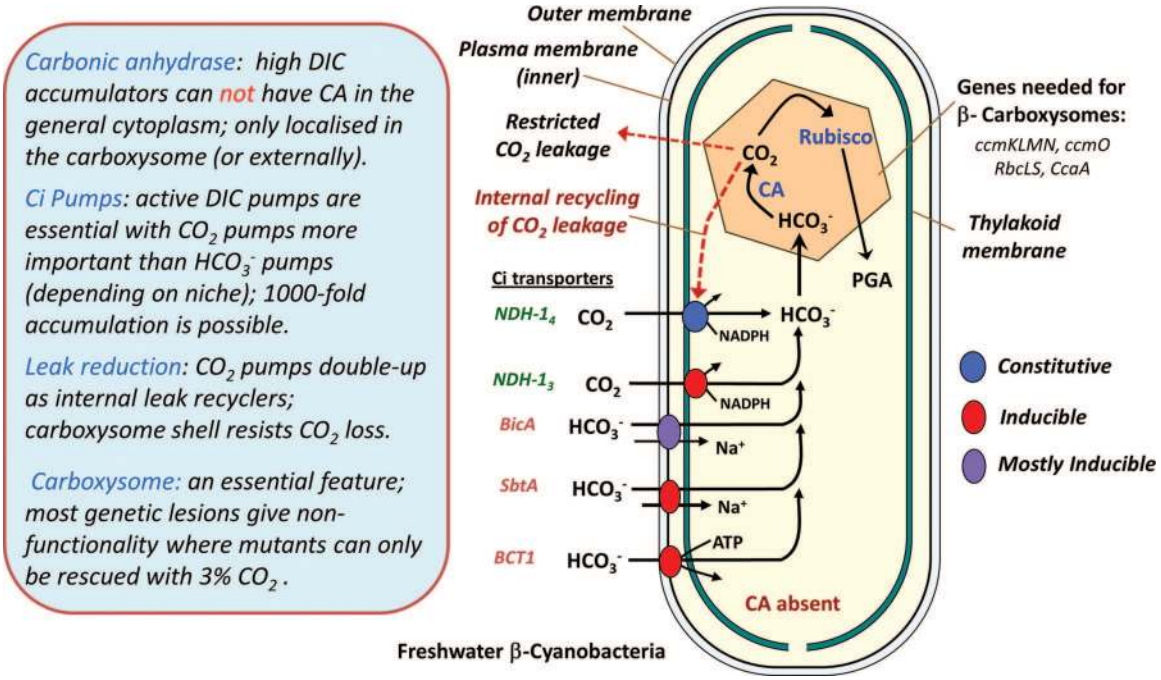

Fig. 1. Basic components of the cyanobacterial CCM of a stylized β-cyanobacterium.

drive conversion of 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 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 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, 2011*a*), 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, 1989*b*; 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 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 kDs 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 dicistonic *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 (*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, 1989*a*). 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

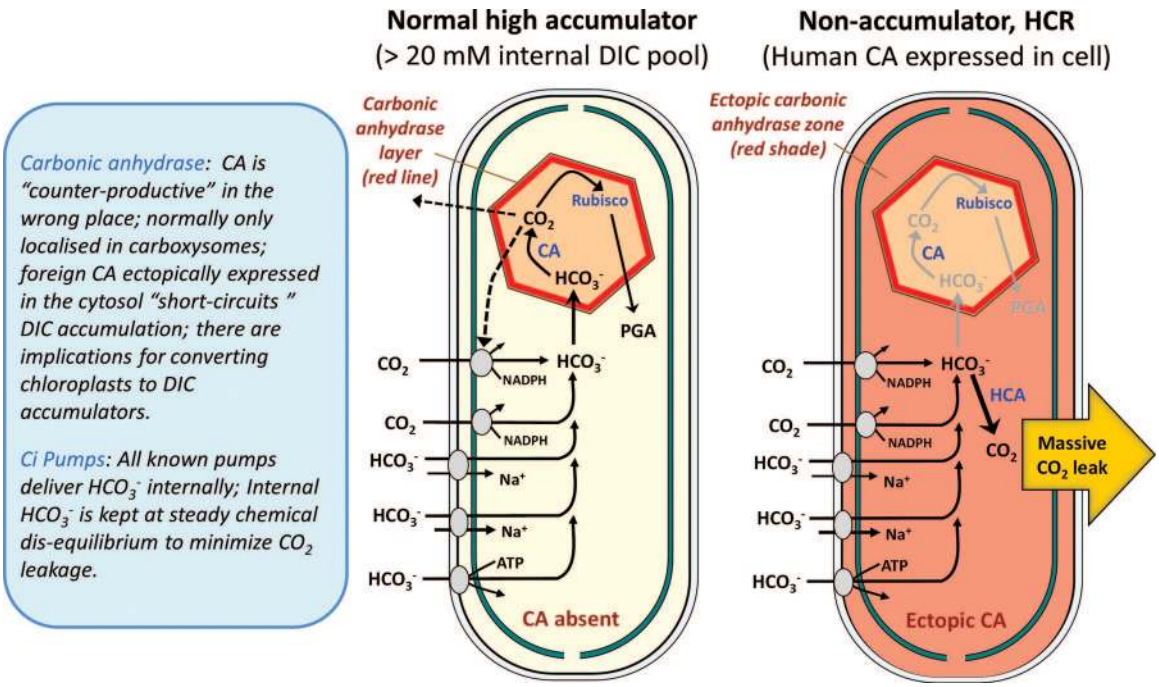

Fig. 2. Carbonic anhydrase (CA) zones in cyanobacteria and the role that CA location has in determining if cells are to be DIC accumulators or non-accumulators.

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 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.

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*., 2011*a*). 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, 1989*b*; 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, 1989*a*), 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.

