

# Can the Cyanobacterial Carbon-Concentrating Mechanism Increase Photosynthesis in Crop Species? A Theoretical Analysis<sup>1</sup>[W][OPEN]

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Experimental elevation of  $[\text{CO}_2]$  around  $\text{C}_3$  crops in the field has been shown to increase yields by suppressing the Rubisco oxygenase reaction and, in turn, photorespiration. Bioengineering a cyanobacterial carbon-concentrating mechanism (CCM) into  $\text{C}_3$  crop species provides a potential means of elevating  $[\text{CO}_2]$  at Rubisco, thereby decreasing photorespiration and increasing photosynthetic efficiency and yield. The cyanobacterial CCM is an attractive alternative relative to other CCMs, because its features do not require anatomical changes to leaf tissue. However, the potential benefits of engineering the entire CCM into a  $\text{C}_3$  leaf are unexamined. Here, a  $\text{CO}_2$  and  $\text{HCO}_3^-$  diffusion-reaction model is developed to examine how components of the cyanobacterial CCM affect leaf light-saturated  $\text{CO}_2$  uptake ( $A_{\text{sat}}$ ) and to determine whether a different Rubisco isoform would perform better in a leaf with a cyanobacterial CCM. The results show that the addition of carboxysomes without other CCM components substantially decreases  $A_{\text{sat}}$  and that the best first step is the addition of  $\text{HCO}_3^-$  transporters, as a single  $\text{HCO}_3^-$  transporter increased modeled  $A_{\text{sat}}$  by 9%. Addition of all major CCM components increased  $A_{\text{sat}}$  from 24 to 38  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Several Rubisco isoforms were compared in the model, and increasing ribulose biphosphate regeneration rate will allow for further improvements by using a Rubisco isoform adapted to high  $[\text{CO}_2]$ . Results from field studies that artificially raise  $[\text{CO}_2]$  suggest that this 60% increase in  $A_{\text{sat}}$  could result in a 36% to 60% increase in yield.

$\text{C}_3$  species include the major grain crops rice (*Oryza sativa*) and wheat (*Triticum aestivum*) and overall accounted for approximately 75% of all primary foodstuff production in 2012 (FAOSTAT, 2013). The yield of many crop species has been substantially improved through breeding and agronomy, but advancement in yield has substantially slowed in many of the major  $\text{C}_3$  crops in the last decade, suggesting that limits on yield improvement using these techniques are being reached and that other approaches are needed (Long and Ort, 2010; Ray et al., 2012).

One largely unexploited approach would be to improve the efficiency of photosynthesis in these species (Zhu et al., 2010). The photosynthetic enzyme Rubisco catalyzes the reaction of  $\text{CO}_2$  with ribulose biphosphate (RuBP), which eventually forms carbohydrates. However, Rubisco will also react with oxygen as the first step in photorespiration. This reaction is considered wasteful, since energy is consumed to recover RuBP and  $\text{CO}_2$  is lost in the process. In  $\text{C}_3$  plants at 25°C and

current atmospheric  $[\text{CO}_2]$ , photorespiration results in an approximately 30% decrease in net carbon assimilation (Zhu et al., 2010). Thus, it is a large inefficiency in carbon uptake and a target for improvement.

Since  $\text{CO}_2$  and oxygen act competitively at Rubisco, photorespiration can be decreased by increasing  $[\text{CO}_2]$  around Rubisco. That this will increase yield is demonstrated by the many studies that have artificially increased atmospheric  $[\text{CO}_2]$  around  $\text{C}_3$  crops growing in the field (Kimball et al., 2002; Long et al., 2006b). Other photosynthetic organisms have evolved mechanisms to internally elevate  $[\text{CO}_2]$  at Rubisco to decrease or eliminate photorespiration. Such carbon-concentrating mechanisms (CCMs) include  $\text{C}_4$  photosynthesis, as in maize (*Zea mays*), and the carboxysome and pyrenoid CCMs of single-celled cyanobacteria and algae. The  $\text{C}_4$  pathway requires the addition of the photosynthetic  $\text{C}_4$  dicarboxylate cycle and inner photosynthetic cells (i.e. bundle sheath), where Rubisco is localized. Converting a  $\text{C}_3$  crop to a  $\text{C}_4$  crop will require a coordination of changes in photosynthetic tissue differentiation and enzyme and transporter localization. In contrast, cyanobacteria achieve the same effect in a single cell by localizing Rubisco to specialized subcellular compartments called carboxysomes, so in theory they would require fewer changes. Carboxysomes are polyhedral bodies with a protein shell that encloses carbonic anhydrase (CA) and Rubisco packed in an ordered or semioordered array (Long et al., 2007; Yeates et al., 2011). Bicarbonate is actively transported from the environment into the cytosol of the cyanobacteria, and  $\text{CO}_2$  in

<sup>1</sup> This work was supported by the National Science Foundation (grant no. EF-1105584) and the Bill and Melinda Gates Foundation.

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[www.plantphysiol.org/cgi/doi/10.1104/pp.113.232611](http://www.plantphysiol.org/cgi/doi/10.1104/pp.113.232611)

the cytosol is actively hydrated to  $\text{HCO}_3^-$  using NADH.  $\text{CO}_2$  is also hydrated to  $\text{HCO}_3^-$ , which serves to increase the  $[\text{CO}_2]$  gradient between the medium and cytosol, increasing  $\text{CO}_2$  flux, and also serves to reflux  $\text{CO}_2$  that leaks from the carboxysome. Since the cytosol lacks CA and the plasma membrane has low permeability to  $\text{HCO}_3^-$ , a high cytosolic  $[\text{HCO}_3^-]$  far from equilibrium with  $[\text{CO}_2]$  is achieved. Bicarbonate diffuses through the protein shell of the carboxysome, and since CA is localized to the inner side of the shell, it is rapidly converted to  $\text{CO}_2$ , given the disequilibrium. The resulting high  $[\text{CO}_2]$  around Rubisco inside the carboxysome accelerates carboxylation and competitively inhibits the oxygenation reaction (for review, see Price et al., 2008, 2011, 2013; Espie and Kimber, 2011).

The  $\text{C}_4$  pathway has been well characterized, and several projects are attempting to engineer it into  $\text{C}_3$  plants with some success (Slewisinski, 2013). However, a primary obstacle has been achieving the necessary two tissue types with the correct localization of the key enzymes (Covshoff and Hibberd, 2012). In this regard, cyanobacterial CCM may provide an attractive parallel approach to eliminating photorespiration in  $\text{C}_3$  plants for two reasons. First, the biochemical, structural, and genetic components of the CCM are well understood, in that all of the necessary proteins and their genes have been identified. Second, chloroplasts of higher plants evolved from a common ancestor with modern cyanobacteria (Raven and Allen, 2003) and, therefore, are structurally similar. Thus, engineering carboxysomes into the chloroplast could involve introducing an operon containing the genes associated with the cyanobacterial CCM into the chloroplast genome.

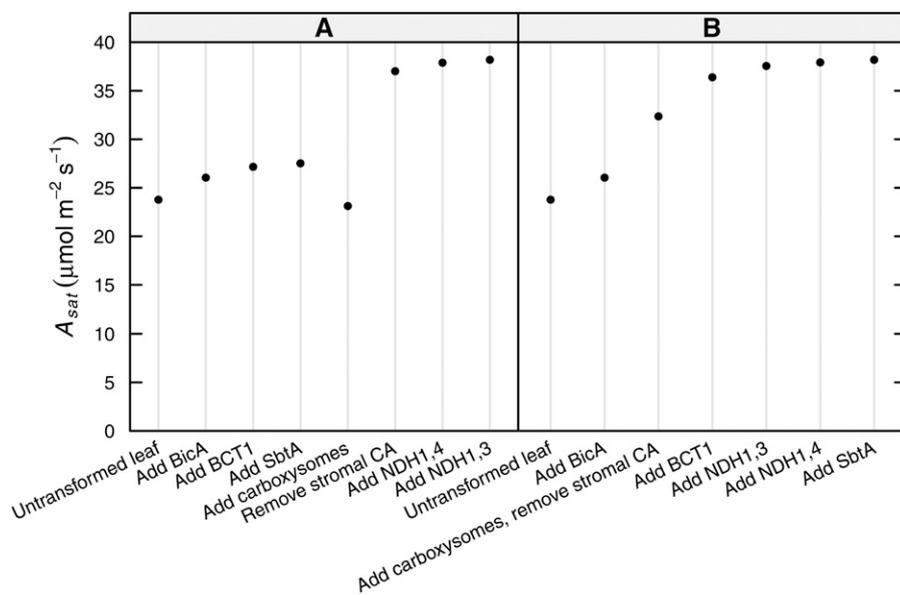
There are, however, multiple proteins in the cyanobacterial CCM, and simultaneously transforming all of the required genes into an organism may be particularly challenging. Furthermore, some of the proteins appear to have a similar function, and others may be deleterious if introduced without the full apparatus. A random sequence of gene stacking would likely be inefficient, while the addition of some genes may simply be unnecessary. However, the biophysical reactions involved are well understood, and there are some measurements of the kinetics of the enzymatic reactions and transporters. With this information, a mathematical model can be created to simulate the CCM. Such a model will allow deduction of the minimal component set necessary for an improvement of leaf photosynthetic efficiency and identify a logical sequence of gene additions to deliver progressive improvements and avoid any lethal effects. This model will also be used to determine which aspects of the CCM exert the most control over the overall process of photosynthetic  $\text{CO}_2$  assimilation, information that can then be used to optimize the CCM, help identify the ideal set of components, or identify parameters that need to be more accurately measured in order to improve the effectiveness of the CCM model. Similar kinetic models have been applied to propose systems optimization of the Calvin cycle when plants are grown

in elevated  $[\text{CO}_2]$  (Zhu et al., 2007), of the whole  $\text{C}_3$  photosynthetic system (Zhu et al., 2013),  $\text{C}_4$  photosynthesis (Yu et al., 2014), and to determine the mechanistic basis of mesophyll conductance (Tholen and Zhu, 2011).

