The Prospect of Using Cyanobacterial Bicarbonate Transporters to Improve Leaf Photosynthesis in C₃ Crop Plants[W]

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The photosynthetic CO₂-fixing enzyme Rubisco arose some 3.5 billion years ago, in an environment when CO₂ was high and oxygen (O₂) was low. Under these conditions, it was CO₂ saturated and presumably performed well (Badger et al., 1998). However, since the advent of oxygenic photosynthesis, the levels of O₂ have risen dramatically and CO₂ has fallen to very low levels. This has gradually created conditions where CO₂ has become limiting for Rubisco and allowed O₂ to act as an alternative inhibitory substrate for the enzyme. To cope with these dramatic environmental changes, two major strategies have evolved to help Rubisco maximize its carboxylation rate at ambient levels of limiting CO₂. First, the enzyme has evolved better kinetic properties, where the Kₘ(CO₂) has decreased and the ability to distinguish against O₂ has increased at the expense of catalytic rate (Badger et al., 1998). Alternatively, many photosynthetic organisms, ranging from cyanobacteria to algae to land plants, have developed active CO₂-concentrating mechanisms (CCMs) to turbo-charge Rubisco’s CO₂ supply at a minor metabolic cost (Badger et al., 1998). Most notably, among plants this has led to the development of C₄ photosynthesis (Sage, 2004).

Most of the important grain crops (rice [Oryza sativa], wheat [Triticum aestivum], barley [Hordeum vulgare], canola [Brassica napus], soybean [Glycine max]), tuber crops, and vegetable crops are C₃ species and have applied the first strategy and lack any form of CCM at the leaf or chloroplast level. Much of the inherent inefficiency in C₃ photosynthesis revolves around the need to gain CO₂ through passive diffusion through the leaf pores (stomata), across cell walls and cytoplasm, and eventually through to the chloroplasts. Diffusive resistance to CO₂ passage results in a drawdown of the effective CO₂ concentration in the chloroplast, and C₃ plants have adopted strategies to maximize the diffusive conductivity for CO₂ by pressurizing chloroplasts against the intracellular airspaces and having large chloroplast surface area-to-leaf area ratios (Evans and von Caemmerer, 1996). Low chloroplast CO₂ concentrations exacerbate the CO₂ limitations and increase the wasteful Rubisco oxygenation reaction of ribulose 1,5-bisphosphate (RuBP) to produce phosphoglycolate, which must be recycled back to RuBP through a complex set of reactions known as the photorespiratory cycle. This is worsened by increased temperature, with the affinity for CO₂ dropping and the oxygenase reaction being relatively enhanced (Kubien and Sage, 2008). To achieve acceptable high rates of photosynthetic CO₂ fixation, typical C₃ species invest up to 30% of soluble protein and some 25% of leaf nitrogen into Rubisco protein. Evolution of the CCM in C₄ plants effectively circumvented a number of the inefficiencies, creating the present-day impetus for attempting to introduce C₄ CCMs into important C₃ crops such as rice (Hibberd et al., 2008).

However, while the C₄ CCM is one approach to elevating CO₂ around Rubisco, drawing from our knowledge of single-cell CCMs in cyanobacteria (Price et al., 2008), there are also opportunities to elevate CO₂ around Rubisco at the individual leaf chloroplast level. These prospects are expanded upon below, but in brief we consider two scenarios. The first, and simplest, approach is to consider the transplantation of cyanobacterial bicarbonate transporters to the C₃ chloroplasts to provide marginal but significant improvement in photosynthetic performance. The second, more elaborate, longer term objective would be to engineer a more functional cyanobacterial CCM in the chloroplast.