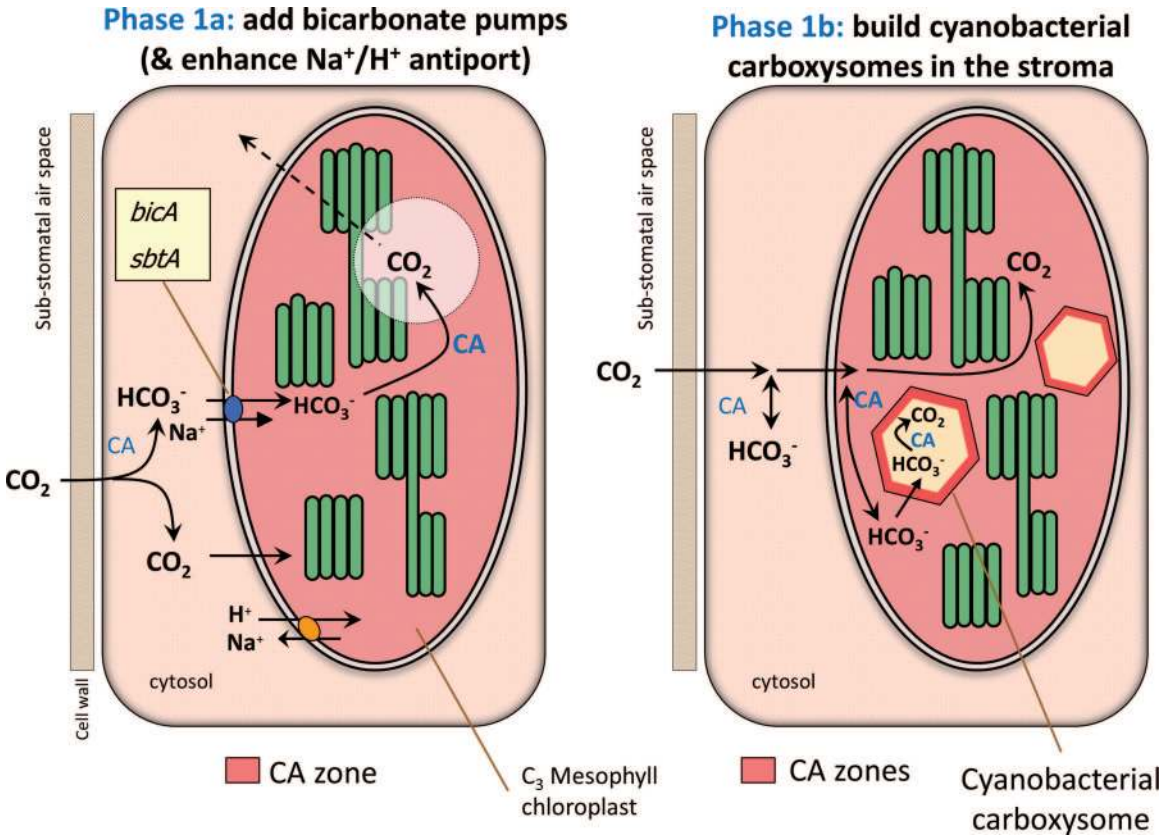

Fig. 3. Phase 1a and 1b engineering—addition of bicarbonate transporters and carboxysomes.

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 HCO_3^- 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 CO_2 fixation to contribute to net photosynthesis in the chloroplast. In addition, the proposed ability of the carboxysomes to limit O_2 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 HCO_3^- concentration should reach 2–3 mM, which would be more than half the concentration to saturate CO_2 fixation in the transferred carboxysomes. In addition, it would reduce CO_2 leakage from transported HCO_3^-. Gene knockouts of chloroplast CA would be the most effective

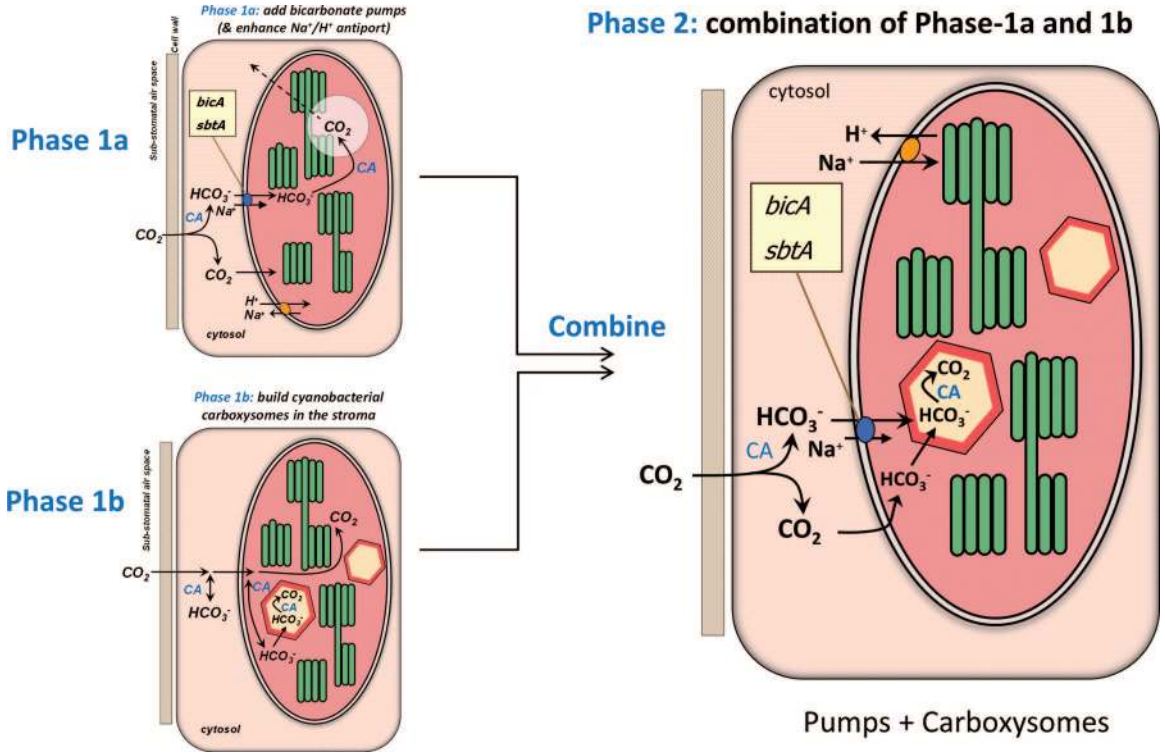

Fig. 4. Phase 2 engineering—combination of 1a and 1b.