This paper develops a kinetic model to determine the potential of the cyanobacterial CCM engineered into  $\text{C}_3$  crops for improving photosynthesis by determining the necessary components and estimating the potential improvement of  $\text{CO}_2$  assimilation rate and efficiency. There are four distinct features to the cyanobacterial CCM: (1) a carboxysome with internally localized Rubisco and CA; (2) active transport of  $\text{HCO}_3^-$  from the medium into the cyanobacterial cytosol (the equivalent compartment in higher plants is the stroma of the chloroplast); (3) active hydration of  $\text{CO}_2$  to  $\text{HCO}_3^-$  within the cytosol; and (4) the absence of CA in the cyanobacterial cytosol, in contrast to the higher plant stroma, which contains high activities of CA. Cyanobacteria have three  $\text{HCO}_3^-$  transporters and two  $\text{CO}_2$  hydration enzymes that have different kinetics and are induced under different conditions (Price et al., 2011). All of these enzymes were examined, giving a total of seven individual components (listed in Fig. 1A) to the full cyanobacterial CCM model. Because of the common ancestry between cyanobacteria and higher plant chloroplasts, the two have homologous membranes and compartments. Proteins were modeled within a  $\text{C}_3$  leaf using locations equivalent to those in cyanobacteria. That is, bicarbonate transporters were localized to the inner chloroplast membrane, and carboxysomes were localized to the stroma. The  $\text{CO}_2$  hydration enzymes are bound to the thylakoid and plasma membranes in cyanobacteria, but the reaction for both occurs within the cyanobacterial cytosol; therefore, in this model, hydration of  $\text{CO}_2$  by these enzymes was localized to the stroma. This model was used to determine the necessity of each of the features noted above, potential improvements in light-saturated  $\text{CO}_2$  uptake ( $A_{\text{sat}}$ ; irradiance of  $1,800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) that would be achieved on incorporation of each feature in turn, a possible sequence of gene additions that would give an incremental improvement, and the key components of the CCM. The model was also used to test the value of using higher plant isoforms of Rubisco versus prokaryotic isoforms adapted to incorporation within the carboxysome. Sources of uncertainty in the model are also defined.

## RESULTS

Incorporating the four key features (composed of seven individual components) of a cyanobacterial CCM into a  $\text{C}_3$  leaf increased  $A_{\text{sat}}$  from 23.8 to  $38 \mu\text{mol m}^{-2} \text{s}^{-1}$  in this model (Fig. 1). The majority of changes that involved the addition of only a single feature decreased  $A_{\text{sat}}$ , sometimes to levels that would greatly reduce plant growth and probably require rescue in high  $[\text{CO}_2]$  (a list of all possible combinations of features



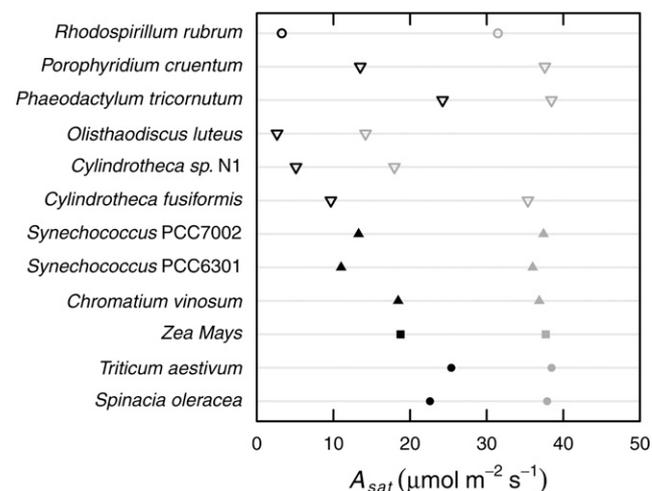
**Figure 1.**  $A_{\text{sat}}$  of leaf models with sequential addition of components of the cyanobacterial CCM. Each point represents a leaf that contains the component listed for that column and all of the components in the columns to the left of it. The columns represent the sequence of transformations that produce the fastest increase in  $A_{\text{sat}}$  assuming that it is not possible to simultaneously add carboxysomes and remove stromal CA and that it is not desirable to add  $\text{CO}_2$  hydration enzymes before removal of stromal CA (A) and assuming that it is possible to simultaneously add carboxysomes and remove stromal CA (B).

and their effects on  $A_{\text{sat}}$  is given in Supplemental Table S1). However, addition of any one of the  $\text{HCO}_3^-$  transporters, bicarbonate uptake A (BicA), bicarbonate transporter 1 (BCT1), or sodium-bicarbonate transporter A (SbtA) alone, improved  $A_{\text{sat}}$ . The addition of carboxysomes without removal of stromal CA or vice versa was detrimental to  $A_{\text{sat}}$  regardless of the inclusion of other features. Thus, unless it is possible to simultaneously add carboxysomes and remove stromal CA, there is no series of transformations that provides increased  $A_{\text{sat}}$  at each stage. Without the ability to make those changes simultaneously, the results here indicate that the best pathway of improvement is to first add the  $\text{HCO}_3^-$  transporters, add carboxysomes, remove stromal CA, and then add the NADH-driven  $\text{CO}_2$  hydration enzymes (Fig. 1A). If it is possible to add carboxysomes and remove stromal CA simultaneously, then a slightly different pathway provides faster initial improvement, as follows: adding (1) BicA, (2) carboxysomes with simultaneous removal of CA, (3) BCT1, (4) NAD(P)H dehydrogenase complex (NDH1,3), (5) NDH1,4, and (6) SbtA (Fig. 1B). The final result is the same regardless of which sequence of transformations is used (last columns in Fig. 1).

At minimum, in order for the carboxysome to be effective requires the removal of stromal CA and the addition of BicA, BCT1, or NDH1,3 (Supplemental Table S1). Individually, neither SbtA nor NDH1,4 increase the stromal  $[\text{HCO}_3^-]$  enough for the carboxysome to be effective. Although at the enzyme concentrations modeled, the  $\text{HCO}_3^-$  transporters are more effective than  $\text{CO}_2$  hydration enzymes at increasing  $A_{\text{sat}}$  since the  $\text{CO}_2$  hydration and  $\text{HCO}_3^-$  transport enzymes use different forms of energy, it may be desirable to include one of each type of enzyme so that the system is more flexible. If only one  $\text{HCO}_3^-$  transporter and one NDH isoform can be incorporated with a carboxysome, the

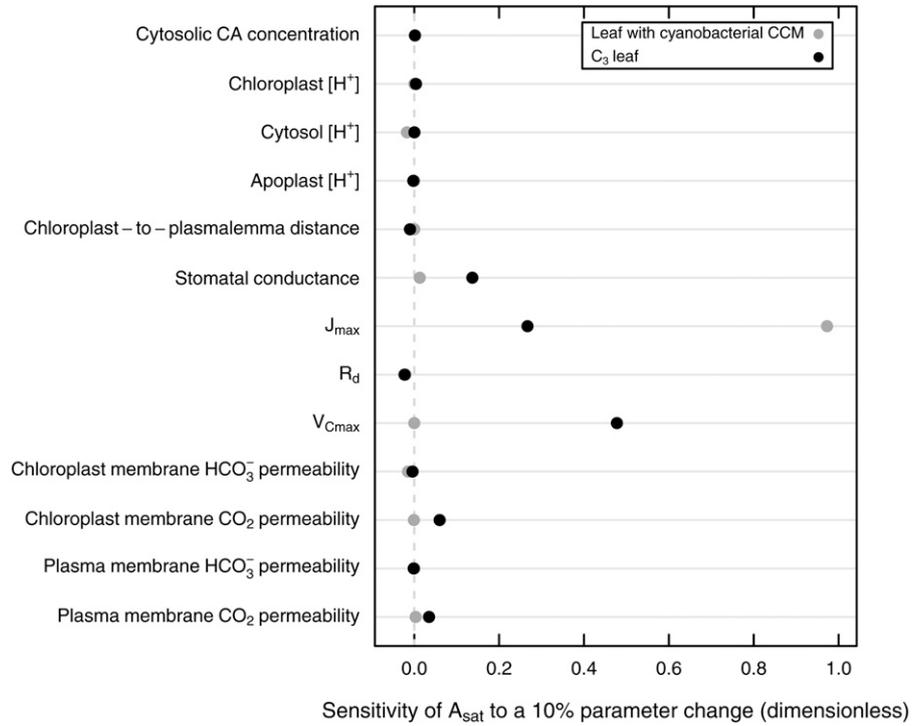
pair that gives the highest  $A_{\text{sat}}$  is BicA with NDH1,3 (Supplemental Table S1).

In the absence of a CCM, replacement of the  $\text{C}_3$  crop Rubisco with a cyanobacterial or algal isoform of Rubisco resulted in a 5% to 50% decrease in  $A_{\text{sat}}$  (Fig. 2). However, when the addition of the CCM is simulated and Rubisco is assumed to be confined to the carboxysome, this disadvantage is eliminated for most of the cyanobacterial, bacterial, and algal isoforms, where, in the high  $[\text{CO}_2]$  of the carboxysome, rates were often similar to, but did not exceed, those of the higher plant isoforms (Fig. 2).



**Figure 2.**  $A_{\text{sat}}$  of leaf models without (black symbols) and with (gray symbols) a cyanobacterial CCM using different isoforms of Rubisco. Species from which the Rubisco isoform parameters were used are listed on the vertical axis. Parameters for the isoforms are given in Supplemental Table S2. Symbols represent the class of species: closed circles,  $\text{C}_3$ ; closed squares,  $\text{C}_4$ ; closed triangles, cyanobacteria; open triangles, nongreen algae form I; and open circles, nongreen algae form II (where form I and form II are as defined by Gibson and Tabita [1977]).

