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Engineering photoautotrophic carbon fixation for enhanced growth and productivity

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Oxygenic photosynthesis is the origin of most organic carbon compounds on Earth and an essential part of the natural carbon cycle. Cyanobacteria, the only oxygenic photoautotrophic prokaryotes, are important in several natural processes: as primary sustainable producers, in providing oxygen to the atmosphere, and in nitrogen fixation. From a biotechnological perspective, cyanobacteria are ideal cell factories since (i) the required energy and carbon source, sunlight and CO_2, are abundant and freely available, (ii) cyanobacteria are capable of producing a variety of natural products, which can be used as fuels, medicines, cosmetics *etc.*, and (iii) metabolic engineering and synthetic biology tools of cyanobacteria are being developed rapidly, making them feasible as host organisms for heterologous production of interesting compounds. However, compared to commercially employed heterotrophic microorganisms, the growth and productivity of cyanobacteria are currently not competitive. Therefore, improving cyanobacterial growth and productivity is an important task to enable commercialization of cyanobacterial bioproducts. Such studies also offer important clues for increasing the photosynthesis and yield of crop plants, which is important in view of providing food for a rapidly increasing world population. There are many strategies targeting this task, such as optimizing cultivation conditions, engineering native pathways, and introducing synthetic pathways based on an understanding of overall metabolic networks. One major limitation of cyanobacterial productivity, however, is the low efficiency of carbon fixation through the Calvin–Benson–Bassham (CBB) cycle. In this review, we introduce and discuss the possibilities to enhance growth and productivity by engineering the CBB cycle. We also give a brief discussion of options to further extend the capabilities of cells to fix inorganic carbon by the introduction of other native and artificial carbon fixation cycles.

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1. Introduction

It is predicted that by 2050, the world population will increase by at least 30% compared to 2015. Together with increased daily food consumption per individual, a 50% increase in food production will be required. It is impossible to meet this demand only by expanding the arable lands. Therefore, high yield crops and/or non-crop food will be needed. Similar to the situation with food, a much larger energy supply will also be required in the future. The energy used today is mainly fossil fuels, usage of which must be diminished due to air pollution and global warming. Therefore, increasing the crop yield and finding alternatives to fossil fuels are two of the major tasks that modern society is facing.

Food and fossil fuels both originate from oxygenic photosynthesis, which consists of a photochemical phase and a second biosynthetic phase. In the photochemical phase, solar energy is absorbed and converted into chemical energy. During this process, water is split, releasing oxygen and protons into the thylakoid lumen. At the same time, the released electrons enter the photosynthetic electron transfer chain. In the electron transfer chain, plastoquinone is reduced to plastoquinol, transferring electrons to the cytochrome b_6f complex. When plastoquinol is oxidized back to plastoquinone, protons are transferred from the cytoplasm to the thylakoid lumen.[1,2] Finally, the electrons are used to reduce $NADP^+$ into NADPH, and the generated proton gradient between the thylakoid lumen and cytoplasm drives ATP synthesis with an ATP synthase. NADPH is subsequently used in the so-called biosynthetic phase, where CO_2 is assimilated to generate the building blocks of amino acids and other carbon-containing molecules, at the same time consuming ATP and NADPH.

One method to obtain food and renewable fuels from the oxygenic photosynthetic process is to supply plant biomass to *Escherichia coli* (*E. coli*), yeasts or other well understood microorganisms for fermentation to get the desired compound(s). However, due to the multiple steps in this process, from energy absorption by plants to fermentation by heterotrophic organisms, the solar energy-to-product efficiency is very low, for example 0.2% in ethanol production from sugarcane fermentation.[3] In addition, *E. coli* and yeasts are not able to ferment all

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types of plant biomass components. For example, the most abundant carbohydrate, lignocellulose from plant materials, needs to be broken down into smaller molecules before it can be used. This breaking down process requires enzymes, high temperature, or/and high pressure.[4] Besides, using crop plants as the source of sugar for fermentation is not a viable alternative on a large scale, as it would lead to less arable land available for food production.

Another method to generate renewable fuels would be to directly obtain them from autotrophic microorganisms like cyanobacteria or microalgae. Using such organisms as green cell factories or cell catalysts has many advantages over traditional fermentation technologies. Cyanobacteria and microalgae use cheap and abundant energy (sunlight) and carbon (CO_2) sources, and also show a higher energy conversion efficiency compared to land plants (*e.g.* 1.5% in oil production from microalgae).[5] Cyanobacteria, being prokaryotes, are relatively easy to engineer compared to eukaryotic organisms. Ideal cyanobacterial cell factories are those that can maintain the cell population, distribute the remaining energy and carbon to the designed product(s), and possess the capability to excrete the product(s) out of the cells. Excreting products will minimize any potential toxicities or other negative effects on the cell metabolism, simplify recovery, and may also increase the yield.[6,7]

While the main advantage of cyanobacteria as cell factories compared to heterotrophic microorganisms is their capability for oxygenic photosynthesis, the main drawback is the relatively low yields achieved per volume and time in bioreactors, requiring large scale facilities and a large surface area.[8–10] The main reason for this is the limitations inherent to photosynthesis, and thus, increasing the photosynthetic efficiency is necessary to enhance the yield and to realize commercialization of cyanobacterial products. The photosynthetic efficiency is determined as the sum efficiency of the photochemical and biosynthetic phases. In this review, we will focus on the biosynthetic phase, discussing the control enzymes of the Calvin–Benson–Bassham (CBB) carbon fixation cycle and other natural and artificial carbon fixation cycles. Some other possible strategies to enhance growth and productivity will also be briefly discussed.

2. The Calvin–Benson–Bassham (CBB) cycle

The CBB cycle, employing one of the most abundant but also one of the least efficient enzymes in nature, ribulose-1,5-bisphosphate carboxylase (RuBisCO), to fix CO_2, is the major carbon fixation cycle in nature. It consists of 13 reactions catalysed by 11 enzymes (Fig. 1). The entire cycle is divided into 3 stages: (i) ribulose-1,5-bisphosphate (RuBP) carboxylation, (ii) 3-phosphoglycerate (PGA) reduction, and (iii) RuBP regeneration. In the carboxylation stage, RuBisCO catalyses one molecule of CO_2 and one molecule of RuBP to form two molecules of PGA. The PGA is converted into glyceraldehyde-3-phosphate (GAP), consuming ATP and NADPH generated in the photochemical phase in the reduction stage. Finally, RuBP is regenerated from GAP through several steps, also consuming ATP in the RuBP regeneration stage. Without considering the side reaction of RuBisCO where the substrate is oxygen (photorespiration), nine molecules of ATP and six molecules of NADPH are required to assimilate three molecules of CO_2 and to generate one molecule of GAP in the CBB cycle.

The cyanobacterial CBB cycle partially overlaps the oxidative pentose phosphate (OPP) pathway, the main NADPH supplier, under heterotrophic growth conditions. However, three steps are specific in the CBB cycle (Fig. 1). They are (i) fixing CO_2 into PGA by RuBisCO (consumption of RuBP), (ii) phosphorylating ribulose-5-phosphate (Ru5P) to RuBP by phosphoribulokinase (PRK, regeneration of RuBP), and (iii) dephosphorylating sedoheptulose-1,7-bisphosphate (SBP) into sedoheptulose-7-phosphate (S7P) by the bi-functional enzyme FBP/SBPase, a step in which photoautotrophic eukaryotes are catalysed by SBPase. Generally, chloroplast FBPase in plants is able to catalyse conversions between SBP and S7P; however, the catalytic rate is too low to support a normal autotrophic growth.[11] Introducing RuBisCO and PRK into a heterotrophic microorganism would theoretically complete the CBB cycle. Together with an appropriate energy supply, introducing RuBisCO and PRK would allow heterotrophic microorganisms to fix CO_2 into carbohydrates.[12] Re-fixing the CO_2 released during the carbon metabolism is a promising strategy to increase the carbon usage efficiency in heterotrophic microorganisms. Therefore, studying the CBB cycle and increasing its efficiency will potentially have beneficial effects on the efficiencies of both autotrophic and heterotrophic cell factories.