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 C_3 Rubisco, either by targeted deletion of the chloroplast *rbcL* gene or by iRNA depression of the SSU gene family, so that CO_2 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 CO_2 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 CO_2 as another actively acquired DIC species and to reduce CO_2 leakage. Since the NDH-1_4 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 CO_2 pump-specific components (NdhF4, D4, and ChpX) and function correctly. For proof-of-concept, a full NDH-1_4 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 C_4 plants. It has been claimed that photorespiration plays a role in nitrogen assimilation in C_3 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 CO_2 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 HCO_3^- and CO_2 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 CO_2 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*., 2011*a*), the objective of reducing the CO_2 drawdown effect in C_3 chloroplasts appears to

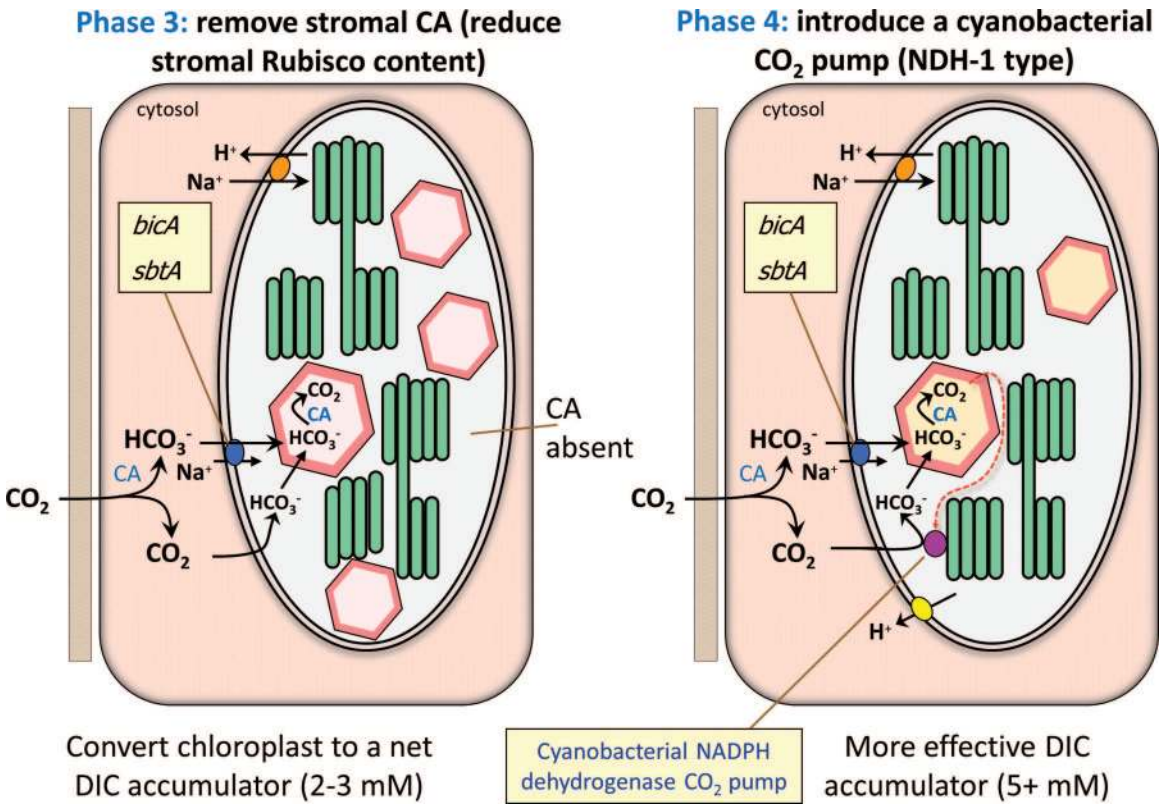

Fig. 5. Phase 3 and 4—progressive addition of CCM components to C_3 chloroplasts and suppression of endogenous CA and Rubisco levels in the chloroplast stroma.

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.*, 2011*a*) 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, 78). 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