**THE CYANOBACTERIAL CCM**

Cyanobacteria have evolved an extremely efficient CCM (Fig. 1; see below), being able to concentrate CO₂ around Rubisco by a factor of up to 1,000-fold. As a result, cyanobacterial CO₂ fixation has been able to retain a Rubisco with a relatively high carboxylation rate, although lower selectivity between CO₂ and O₂, compared with the Rubisco in C₃ plants (Badger et al., 1998). Cyanobacterial cells also have high nitrogen use efficiency, as less nitrogen is devoted to Rubisco than in a C₃ plant (Badger et al., 1998). In addition, Rubisco within a cyanobacterium operates at near CO₂ saturation due to the action of the CCM, such that wasteful photorespiration is largely eliminated.
BCT1, and NDH-I3 uptake systems are only induced under DIC-limiting activity is only present in the carboxysomes. In general, BicA, SbtA, the absence of CA in the general cytoplasm. A specific, low level of CA to the recycling of internally generated CO2 through the CO2 pumps and this is kept in a state of dynamic disequilibrium favoring HCO3^-.

The carboxysomes are typically 90 to 200 nm in diameter (enlarged in Figure 1. The cyanobacterial CCM utilizes up to five uptake systems for DIC and the polyhedral microcompartments known as carboxysomes, which contain the cell’s complement of Rubisco and act as a localized site for the elevation of CO2 around Rubisco. A key operational feature is that all uptake systems deliver HCO3^- to the general cytosol and that they act as the site of CO2 elevation within the cell, with the supply rate of CO2 from accumulated HCO3^- being catalyzed by a carboxysome-located carbonic anhydrase (CA). The carboxysome shell in these cyanobacteria is composed of just six to eight proteins that are composed of 20 equilateral triangular sides is that they act as the site of CO2 elevation within the cell, with the supply rate of CO2 from accumulated HCO3^- being catalyzed by a carboxysome-located carbonic anhydrase (CA). The carboxysome shell in these cyanobacteria is composed of just six to eight proteins (Price et al., 2008), and the average unicellular cyanobacterial cells would normally possess five to 15 carboxysomes per cell. The key to the efficiency of any CCM revolves around the ability to minimize the loss of CO2 from the elevation zone. In model cyanobacteria, this is accomplished by a combination of (1) the accumulation of the ionic form of DIC, which is less membrane permeable than CO2, (2) the complete elimination of CA activity from the general cytosol to help reduce CO2 leakage out of the cell, (3) the special properties of the carboxysome protein shell acting to retard CO2 leakage, and (4) the action of the CO2 pumps in recycling CO2 leakage from the carboxysome back into the HCO3^- pool (Maeda et al., 2002; Price et al., 2008).

The localization of CA, which catalyzes the reversible hydration and dehydration of CO2 and HCO3^-, is a key element of cyanobacterial CCMs. The absence of CA in the cytosol, and the action of the directional CO2 uptake systems that convert CO2 to HCO3^- at the
thylakoid membrane, allow the cell to accumulate HCO$_3^-$ and keep it out of rapid chemical equilibrium with CO$_2$. This is very effective in minimizing the concentration of the diffusible CO$_2$ molecule owing to the slow dehydration of HCO$_3^-$ in the absence of CA (Walker et al., 1980). The importance of accumulating HCO$_3^-$ in the cytosol, and maintaining an internal HCO$_3^-$ pool out of chemical equilibrium, was shown by an experiment where human CA was expressed in the cytoplasm of a model cyanobacterium, Synechococcus elongatus PCC7942. The ectopic expression caused complete 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). This is very different from the situation in C$_3$ chloroplasts, where CA is highly abundant in the stroma in order to maximize the diffusion of CO$_2$ across the envelope and throughout the chloroplast (Badger and Price, 1994).