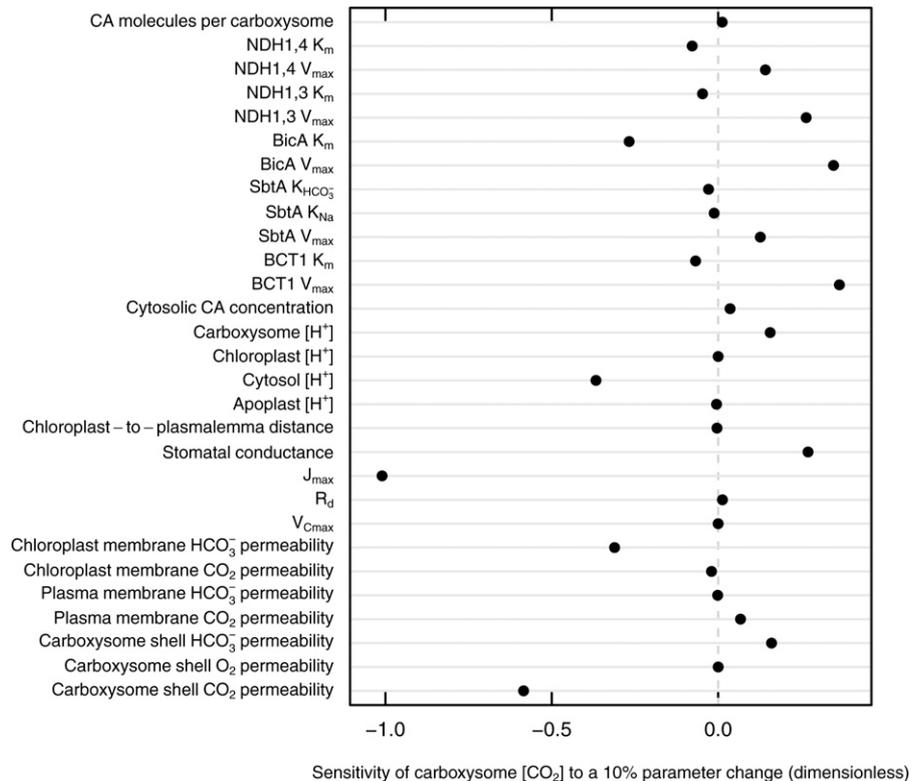
**Figure 3.** Sensitivity of the predicted  $A_{\text{sat}}$  to model parameters for leaf  $C_3$  photosynthesis without (black circles) or with (gray circles) the addition of the cyanobacterial CCM.  $R_d$ , Mitochondrial respiration in the light.



In order to determine how limitations to photosynthesis are changed in a leaf with a CCM, the sensitivity of  $A_{\text{sat}}$  to several parameters in the model was compared between an untransformed leaf and a leaf with all seven of the changes listed in Figure 1A. In the

transformed leaf,  $A_{\text{sat}}$  was very sensitive to the maximum rate of electron transport ( $J_{\text{max}}$ ), with slight sensitivity to stomatal conductance and cytosolic [H<sup>+</sup>] (Fig. 3). Following Farquhar et al. (1980), we use the term  $J_{\text{max}}$ . During the development of that model, it was

**Figure 4.** Sensitivity of carboxysomal [CO<sub>2</sub>] to variation in model parameters for leaf  $C_3$  photosynthesis with the simulated addition of the cyanobacterial CCM.  $R_d$ , Mitochondrial respiration in the light.



**Table 1.** Parameters, values, and their sources

Name	Symbol	Value	Units	References
Temperature	$T$	298.15	K	25°C
Pressure	$pressure$	101,325	Pa	Atmospheric pressure
Gas constant	$R$	8.31446	$J mol^{-1} K^{-1}$	Universal gas constant
Atmospheric $[CO_2]$	$C_a$	$390 \times 10^{-6}$	$mol mol^{-1}$	Approximate atmospheric $[CO_2]$ in 2012
Oxygen partial pressure	$O$	$pressure \times 0.208$	Pa	21% oxygen
Solubility of $CO_2$	$H_{CO_2}, H_{CO_2}$	2,994.012	$m^3 Pa^{-1} mol^{-1}$	von Caemmerer (2000)
Solubility of oxygen	$H_{O_2}$	79,365.1	$m^3 Pa^{-1} mol^{-1}$	von Caemmerer (2000)
$CO_2$ diffusivity in water	$D_{CO_2, H_2O}$	$1.7 \times 10^{-9}$	$m^2 s^{-1}$	Terashima et al. (2011)
$HCO_3^-$ diffusivity in water	$D_{HCO_3^-, H_2O}$	$D_{CO_2, H_2O} \times 0.56$	$m^2 s^{-1}$	Evans et al. (2009)
Molarity of air	$n_{air}$	40.87	$mol m^{-3}$	Calculated from $T$ , $R$ , and $pressure$ using the ideal gas law.
Boundary thickness	$l_b$	0.0005	m	Nobel (1983); 10-cm leaf at about $1 m s^{-1}$ wind speed
Cell wall thickness	$l_w$	$0.32 \times 10^{-6}$	m	Pritchard et al. (1986)
Chloroplast-to-plasmalemma distance	$l_{thin\ cytosol}$	$0.1 \times 10^{-6}$	m	Evans et al. (2009)
Boundary layer conductance	$g_b$	1.53	$mol m^{-2} s^{-1}$	Calculated from $l_b$
Stomatal conductance	$g_s$	0.4375	$mol m^{-2} s^{-1}$	Bernacchi et al. (2006)
Mesophyll surface area fraction	$S_m$	15	$m^2 m^{-2}$ (exposed mesophyll surface area per one-sided leaf area)	Slaton and Smith (2002)
Carboxysome surface area fraction	$S_{csome}$	72.75	$m^2 m^{-2}$ (surface area per one-sided leaf area)	Long et al. (2007) <sup>a</sup>
$CO_2$ transfer coefficient	$k_{tr}$	$\frac{D_{CO_2, H_2O}}{l_w/10}$	$m s^{-1}$	Assumed that $CO_2$ dissolution into the cell wall only occurs in the one-tenth of the wall adjacent to the airspace
Cell wall porosity	$\phi_{apo}$	0.2	Dimensionless	Evans et al. (2009)
Cell wall tortuosity	$\tau_{apo}$	1	Dimensionless	Evans et al. (2009)
Plasmalemma permeability to $CO_2$	$P_{p, CO_2}$	0.0035	$m s^{-1}$	Gutknecht et al. (1977)
Plasmalemma permeability to $HCO_3^-$	$P_{p, HCO_3^-}$	$0.5 \times 10^{-6}$	$m s^{-1}$	Tholen and Zhu (2011)
Chloroplast envelope permeability to $CO_2$	$P_{chl, CO_2}$	$P_{p, CO_2}/2$	$m s^{-1}$	Calculated using two membrane layers of the envelope
Chloroplast envelope permeability to $HCO_3^-$	$P_{chl, HCO_3^-}$	$P_{p, HCO_3^-}/2$	$m s^{-1}$	Calculated using two membrane layers of the envelope
Mitochondrial membrane permeability to $CO_2$	$P_{m, CO_2}$	$P_{p, CO_2}/2$	$m s^{-1}$	Calculated using two membrane layers of the mitochondrion
Mitochondrial membrane permeability to $HCO_3^-$	$P_{m, HCO_3^-}$	$P_{p, HCO_3^-}/2$	$m s^{-1}$	Calculated using two membrane layers of the mitochondrion
Carboxysome shell permeability to $CO_2$	$P_{csome, CO_2}$	$D_{glut, O_2}/l_{shell}$	$m s^{-1}$	Assumed
Carboxysome shell permeability to oxygen	$P_{csome, O_2}$	$D_{glut, O_2}/l_{shell}$	$m s^{-1}$	Assumed
Carboxysome shell permeability to $HCO_3^-$	$P_{csome, HCO_3^-}$	$P_{p, CO_2}$	$m s^{-1}$	Assumed
Gluten film oxygen diffusivity	$D_{glut, O_2}$	$0.0914 \times 10^{-12}$	$m^2 s^{-1}$	Perez-gago and Krochta (2001)

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**Table I.** (Continued from previous page.)