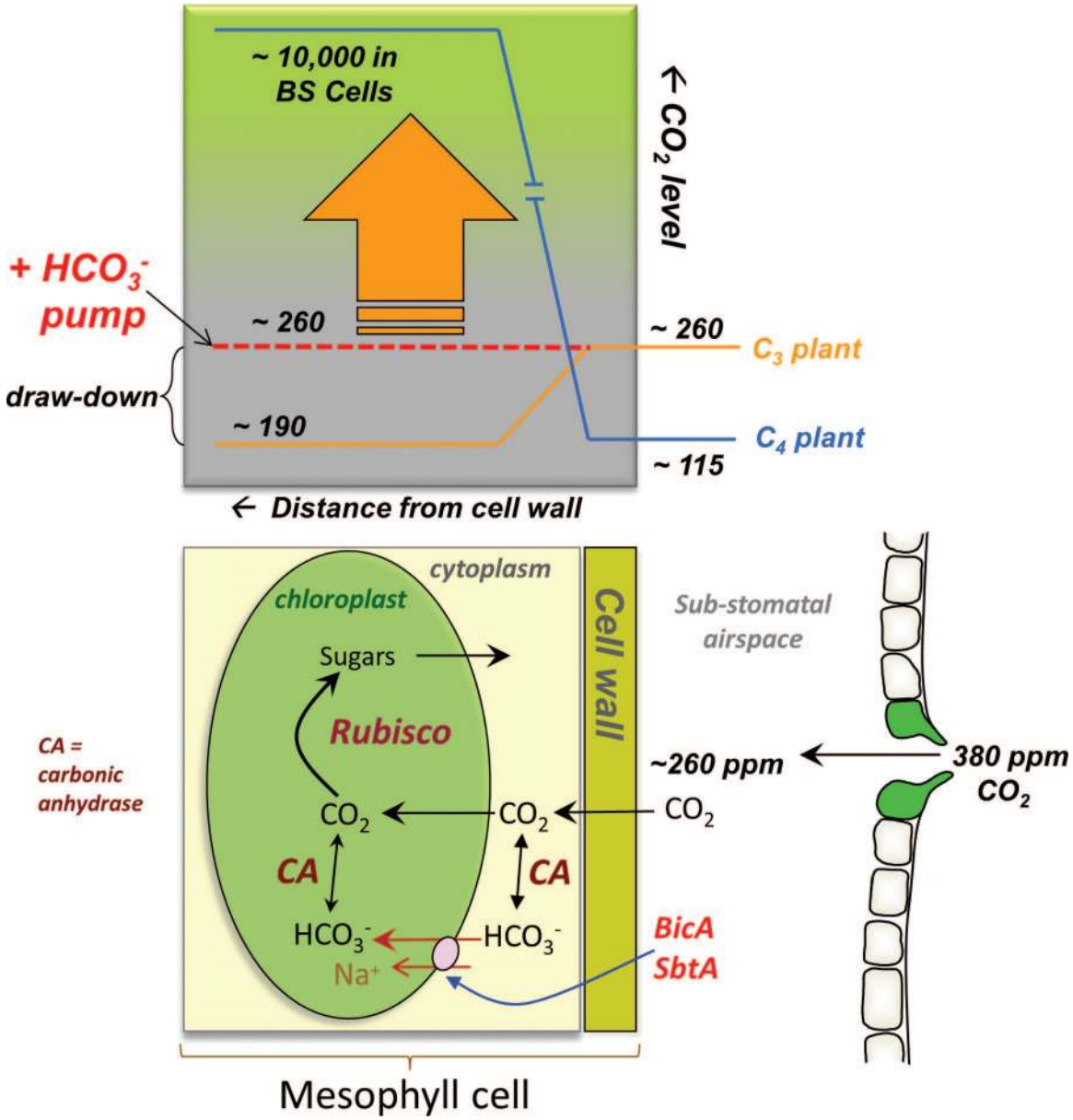

Fig. 6. Schematic showing the existence of a downhill diffusion gradient for CO_2 (drawdown) from the ambient air (380 ppm CO_2) to the chloroplast stroma (typically ~190 ppm CO_2). The CO_2 gradient from the substomatal cavity (C_i; ~260 ppm CO_2) to the chloroplast stroma is the first target of phase 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 ¼ ATP per HCO_3^- transported by BicA and ½ 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*., 2011*a*).

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

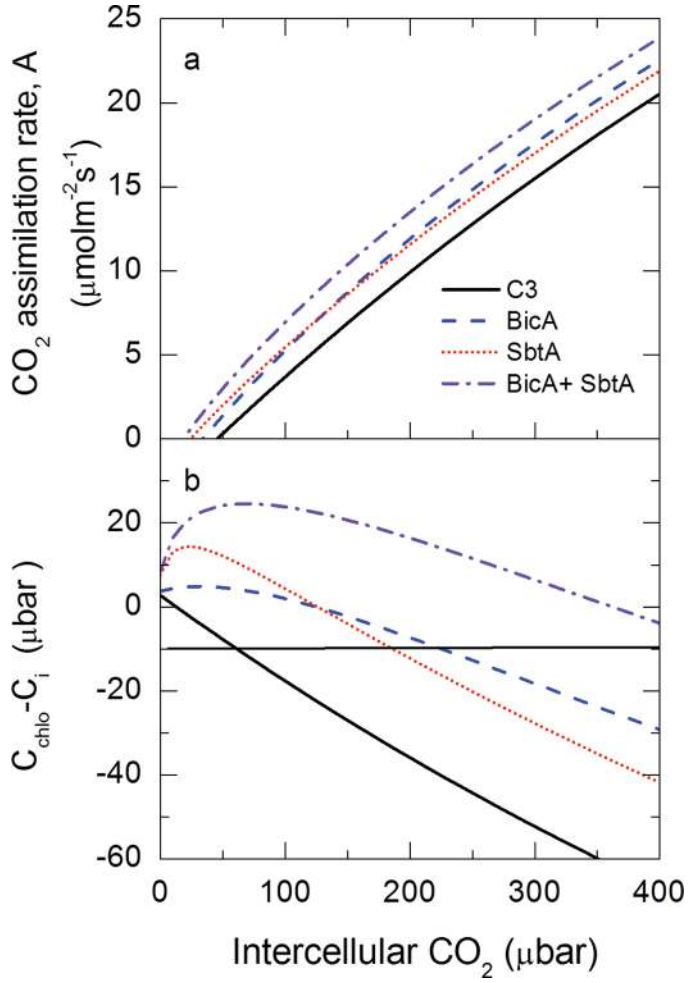

Fig. 7. Modelled data, replotted from Price *et al*. (2011*a*), showing the theoretical increase in the rate of CO_2 assimilation (a) and the reduction in the magnitude of the substomatal to stromal drawdown (b; C_i minus C_{chlo}) for plants expressing BicA or SbtA, or both. For details of the model and parameters used see Price GD, Badger MR, von Caemmerer S. 2011. The prospect of using cyanobacterial bicarbonate transporters to improve leaf photosynthesis in C_3 crop plants. *Plant Physiology* **155,** 20–26. www.plantphysiol.org 'Copyright American Society of Plant Biologists.'