Five distinct transport systems for DIC uptake have been identified in cyanobacteria (Fig. 1; Table I; for more details and related references, see Price et al., 2008). (1) BCT1, which is inducible under DIC limitation and is a high-affinity HCO$_3^-$ transporter (uniporter) belonging to the traffic ATPase family. (2) SbtA, an inducible, high-affinity Na$^+$/HCO$_3^-$ symporter (Price et al., 2004; Shibata et al., 2002) that apparently acts as a Na$^+$/HCO$_3^-$ symporter with relatively low flux rate. (3) BicA, a low-affinity, high-flux, Na$^+$/HCO$_3^-$ transporter belonging to the widespread Sulf (sulphur) family and related to the human SLC26 family of anion transporters (Price et al., 2004); BicA is a probable Na$^+$/HCO$_3^-$ symporter. (4) NDH-I$_y$, a constitutive CO$_2$ uptake system based on a specialized NADPH dehydrogenase (NDH-I) complex; this system uses NADPH as an electron donor to drive the conversion of CO$_2$ to HCO$_3^-$ during the uptake step (Price et al., 2002). Each complex is composed of 10 core subunits that are common to the respiratory NDH-I complex and three specialized subunits required for CO$_2$ uptake. Interestingly, NDH-I-type CO$_2$ uptake systems appear to be 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$_3^-$. (5) NDH-I$_v$, a second CO$_2$ uptake system based on a modified NDH-I complex that is inducible under DIC limitation and is of higher uptake affinity than NDH-I$_y$, located on the thylakoid membranes in Synechocystis PCC6803.

**Figure 2.** A timeline indicating that CCMs possibly arose in cyanobacteria and microalgae at around 400 to 350 million years ago, well after the evolution of early land plants.

**Table I.** A summary of the properties of cyanobacterial DIC transporters

<table>
<thead>
<tr>
<th>Transport Type</th>
<th>Mechanism</th>
<th>Substrate Affinity</th>
<th>Flux Rate</th>
<th>Photosynthetic Affinity ($k_{\text{aff}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BicA</td>
<td>Na$^+$/HCO$_3^-$ uptake</td>
<td>Low-medium</td>
<td>High</td>
<td>90–170 $\mu$M HCO$_3^-$</td>
</tr>
<tr>
<td>SbtA</td>
<td>Na$^+$/HCO$_3^-$ uptake</td>
<td>High</td>
<td>Low</td>
<td>&lt;5 $\mu$M HCO$_3^-$</td>
</tr>
<tr>
<td>BCT1</td>
<td>Traffic ATPase, HCO$_3^-$ uptake</td>
<td>High</td>
<td>Low</td>
<td>10–15 $\mu$M HCO$_3^-$</td>
</tr>
<tr>
<td>NDH-I$_y$</td>
<td>NADPH-driven CO$_2$ uptake via conversion to HCO$_3^-$</td>
<td>Medium</td>
<td>High</td>
<td>10–15 $\mu$M CO$_2$</td>
</tr>
<tr>
<td>NDH-I$_v$</td>
<td>NADPH-driven CO$_2$ uptake via conversion to HCO$_3^-$</td>
<td>High</td>
<td>Low</td>
<td>1–2 $\mu$M CO$_2$</td>
</tr>
</tbody>
</table>

**ADDING A HCO$_3^-$ TRANSPORTER TO THE CHLOROPLAST ENVELOPE OF CROP PLANTS**

With the objective of attaining a modest elevation of CO$_2$ levels in the C$_3$ chloroplast, the simplest approach would be to express a cyanobacterial HCO$_3^-$ transporter on the inner envelope of the C$_3$ chloroplast (Fig. 3). Single-subunit HCO$_3^-$ transporters such as BicA and SbtA are the most obvious initial candidates. However, within technical restraints, the transfer of multisubunit transporters such as the BCT1 HCO$_3^-$ transporter (four genes) is also possible. Additionally, the use of HCO$_3^-$ transporters from microalgae such as Chlamydomonas can also be considered as viable candidates (Duanmu et al., 2009). From a technical viewpoint, the addition of DIC transporters mentioned above would be dependent on host genome transformation techniques using Agrobacterium tumefaciens, which are generally available for a range of important crop species. Chloroplast transformation techniques would not be required for this approach, and this is especially important because chloroplast transformation in crop species in not yet available. As can be seen from the associated modeling presented in this report (Fig. 4), the approach of installing BicA and/or SbtA transporters into the chloroplast inner envelope could achieve a 5% to 15% improvement in photosynthetic CO$_2$ fixation rates at constant submatal CO$_2$ levels (see below).