Name	Symbol	Value	Units	References
Carboxysome shell thickness	$l_{\text{shell}}$	$0.03 \times 10^{-6}$	m	Long et al. (2007)
Chloroplast surface area fraction	$S_{\text{chl}}$	0.75	$\text{m}^2 \text{m}^{-2}$ (surface area appressed to the plasmalemma per mesophyll surface area)	Tomás et al. (2013)
Mitochondria surface area fraction	$S_{\text{mito}}$	0.12	$\text{m}^2 \text{m}^{-2}$ (surface area appressed to the plasmalemma per mesophyll surface area)	Tholen and Zhu (2011)
Cytosol viscosity	$\nu_{\text{cytosol}}$	2	Dimensionless (relative to water)	Tholen and Zhu (2011); compare with Scalettar et al. (1991) and Köhler et al. (2000)
Stroma viscosity	$\nu_{\text{stroma}}$	10	Dimensionless (relative to water)	Tholen and Zhu (2011); compare with Köhler et al. (2000)
Carboxysome viscosity	$\nu_{\text{csome}}$	$\nu_{\text{stroma}}$	Dimensionless (relative to water)	Assumed
CA $k_{\text{eq}}$ ( $[\text{HCO}_3^-] \times [\text{H}^+]/[\text{CO}_2]$ )	$k_{\text{eq}}$	$0.56 \times 10^{-6}$	$\text{mol m}^{-3}$	Pocker and Miksch (1978)
CA $k_{\text{cat}}$	$k_{\text{h,cat}}$	$0.3 \times 10^6$	$\text{s}^{-1}$	Larsson et al. (1997)
CA $K_{\text{m,CO}_2}$	$K_{\text{h,c}}$	1.5	$\text{mol m}^{-3}$	Pocker and Ng (1973)
CA $K_{\text{m,HCO}_3}$	$K_{\text{h,b}}$	34	$\text{mol m}^{-3}$	Pocker and Miksch (1978)
Rubisco maximum rate of carboxylation	$V_{\text{c,max}}$	7.6	$\text{mol m}^{-3} \text{s}^{-1}$	Bernacchi et al. (2005) <sup>b</sup>
Rubisco carboxylation rate constant	$k_{\text{c,cat}}$	2.87	$\text{site}^{-1} \text{s}^{-1}$	von Caemmerer (2000)
Rubisco Michaelis-Menten constant for $\text{CO}_2$	$K_{\text{c}}$	0.0086	$\text{mol m}^{-3}$	von Caemmerer (2000) <sup>c</sup>
Rubisco Michaelis-Menten constant for oxygen	$K_{\text{o}}$	0.215	$\text{mol m}^{-3}$	von Caemmerer (2000) <sup>c</sup>
$\text{CO}_2$ photocompensation point	$\Gamma^*$	$1.29 \times 10^3$	$\text{mol m}^{-3}$	von Caemmerer (2000) <sup>c</sup>
Maximum rate of RuBP regeneration	$J_{\text{max}}$	13.7	$\text{mol m}^{-3} \text{s}^{-1}$	Bernacchi et al. (2002) at $25^\circ\text{C}^{\text{b,d}}$
SbtA Michaelis-Menten constant for Na	$K_{\text{Na}}$	1.048	$\text{mol m}^{-3}$	Shibata et al. (2002) <sup>e</sup>
SbtA Michaelis-Menten constant for $\text{HCO}_3^-$	$K_{\text{HCO}_3^-}$	0.019	$\text{mol m}^{-3}$	Shibata et al. (2002) <sup>e</sup>
SbtA maximum rate of $\text{HCO}_3^-$ transport	$V_{\text{max,sbta}}$	$22.48 \times 10^{-6}$	$\text{mol m}^{-2} \text{s}^{-1}$	Shibata et al. (2002) <sup>e,f</sup>
BicA Michaelis-Menten constant for $\text{HCO}_3^-$	$K_{\text{m,bica}}$	0.217	$\text{mol m}^{-3}$	Price et al. (2004)
BicA maximum rate of $\text{HCO}_3^-$ transport	$V_{\text{max,bica}}$	$185 \times 10^{-6}$	$\text{mol m}^{-2} \text{s}^{-1}$	Price et al. (2004) <sup>f</sup>
BCT1 Michaelis-Menten constant for $\text{HCO}_3^-$	$K_{\text{m,bct1}}$	0.015	$\text{mol m}^{-3}$	Omata et al. (2002)
BCT1 maximum rate of $\text{HCO}_3^-$ transport	$V_{\text{max,bct1}}$	$55 \times 10^{-6}$	$\text{mol m}^{-2} \text{s}^{-1}$	Omata et al. (1999) <sup>f</sup>
NDH1,3 Michaelis-Menten constant for $\text{CO}_2$	$K_{\text{m,ndh13}}$	0.012	$\text{mol m}^{-3}$	Price et al. (2011)
NDH1,3 maximum rate of $\text{CO}_2$ hydration	$V_{\text{max,ndh13}}$	0.075	$\text{mol m}^{-2} \text{s}^{-1}$	Berner (1993) <sup>g</sup> ; David et al. (2002)

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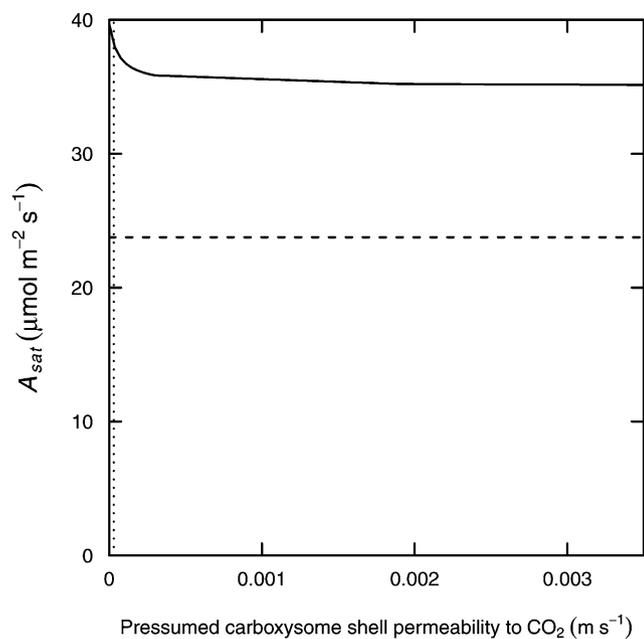
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Name	Symbol	Value	Units	References
NDH1,4 Michaelis-Menten constant for CO <sub>2</sub>	$K_{m,ndh14}$	0.002	mol m <sup>-3</sup>	Price et al. (2011)
NDH1,4 maximum rate of CO <sub>2</sub> hydration	$V_{max,ndh14}$	$V_{max,ndh13}$	mol m <sup>-2</sup> s <sup>-1</sup>	Berner (1993) <sup>g</sup> ; David et al. (2002)
Mitochondrial respiration in the light	$R_d$	1.98	mol m <sup>-3</sup> s <sup>-1</sup>	Bernacchi et al. (2002) at 25°C
Stromal CA concentration	$[CA]_{stroma}$	0.27	mol m <sup>-3</sup>	Tholen and Zhu (2011); compare with Gillon and Yakir (2000)
Mitochondrial CA concentration	$[CA]_{mito}$	$[CA]_{stroma}$	mol m <sup>-3</sup>	Assumed
Cytosolic CA concentration	$[CA]_{cyto}$	$[CA]_{stroma}/2$	mol m <sup>-3</sup>	Tholen and Zhu (2011)
Apoplasmic CA concentration	$[CA]_{apo}$	$[CA]_{stroma}/2$	mol m <sup>-3</sup>	Assumed
Stroma mesophyll volume fraction	$f_{stroma}$	0.147	m <sup>3</sup> m <sup>-3</sup> (volume per mesophyll cell volume)	Winter et al. (1994)
Cytosol mesophyll volume fraction	$f_{cytosol}$	0.054	m <sup>3</sup> m <sup>-3</sup> (volume per mesophyll cell volume)	Winter et al. (1994)
Airspace volume fraction	None	0.5	m <sup>3</sup> m <sup>-3</sup> (volume per bulk volume of leaf)	Byott (1976)
Stroma volume fraction	None	$0.87 \times 10^{-6}$	m <sup>3</sup> m <sup>-2</sup> (volume per mesophyll surface area)	Tholen and Zhu (2011)
Mitochondria volume fraction	None	$0.027 \times 10^{-6}$	m <sup>3</sup> m <sup>-2</sup> (volume per mesophyll surface area)	Tholen and Zhu (2011)
Leaf chlorophyll	None	0.5	g m <sup>-2</sup>	Daughtry et al. (2000)

<sup>a</sup>The number of carboxysomes was chosen so that the concentration of Rubisco as mol m<sup>-2</sup> was the same as a leaf without carboxysomes. <sup>b</sup>Converted to a volumetric basis from a leaf-area basis using values of 100 and 180 μmol m<sup>-2</sup> s<sup>-1</sup> for  $V_{c,max}$  and  $J_{max}$ . <sup>c</sup>Calculated from Table 1.3 of von Caemmerer (2000) using solubilities of 0.0034 and 0.00126 mol L<sup>-1</sup> bar<sup>-1</sup> for CO<sub>2</sub> and oxygen, respectively. <sup>d</sup>When the model of Farquhar et al. (1980) was developed, the rate of RuBP regeneration was assumed to be entirely limited by the rate of electron transport. Thus, the symbol  $J_{max}$  was used. It is now known that processes other than electron transport can limit RuBP regeneration. Here, we use the same symbol for continuity, but with the understanding that it represents RuBP regeneration, potentially being limited by several factors. <sup>e</sup>Values were determined from Shibata et al. (2002) by fitting data to a two-substrate sequential enzyme reaction model. <sup>f</sup>Assuming the same amount of protein per chlorophyll in cyanobacteria and leaf chlorophyll concentration from Daughtry et al. (2000). <sup>g</sup>Values for protein concentration in cyanobacteria for these enzymes were obtained through personal communication with G. Dean Price. Values were calculated assuming that higher plants would have the same protein concentration per area of thylakoid membrane as in cyanobacteria. There were multiple estimates of  $V_{max}$  and thylakoid surface area. To represent a typical value, the mean of the highest and lowest estimates was used.

assumed that RuBP regeneration was limited solely by electron transport, but it is now known that enzyme activity in the Calvin cycle also limits RuBP regeneration (Lefebvre et al., 2005). Thus, when we refer to  $J$  (for rate of RuBP regeneration) and  $J_{max}$ , this actually represents RuBP regeneration with the understanding that it may be limited by Calvin cycle activity or electron transport rate. Because assimilation is saturated at high [CO<sub>2</sub>],  $A_{sat}$  in the transformed leaf will be insensitive to the many factors that limit CO<sub>2</sub> transfer and [CO<sub>2</sub>] at Rubisco in the untransformed leaf. Therefore, the sensitivity of [CO<sub>2</sub>] within the carboxysome was also determined to assess which parameters most influence the CCM (Fig. 4). In this case, the carboxysomal [CO<sub>2</sub>] in the transformed leaf was similarly most sensitive to  $J_{max}$ , but several other parameters exerted strong influence, in particular the carboxysome shell permeability to CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>, the carboxysomal and cytosolic pH, the  $K_m$  of BicA, and the  $V_{max}$  of BicA, BCT1, and NDH1,3 (Fig. 4).