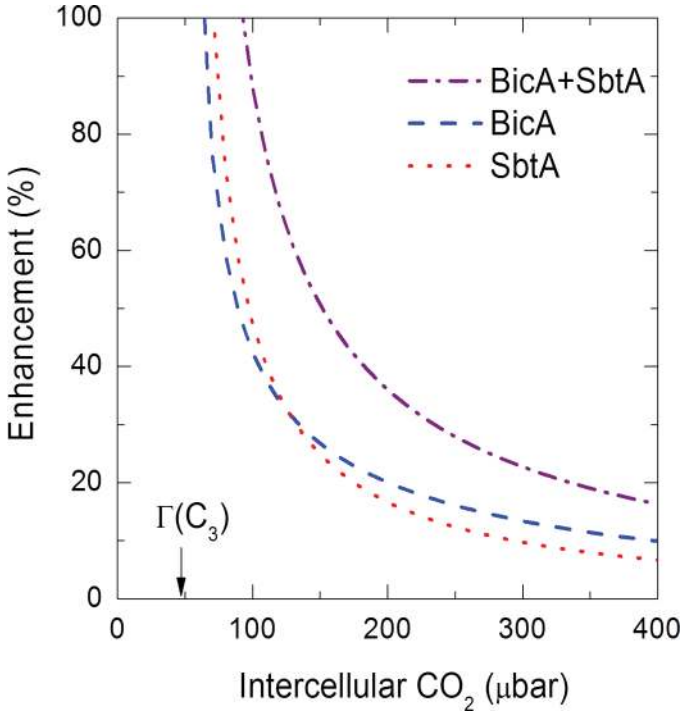

Fig. 8. The modelled percentage improvement in assimilation rate due to addition of bicarbonate transporters as relative to the base C_3 rate (assuming constant C_i values for each scenario). Data calculated from the data in Fig 7a. Γ refers to the position of the CO_2 compensation point for C_3.

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 C_3 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*., 2011*b*), 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.

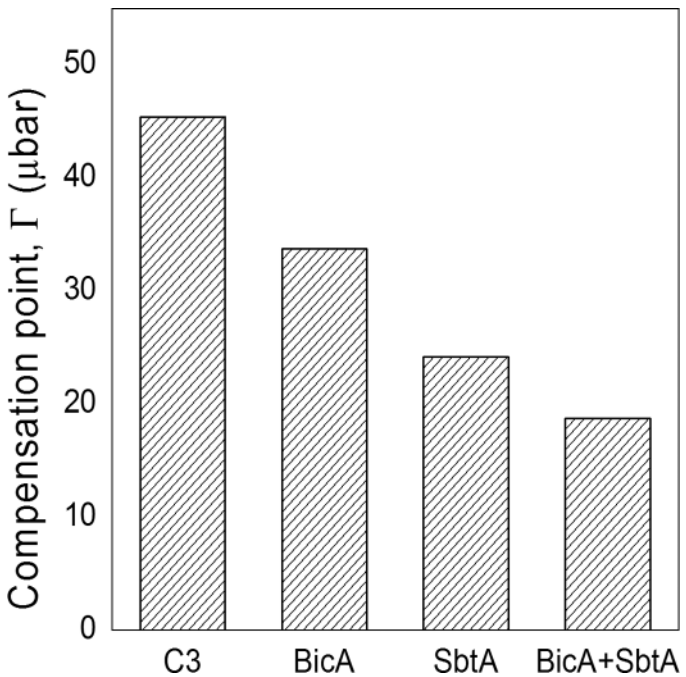

Fig. 9. The modelled reduction in the leaf CO_2 compensation point (Γ) due to addition of bicarbonate transporters, as compared with the base C_3 rate. Data calculated from the data in Fig 7a.

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 HCO_3^- 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 HCO_3^- 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 HCO_3^- transporter is present in the whole-membrane-enriched fraction (using immunodetection).
6. **Envelope located**: fractionate chloroplast membranes and verify that the HCO_3^- 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 HCO_3^- transporter is correctly activated and capable of taking up HCO_3^- in isolated chloroplasts under illumination (silicone oil centrifugation filtration).
8. **Reduced compensation point**: verify that the leaf CO_2 compensation point is reduced by a statistically significant amount (leaf gas exchange measurements over a range of O_2 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 ^{13}C 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*., 2011*b*), HCO_3^- levels in the cytoplasm of a typical C_3 cell appear sufficient to supply both types of HCO_3^- transporters with substrate at levels above the affinity of each transporter. It is estimated that at least 250 µM HCO_3^- is present in the cytosol of a leaf cell in ambient air (Evans and von Caemmerer, 1996), which is well above the substrate affinities ($K_{0.5}$ HCO_3^-) of SbtA (low flux rate) and BicA (high flux rate) for HCO_3^-, 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 C_4 and C_3 *Cleome* species (Brautigam *et al*., 2011) that C_4 *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 HCO_3^- 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, CO_2 uptake is fully activated within a few seconds, whereas full activation of HCO_3^- 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 HCO_3^- 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*., 2011*b*). 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 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.

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

Agarwal PK, Agarwal P, Reddy MK, Sopory SK. 2006. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Reports* **25,** 1263–1274.

Badger MR, Andrews TJ. 1982. Photosynthesis and inorganic carbon usage by the marine cyanobacterium, *Synechococcus* sp. *Plant Physiology* **70,** 517–523.

Badger MR, Andrews TJ, Whitney SM, Ludwig M, Yellowlees DC, Leggat W, Price GD. 1998. The diversity and coevolution of rubisco, plastids, pyrenoids, and chloroplast-based CO_2-concentrating mechanisms in algae. *Canadian Journal of Botany* **76,** 1052–1071.

Badger MR, Hanson D, Price GD. 2002. Evolution and diversity of CO_2 concentrating mechanisms in cyanobacteria. *Functional Plant Biology* **29,** 161–173.

Badger MR, Price GD. 1994. The role of carbonic anhydrase in photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **45,** 369–392.

Badger MR, Price GD. 2003. CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. *Journal of Experimental Botany* **54,** 609–622.

Badger MR, Price GD, Long BM, Woodger FJ. 2006. The environmental plasticity and ecological genomics of the cyanobacterial CO_2 concentrating mechanism. *Journal of Experimental Botany* **57,** 249–265.

Bloom AJ, Burger M, Asensio JSR, Cousins AB. 2010. Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. *Science* **328,** 899–903.