It is clear that a CO$_2$ diffusion gradient or drawdown exists between the CO$_2$ level in the substomatal cavity of the leaf (C$_i$) and the steady-state level of CO$_2$ in chloroplast (C$_{chlo}$), with the magnitude of this gradient...
sitting about 40% below $C_i$ at high irradiance (Evans and von Caemmerer, 1996). It is important to recognize that in the first instance, the primary objective of adding a $\text{HCO}_3^-$ pump would be to diminish the size of this $\text{CO}_2$ drawdown at the chloroplast and not to elevate it significantly above the external $\text{CO}_2$ concentration. This minimizes the risk of wasteful $\text{CO}_2$ leakage. Such a situation is very similar to the concept of introducing a C4 cycle into C3 cells (Matsuoka et al., 2001), which has been modeled for transplantation into a typical C3 chloroplast (von Caemmerer, 2003) and found to be theoretically capable of raising the steady-state $\text{CO}_2$ level within the chloroplast. More specific modeling data on the theoretical engineering of BicA into a chloroplast is shown in Figure 4 and discussed below.

![Figure 3](https://plantphysiol.org)

Figure 3. Schematic representations illustrating the concepts of adding a cyanobacterial $\text{HCO}_3^-$ transporter to the chloroplast envelope of a notional C3 leaf chloroplast (A) and the longer term prospect of constructing a more fully functional cyanobacterial or microalgal CCM in the C3 chloroplast (B). The diagrams show $\text{CO}_2$ moving from the intracellular airspace (IAS; substomatal cavity) of a mesophyll leaf cell through the cell wall to the cytoplasm (Cyt) before entering the chloroplast by $\text{CO}_2$ diffusion or via entry through a $\text{HCO}_3^-$ transporter. The hexagonal structure represents the icosahedral carboxysomes that would contain the full complement of Rubisco in the chloroplast, with a specific CA partitioned to this compartment and stromal CA removed. Linkages between the carbon reduction in the chloroplast and photorespiration involving peroxisomes (P) and mitochondria (M) are also shown.

In terms of establishing active $\text{HCO}_3^-$ uptake across the chloroplast envelope, the question arises as to whether a Na$^+$-dependent $\text{HCO}_3^-$ pump could function in a chloroplast. Estimates indicate that at least 250 $\mu$M $\text{HCO}_3^-$ is present in the cytosol of a leaf cell in ambient air (Evans and von Caemmerer, 1996), and this appears to be maintained by cytosolic CA activity. The uptake affinities of SbtA (low flux rate) and BicA (high flux rate) for $\text{HCO}_3^-$ in cyanobacteria are 5 to 15 $\mu$M and 90 to 170 $\mu$M, respectively (Shibata et al., 2002; Price et al., 2004) and would indicate that either transporter would operate well above its intrinsinc $K_m$. An additional concern relates to the question of energization and the involvement of Na$^+$ gradients across the chloroplast envelope. Both SbtA and BicA require about 1 mM Na$^+$ for half-maximal activity in the form of a standing inward gradient for Na$^+$ (Shibata et al., 2002; Price et al., 2004). The leaf cytosol possesses 1 to 3 mM Na$^+$ (Karley et al., 2000), and proteomic analyses have revealed that the Arabidopsis chloroplast envelope possesses several potential Na$^+$-coupled transporters and Na$^+$/$\text{H}^+$ antiporters that are homologous to cyanobacterial forms (Rolland et al., 2003). Thus, there are good prospects that the chloroplast possesses and maintains an inwardly directed Na$^+$ gradient. As a potential enhancement, the transfer of a cyanobacterial Na$^+$/$\text{H}^+$ antiporter (or a version from the C4 chloroplast) could also be considered if this Na$^+$ gradient needed to be augmented, perhaps at the expense of any existing $\text{H}^+$ gradient (proton motive force) inferred from the existence of $\text{H}^+$-coupled transporters in the envelope (Weber et al., 2005). Any possible perturbation of osmotic and pH homeostasis in the chloroplast by elevating steady-state $\text{HCO}_3^-$ levels by up to 15%, or even by as much as 25-fold relative to air-exposed leaves, would be expected to be minimal (Wagner et al., 1990).