To determine how the permeability of the protein shell to CO<sub>2</sub> affects the effectiveness of the CCM, the coefficient of permeability was varied across a range of plausible values. Although low permeability to CO<sub>2</sub> is a key characteristic of the protein shell (Dou et al., 2008; Cai et al., 2009), the actual permeability to CO<sub>2</sub> has never been quantitatively measured directly or indirectly, and in this model it could only be assumed that it was similar to thin films of other proteins, in this case, whey protein isolate (Perez-gago and Krochta, 2001). Since there is such uncertainty in this parameter, it is desirable to know whether it could realistically have a value at which the cyanobacterial CCM would not be effective if introduced into a C<sub>3</sub> leaf. The lower limit is zero permeability, and a reasonable upper limit is the same permeability to CO<sub>2</sub> as lipid bilayer membranes, which are relatively permeable to small, uncharged molecules such as CO<sub>2</sub> compared with protein layers ( $P_{p,CO_2}$  compared with  $P_{csome,CO_2}$  in Table I). A modeled



**Figure 5.** Predicted  $A_{\text{sat}}$  for a  $C_3$  leaf with the simulated addition of the cyanobacterial CCM plotted against varying assumed permeabilities of the carboxysome protein shell to  $\text{CO}_2$ . The range of permeabilities was chosen so that it would encompass all plausible values. The lower limit is zero permeability, and a reasonable upper limit is the same permeability to  $\text{CO}_2$  as lipid bilayer membranes, which are quite permeable to small, uncharged molecules such as  $\text{CO}_2$ . The vertical dotted line is the best estimate for permeability, which was used in all other predictions (Figs. 1–5 and 7). The horizontal dashed line is the modeled assimilation rate of a  $C_3$  leaf without a CCM.

leaf with a CCM had higher values of  $A_{\text{sat}}$  compared with a modeled leaf without a CCM regardless of the assumed permeability of the shell to  $\text{CO}_2$  (Fig. 5). The CCM becomes more effective as shell  $\text{CO}_2$  permeability decreases, while  $A_{\text{sat}}$  shows high sensitivity to  $\text{CO}_2$  permeability around the best estimate value for this parameter (Fig. 5).

Even though  $A_{\text{sat}}$  is not improved by using a cyanobacterial Rubisco isoform due to RuBP-regeneration limitation, carboxysomal  $[\text{CO}_2]$  is nearly high enough to take advantage of the higher  $k_{\text{cat}}$  of that isoform if the RuBP regeneration rate can be increased (Fig. 6). In saturating light, a modeled leaf with a cyanobacterial CCM was more efficient than a modeled  $C_3$  leaf in terms of ATP use per net  $\text{CO}_2$  fixation by Rubisco at internal airspace  $[\text{CO}_2]$  ( $C_i$ ) below about  $300 \mu\text{mol mol}^{-1}$  (Fig. 7), whereas in light-limited conditions, the CCM was more ATP efficient at  $C_i$  below about  $150 \mu\text{mol mol}^{-1}$  (Fig. 7).

## DISCUSSION

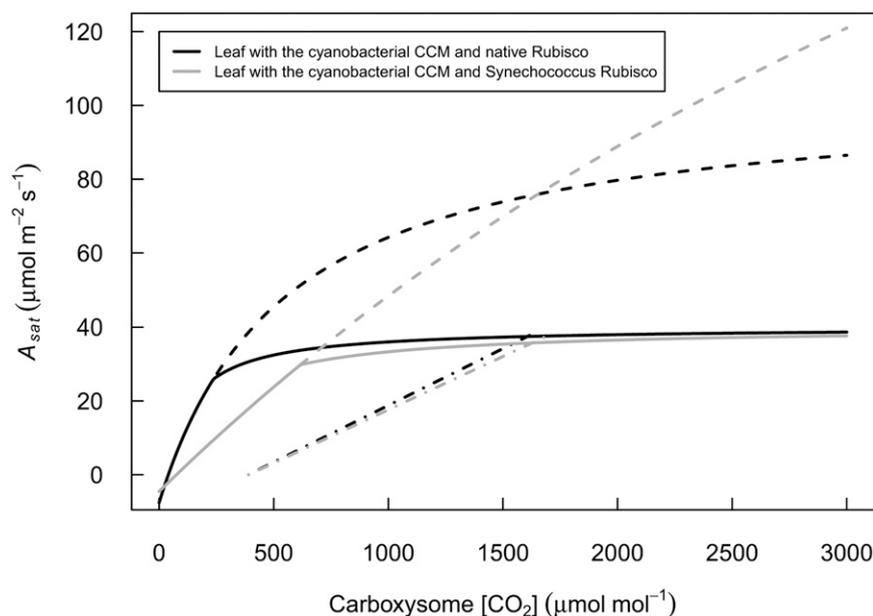
### Potential Improvement to $A_{\text{sat}}$

The increase in  $A_{\text{sat}}$  from  $23.8$  to  $38 \mu\text{mol m}^{-2} \text{s}^{-1}$  shown here from theory due to engineering a cyanobacterial CCM into  $C_3$  species could have remarkable benefits for crop production, including increased yield,

nitrogen use efficiency, and water use efficiency. Increasing leaf photosynthesis by 25% through artificial elevation of  $[\text{CO}_2]$  under open-air conditions in the field increased the yield of soybean (*Glycine max*) by 15% and water use efficiency by 20% to 40% (Long et al., 2006b; Bernacchi et al., 2007). In our simulation here, the 60% increase in  $A_{\text{sat}}$  that is predicted would by analogy allow a 36% increase in yield. The smaller increase in yield than in photosynthesis for crops grown in the field under elevated  $[\text{CO}_2]$  is suggested to result from sink limitation (i.e. the ability of the crop to make full use of the available additional photo-assimilate; Ainsworth et al., 2004; Long et al., 2006a). Identifying germplasm with sufficient capacity to form grain or seed is considered less challenging than the generation of more resource to fill those grains or seed (Sheehy et al., 2001). Therefore, it might be expected that provision of an increase in photosynthetic efficiency of 60% could, with some breeding, be matched by an equal yield increase.

The prediction here is for  $A_{\text{sat}}$  (i.e. light-saturated photosynthesis). Typically, about half of crop carbon gain is from light-limited photosynthesis (Baker et al., 1988). Here, though, an increase in photosynthesis would also be expected, since the maximum efficiency of carbon assimilation under light-limiting conditions is determined by the ratio of carboxylations to oxygenations at Rubisco. The model results indicate that the CCM was more ATP use efficient in light-limited conditions for  $C_i$  below about  $150 \mu\text{mol mol}^{-1}$  (Fig. 7). The difference between light conditions occurs because in RuBP regeneration-limited photosynthesis,  $\text{CO}_2$  uptake saturates at a lower  $C_i$  than  $\text{HCO}_3^-$  transport. Therefore, as  $C_i$  increases,  $\text{HCO}_3^-$  transport increases more than  $\text{CO}_2$  uptake and ATP/ $\text{CO}_2$  increases. In low light, photosynthesis is limited by RuBP regeneration at a lower  $C_i$ , making this effect apparent at approximately  $50 \mu\text{mol mol}^{-1} \text{CO}_2$  (Fig. 7). Although initially this suggests that the CCM is not as beneficial in light-limited conditions, if  $\text{HCO}_3^-$  transport were regulated to be proportional to light levels or inversely proportional to stromal  $[\text{HCO}_3^-]$ , the efficiency in light-limited conditions would be higher than presented here. Regulation of these transporters has not been thoroughly studied, but some are inducible (Price et al., 2013), and it is reasonable to assume that they are disabled in the dark in the cyanobacterial system or that there would be futile cycling of  $\text{CO}_2$  in the dark. That suggests that their activity could also be proportional to light intensity. Therefore, the concentration of  $\text{CO}_2$  at Rubisco would be expected to increase not just light-saturated photosynthesis but also canopy photosynthesis to a similar degree.

Cyanobacterial and algal Rubisco isoforms performed very poorly under conditions assumed to operate in the normal  $C_3$  plastid (i.e. without a CCM; Fig. 2). This is to be expected, given that they presumably reflect millions of years of evolution to operate in a high  $[\text{CO}_2]$  environment, with their low specificity resulting in high oxygenation rates in the

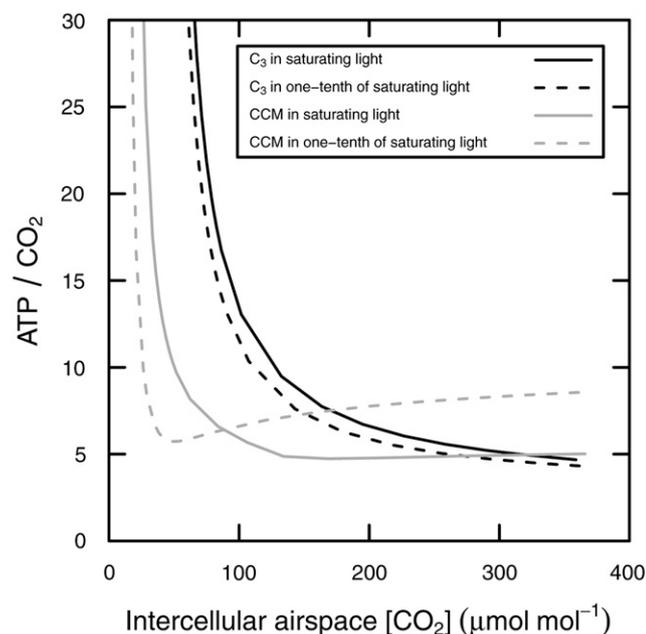


**Figure 6.** Predicted  $A_{\text{sat}}$  versus carboxysome  $[\text{CO}_2]$  for  $\text{C}_3$  (black lines) and cyanobacterial (gray lines) isoforms of Rubisco. Solid lines are modeled  $A_{\text{sat}}$ . Dashed lines are the net carboxylation rate of Rubisco if RuBP regeneration were not limiting. Dotted-dashed lines are the supply function from the atmosphere to the carboxysome. The  $[\text{CO}_2]$  at the x-intercept is atmospheric  $[\text{CO}_2]$ , where the supply function intercept assimilation rate is the  $[\text{CO}_2]$  in the carboxysome and  $A_{\text{sat}}$  in atmospheric  $[\text{CO}_2]$  ( $390 \mu\text{mol mol}^{-1}$ ).