Bonacci W, Teng PK, Afonso B, Niederholtmeyer H, Grob P, Silver PA, Savage DF. 2012. Modularity of a carbon-fixing protein organelle. *Proceedings of the National Academy of Sciences, USA* **109,** 478–483.

Bonfil DJ, Ronentarazi M, Sultemeyer D, Liemanhurwitz J, Schatz D, Kaplan A. 1998. A putative HCO_3– transporter in the cyanobacterium *Synechococcus* sp. strain PCC7942. *FEBS Letters* **430,** 236–240.

Brautigam A, Kajala K, Wullenweber J, *et al.* 2011. An mRNA blueprint for C_4 photosynthesis derived from comparative transcriptomics of closely related C_3 and C_4 species. *Plant Physiology* **155,** 142–156.

Cannon GC, Baker SH, Soyer F, Johnson DR, Bradburne CE, Mehlman JL, Davies PS, Jiang QL, Heinhorst S, Shively JM. 2003. Organization of carboxysome genes in the thiobacilli. *Current Microbiology* **46,** 115–119.

Cot SSW, So AKC, Espie GS. 2008. A multiprotein bicarbonate dehydration complex essential to carboxysome function in cyanobacteria. *Journal of Bacteriology* **190,** 936–945.

Espie GS, Kimber MS. 2011. Carboxysomes: cyanobacterial RubisCO comes in small packages. *Photosynthesis Research* **109,** 7–20.

Evans JR. 1989. Photosynthesis and nitrogen relationships in leaves of C_3 plants. *Oecologia* **78,** 9–19.

Evans JR, Kaldenhoff R, Genty B, Terashima I. 2009. Resistances along the CO_2 diffusion pathway inside leaves. *Journal of Experimental Botany* **60,** 2235–2248.

Evans JR, von Caemmerer S. 1996. Carbon dioxide diffusion inside leaves. *Plant Physiology* **110,** 339–346.

Ferro M, Brugiere S, Salvi D, *et al.* 2010. AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. *Molecular and Cellular Proteomics* **9,** 1063–1084.

Field CB, Behrenfeld MJ, Randerson JT, Falkowski P. 1998. Primary production of the biosphere—integrating terrestrial and oceanic components. *Science* **281,** 237–240.

Flexas J, Ribas-Carbo M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenhoff R. 2006. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 *in vivo*. *The Plant Journal* **48,** 427–439.

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 CO_2 in air. *Journal of Bacteriology* **171,** 6069–6076.

Fukuzawa H, Suzuki E, Komukai Y, Miyachi S. 1992. A gene homologous to chloroplast carbonic anhydrase (*icfA*) is essential to photosynthetic carbon dioxide fixation by *Synechococcus* PCC7942. *Proceedings of the National Academy of Sciences, USA* **89,** 4437–4441.

Furumoto T, Yamaguchi T, Ohshima-Ichie Y, *et al.* 2011. A plastidial sodium-dependent pyruvate transporter. *Nature* **476,** 472–475.

Hibberd JM, Sheehy JE, Langdale JA. 2008. Using C-4 photosynthesis to increase the yield of rice—rationale and feasibility. *Current Opinion in Plant Biology* **11,** 228–231.

Kanevski I, Maliga P, Rhoades DF, Gutteridge S. 1999. Plastome engineering of ribulose-1,5-bisphosphate carboxylase/oxygenase in tobacco to form a sunflower large subunit and tobacco small subunit hybrid. *Plant Physiology* **119,** 133–141.

Karley AJ, Leigh RA, Sanders D. 2000. Where do all the ions go? The cellular basis of differential ion accumulation in leaf cells. *Trends in Plant Science* **5,** 465–470.

Kerfeld CA, Heinhorst S, Cannon GC. 2010. Bacterial microcompartments. *Annual Review of Microbiology* **64,** 391–408.

Kinney JN, Axen SD, Kerfeld CA. 2011. Comparative analysis of carboxysome shell proteins. *Photosynthesis Research* **109,** 21–32.

Kubien DS, Sage RF. 2008. The temperature response of photosynthesis in tobacco with reduced amounts of Rubisco. *Plant, Cell and Environment* **31,** 407–418.

Lieman-Hurwitz J, Rachmilevitch S, Mittler R, Marcus Y, Kaplan A. 2003. Enhanced photosynthesis and growth of transgenic plants that express *ictB*, a gene involved in HCO_3– accumulation in cyanobacteria. *Plant Biotechnology Journal* **1,** 43–50.

Liu HB, Landry MR, Vaulot D, Campbell L. 1999. *Prochlorococcus* growth rates in the central equatorial Pacific: an application of the f(max) approach. *Journal of Geophysics Research–Oceans* **104,** 3391–3399.

Liu HB, Nolla HA, Campbell L. 1997. *Prochlorococcus* growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean. *Aquatic Microbial Ecology* **12,** 39–47.

Long BM, Badger MR, Whitney SM, Price GD. 2007. Analysis of carboxysomes from Synechococcus PCC7942 reveals multiple Rubisco complexes with carboxysomal proteins CcmM and CcaA. *Journal of Biological Chemistry* **282,** 29323–29335.

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 (CcaA) and M58 content. *Photosynthesis Research* **109,** 33–45.

Long BM, Tucker L, Badger MR, Price GD. 2010. Functional cyanobacterial beta-carboxysomes have an absolute requirement for both long and short forms of the CcmM protein. *Plant Physiology* **153,** 285–293.

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, Sultemeyer D, Price GD. 2000. Isolation of *ccmKLMN* genes from the marine cyanobacterium, *Synechococcus* sp PCC7002 (Cyanobacteria), and evidence that CcmM is essential for carboxysome assembly. *Journal of Phycology* **36,** 1109–1118.

Maeda S, Badger MR, Price GD. 2002. Novel gene products associated with NdhD3/D4-containing NDH-1 complexes are involved in photosynthetic CO2 hydration in the cyanobacterium, *Synechococcus* sp. PCC7942. *Molecular Microbiology* **43,** 425–435.