The CBB cycle is one of the limitations to higher yield and some of its enzymes are more critical than others for the overall efficiency. Therefore, identifying these steps and the corresponding enzymes is the first step towards enhancing the CBB cycle efficiency and subsequently productivity, and technologies to achieve this have been developed. Generally, increasing or decreasing the catalytic efficiency of enzyme(s) will accordingly increase or decrease the flux through the pathway. Analysis of the metabolism following enzyme engineering is one of the methods to determine the critical step(s). In this method, the term "flux control co-efficient" is used, which is determined by calculating the ratio between the change of flux and the change of specific enzyme activity under certain growth conditions.[13] The value varies between 0 (no control) and 1 (full control). In many cases, the full control comes from several enzymes, of which one of them is critical. Two things should be kept in mind: (i) specific growth conditions may be required for an enzyme to show control on the flux through the pathway, and the flux control co-efficient varies under different cultivation conditions; (ii) significant changes of the enzyme are required to detect the flux change when the capacity of the enzyme is much higher than the natural capacity of the pathway. In this case, a small flux control coefficient is expected; in other words, the enzyme is not a flux-controlling enzyme under many cultivation conditions. In the early 1990s, numerous studies on antisense plants with deduced CBB cycle enzymes explored the flux control coefficient and physiological features under different growth conditions. Table 1 summarises these studies.

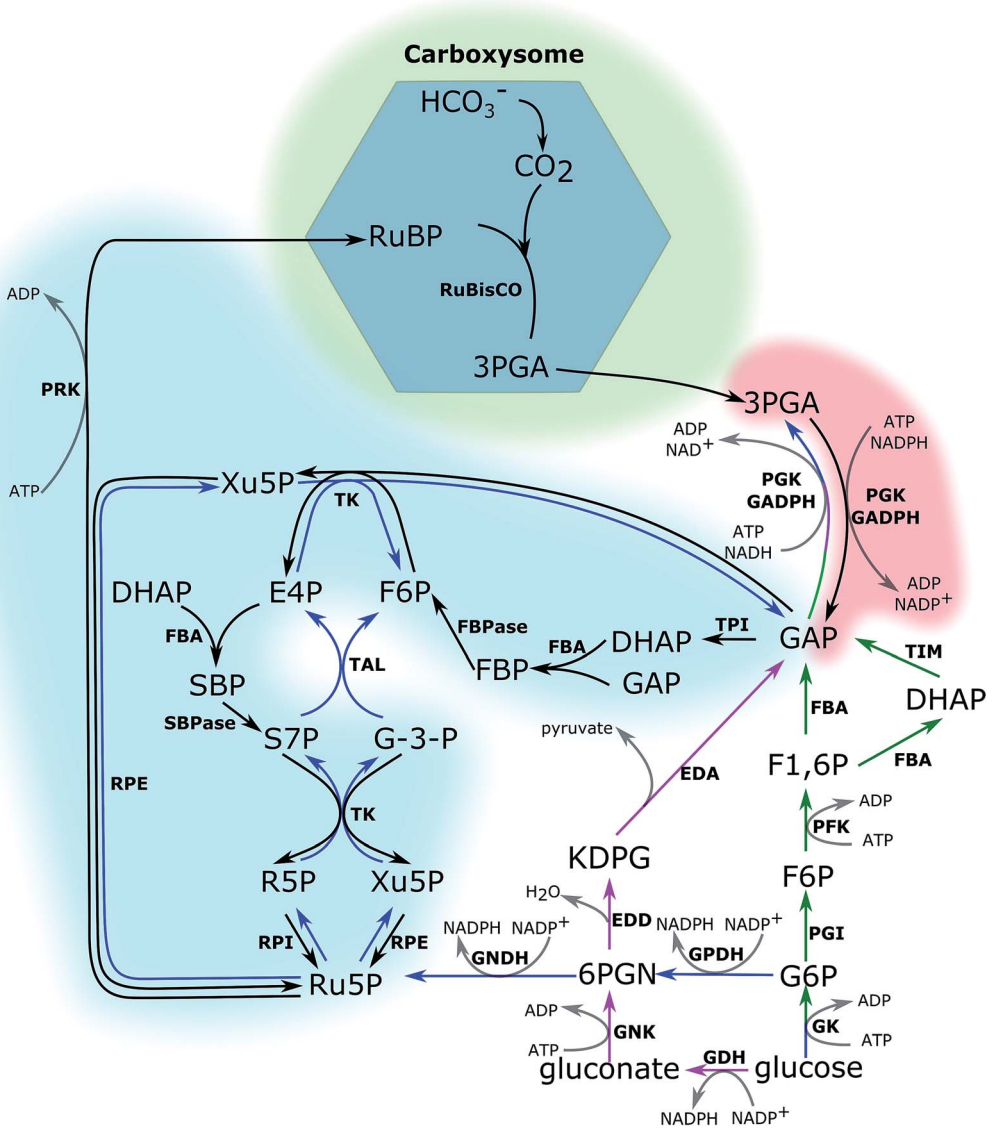

Fig. 1 Central carbon metabolic pathways in cyanobacteria. The Calvin–Benson–Bassham (CBB) cycle (black), Oxidative Pentose Phosphate (OPP) pathway (blue), glycolysis pathway (green), and Entner–Doudoroff (ED) pathway (purple). Green, red and blue shadows indicate carboxylation, PGA reduction and RuBP regeneration stages of the CBB cycle, respectively. RuBP, ribulose-1,5-bisphosphate; 3PGA, 3-phosphoglycerate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; E4P, erythrose-4-phosphate; SBP, sedoheptulose-1,7-bisphosphate; S7P, sedoheptulose-7-phosphate; R5P, ribose-5-phosphate; Xu5P, xylulose-5-phosphate; Ru5P, ribulose-5-phosphate; 6PGN, 6-phosphate gluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; G6P, glucose-6-phosphate. RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; PGK, phosphoglycerate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPI, triose phosphate isomerase; FBA, aldolase; FBPase, fructose-1,6-biphosphatase; TK, transketolase; SBPase, sedoheptulose-1,7-biphosphatase; RPI, ribose phosphate isomerase; RPE, ribose phosphate epimerase; PRK, phosphoribulokinase; TAL, transaldolase; EDA, KDPG aldolase; EDD, phosphogluconate dehydratase; GDH, glucose dehydrogenase; GNK, gluconate kinase; GK, glucose kinase; PFK, 6-phosphofructokinase; PGI, phosphoglucose isomerase; TIM, triosephosphate isomerase; GPDH, glucose-6-phosphate dehydrogenase; GNDH, gluconate-6-phosphate dehydrogenase.

2.1 RuBP carboxylation

2.1.1 Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39). In the CBB cycle, RuBisCO catalyses the first reaction in the carboxylation stage. This is the predominant way for atmospheric CO_2 to enter the biological cycle. RuBisCO is responsible for assimilating 90% of the carbon found in biomass on Earth. However, RuBisCO is well known as one of the most inefficient enzymes in nature. It cannot distinguish O_2 from CO_2 and takes one molecule of O_2 as the substrate for every four molecules of CO_2 fixed. When it uses O_2 as the substrate, a process referred to as photorespiration, 2-phosphoglycolate, which is toxic to the cell, is produced. To convert 2-phosphoglycolate into nontoxic compounds, ATP is consumed and the fixed carbon is re-released. Thus, the energy conversion and the carbon fixation efficiency of the CBB cycle decreases. However, if the photorespiration pathway is deleted,

Table 1 Physiological features of plants with deduced CBB cycle enzymes and flux control coefficient of the CBB cycle enzymes under various cultivation conditions