low  $[\text{CO}_2]$  of the  $\text{C}_3$  leaf chloroplast. Although the performance of these isoforms of Rubisco was increased greatly by the addition of the cyanobacterial CCM, none of these isoforms substantially outperformed the higher plant isoforms, despite their higher turnover number ( $k_{\text{cat}}$ ) per active site. This resulted because in the modeled system, the rate of RuBP regeneration and not the rate of carboxylation was limiting, preventing a stronger response in  $A_{\text{sat}}$  and explaining the high sensitivity of the system to  $J$  (Figs. 3 and 4). Thus, at elevated  $[\text{CO}_2]$ , most Rubisco isoforms support a similar  $A_{\text{sat}}$  as simulated (Fig. 2). However, replacing the  $\text{C}_3$  crop Rubisco with a cyanobacterial isoform could still be of value. First, cyanobacterial Rubisco can assemble in a lattice form, and the carboxysome shell can enclose this. It is not known whether this is possible with higher plant or any eukaryotic Rubisco, so introducing the cyanobacterial Rubisco along with the carboxysome shell structure into the chloroplast would remove this uncertainty. Second, model results show that this CCM is capable of increasing carboxysomal  $[\text{CO}_2]$  to levels high enough to take advantage of the higher  $k_{\text{cat}}$  of the cyanobacterial Rubisco isoform (Fig. 6), which could be utilized in two ways: increasing  $A_{\text{sat}}$  by increasing the capacity for RuBP regeneration or decreasing Rubisco concentration while maintaining the same  $A_{\text{sat}}$ . Recent theoretical and practical evidence suggests that reoptimization of investment within the Calvin cycle could increase the rate of RuBP regeneration (Lefebvre et al., 2005; Zhu et al., 2007). RuBP regeneration might also be increased by increasing the maximum rate of whole-chain electron transport, which is suggested to be limited by the amount of cytochrome  $b_6/f$  complex (Price et al., 1998; Ruuska et al., 2000). However, even with RuBP regeneration

limiting photosynthesis, at high carboxysomal  $[\text{CO}_2]$ , a Rubisco isoform with a high  $k_{\text{cat}}$  would allow for lower Rubisco concentrations than with a higher plant  $\text{C}_3$  isoform but still maintain the same photosynthetic rate, improving nitrogen use efficiency (Long et al., 2004).

The cyanobacterial Rubisco isoforms saturate at much higher  $[\text{CO}_2]$  than  $\text{C}_3$  isoforms ( $K_{\text{m,CO}_2}$  of 180 compared with  $10 \text{ mmol m}^{-3}$ ; Fig. 6). The results here show that they are not saturated even at the simulated high  $[\text{CO}_2]$  of  $1,700 \mu\text{mol mol}^{-1}$  achieved with the CCM here. Therefore, further increases in  $[\text{CO}_2]$  would still be beneficial. The sensitivity of carboxysomal  $[\text{CO}_2]$  to different system features indicates that the primary targets increase  $[\text{CO}_2]$  further. Here, parameters with sensitivity coefficients greater than 0.1 were considered important (i.e. a 1% change in the parameter resulted in a greater than 0.1% change in carboxysomal  $[\text{CO}_2]$ ). Carboxysomal  $[\text{CO}_2]$  is sensitive to the  $V_{\text{max}}$  of BicA, BCT1, NDH1,3, and NDH1,4, suggesting that improving effectiveness may be as simple as increasing the activities of these enzymes (Fig. 4). In addition, the model is also sensitive to pH in both the carboxysome and cytosol (Fig. 4). The pH in the carboxysome will most likely be the same as in the stroma, since protons can readily cross the carboxysome shell (Menon, 2010), so altering carboxysomal pH would require changing stromal pH as well. However, it is not known how the diffusion of protons within the lattice of Rubisco will affect pH in the carboxysome. Since pH in both the cytosol and stroma is regulated, there are limits to altering pH in those compartments, but because sensitivity to these parameters is so high, even small changes should have a substantial effect. In addition to these factors, there are numerous anatomical features that are likely not optimized for a CCM, such as average cell volume, the exposed surface area of mesophyll



**Figure 7.** ATP use per net carboxylation by Rubisco ( $v_{c,net} = v_c - v_o/2$ ) versus intercellular airspace  $[CO_2]$  in light-saturated (solid lines;  $1,800 \mu\text{mol}$  photosynthetically active radiation  $\text{m}^{-2} \text{s}^{-1}$ ) and light-limited (dashed lines;  $180 \mu\text{mol}$  photosynthetically active radiation  $\text{m}^{-2} \text{s}^{-1}$ ) conditions for a modeled  $C_3$  leaf without (black lines) or with (gray lines) a cyanobacterial CCM.

cells, and cell wall thickness. Although altering these traits offers another route to improve the effectiveness of the CCM, examining all of them was beyond the scope of this study, whose primary aim was to assess the benefit of engineering the cyanobacterial CCM into the  $C_3$  crop chloroplast.

#### Necessary Components and an Optimal Gene-Stacking Sequence

The CCM involves many components, some of which may prove more difficult to introduce than others. For example, two of the  $HCO_3^-$  pumps, BicA and SbtA, require single transformations targeted at the plastid envelope and should be relatively easy transformations (Burnell, 2011). The carboxysome shell, by contrast, is encoded by up to nine genes, one with multiple products, that must be expressed in correct proportions, and it requires the assembly of the shell around Rubisco and CA (Price et al., 1993; Ludwig et al., 2000; Long et al., 2007, 2010; Yeates et al., 2008; Kinney et al., 2011; Rae et al., 2012). It may also be difficult to attach effective transit peptides to each component, making plastid transformation a better option. However, plastid transformation has succeeded so far in only a few species and, as yet, in none of the major grain and seed crops (Maliga, 2004; Meyers et al., 2010). Of the four key features of the cyanobacterial CCM, the  $HCO_3^-$  transporters improve

photosynthesis independently of other features (Fig. 1; Supplemental Table S1). In contrast, carboxysomes and removal of stromal CA are mutually dependent, and both have a strongly detrimental effect on simulated  $A_{sat}$  in the absence of the  $HCO_3^-$  transporters. This work suggests that while stacking all four major features of the cyanobacterial CCM would give by far the greatest benefit, an increase of 9% could be achieved by simply introducing the BicA transporter and an increase of 16% could be achieved by introducing BicA, BCT1, and SbtA together (Fig. 1A). This work also shows that this would be a valuable first step, since introducing the carboxysome and knocking out the stromal CA would be ineffective without  $HCO_3^-$  transporters. The total  $V_{max}$  for transport of  $HCO_3^-$  across the membrane is the sum of the  $V_{max}$  of each transporter. Thus, adding multiple transporters is more effective than adding a single transporter because it increases the  $V_{max}$  of total transport. In that respect, it could be equally as effective to simply express more of a single transporter than to introduce multiple transporters. However, the different affinities (Table I) suggest that the transporters allow for uptake at a wide range of  $[HCO_3^-]$ , allowing the CCM to be effective across a wider range of  $C_i$ . This would be particularly useful if the inducibility of the transporters seen in cyanobacteria (Price et al., 2013) is preserved in the  $C_3$  leaf, since that would allow for specific transporters to be inactivated at  $[HCO_3^-]$  where they are ineffective.