Majeau N, Arnoldo MA, Coleman JR. 1994. Modification of carbonic anhydrase activity by antisense and over-expression constructs in transgenic tobacco. *Plant Molecular Biology* **25,** 377–385.

McGinn PJ, Price GD, Maleszka R, Badger MR. 2003. Inorganic carbon limitation and light control the expression of transcripts related to the CO_2-concentrating mechanism in the cyanobacterium *Synechocystis* sp strain PCC6803. *Plant Physiology* **132,** 218–229.

Mozes-Koch R, Gover O, Tanne E, Peretz Y, Maori E, Chernin L, Sela I. 2012. Expression of an entire bacterial operon in plants. *Plant Physiology* **158,** 1883–1892.

Nishimura T, Takahashi Y, Yamaguchi O, Suzuki H, Maeda SI, Omata T. 2008. Mechanism of low CO_2-induced activation of the cmp bicarbonate transporter operon by a LysR family protein in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. *Molecular Microbiology* **68,** 98–109.

Parry MAJ, Hawkesford MJ. 2010. Food security: increasing yield and improving resource use efficiency. *Proceedings of the Nutrition Society* 69, 592–600.

Parry MAJ, Reynolds M, Salvucci ME, Raines C, Andralojc PJ, Zhu XG, Price GD, Condon AG, Furbank RT. 2011. Raising yield potential of wheat. II. Increasing photosynthetic capacity and efficiency. *Journal of Experimental Botany* **62,** 453–467.

Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K, Hoisington D. 2004. Stress-induced expression in wheat of the Arabidopsis thaliana DREB1A gene delays water stress symptoms under greenhouse conditions. *Genome* **47,** 493–500.

Pena KL, Castel SE, de Araujo C, Espie GS, Kimber MS. 2010. Structural basis of the oxidative activation of the carboxysomal gamma-carbonic anhydrase, CcmM. *Proceedings of the National Academy of Sciences, USA* **107,** 2455–2460.

Peng LW, Yamamoto H, Shikanai T. 2011. Structure and biogenesis of the chloroplast NAD(P)H dehydrogenase complex. *Biochimica et Biophysica Acta* **1807,** 945–953.

Price GD. 2011. Inorganic carbon transporters of the cyanobacterial CO_2 concentrating mechanism. *Photosynthesis Research* **109,** 47–57.

Price GD, Badger MR. 1989*a*. Expression of human carbonic anhydrase in the cyanobacterium *Synechococcus* PCC7942 creates a high CO_2-requiring phenotype. Evidence for a central role for carboxysomes in the CO_2 concentrating mechanism. *Plant Physiology* **91,** 505–513.

Price GD, Badger MR. 1989*b*. 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 CO_2 within the carboxysome. *Plant Physiology* **91,** 514–525.

Price GD, Badger MR, von Caemmerer S. 2011*a*. The prospect of using cyanobacterial bicarbonate transporters to improve leaf photosynthesis in C_3 crop plants. *Plant Physiology* **155,** 20–26.

Price GD, Badger MR, Woodger FJ, Long BM. 2008. Advances in understanding the cyanobacterial CO_2-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. *Journal of Experimental Botany* **59,** 1441–1461.

Price GD, Howitt SM. 2011. The cyanobacterial bicarbonate transporter BicA: its physiological role and the implications of structural similarities with human SLC26 transporters. *Biochemistry and Cell Biology* **89,** 178–188.

Price GD, Maeda S, Omata T, Badger MR. 2002. Modes of active inorganic carbon uptake in the cyanobacterium, *Synechococcus* sp PCC7942. *Functional Plant Biology* **29,** 131–149.

Price GD, Shelden MC, Howitt SM. 2011*b*. Membrane topology of the cyanobacterial bicarbonate transporter, SbtA, and identification of potential regulatory loops. *Molecular Membrane Biology* **28,** 265–275.

Price GD, Sültemeyer D, Klughammer B, Ludwig M, Badger MR. 1998. The functioning of the CO_2 concentrating mechanism in several cyanobacterial strains—a review of general physiological characteristics, genes, proteins, and recent advances. *Canadian Journal of Botany* **76,** 973–1002.

Price GD, von Caemmerer S, Evans JR, Yu JW, Lloyd J, Oja V, Kell P, Harrison K, Gallagher A, Badger MR. 1994. Specific reduction of chloroplast carbonic anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO_2 assimilation. *Planta* **193,** 331–340.

Price GD, Woodger FJ, Badger MR, Howitt SM, Tucker L. 2004. Identification of a SulP-type bicarbonate transporter in marine cyanobacteria. *Proceedings of the National Academy of Sciences, USA* **101,** 18228–18233.

Rae BD, Forster B, Badger MR, Price GD. 2011. The CO_2-concentrating mechanism of *Synechococcus* WH5701 is composed of native and horizontally-acquired components. *Photosynthesis Research* **109,** 59–72.

Rae BD, Long BM, Badger MR, Price GD. 2012. Structural determinants of the outer shell of β-carboxysomes in *Synechococcus elongatus* PCC 7942: roles for CcmK2, K3-K4, CcmO, and CcmL. *PLoS ONE* **7,** e43871.

Raven JA, Giordano M, Beardall J, Maberly SC. 2011. Algal and aquatic plant carbon concentrating mechanisms in relation to environmental change. *Photosynthesis Research* **109,** 281–296.

Rolland N, Ferro M, Seigneurin-Berny D, Garin J, Douce R, Joyard J. 2003. Proteomics of chloroplast envelope membranes. *Photosynthesis Research* **78,** 205–230.

Sage RF. 2004. The evolution of C_4 photosynthesis. *New Phytologist* **161,** 341–370.

Sage RF, Zhu XG. 2011. Exploiting the engine of C-4 photosynthesis. *Journal of Experimental Botany* **62,** 2989–3000.

Sawaya MR, Cannon GC, Heinhorst S, Tanaka S, Williams EB, Yeates TO, Kerfeld CA. 2006. The structure of beta-carbonic anhydrase from the carboxysomal shell reveals a distinct subclass with one active site for the price of two. *Journal of Biological Chemistry* **281,** 7546–7555.