Enzyme	Organisms	Conditions	Effects or flux control coefficient	Reference
RuBisCO	Tobacco (*Nicotiana tabacum* L.)	15 h/9 h light/dark cycle (25 °C/20 °C), 300 μmol photons m^{-2} s^{-1}	Antisense lines (20% RuBisCO left) have longer senescence phase	14
	Tobacco (*Nicotiana tabacum* L.)	500 μmol photons m^{-2} s^{-1} irradiance (12 h light/12 h dark)	Decreased photosynthesis and nitrogen metabolism especially under higher nitrate (12 mM) or ammonium nitrate (6 mM) conditions with less than 60% RuBisCO left	15
	Tobacco (*Nicotiana tabacum* L.)	Lower than 1000 μmol m^{-2} s^{-1} irradiance, ambient CO_2	>0.2 flux control coefficient; 0.01–0.03 flux control coefficient with 100 μmol m^{-2} s^{-1} irradiance	16
		Higher than 1500 μmol m^{-2} s^{-1} irradiance, ambient CO_2	0.8–0.9 flux control coefficient	
		Limited inorganic nitrogen	0.5 flux control coefficient; photosynthesis was largely reduced while growth was hardly affected	
	Tobacco (*Nicotiana tabacum* L.)	12 h day/l2 h night cycle (irradiance 340 μmol m^{-2} s^{-1}, 20 °C, 65% relative humidity)	Reduced growth and photosynthesis, increased leaf area ratio and increased shoot-to-root ratio when less than 50% RuBisCO is left	17
	Rice (*Oryza sativa* L.)	Saturated light conditions	Strains with 65% RuBisCO activity showed a 20% lower photosynthesis rate in ambient CO_2 and a 5% to 15% higher photosynthesis rate in 100–115 Pa CO_2	18
	Rice (*Oryza sativa* L.)	16–22 °C and 28 Pa intercellular CO_2	Flux control coefficient (>0.88) detected	19
	Flaveria bidentis	Day/night growth temperatures were 28/15 °C	With less than 40% RuBisCO left, CO_2 assimilation and photosynthesis was reduced at high light irradiance but unaffected under low light condition (less than 100 μmol m^{-2} s^{-1}) on a wide range of CO_2 concentration	20
	Tobacco (*Nicotiana tabacum*)	1000 μmol m^{-2} s^{-1}, 350 microbars CO_2, and 25 °C	With 18% RuBisCO left, soluble protein content reduced the same level as RuBisCO, little change on other photosynthesis proteins. 63% reduction of CO_2 assimilation	21
	Arabidopsis thaliana	Low (200 μmol m^{-2} s^{-1}) and high (600 μmol m^{-2} s^{-1})	With 30 to 40% RuBisCo activity, photosynthesis, growth and above ground biomass are all reduced	22
	Flaveria bidentis	28/20 °C day/night temperature, 700 μmol m^{-2} s^{-1}, and a 16 h photoperiod or a naturally lit greenhouse	With 40% RuBisCO activity left, photosynthesis decreased but the H_2O exchange rate was similar to that of the wild type	23
	Flaveria bidentis	28/20 °C day/night temperature, 700 μmol m^{-2} s^{-1}, and a 16 h photoperiod or a naturally lit greenhouse	Quantum yield of PSI to PSII, PSI to CO_2 fixation and PSII to CO_2 fixation increased with enhanced irradiance in the RuBisCO decreased plant	24
SBPase	Tobacco (*Nicotiana tabacum*)	14 h light/10 h dark, 26 °C/18 °C, 600–1200 μmol m^{-2} s^{-1}	With less than 20% SBPase left, there were no changes in other CBB cycle enzymes but starch accumulation was reduced. With 57% SBPase left, the CO_2 assimilation and quantum yield of PSII reduced	25
	Tobacco (*Nicotiana tabacum*)	Saturated light conditions	Flux control coefficient: 0.31 under ambient CO_2 conditions and 0.54 under saturated CO_2 conditions	26
	Tobacco (*Nicotiana tabacum*)	Greenhouse in light levels between 400 and 1500 μmol m^{-2} s^{-1}, a 16 h photoperiod at a minimum of 25 °C light/18 °C dark and a maximum of 35 °C light/25 °C dark	Reduced growth and shoot biomass with 75% SBPase activity. SBPase decreased resulting in a shorter plant	27

Table 1 (*Contd.*)

Enzyme	Organisms	Conditions	Effects or flux control coefficient	Reference
	Tobacco (*Nicotiana tabacum* L.)	Greenhouse with a 14 h photoperiod at 25 °C light/18 °C dark, 700 to 1200 μmol m^{-2} s^{-1}	−0.2 flux control coefficient in the youngest developing leaves, 0.3–0.5 flux control coefficient in mature leaves, and decreased photosynthesis and starch accumulation in mature leaves	28
	Tobacco (*Nicotiana tabacum* L.)	Greenhouse 14 h/10 h (26 °C/18 °C) light/dark period, 1000–1400 μmol m^{-2} s^{-1}	Reductions in SBPase activity between 9% and 60%, maximum RuBP regeneration capacity declined linearly ($r^2 = 0.79$) and no significant change in Rubisco activity	29
	Rice (*Oryza sativa* L. *ssp. japonica*)	Greenhouse at 25 ± 2 °C with a photosynthetic photon flux density of 300 μmol m^{-2} s^{-1}, a relative humidity of 70% to 80%, and a photoperiod of 14 h/10 h light/dark	Stronger effects on reducing growth, photosynthesis and starch accumulation under low N than high N conditions when SBPase was deduced	30
Chloroplast FBPase	Potato (*Solanum tuberosum*)	16 h light (250 μmol m^{-2} s^{-1}, 22 °C)/8 h dark (15 °C) under greenhouse conditions	Unchanged tuber yield and reduced photosynthetic rate when FBPase activity decreased to 36% of that of the wild type. Reduced growth and photosynthetic rate when FBPase activity is below 15% of that of the wild type. <0.2 flux control coefficient	31
PRK	Tobacco (*Nicotiana tabacum* L.)	330 μmol photons m^{-2} s^{-1}, 350 ppm CO_2, 25 °C	With less than 85% PRK activities left, CO_2 assimilation was reduced. Almost 0 flux control coefficient under low light conditions	32
	Tobacco (*Nicotiana tabacum* L.)	330 μmol photons m^{-2} s^{-1}, 350 μmol CO_2 mol^{-1}, 25 °C	94% decrease in PRK has stronger inhibition on photosynthesis (by 35%) with 0.4 mM NH_4NO_3 than with 0.5 mM NH_4NO_3 (by 20%)	33
	Tobacco (*Nicotiana tabacum* L.)	330 μmol photons m^{-2} s^{-1}	0.25 flux control coefficient to CO_2 assimilation	34
		800 μmol photons m^{-2} s^{-1}	0 flux control coefficient to CO_2 assimilation	
GAPDH	Tobacco (*Nicotiana tabacum* L.)	Ambient CO_2	With less than 35% GAPDH left, photosynthesis was inhibited (900 and 1500 μmol m^{-2} s^{-1}). RuBP was reduced linearly with reduction of GAPDH. <0.2 flux control coefficient	35
TK	Tobacco (*Nicotiana tabacum* L.)	170 μmol m^{-2} s^{-1}, ambient CO_2	0.07 flux control coefficient	36
		700 μmol m^{-2} s^{-1}, ambient CO_2	0.32 flux control coefficient	
		Saturating light and CO_2	Almost 1 flux control coefficient	
FBA	Potato (*Solanum tuberosum*)	70 μmol m^{-2} s^{-1} or 390 μmol m^{-2} s^{-1}, ambient CO_2 or 800 ppm CO_2	Decrease of FBA has stronger inhibition on photosynthesis and growth under higher light and CO_2 conditions than lower light and CO_2 conditions	37
	Potato (*Solanum tuberosum*)	70 μmol m^{-2} s^{-1}, ambient CO_2	0.15 flux control coefficient	38
		390 μmol m^{-2} s^{-1} light and 400 ppm CO_2	0.21 flux control coefficient	
		390 μmol m^{-2} s^{-1} light and 800 ppm CO_2	0.55 flux control coefficient	