Other problems in establishing SbtA or BicA in C3 chloroplasts would include ensuring the correct targeting to the chloroplast envelope and uncertainty about whether these transporters need to be post-translationally activated. In the case of targeting, we expect that SbtA and BicA can be fused to the cDNAs for known envelope-targeted proteins such that details on targeting are not initially required. We have determined the membrane topology structure of BicA and SbtA as an initial step in identifying the most likely cytoplasmic regulatory domains in these transporters (Shelden et al., 2010).
The addition of bicarbonate transporters to a C3 chloroplast is based on previous approaches used to consider the theoretical addition of a CO2 pump of single-cell C4 type (von Caemmerer, 2003; von Caemmerer and Furbank, 2003); equations and parameters used in the simulations shown in Figure 4 are detailed in the Supplemental Equations S1 and Supplemental Table S1. Much of the discussion of the benefits of introduction of single-cell C4 photosynthesis into a C3 leaf applies to the introduction of bicarbonate transporters (von Caemmerer, 2003). The key point issuing from the modeling is that the addition of either HCO3− transporter, BicA or SbtA, can lead to an increase in the rate of light-saturated CO2 assimilation at ambient and low intercellular CO2 partial pressures (Ci). The magnitude of the increase will be very much dependent on the kinetic properties of the transporters and the conductance to CO2 diffusion of the chloroplast envelope (von Caemmerer, 2003). The introduction of a transporter elevates chloroplast CO2 partial pressures (Cchlo) above Ci at low Ci values, resulting in a reduced CO2 compensation point. The addition of the high-affinity SbtA transporter is more effective at reducing the compensation point than the BicA transporter because of its lower Knv and introduction of both can be more effective again. At higher Ci levels, transporters serve to reduce the drawdown in CO2 between intercellular CO2 and the chloroplast (Fig. 4). At a constant Ci level of 250 μbar, a theoretical transgenic plant, with the assumed activities of both HCO3− uptake systems, could display an indicative assimilation rate greater than 15% higher than wild-type C3.

One of the disadvantages of C4 photosynthesis is the requirement of two ATPs per CO2 fixed in the C4 cycle, which makes the introduction of a C4 cycle into current C3 leaves energetically inefficient (von Caemmerer, 2003), although there are examples of a number of single-cell C4 species that have overcome the anatomical limitations inherent in C3 leaves (Edwards et al., 2004). It is likely that bicarbonate transport is less expensive. Considering the negative electrogenicity of HCO3− uptake in cyanobacteria (Ritchie et al., 1996)
and the likely Na\(^+\):proton equivalence, we estimate a required 0.25 ATP per HCO\(_3\)\(^-\) transported by BicA and 0.5 ATP per HCO\(_3\)\(^-\) for SbtA. On this basis, the ATP requirement per net CO\(_2\) assimilation rate drops with increasing C\(_i\) during C\(_3\) photosynthesis as the cost of photorespiration decreases (Fig. 4). The introduction of bicarbonate 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\(_i\) (Fig. 4).