Shelden MC, Howitt SM, Price GD. 2010. Membrane topology of the cyanobacterial bicarbonate transporter, BicA, a member of the SulP (SLC26A) family. *Molecular Membrane Biology* **27,** 12–23.

Shibata M, Katoh H, Sonoda M, Ohkawa H, Shimoyama M, Fukuzawa H, Kaplan A, Ogawa T. 2002. Genes essential to sodium-dependent bicarbonate transport in cyanobacteria—function and phylogenetic analysis. *Journal of Biological Chemistry* **277,** 18658–18664.

So AKC, Espie GS, Williams EB, Shively JM, Heinhorst S, Cannon GC. 2004. A novel evolutionary lineage of carbonic anhydrase (epsilon class) is a component of the carboxysome shell. *Journal of Bacteriology* **186,** 623–630.

So AKC, John-McKay M, Espie GS. 2002. Characterization of a mutant lacking carboxysomal carbonic anhydrase from the cyanobacterium *Synechocystis* PCC6803. *Planta* **214,** 456–467.

Sonoda M, Katoh H, Vermaas W, Schmetterer G, Ogawa T. 1998. Photosynthetic electron transport involved in *pxcA*-dependent proton extrusion in *Synechocystis* sp. strain PCC6803—effect of *pxcA* inactivation on CO_2, HCO_3–, and NO_3– uptake. *Journal of Bacteriology* **180,** 3799–3803.

Spalding MH. 2008. Microalgal carbon-dioxide-concentrating mechanisms: *Chlamydomonas* inorganic carbon transporters. *Journal of Experimental Botany* **59,** 1463–1473.

Sültemeyer D, Klughammer B, Badger MR, Price GD. 1998. Fast induction of high-affinity HCO_3– transport in cyanobacteria. *Plant Physiology* **116,** 183–192.

Tabita FR. 1999. Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a different perspective. *Photosynthesis Research* **60,** 1–28.

Tanaka S, Kerfeld CA, Sawaya MR, Cai F, Heinhorst S, Cannon GC, Yeates TO. 2008. Atomic-level models of the bacterial carboxysome shell. *Science* **319,** 1083–1086.

Tcherkez GGB, Farquhar GD, Andrews TJ. 2006. Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proceedings of the National Academy of Sciences, USA* **103,** 7246–7251.

Urade R. 2009. The endoplasmic reticulum stress signaling pathways in plants. *Biofactors* **35,** 326–331.

von Caemmerer S. 2003. C_4 photosynthesis in a single C_3 cell is theoretically inefficient but may ameliorate internal CO_2 diffusion limitations of C_3 leaves. *Plant, Cell and Environment* **26,** 1191–1197.

von Caemmerer S, Furbank RT. 2003. The C-4 pathway: an efficient CO2 pump. *Photosynthesis Research* **77,** 191–207.

Wagner U, Kolbowski J, Oja V, Laisk A, Heber U. 1990. pH homeostasis of the chloroplast stroma can protect photosynthesis of leaves during the influx of potentially acidic gases. *Biochimica et Biophysica Acta* **1016,** 115–120.

Walker NA, Smith FA, Cathers IR. 1980. Bicarbonate assimilation by freshwater charophytes and higher-plants. 1. Membrane-transport of bicarbonate ions is not proven. *Journal of Membrane Biology* **57,** 51–58.

Wang HL, Postier BL, Burnap RL. 2004. Alterations in global patterns of gene expression in *Synechocystis* sp PCC 6803 in response to inorganic carbon limitation and the inactivation of *ndhR*, a LysR family regulator. *Journal of Biological Chemistry* **279,** 5739–5751.

Wang P, Duan W, Takabayashi A, Endo T, Shikanai T, Ye JY, Mi HL. 2006. Chloroplastic NAD(P)H dehydrogenase in tobacco leaves functions in alleviation of oxidative damage caused by temperature stress. *Plant Physiology* **141,** 465–474.

Whitney SM, Houtz RL, Alonso H. 2011. Advancing our understanding and capacity to engineer nature's CO2-sequestering enzyme, Rubisco. *Plant Physiology* **155,** 27–35.

Woodger FJ, Badger MR, Price GD. 2003. Inorganic carbon limitation induces transcripts encoding components of the CO_2-concentrating mechanism in *Synechococcus* sp PCC7942 through a redox-independent pathway. *Plant Physiology* **133,** 2069–2080.

Woodger FJ, Badger MR, Price GD. 2005. Sensing of inorganic carbon limitation in *Synechococcus* PCC7942 is correlated with the size of the internal inorganic carbon pool and involves oxygen. *Plant Physiology* **139,** 1959–1969.

Woodger FJ, Bryant DA, Price GD. 2007. Transcriptional regulation of the CO_2-concentrating mechanism in a euryhaline, coastal marine cyanobacterium, *Synechococcus* sp strain PCC 7002: role of NdhR/CcmR. *Journal of Bacteriology* **189,** 3335–3347.

Xu M, Bernat G, Singh A, Mi HL, Rogner M, Pakrasi HB, Ogawa T. 2008. Properties of mutants of *Synechocystis* sp strain PCC6803 lacking inorganic carbon sequestration systems. *Plant and Cell Physiology* **49,** 1672–1677.

Yeates TO, Kerfeld CA, Heinhorst S, Cannon GC, Shively JM. 2008. Protein-based organelles in bacteria: carboxysomes and related microcompartments. *Nature Reviews Microbiology* **6,** 681–691.

Yeates TO, Thompson MC, Bobik TA. 2011. The protein shells of bacterial microcompartment organelles. *Current Opinion in Structural Biology* **21,** 223–231.

Yu JW, Price GD, Song L, Badger MR. 1992. Isolation of a putative carboxysomal carbonic anhydrase gene from the cyanobacterium *Synechococcus* PCC7942. *Plant Physiology* **100,** 794–800.