engineered cyanobacterial strains show a high CO_2-requiring phenotype.[39] One explanation for this is that a high concentration of CO_2 decreases the formation of toxic 2-phosphoglycolate. Furthermore, the photorespiration pathway plays a role in balancing the ATP/NADPH ratio and in synthesis of glycine and serine, which means there are physiological benefits for the cell even though photorespiration does decrease the carbon fixation efficiency.[39–41] Furthermore, the turnover rate for CO_2 fixation of RuBisCO is only 1–10 s^{-1}. From an evolutionary aspect, there are some clues to the low efficiency of RuBisCO. RuBisCO started to evolve about 3.8 billion years ago, even before the appearance of cyanobacteria and the CBB cycle. At

that time, RuBisCO's substrate CO_2 was the dominant atmospheric gas and there was no O_2 in the air. Therefore, photorespiration was not an issue. However, RuBisCO failed to evolve into a more efficient enzyme, regardless the appearance and increasing concentration of atmospheric O_2. This may be due to the balance between biochemical constraint and evolutionary pressure,[42] carboxylation velocity and CO_2 affinity,[43] and holoenzyme stability and activity.[44] Even though there has been no experimental breakthrough on modifying RuBisCO for obtaining *e.g.* a faster turnover rate and higher CO_2 affinity simultaneously so far, computer-based modelling has shown that it is possible to improve RuBisCO specificity and the turnover rate simultaneously.[45] Actually, engineered RuBisCO with an enhanced turnover rate without affecting CO_2 affinity was reported in *Synechocystis* PCC 6803 and *E. coli*.[46] One of the engineered *Synechocystis* PCC 6803 strains (Rbc^{F140I}) showed a faster photosynthetic rate than wild-type cells.

Photoautotrophic organisms have developed other strategies to deal with the issue of the changed atmospheric ratio of CO_2 and O_2, adapted to different habitation conditions. Green algae and cyanobacteria have developed special carbon concentrating mechanisms, pyrenoids in green algae and carboxysomes in cyanobacteria, to increase the CO_2 concentration near RuBisCO. Pyrenoids are also found in hornworts.[47,48] In C4 and crassulacean acid metabolism (CAM) plants, the carbon fixation process uses the relatively more efficient carboxylase phosphoenolpyruvate carboxylase (PEPc) to fix atmospheric CO_2 into a C4 acid. This C4 acid then in a later step undergoes decarboxylation to release CO_2 near RuBisCO, resulting in a high concentration of CO_2 in the vicinity of the enzyme. In another word, the initial CO_2-fixation step is separated from the RuBisCO step either in space, as is the case of C4 plants with different cell types, or in time, as in CAM plants where the reactions take place at different phases of the day/night cycles.[49] In contrast, C3 plants do not have similar compartments or mechanisms to concentrate CO_2. They instead contain a very high concentration of RuBisCO, about a quarter of the soluble proteins in leaves, consuming almost half of the available nitrogen in the cells, to compensate for the low catalytic performance.

Due to the adaption to different growth environments and available CO_2 concentrations in cells, RuBisCO enzymes purified from different sources have varied carboxylation velocity and CO_2 affinity (Fig. 2). For instance, RuBisCO in C3 plants (without carbon-concentrating mechanisms) has relatively higher CO_2 affinity and a lower carboxylation velocity compared to RuBisCO in organisms with carbon-concentrating mechanisms (C4 plants, CAM plants, hornworts, green algae and cyanobacteria).[50] Since the kinetic parameters are so different, heterologous RuBisCO expression will be a promising strategy to improve RuBisCO performance and to increase the carbon fixation efficiency, for example, introducing a higher CO_2 affinity RuBisCO into cyanobacteria. However, RuBisCO folding and assembly are complex processes involving chaperons that vary between species.[51,52] Therefore, this strategy is highly restricted.[53] In one example, a functional RuBisCO from *Synechococcus elongatus* PCC 7942 was heterologously expressed in tobacco. Even though the transformed lines could only grow autotrophically at an elevated CO_2 concentration (3% CO_2), the CO_2 fixation rate per RuBisCO protein was significantly increased.[54] Later, it was further demonstrated that co-expression of the chaperone RbcX or the carboxysome protein CcmM35 from *Synechococcus elongatus* PCC 7942 was not essential for a functional cyanobacterial RuBisCO in tobacco.[55] This indicates that *Synechococcus elongatus* PCC 7942 RuBisCO is able to fold and assemble properly with tobacco chaperons. In contrast, RuBisCO from the rhodophyte *Galdieria sulphuraria* and the diatom *Phaeodactylum tricornutum* failed to fold and assemble into functional enzymes in tobacco plants.[53] This may be mainly due to the incompatibility of RuBisCO with the assembly chaperon RbcX, since RbcX requires specific interactions with its corresponding RuBisCO and acts as a gatekeeper for formation of the holoenzyme. In contrast, folding chaperons are nonspecific and did not show negative effects on mutated RuBisCO.[46] *Synechococcus elongatus* PCC 7942 is evolutionarily closer to tobacco than the other two species. This indicates that choosing a more closely related RuBisCO to transfer to host cells may be beneficial. Following this line, heterologous expression of RuBisCO in the same groups *e.g.* from cyanobacteria to cyanobacteria is a strategy worth trying. Many studies have shown positive effects on target bioproduct formation using this strategy in host cells (Table 2). For example, overexpressing *Synechococcus elongatus* PCC 6301 RuBisCO in the very closely related strain *Synechococcus elongatus* PCC 7942 resulted in increased RuBisCO activity and

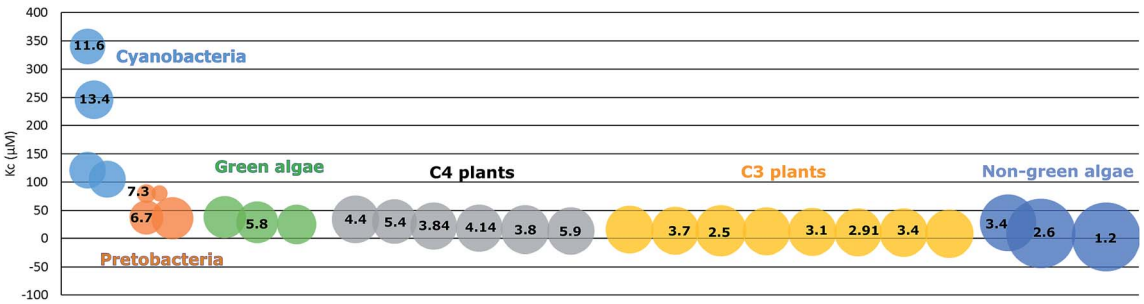

Fig. 2 Variation of RuBisCO kinetics. The size of an individual circle represents specificity S (K_oV_c/K_cV_o), ranging from 9 to 167. K_c and K_o represent CO_2 and O_2 affinity, respectively. V_c and V_o indicate the catalytic rate of carboxylation and oxygenation, respectively. The number in the individual circle represent V_c (S^{-1}) (modified from Savir *et al.* 2010).

isobutyraldehyde production.[56] Heterologous expression may avoid some of the negative regulations of protein expression and/or activity, but at the same time still use the chaperones of the host cells to fold and assemble into a functional enzyme.

2.1.2 Carbon-concentrating mechanisms. Another obvious obstacle to increase RuBisCO performance is the low concentration of CO_2 near RuBisCO. A direct method targeting this problem is to supply CO_2 or $NaHCO_3$ to the culture. Another promising method is engineering carbon-concentrating mechanisms. Increasing *in vivo* CO_2 concentration may increase RuBisCO carbon fixation for two reasons: (i) allowing the carboxylation rate to be close to the maximum turnover rate; (ii) reducing photorespiration.