#### Key Model Parameters

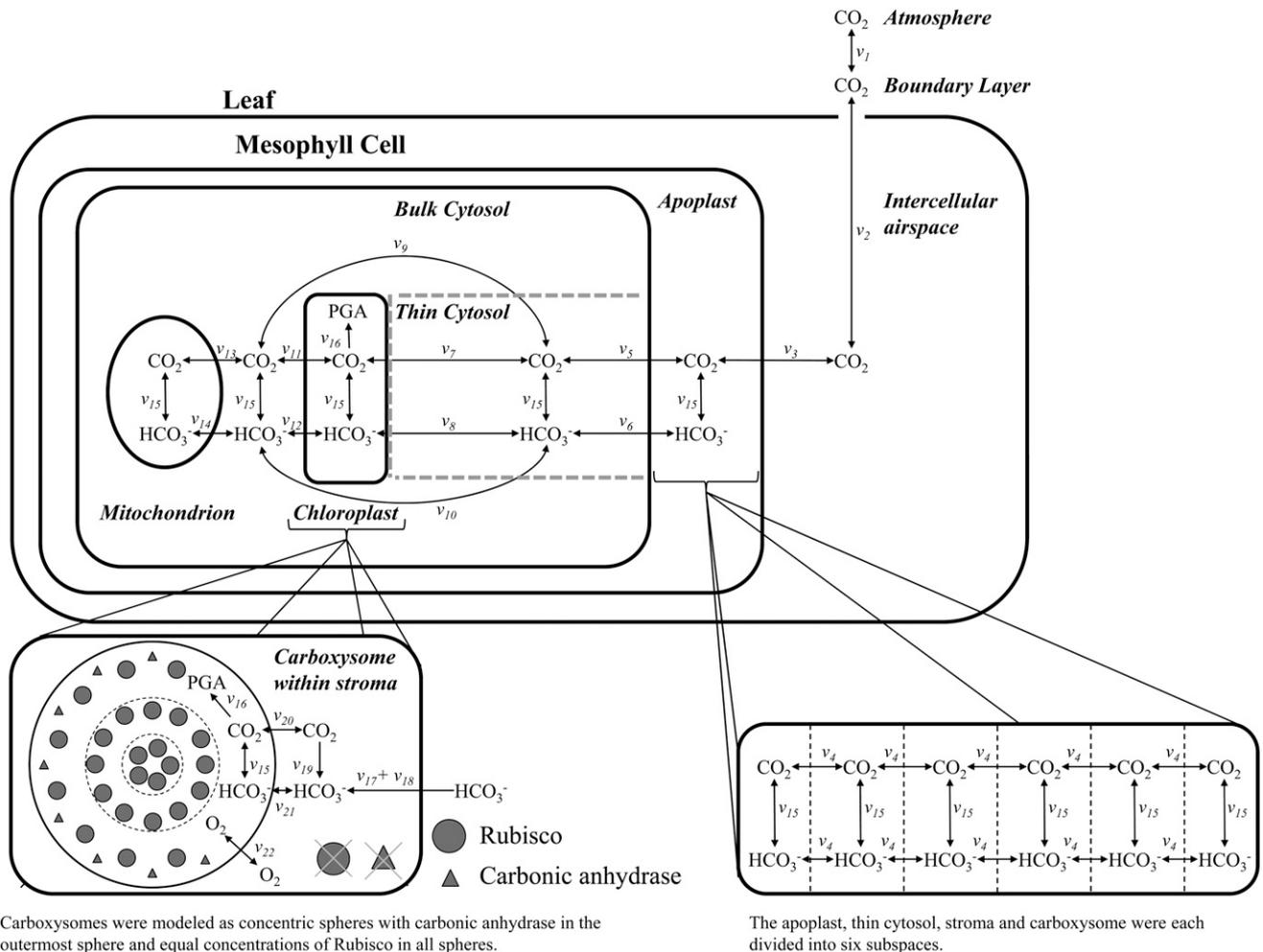
The sensitivity of modeled  $C_3$  and CCMs suggests that transformation of the complete cyanobacterial CCM into the chloroplast would result in the control of  $A_{sat}$  by stomatal conductance, the maximum rate of carboxylation by Rubisco ( $V_{c,max}$ ), and the  $CO_2$  permeabilities of the chloroplast envelope and plasma membranes would shift to  $J$ , which here refers to RuBP regeneration that can be limited by electron transport or the activity of enzymes in the Calvin cycle. As discussed earlier, increasing the amount of cytochrome  $b_6/f$  complex may be one approach to increase  $J_{max}$  (Price et al., 1998; Ruuska et al., 2000). Increasing  $J_{max}$  in  $C_3$  species certainly seems possible, since  $C_4$  species tend to have higher values of  $J_{max}$  than  $C_3$  species (Wullschleger, 1993; Massad et al., 2007). There is also evidence that, in addition to electron transport, RuBP regeneration is colimited by enzyme activity in the Calvin cycle, specifically the amount of sedoheptulose-1,7-bisphosphatase (SBPase; Harrison et al., 2001), and increasing the concentration of SBPase has been shown to increase carbon assimilation and biomass in current atmospheric  $[CO_2]$  (Lefebvre et al., 2005). Moreover, the increase in biomass in an SBPase-overexpressing line compared with the wild type was larger in elevated  $[CO_2]$  compared with control  $[CO_2]$  (Rosenthal et al., 2011), which would be expected since in saturating light, rising  $[CO_2]$  shifts control from Rubisco to

the rate of RuBP regeneration (Long et al., 2004). This would make SBPase overexpression a target to add to a plant containing a successful cyanobacterial CCM.

Assessment of the sensitivity of predicted carboxysome  $[\text{CO}_2]$  to variation in parameters in the model (Fig. 4) had two purposes: (1) identifying the most effective components of the CCM and (2) identifying parameters in the model in most need of improved constraint, to focus effort to better understand the potential value of inclusion of the cyanobacterial CCM. The high sensitivity of the system to the kinetic properties of BicA and BCT1 underline the finding that the simulated inclusion of these  $\text{HCO}_3^-$  transporters alone will apparently increase  $A_{\text{sat}}$ , making them primary first targets for transformation. The complete CCM was also sensitive to the kinetic properties of the  $\text{CO}_2$ -hydrating enzyme NDH1.3, although this will depend on the diffusivity of  $\text{CO}_2$  across the carboxysome shell. These enzymes serve the purpose of greatly steepening

the  $\text{CO}_2$  gradient between the atmosphere and stroma, thus accelerating  $\text{CO}_2$  uptake into the chloroplast. In theory, the effect of this steepened gradient should be far more effective in the leaf than in a cyanobacterium, given that diffusivity is orders of magnitude greater in air than in water. There are few estimates of kinetic parameters for these transporters, though, so there is substantial uncertainty regarding their true kinetic properties and even amounts. This highlights a key need moving forward in assessing the real value of pursuing the addition of these proteins to crop plant plastids.

Equally, the system proved very sensitive to the assumed permeability of the protein shell (Figs. 4 and 5). The CCM is beneficial in terms of increasing  $A_{\text{sat}}$  across the plausible range of permeabilities (Fig. 5), indicating that, although there is little certainty in this value, the CCM provides a benefit regardless of the true permeability of the shell. Nonetheless, predictions of  $A_{\text{sat}}$  vary strongly depending on the value used and



**Figure 8.** Compartments with the fluxes between pools in each compartment. The nine biological compartments of the model are labeled in boldface italic letters. The apoplast, thin cytosol, chloroplast stroma, and carboxysome compartments were each divided into size subcompartments to allow for nonlinear reactions of Rubisco and CA with their substrates. Pools are given in roman letters. Fluxes are labeled in italics, and rate equations for each flux are given in Supplemental Appendix S1.

are very sensitive to shell CO<sub>2</sub> permeability at the value used in this model (Figs. 4 and 5). Thus, better constraint of this parameter will be crucial to a more accurate quantification of the benefit of adding carboxysomes and the potential value of engineering the shell to decrease CO<sub>2</sub> permeability. The same argument can be made for the movement of HCO<sub>3</sub><sup>-</sup> through the shell, particularly understanding whether this is a gated transfer (Klein et al., 2009). In contrast, the model is completely insensitive to permeability of the protein shell to oxygen. This is because oxygen in the atmosphere is very high at 21% and diffuses rapidly in air, so regardless of the permeability, large amounts of oxygen would diffuse into the carboxysome. Therefore, despite the fact that the true permeability to oxygen is also highly uncertain, obtaining better estimates will do little to improve the accuracy of model predictions.

**CONCLUSION**

This simulation of the addition of the cyanobacterial CCM to a C<sub>3</sub> crop leaf suggests that a nearly 60% improvement in net leaf CO<sub>2</sub> uptake could be achieved without any modification of leaf anatomy. By analogy to the artificial elevation of [CO<sub>2</sub>] in crop fields driving increased leaf CO<sub>2</sub> uptake, this could lead to a 36% to 60% increase in C<sub>3</sub> crop yield. The simulations suggest that the addition of the single BicA transporter would alone increase photosynthesis by 9% or 16% if all transporters are added. Beyond this, there is no benefit to introducing other components of the cyanobacterial CCM unless the complete CCM is added. Addition of carboxysomes and localization of Rubisco within this microcompartment would itself significantly depress leaf CO<sub>2</sub> uptake; their benefit is only achieved by minimally adding a plastid envelope HCO<sub>3</sub><sup>-</sup> transporter such as BicA or a stromal CO<sub>2</sub> hydration enzyme such as NDH1,3 and complete removal of CA activity from the stroma. Introducing BicA and other transporters could be achieved by nuclear transformation and the inclusion of chloroplast envelope-targeting sequences. Achieving the full benefit of the cyanobacterial CCM will be considerably more challenging and most probably will depend on plastid transformation for expression of the shell proteins and an isoform of Rubisco adapted to packaging within the carboxysome.

**MATERIALS AND METHODS**

Carboxylation by Rubisco was simulated using the steady-state biochemical model of leaf photosynthesis of Farquhar et al. (1980):

$$v_c = \min \left( \frac{V_{c,max} \cdot [CO_2]}{[CO_2] + K_c \left( 1 + \frac{[O_2]}{K_o} \right)}, \frac{J \cdot [CO_2]}{4[CO_2] + 8\Gamma^*} \right) \quad (1)$$

where  $v_c$  is the rate of carboxylation by Rubisco,  $V_{c,max}$  (mol m<sup>-3</sup> s<sup>-1</sup>) and [CO<sub>2</sub>] (mol m<sup>-3</sup>) are as defined above, [O<sub>2</sub>] (mol m<sup>-3</sup>) is the oxygen concentration,  $K_c$  (mol m<sup>-3</sup>) and  $K_o$  (mol m<sup>-3</sup>) are the affinities of Rubisco for CO<sub>2</sub> and oxygen,  $J$  (mol m<sup>-3</sup> s<sup>-1</sup>) is as defined above, assumed to be limited by electron transport, and  $\Gamma^*$  (mol m<sup>-3</sup>) is the CO<sub>2</sub> photocompensation point. Values for all parameters, and their sources, are given in Table I.

Oxygenation by Rubisco was calculated as by von Caemmerer (2000):

$$v_o = v_c \cdot \frac{2 \cdot \Gamma^*}{[CO_2]} \quad (2)$$

where  $v_o$  is the rate of oxygenation by Rubisco. CO<sub>2</sub> was released in the mitochondria at a rate of  $v_o/2$ .