We have only considered the implications for light-saturated photosynthesis here. For healthy crops, about half the canopy will be operating under these conditions, which should make the introduction of bicarbonate transporters a productive strategy. Enhancing leaf photosynthetic rates also has the potential of increasing leaf WUE, depending on stomatal responses. Bicarbonate transporters provide the largest benefit of a low C\(_i\) and the stomata of a plant with HCO\(_3\)\(^-\) pump enhancement could afford to be less open while providing the same rate of assimilation, thereby resulting in less loss of water from the leaf stomates. The SbtA transporter could be capable of improving WUE under dry-air conditions more effectively than BicA, and the addition of both transporters is likely to be even better. If SbtA or SbtA + BicA can be successfully introduced into C\(_3\) plants, such species might be able to better survive transient episodes of high water deficit under high light and moderate temperature stress.

**ADDING A MORE ELABORATE CYANOBACTERIAL CCM TO THE CHLOROPLAST**

A longer term objective, involving the greater technical difficulty of the introduction of multiple genes, could be to establish a more elaborate form of the cyanobacterial CCM in the chloroplast (Fig. 3). This could involve the transfer of one or two functionally active HCO\(_3\)\(^-\) transporters to the inner envelope membrane combined with the transfer of a CO\(_2\) uptake system to the thylakoid membranes and a Rubisco microcompartment such as the carboxysomes. The C\(_3\) chloroplast would also need to be, like the cyanobacterial cytosol, converted to a HCO\(_3\)\(^-\)-accumulating organelle where the HCO\(_3\)\(^-\) pool is held in a state of slow chemical interconversion. To do this, it would be necessary to reorganize chloroplastic C\(_3\) Rubisco into effective carboxysome structures and devise an effective means of removing the highly abundant chloroplastic CA so that HCO\(_3\)\(^-\) accumulation can be optimized. Certainly, it has been possible to remove up to 99% of chloroplastic CA activity in tobacco leaves by antisense RNA approaches (Price et al., 1994). Ideally, a complete removal of CA from the stroma would be more desirable, except for retaining the critically important CA in transferred carboxysomes. One of the most significant uncertainties relates to the conductance of the envelope to CO\(_2\) diffusion, with a range of estimates available (Flexas et al., 2008).

Aquaporins seem to play a role in CO\(_2\) conductance; thus, a useful enhancement might be to reduce aquaporin levels in the envelope by RNA interference technology, since lower conductance would aid in reducing CO\(_2\) leakage (Flexas et al., 2006).

Our understanding of carboxysome assembly and function has improved greatly in recent years, to the point where engineering the assembly of a carboxysome in the chloroplast is approaching feasibility. This has been aided by advances in determining the crystal structures of some key components of the shell (Yeates et al., 2008) and our own efforts to identify proteins required as key Rubisco- and shell-organizing proteins (Long et al., 2007, 2010). The remarkable feature of the small shell proteins is an ability to self-assemble (Yeates et al., 2008). This property, shared with some virus coat proteins, could greatly aid the final goal of assembling functional carboxysomes within the chloroplast. The longer term objective of engineering a more potent form of the cyanobacterial CCM into the chloroplast may provide greater photosynthetic enhancements than the introduction of bicarbonate transporters alone.

**CONCLUSION**

Modeling indicates that the addition of cyanobacterial (or microalgal) HCO\(_3\)\(^-\) pumps at the chloroplast envelope of a typical C\(_3\) plant could provide a significant boost to the photosynthetic performance of leaf photosynthesis, either as increased assimilation rate or as improved WUE. A next research focus is to target BicA and SbtA transporters to the chloroplast of a model C\(_3\) plant and to extend our understanding of activation and energization processes for cyanobacterial HCO\(_3\)\(^-\) transporters. However, it should also be clear that parallel transgenic strategies, such as improving the performance of Rubisco or raising capacities for RuBP generation or light interception, would provide complementary improvements to crop performance, as discussed in other articles in this issue.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Equations S1.** Modeling equations used in generation of data in Figure 4.

**Supplemental Table S1.** Photosynthetic parameters used in the model simulation.

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**LITERATURE CITED**