In cyanobacteria, carbon-concentrating mechanisms include the inorganic carbon transporters (of CO_2 and HCO_3^-) and the special compartment named carboxysome. There are five inorganic carbon transporters in cyanobacteria; BCT1, SbtA, BicA, NDH-I_4 and NDH-I_3. The five transporters are different in expression, substrate affinity, and location[57] (Table 3). Among the five inorganic transporters, native BicA has been overexpressed in *Synechocystis* PCC 6803. It was found that in the engineered strains, the growth rate was two times faster than that of the wild-type strain, and a higher amount of biomass was accumulated.[58] When supplied with a higher CO_2 concentration (0.5%), growth of the BicA-overexpressing strains was further enhanced. This means that under atmospheric CO_2 conditions, the growth was still restricted by the carbon concentration even though BicA was overexpressed. This may be due to the low CO_2 affinity of BicA.[58] Therefore, overexpressing higher CO_2 affinity transporters may be a better way to increase growth under ambient CO_2 conditions. However, each of the other four transporters is encoded by a long operon consisting of several genes, increasing the cloning difficulties. Another promising strategy is to change low Ci induced transporters, like SbtA, BCT1 and NDH-I_3, into constitutively expressed transporters. This does not only involve controlling the transcription of the corresponding genes, but may also be related to translation and/or post-translation modifications, depending on the induction mechanisms.

In cyanobacteria, another crucial parameter in the carbon-concentrating mechanism is the special micro-compartment called carboxysome.[59] Carboxysomes encapsulate RuBisCO and carbonic anhydrase. Carbonic anhydrase converts bicarbonate into CO_2, leading to an order of magnitude enhanced CO_2 concentration near RuBisCO. Since C3 plants do not have similar compartments, transplanting carboxysomes or compartments with similar functions into C3 plants is regarded as one potential way to increase carbon fixation and plant productivity. Cyanobacteria have two types of carboxysomes, α and β carboxysomes, encapsulating form 1A and 1B RuBisCOs, respectively. Since plants have form 1B RuBisCO, it would make sense to attempt transplanting β carboxysomes into plants. Even though there are reports of successfully assembling carboxysomes in *E. coli* cells,[60] doing the same thing in plant chloroplasts is still challenging given the higher complexity of an eukaryotic cell. The assembly process of β carboxysomes in *Synechococcus elongatus* PCC 7942 has been elucidated, increasing the chance to engineer a functional carboxysome in foreign cells.[61] Functional carboxysomes with truncated proteins have also been reported.[62] This work benefits carboxysome transplanting, since shorter and/or fewer genes are required for a functional carboxysome in this case. A smaller carboxysome will also conserve carbon, nitrogen and other resources for other usages in the cell. Carbonic anhydrases also localize differently in plants and cyanobacteria, in the cytosol in plants and in the carboxysome in cyanobacteria. Therefore, how to direct a cytosolic carbonic anhydrase into a carboxysome is another issue. One possible strategy is to inactivate or delete the native cytosolic carbonic anhydrase and at the same time express a carboxysome carbonic anhydrase together with the carboxysome.

2.1.3 RuBisCO activase. RuBisCO has a complicated catalytic process. Both carboxylation and oxygenation start with the carboxylation of a specific lysine in the N-terminal domain by a non-substrate CO_2 (carbamylation) followed by Mg^{2+} binding to stabilize the protein. Then, RuBP binds to the active site and is enolized to an enediol, followed by the structural change of loop 6 and the flexible tail of the C-terminal domain. At this point, the active site is ready to bind the substrate CO_2 or O_2. During the multi-step catalytic process, RuBisCO is inhibited by sugar phosphates, like the substrate RuBP, and 2-carboxy-D-arabinitol-1-phosphate (CA1P, night-time inhibitor of RuBisCO in plants). To release the inhibitors and to reactivate RuBisCO, an AAA+ (ATPase associated with various cellular activities) protein, RuBisCO activase (Rca), is required. Rca exists in a hexameric form, changes RuBisCO active site structures and releases the inhibitors at the expense of ATP.[63] It has been reported that Rca repairs one active site at one time with minimal structural perturbation.[64] Even though studies of Rca in plants began in the 1980s, knowledge of Rca in cyanobacteria is limited. Until now, Rca has been found in some heterocystous *Anabaena* strains, but not in any unicellular or non-heterocystous cyanobacteria, even though cell extracts of *Synechococcus elongatus* PCC 7942 were reported to cross-react with antibodies raised against RuBisCO activase.[65] Therefore, Rca may not be an essential factor to maintain an active cyanobacterial RuBisCO in unicellular cyanobacteria. If this is the case, this means that some cyanobacterial RuBisCO does not require Rca for reactivation, and thus transplanting these cyanobacterial RuBisCOs into plant cells may not require expression of Rca in those cells.

2.2 3-Phosphoglycerate (PGA) reduction

Two enzymes are involved in the process of PGA reduction, a phosphoglycerate kinase (PGK, EC 2.7.2.3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.13). In the CBB cycle, NADPH and two thirds of the ATP used in the CBB cycle are consumed in these two steps. The product of this process, glyceraldehyde-3-phosphate (GAP), is one of the main outputs of the CBB cycle. In the cyanobacterial model strain *Synechocystis* PCC 6803, there are two types of GAPDH, GAPDH1 and GAPDH2, encoded by *gap1* and *gap2*, respectively.[66] GAPDH2 catalyses the reaction in the CBB cycle and is redox regulated through associating and dissociating with the small

Table 2 Effects of overexpressing the CBB cycle enzymes in cyanobacteria and plants reported in the literature

Enzyme	Host	Source	Main effects	Reference
RuBisCO	*Synechococcus elongatus* PCC 7942	*Allochromatium vinosum*	1.5 to 4 fold increase of RuBisCO activity; 1.6 times higher total photosynthesis activity	77
	Synechococcus elongatus PCC 7942	*Synechococcus elongatus* PCC 6301	1.4 fold increase in total RuBisCO activity; unchanged photosynthetic O_2 production; 2 fold higher isobutyraldehyde production	56
	Synechococcus elongatus PCC 7942	*Synechococcus elongatus* 7942	Unchanged oxygen evolution and free fatty acid production	78
	Synechococcus PCC 7002	*Synechococcus elongatus* PCC 7942	3 fold increase of free fatty acid production	79
	Tobacco, *Nicotiana tabacum*	*Synechococcus elongatus* PCC 7942	Higher rates of CO_2 fixation per unit of enzyme, autotrophic growth only rely on the cyanobacterial RuBisCO under 3% CO_2 conditions	54
	Synechococcus elongatus PCC 7942	*Synechococcus* PCC 7002	Positive effects on growth and 2,3-butanediol production using 10 g L^{-1} glucose in the BG11 medium when cooverexpressed with PRK and OPP enzymes, and the galactose transporter	80
	Synechococcus elongatus PCC 7942	*Synechococcus elongatus* PCC 7942	No significant effects on growth and 2,3-butanediol production using 10 g L^{-1} glucose in the BG11 medium	80
	Synechocystis PCC 6803	*Synechocystis* PCC 6803	Increased RuBisCO content when the FLAG tag or *ccmM* gene was used; increased growth, biomass accumulation and oxygen evolution under 100 μmol photons m^{-2} s^{-1} light intensity; increased ethanol production with air	81,82
	Synechococcus PCC 7002	*Synechococcus* PCC 7002	No impact on growth and O_2 evolution, increased protein content of pyruvate metabolism and fatty acid biosynthesis	83
Chloroplastic FBPase	*Anabaena* PCC 7120	Wheat	1.4 fold higher FBPase activity, increased net photosynthesis (117.2%) and true photosynthesis (122.5%), faster growth and more chlorophyll a under atmospheric conditions (360 μmol mol^{-1} CO_2)	84
	Chlamydomonas reinhardtii	*Chlamydomonas reinhardtii*	1.4 fold higher FBPase activity and slower photoautotrophic growth with or without elevated CO_2 levels	85
Form II FBPase	Tobacco, *Nicotiana tabacum cv. Xanthi*	*Synechococcus elongatus* PCC 7942	Transformant line with 2.3 fold higher FBPase activity had higher dry matter under atmospheric conditions, photosynthetic activity at saturated light intensity, RuBP level, *in vivo* RuBisCO activation state, and hexose, sucrose and starch concentrations in upper and lower leaves	86
FBP/SBPase	Tobacco	*Synechococcus elongatus* PCC 7942	Increased photosynthetic CO_2 fixation, photosynthesis, growth, RuBP and GAP concentrations, initial RuBisCO activity (probably due to the higher RuBP concentration) under atmospheric conditions	87
	Euglena gracilis	*Synechococcus elongatus* PCC 7942	Larger cell volume at 100 μmol photons m^{-2} s^{-1} and 0.04% CO_2, enhanced biomass and photosynthetic activity under high light and high CO_2 conditions, increased wax ester production on anaerobiosis	88