$J$  was calculated as a function of the amount of light absorbed by PSII ( $I_2$ ),  $J_{max}$  and a curvature factor ( $\theta$ ), using a nonrectangular hyperbolic relationship of  $J$  to  $I_2$ :

$$J = \frac{I_2 + J_{max} - \sqrt{(I_2 + J_{max})^2 - 4\theta \cdot I_2 \cdot J_{max}}}{2\theta} \quad (3)$$

The amount of light absorbed by PSII ( $I_2$ ) was calculated using an irradiance ( $I$ ) of 1,800 μmol m<sup>-2</sup> s<sup>-1</sup>, leaf absorptance ( $\alpha$ ), and a light quality correction factor ( $f$ ):

$$I_2 = I \cdot \frac{\alpha}{2} \cdot (1 - f) \quad (4)$$

Reversible conversion between HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> by CA was modeled using Michaelis-Menten kinetics:

$$v_h = \frac{k_{h,cat} \cdot [CA] \cdot \left( [CO_2] - \frac{[HCO_3^-] \cdot [H^+]}{K_{eq}} \right)}{K_{h,c} + \frac{K_{h,c}}{K_{h,b}} \cdot [HCO_3^-] + [CO_2]} \quad (5)$$

where  $k_{h,cat}$  (s<sup>-1</sup>) is the hydration rate constant, the concentrations ([CA], [HCO<sub>3</sub><sup>-</sup>], and [H<sup>+</sup>]) are measured in mol m<sup>-3</sup>, and  $K_{h,c}$  (mol m<sup>-3</sup>) and  $K_{h,b}$  (mol m<sup>-3</sup>) are the affinities of CA for CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>.

Bicarbonate transport across the chloroplast envelope by BCT1 and BicA was calculated using one-substrate Michaelis-Menten kinetics as:

$$v_t = \frac{V_{max} \cdot [HCO_3^-]}{[HCO_3^-] + K_m} \quad (6)$$

where  $V_{max}$  is the maximum rate of transport for BCT1 or BicA and  $K_m$  is the Michaelis-Menten constant of BCT1 or BicA for HCO<sub>3</sub><sup>-</sup>.

Bicarbonate transport across the chloroplast envelope by SbtA was calculated using the two-substrate, sequential reaction Michaelis-Menten kinetics as from Alberty (1953):

$$v_t = \frac{V_{max} \cdot [Na^+] \cdot [HCO_3^-]}{[Na^+] \cdot [HCO_3^-] + K_{HCO_3^-} \cdot [Na^+] + K_{Na^+} \cdot [HCO_3^-] + K_{HCO_3^-} \cdot K_{Na^+}} \quad (7)$$

where  $V_{max}$  is the maximum rate of transport for SbtA and  $K_{HCO_3^-}$  and  $K_{Na^+}$  are the Michaelis-Menten constants for HCO<sub>3</sub><sup>-</sup> and Na<sup>+</sup>, respectively. Parameters for this enzyme were determined by fitting this model to data from Shibata et al. (2002).

Diffusion of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> was modeled using various forms of Fick's law depending on the compartment. For diffusion across the boundary layer and intercellular space, a conductance was used:

$$\frac{dx}{dt} = g \cdot (x_1 - x_2) \quad (8)$$

For diffusion across a membrane, a permeability was used:

$$\frac{dx}{dt} = P \cdot S \cdot (x_1 - x_2) \quad (9)$$

And for diffusion within a gas or aqueous solution, a diffusivity was used:

$$\frac{dx}{dt} = \frac{D \cdot S}{v \cdot l} \cdot (x_1 - x_2) \quad (10)$$

where  $x_1$  and  $x_2$  are concentrations of CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> in adjacent compartments,  $g$  (mol m<sup>-2</sup> s<sup>-1</sup>) is conductance of CO<sub>2</sub> across the stomata or boundary layer,  $P$  (m s<sup>-1</sup>) is permeability of the membrane through which either CO<sub>2</sub> or HCO<sub>3</sub><sup>-</sup> is diffusing,  $S$  (m<sup>2</sup>) is the surface area of this membrane,  $D$  (m<sup>2</sup> s<sup>-1</sup>) is diffusivity of HCO<sub>3</sub><sup>-</sup> through liquid or CO<sub>2</sub> through liquid or air depending on the compartment,  $v$  (dimensionless) is the relative viscosity of the solution compared with water, and  $l$  (m) is the length of the diffusive path. Parameters were obtained by a literature search, and for anatomical characteristics, values for soybean (*Glycine max*) or similar species were used when available.

Diffusion was modeled from the atmosphere to the site of Rubisco via a potential nine compartments, as illustrated in Figure 8. The apoplast, thin cytosol, stroma, and carboxysome were each further divided into six sub-compartments (for a total of 29 distinct compartments) in order to correctly estimate nonlinear reaction rates as described below. Since the majority of CO<sub>2</sub> in the cytosol diffuses via a short path to the chloroplast (Terashima et al., 2011), the cytosol was modeled as two compartments: the thin portion of cytosol (labeled “thin cytosol” in Fig. 8) between the plasmalemma and chloroplast envelope and the bulk cytosol comprising the rest of the cytosol.

For the model, the carboxysome was assumed to approximate a sphere, with a layer of CA on the immediate inside of the shell and then concentric layers of Rubisco filling the remainder of the space. The permeability of the protein shell to CO<sub>2</sub> and oxygen has not been determined quantitatively. In the absence of these measurements, it was assumed to be similar to a protein thin film, in this case whey protein isolate (Perez-gago and Krochta, 2001), corrected for the known thickness of the carboxysome protein shell (Long et al., 2007). Similarly, the permeability of the shell to HCO<sub>3</sub><sup>-</sup> is unknown. Although HCO<sub>3</sub><sup>-</sup> does not permeate lipid membranes easily, it is thought that the hexamers of the carboxysome shell form gated pores that allow selective permeation by HCO<sub>3</sub><sup>-</sup> (Kerfeld et al., 2005; Klein et al., 2009). As a basis of comparison, it was reasoned that the shell should be as permeable to HCO<sub>3</sub><sup>-</sup> as lipid membranes are to dissolved gases, so it was assumed that the permeability of the shell to HCO<sub>3</sub><sup>-</sup> was similar to the permeability of CO<sub>2</sub> through a lipid bilayer. The consequence of uncertainty in these parameters was evaluated using a sensitivity analysis (Figs. 3–5). The number of carboxysomes was chosen so that they would contain the same number of Rubisco molecules in the C<sub>3</sub> leaf stroma, assuming 3,351 Rubisco molecules per carboxysome (Long et al., 2007), giving 2,700 carboxysomes per chloroplast. Carboxysomes are small compared with chloroplasts, and this number of carboxysomes accounts for only about 15% of the stromal volume.

Conversion between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> via CA was modeled in all aqueous compartments except in model runs that explicitly remove CA. Since the reaction rates of CA and Rubisco are nonlinear with respect to substrate concentration, the mean concentration of substrate in the compartment cannot be used to calculate reaction rate, because substrate concentration decreases along the path of net diffusion. To allow for this nonlinear activity, the apoplast, chloroplast, thin cytosol, and carboxysome were each divided into six subcompartments. The limit of the reaction rate as the number of subcompartments increases infinitely is the actual reaction rate, but there was little change in the estimated reaction rate past six subcompartments, so that number of subcompartments was deemed sufficient.

The system of differential equations (Supplemental Appendix S1) was solved by numerical integration using the Isodes algorithm from the deSolve package (Soetaert et al., 2010; version 1.10-4) in the R statistical computing language (R Development Core Team, 2012; version 2.15.2). This algorithm is part of the ODEPACK collection of differential equation solvers and was chosen for its ability to solve stiff sets of equations (Hindmarsh, 1983; Radhakrishnan and Hindmarsh, 1993). The simulation time was 20 s, which was long enough for the system to reach steady state. The maximum time step size was set to 0.02 s, and the relative error tolerance was set to  $1 \times 10^{-7}$ . Source codes to run the model are provided in the supplemental material (Supplemental Data S1 and S2).

Sensitivity was calculated as  $\frac{\Delta A_{\text{sat}}}{\Delta x} \cdot \frac{x}{A_{\text{sat}}}$ , where  $x$  is the value of the parameter. Although the true sensitivity should be calculated using the derivative  $\frac{dA_{\text{sat}}}{dx} \cdot \frac{x}{A_{\text{sat}}}$ , this is not possible here because the equations were solved numerically. Therefore, to estimate the derivative, for each parameter, the model was run three times using the literature value of the parameter (Table I), a value 10% lower than the literature value, and a value 10% higher than the literature value, producing three estimates of  $A_{\text{sat}}$ . For each parameter, a linear regression was fit to the three resulting points, with the value of the parameter as the dependent variable and  $A_{\text{sat}}$  as the independent variable. The slope of that fit was used as  $\frac{\Delta A_{\text{sat}}}{\Delta x}$ . For all parameters, a 10% change from the value used in the model was small enough that the response of  $A_{\text{sat}}$  versus  $x$  was linear. Thus,  $\frac{\Delta A_{\text{sat}}}{\Delta x}$  provided a good estimate of  $\frac{dA_{\text{sat}}}{dx}$ . A similar procedure was used to determine the sensitivity of carboxysomal [CO<sub>2</sub>] to parameters in the model using carboxysomal [CO<sub>2</sub>] as the independent variable instead of  $A_{\text{sat}}$ .

To examine the performance of different Rubisco isoforms, kinetics for different isoforms (Supplemental Table S2) were used in a model of a normal C<sub>3</sub> leaf, again with the same leaf but with the complete cyanobacterial CCM added, and with the Rubisco confined to the carboxysome.

To determine the ATP use per net carboxylation, stoichiometries for ATP per HCO<sub>3</sub><sup>-</sup> of 1, 0.5, and 0.25 were used for BCT1, SbtA, and BicA, respectively (Price et al., 2011). Net carboxylation by Rubisco was calculated as:

$$v_{c,\text{net}} = v_c - \frac{v_o}{2} \quad (11)$$

## Supplemental Data

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

**Supplemental Table S1.** Modeled  $A_{\text{sat}}$  using all combinations of cyanobacterial CCM components.

**Supplemental Table S2.** Kinetic parameters for Rubisco isoforms.

**Supplemental Data S1.** Model source code.

**Supplemental Data S2.** The odemodel package for R.

**Supplemental Appendix S1.** Rate equations.

## ACKNOWLEDGMENTS

We thank G. Dean Price for providing data for protein concentrations for NDH1,3 and NDH1,4. The visual table of contents icon was derived from an image created by Todd Yeates.

Received December 13, 2013; accepted February 16, 2014; published February 18, 2014.

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