Table 2 (*Contd.*)

Enzyme	Host	Source	Main effects	Reference
	Soybean, *Glycine max*	Cyanobacteria	Increased carbon assimilation (4–14%), RuBP regeneration capacity (4–8%), and maximum carboxylation rate of the RuBisCO (5–8%); maintained seed yield at an elevated CO_2 level (600 ppm) and higher temperature	89
	Synechocystis PCC 6803	*Synechocystis* PCC 6803, *Synechococcus elongatus* 7942, and *Synechococcus* PCC 7002	Increased growth, biomass accumulation and oxygen evolution at 100 μmol photons m^{-2} s^{-1} light intensity; increased ethanol production with air	72,82
	Synechococcus PCC 7002	*Synechococcus* PCC 7002	Enhanced FBP/SBPase, RuBisCO, and FBA activity, increased cell volume, growth, O_2 evolution rate, CO_2 assimilation and non-storage carbohydrate accumulation	83
SBPase	Tobacco, *Nicotiana tabacum*	*Arabidopsis thaliana*	Higher photosynthetic capacity, RuBP regeneration capacity, sucrose and starch accumulation, leaf area at the 4–5 leaf stage, and carbon fixation (6–12%)	90
	Tobacco, *Nicotiana tabacum cv. Xanthi*	*Chlamydomonas*	Increased SBPase activity (1.6 or 4.3 fold), dry matter, photosynthetic CO_2 fixation, growth rate, RuBP contents and RuBisCO activation state	86
	Rice, *Oryza sativa* L.	Rice, *Oryza sativa* L.	Increased CO_2 assimilation at 30 °C and maintained CO_2 assimilation above 30 °C	91
	Tobacco, *Nicotiana tabacum*	*Arabidopsis thaliana*	Enhanced carbon assimilation and RuBP regeneration capacity under elevated CO_2 conditions (585 ppm)	92
	Arabidopsis thaliana	*Arabidopsis thaliana*	Increased SBPase activity, leaf area, and photosynthetic carbon fixation rate	93
FBA	Tobacco, *Nicotiana tabacum*	*Arabidopsis thaliana*	Enhanced growth and increased biomass especially at a high CO_2 concentration	94
	Arabidopsis thaliana	*Arabidopsis thaliana*	Increased FBA and FBA activity, photosynthetic CO_2 assimilation rate and more seeds and aerial biomass	95
	Tobacco, *Nicotiana tabacum*	*Arabidopsis thaliana*	Enhanced CO_2 assimilation rate, electron transporting rate, photosynthesis and leaf area (even combined with SBPase overexpression)	96
	Arabidopsis thaliana	*Arabidopsis thaliana*	Increased FBA activity, PSII efficiency, maximum CO_2 fixation efficiency, and leaf area	93
	Chlorella vulgaris	*Synechocystis* PCC 6803	FBA activity increased about 1.3 fold; increased CO_2 fixation rate, growth rate and biomass accumulation	97
	Synechocystis PCC 6803	*Synechocystis* PCC 6803	Increased growth, biomass accumulation and oxygen evolution at 100 μmol photons m^{-2} s^{-1} light intensity; increased ethanol production with air	72,82
TK	Tobacco, *Nicotiana tabacum*	*Arabidopsis thaliana*	Increased TK activity, decreased thiamine levels and leaf area	98
	Synechocystis PCC 6803	*Synechocystis* PCC 6803	Increased biomass accumulation at 15 and 100 μmol photons m^{-2} s^{-1} light intensity; increased ethanol production with air	72,82
RuBisCO (the small subunit) and TK	Rice, *Oryza sativa*	Rice, *Oryza sativa*	Increased RuBisCO and TK amounts,; similar CO_2 assimilation rate to the wild type	99

redox-sensitive protein CP12 and phosphoribulokinase (PRK, EC 2.7.1.19).[67] The three proteins form a complex when the NAD(P)H level is low (for example in darkness).[67] Formation of the complex is essential, specifically in mixotrophic growth or light/dark cycles due to the competition between the CBB cycle and OPP pathway. Ribulose-5-phosphate is an intermediate in

both the CBB cycle (substrate of PRK) and the OPP pathway (substrate of ribulose-5-phosphate epimerase/isomerase). This means that PRK competes for ribulose-5-phosphate with ribulose-5-phosphate epimerase/isomerase. In darkness, cyanobacteria rely on the OPP pathway to generate reducing equivalents (mainly NADPH). If the OPP pathway is inhibited in darkness (by PRK assimilating ribulose-5-phosphate in this case), there will not be enough reducing equivalents and cell growth will be hampered. However, it is not the same situation when cells are grown in continuous light, since the photochemical phases will supply cells with enough NADPH. Therefore, the role of GAPDH should attract attention, especially when cells are exposed to light/dark cycles. However, in anti-sense tobacco plants with reduced levels of chloroplast GAPDH, carbon assimilation under ambient conditions was not inhibited unless less than 30–40% of the wild-type GAPDH activity was left.[35] This means that GAPDH does not have a significant flux control co-efficient for the CBB cycle.

2.3 Ribulose-1,5-biphosphate (RuBP) regeneration

As discussed above, there are 11 enzymes in the CBB cycle and most of them are in the stage called "RuBP regeneration". This stage is crucial to maintain the carbon fixation rate especially under relatively low light conditions. This concept was first suggested in the 1980s, from a CBB cycle model of C3 plants.[68] In this stage, the last reaction, catalysed by PRK, consumes the remaining one-third of the ATP used in the whole CBB cycle. Interestingly, reducing the expression levels of PRK in anti-sense tobacco plants did not affect carbon assimilation and growth until the activity of PRK was decreased by more than 85%[32] even though it catalyses an irreversible reaction. This indicates only minor control on the carbon flux by PRK.

Three enzymes which have been reported to play important roles in controlling the CBB cycle flux are aldolase, fructose-1,6/sedoheptulose-1,7-biphosphatase and transketolase.[69,70] Aldolase (FBA, EC 4.1.2.13) catalyses the aldol condensation of dihydroxyacetone phosphate (DHAP) and GAP to fructose-1,6-bisphosphate (FBP) or DHAP and erythrose-4-phosphate (E4P) into sedoheptulose-1,7-bisphosphate (SBP) in the CBB cycle. Since SBP is not an OPP pathway intermediate, one can say that FBA catalyses a reversible reaction between FBP and DHAP and GAP and an irreversible reaction between SBP and DHAP and E4P. There are two classes (I and II) of FBA, defined based on the catalytic mechanisms and distributions in the biosphere. Their respective genes do not show DNA sequence homology, which implies separate evolution paths. Class II FBA catalyses reactions in the CBB cycle in both *Synechocystis* PCC 6803 and plants. The function of class II FBA cannot be replaced by class I FBA in *Synechocystis* PCC 6803 under autotrophic growth conditions, indicating that class I FBA is not able to catalyse the reactions in the CBB cycle.[71]

Early studies of FBA in controlling photosynthesis and growth of potato plants showed that reduction of plastid FBA activity by more than 30% inhibits photosynthesis under 400 μmol photons m^{-2} s^{-1} irradiance and ambient carbon conditions.[37,38] The slow regeneration of RuBP is responsible for the phenomenon. Activities of plastid FBPase and PRK are also reduced in FBA antisense potato lines. These results indicate that even though FBA catalyses reversible reactions, it has significant control over the CBB cycle carbon flux. This is further confirmed in other plants, microalgae and cyanobacteria (Table 2). When class II FBA is overexpressed, growth, biomass accumulation, and/or CO_2 assimilation are increased under certain growth conditions. Recently, a report showed that overexpressing FBA in engineered *Synechocystis* PCC 6803 increases the production of ethanol.[72]

The next metabolic steps in the CBB cycle after FBA are catalyzed by plastid FBPase (EC 3.1.3.12), dephosphorylating FBP into fructose-6-phosphate (F6P) and SBPase (EC 3.1.3.37), and dephosphorylating SBP into sedoheptulose-7-phosphate (S7P) in plants. Plastid FBPase and SBPase have very different evolutionary origins. Plastid FBPase originated from bacteria and was transferred into plant chloroplasts by endosymbiosis, while SBPase originated from archaea.[11] Regardless of the different evolution paths, the two enzymes share high structural homology, with differences only on the solvent-exposed surface area. Plant SBPase and most plastid FBPases, except some later emerging plastid FBPases in land plants,[73] contain the redox regulation target, cysteine.[74] The major redox regulator is thioredoxin *f*, which is reduced by ferredoxin. Therefore, the activities of FBPase and SBPase are regulated by light. They are also inhibited by glycerate.[75] These regulatory mechanisms allow dedicated regulation of plastid FBPase and SBPase. In cyanobacteria, the bi-functional enzyme FBP/SBPase plays the same role as plastid FBPase and SBPase. However, cyanobacterial FBP/SBPase does not share amino acid homology with plant plastid FBPase and SBPase. Therefore, existence and mechanisms of the redox-regulations, if there are any, of cyanobacterial FBP/SBPase are unclear.

Products of FBPase and SBPase are points where carbon leaves the CBB cycle in the form of carbohydrate. This makes these enzymes important in keeping the input and output of the CBB cycle balanced. A C3 plant model shows that the plastid FBPase and SBPase contents are very low compared to levels of other CBB cycle enzymes.[68] Concentrations of most of the CBB cycle enzymes are higher than those of their corresponding substrates, while FBPase and SBPase are present in similar concentrations to their substrates in *Chlamydomonas reinhardtii*.[76] It has been shown that dramatic reduction of the levels of plastid FBPase in potato (less than 15% activity remained) and SBPase in tobacco (less than 20% activity remained) have negative effects on plant growth.[25,31]

To summarize, plastid FBPase and SBPase are redox regulated, catalyse irreversible reactions, and occur at low levels compared to the other CBB cycle enzymes, and these reduced levels result in growth deficiencies. Therefore, plastid FBPase and SBPase may be crucial enzymes in controlling the carbon flux through the CBB cycle, maybe by affecting the RuBP regeneration. Furthermore, there are plenty of experimental reports where increasing FBPase and/or SBPase through genetic engineering in plants, algae or cyanobacteria led to enhanced growth and/or photosynthesis (Table 2). In *Synechocystis* PCC 6803, increasing the content of FBP/SBPase, either a native or

Table 3 Inorganic carbon transporters in cyanobacteria

Transporter	Category	Expression	Features	Location
BCT1	Traffic ATPase, HCO_3^-	Induced under Ci limited conditions, enhanced transcription under high light	High affinity (5 μM *Synechococcus elongatus* PCC 7942 BCT1); medium to low flux rate	Plasma membrane
SbtA	Na^+-dependent HCO_3^- transporter	Induced under Ci limited conditions	High affinity (2 μM *Synechococcus* PCC 7002 SbtA); low flux rate	Plasma membrane
BicA	Na^+-dependent HCO_3^-transporter	Induced under Ci limited conditions in *Synechococcus* PCC 7002; constitutively expressed in *Synechocystis* PCC 6803	Low affinity (≈38 μM of *Synechococcus* PCC 7002 BicA); fast flux rate	Plasma membrane
NDH-I_4	CO_2 uptake	Constitutively expressed	Relatively high affinity (≈10 μM)	Plasma membrane
NDH-I_3	CO_2 uptake	Induced under Ci limited conditions	High affinity (1–2 μM)	Thylakoid membrane

a non-native version, resulted in increased ethanol production in the engineered strains (Table 2).

The next step in regeneration of RuBP is catalysed by transketolase (TK, EC 2.2.1.1). In general, reactions catalysed by transketolase are reversible. It uses GAP and S7P to produce xylulose-5-phosphate (Xu5P) and ribose-5-phosphate (R5P), or GAP and F6P to produce Xu5P and erythrose 4-phosphate (E4P). Activation of TK requires the cofactor thiamine diphosphate (TPP), the active form of thiamine (vitamin B1). The synthesis of TPP in turn requires GAP and R5P as substrates. Therefore, there may be some circuit regulation on TK activity and TPP levels. E4P is also one of the precursors of the shikimic acid pathway, which is one of the connections between central and secondary carbon metabolisms. Thus, TK is another branch point where fixed carbon leaves the CBB cycle. The indirect regulation by its own product (R5P) and substrate (GAP) and the role in connecting the secondary metabolism to the central carbon metabolism make TK one of the key enzymes in controlling the carbon flux through the CBB cycle. In tobacco anti-sense plants, a 20% reduction of TK activity resulted in decreased photosynthesis and growth. Formation of aromatic amino acids and the phenylpropanoid metabolism pathway are also inhibited.[36] Under saturated light (700 μmol photons m^{-2} s^{-1}) and CO_2 (5000 ppm) conditions, it was found that TK had full control over the maximum photosynthesis rate, meaning that photosynthesis was linear with TK activity under these conditions. In contrast, RuBisCO does not exert any control over the carbon flux under these conditions. Even though it is unexpected that TK would have a strong control over the CBB cycle carbon flux since it catalyses reversible reactions and is regarded as a non-finely regulated enzyme, positive effects on carbohydrate accumulation, leaf size or end product yield are observed by overexpressing this enzyme (Table 2).

3. Alternative carbon fixation cycles

Organisms that utilize CO_2 (or HCO_3^-) as the sole carbon source are defined as autotrophic organisms. The energy source of autotrophic organisms can be solar energy (like cyanobacteria and plants, photoautotrophs) or chemical energy (chemolithoautotrophs). The CBB cycle is found in most photoautotrophs. Since efforts at optimizing RuBisCO performance and improving the CBB cycle efficiency have shown only limited success, interest has been redirected towards other carbon fixation cycles and carboxylases. Alternative carbon fixation cycles found in archaea include the reductive acetyl-CoA cycle, the reductive TCA (rTCA) cycle, the 3-hydroxypropionate (3-HP) cycle, and the 4-hydroxybutyrate cycles (3-hydroxypropionate/4-hydroxybutyrate, 3-HP/4-HB cycle, and dicarboxylate–4-hydroxybutyrate, DC/4-HB cycle[100]) (Table 4). In some archaea, there is form III RuBisCO. However, form III RuBisCO does not support autotrophic growth and no complete CBB cycle has been found in these archaea.

One standard to evaluate carbon fixation cycles is the carbon fixation efficiency, which is mainly estimated from reaction kinetics and ATP requirements. ATP requirements in a carbon fixation cycle are largely affected by the carbon fixation enzyme(s).[101] Some carboxylases directly or indirectly catalyse the assimilation of CO_2 associated with ATP hydrolysis. For example, in the CBB cycle, the CO_2 assimilation using RuBisCO is associated with ATP usage in the step catalysed by phosphoglycerate kinase. In contrast, α-ketoglutarate:ferredoxin oxidoreductase in the reductive TCA cycle assimilates CO_2 associated with reduction of ferredoxin. This will reduce the overall ATP requirement in the cycle. Therefore, to synthesize one molecule of acetyl-CoA, 7 *versus* 2 ATPs are required in the CBB cycle and the reductive TCA cycle.[102] Among all the native cycles, the reductive acetyl-CoA pathway is special in respect to the carbon fixation reactions. In the reductive acetyl-CoA pathway, five sixths of the carbon is assimilated by formate dehydrogenase and CO dehydrogenase/acetyl-CoA synthase. Therefore, the carbon assimilation in this cycle is more reduction dependent, which makes this cycle more energy efficient, and it is predicted to have a higher biomass yield.[103] In general, the ATP efficient cycles are naturally found under anaerobic conditions while aerobic archaea usually have the ATP

Table 4 Native biological carbon fixation cycles with individual specific carbon assimilation enzyme(s)

Pathway	Electron donor	Carbon fixation enzyme(s)	Oxygen sensitivity
CBB cycle	H_2O	RuBisCO	−
Reductive acetyl-CoA pathway	H_2	Formate dehydrogenase	+
		CO dehydrogenase/acetyl-CoA synthase	
Reductive TCA cycle	H_2S	Isocitrate dehydrogenase	−
		α-Ketoglutarate:ferredoxin oxidoreductase	+
		Pyruvate:ferredoxin oxidoreductase	+
		Phosphoenolpyruvate carboxylase	−
3-HP bicycle	S_2-, H_2	Acetyl-CoA/propionyl-CoA carboxylase	−
3-HP/4-HB cycle	FeS_2	Acetyl-CoA/propionyl-CoA carboxylase	−
DC/4-HB cycle	H_2	Pyruvate:ferredoxin oxidoreductase	+
		Phosphoenolpyruvate carboxylase	−

inefficient cycles. This makes it challenging to transplant ATP efficient cycles into aerobic organisms, such as cyanobacteria or *E. coli*. Of course, intermediate compatibility is another challenge, since foreign intermediates due to non-native gene expression may interfere with the native cell metabolism.

Recently, another potential CO_2 assimilation cycle was identified in the dissimilatory phosphite oxidation (DPO) bacterium *Candidatus phosphitivorax*.[104] In this process, phosphite is oxidized, thereby providing reducing equivalents, and NADP-dependent formate dehydrogenase assimilates CO_2 into the formate. Afterwards, the formate is converted into pyruvate through the reductive glycine pathway.[105] Since phosphite oxidation to phosphate is an efficient electron providing process, this process may drive cell growth under energy limited conditions. The carbon fixation during this process is mainly reduction dependent, which means it is more ATP efficient than the CBB cycle. If an oxygen insensitive formate dehydrogenase was applied, the reductive glycine pathway will be oxygen tolerant. This makes it promising to be transplanted into aerobic organisms.

Instead of understanding, transplanting and engineering native carbon fixation cycles, assembling an artificial carbon fixation cycle with potentially more efficient carbon fixation enzymes is another attractive strategy to improve the carbon fixation efficiency. Bar-Even *et al.* examined 5000 native existing enzymes for alternative synthetic carbon fixation cycles.[101] Focusing on the cycle kinetics, thermodynamic feasibility, oxygen tolerance, and high energy efficiency (the amount of NADPH equivalents and ATP equivalents to produce one molecule of the product), they modelled a pathway using PEPc to fix CO_2, generating glyoxylate, which was named the malonyl-CoA-oxaloacetate-glyoxylate (MOG) pathway. The starting reactions of this pathway overlap with the C4 carbon fixation pathway in the plant mesophyll cell, but the MOG pathway does include the reactions in the bundle-sheath cells, therefore avoiding the usage of RuBisCO. Computational modelling indicated that this pathway is potentially 2- to 3-fold faster at fixing carbon than the CBB cycle.[101] Although the whole cycle has not been tested experimentally yet, it has been reported that overexpressing PEPc in *Synechocystis* PCC 6803 enhanced cell growth under low light conditions.[106]

There is another synthetic carbon fixation cycle, crotonyl-coenzyme A (CoA)/ethylmalonyl-CoA/hydroxybutyryl-CoA (CETCH) cycle, which has been examined *in vitro*.[107] The CETCH cycle uses enoyl-CoA carboxylases/reductases, which are not present in autotrophic CO_2 fixing organisms, to carboxylate CO_2. After enzyme modification or replacement, a well functional version of this cycle, CETCH 5.4, was generated and tested *in vitro*. This cycle includes 13 enzymes in the carbon fixation cycle and 5 enzymes for auxiliary proofreading (converting the CO_2 fixation product glyoxylate into malate) and cofactor regeneration. CETCH 5.4, in which two CO_2 molecules were fixed into glyoxylate at the expense of two ATPs and three NADPH, is more energy-efficient than the CBB cycle.[107]

Both the MOG cycle and the CETCH 5.4 are promising CBB cycle alternatives. However, introducing synthetic pathways into living organisms is challenging because of the unpredictable interference between the artificial metabolism and the background native metabolism of the host. Testing the function of the synthetic pathways *in vitro* while considering and mimicking *in vivo* conditions can provide useful guides on how to introduce complicated synthetic pathways into living organisms. Even though there are no reports about introducing new complete CO_2 fixation pathways into photoautotrophic organisms, there is one example of an artificial pathway to compensate the CO_2 loss when pyruvate is converted into acetyl-CoA in *Synechococcus elongates* PCC 7942.[108]

4. Further strategies to improve the photosynthetic efficiency

One of the main reasons that oxygenic photoautotrophic organisms (plants, cyanobacteria and algae) are promising cell factories is that they can directly convert solar energy into chemical energy. Solar energy reaching the earth in one hour is enough for global human activities in one year. If sustainable methods are developed to capture, convert and store solar energy, it will be the infinite fuel for the future. Solar panels are a well-developed technology in capturing and converting solar energy into electricity. However, they do not capture CO_2. Photoautotrophic organisms like cyanobacteria and algae are more sustainable in this aspect. However, the solar energy conversion efficiency of even the most efficient photoautotrophic organisms is much lower than that of current commercial solar cells (4.6–6% *versus* 18%).[102]

In photoautotrophic organisms, the antenna complexes absorb and transfer solar energy to the photochemical reaction

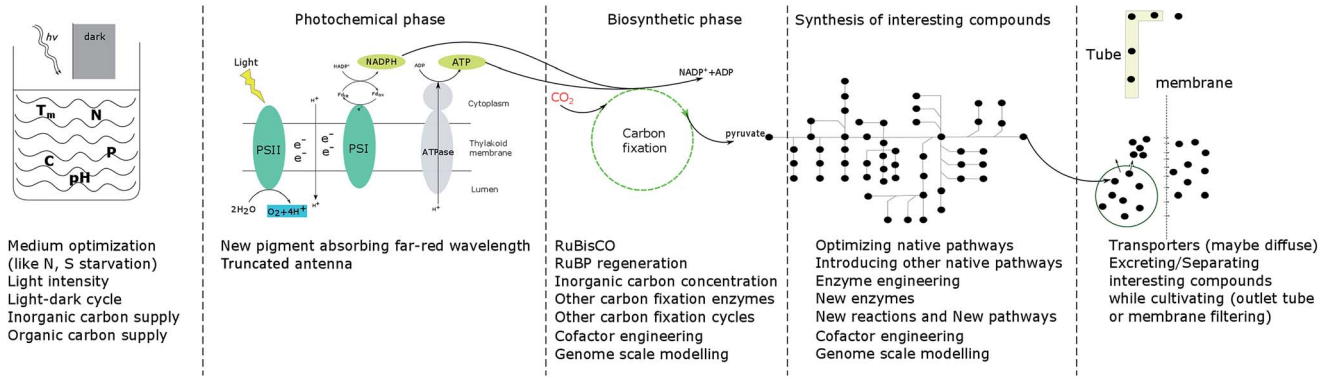

Fig. 3 Summary of strategies to enhance cell growth and/or product yields in photoautotrophic organisms.

centres, photosystems II and I. During this process, excess absorbed solar energy is dissipated as heat. This often happens in the leaves on the top of the canopies and cells in the surface facing the light source of the algal and cyanobacterial cultures, especially under high light conditions. At the same time, leaves at the bottom of the canopies and cells further from the surface of algal and cyanobacterial cultures will not get enough light. As a result, the energy conversion efficiency of the entire population is low. Therefore, a strategy, using "truncated light-harvesting antenna" (TLA) strains, was developed and used in plants, algae and cyanobacteria to increase the total solar energy conversion efficiency.[109–111] The strategy is to decrease the antenna size to prevent the top leaves or cells from absorbing excess solar energy. There will be two positive outcomes, one is to reduce light inhibition of the top leaves or surface cells, and the other is to increase light penetration thereby allowing the lower leaves and cells to receive more solar energy. Therefore, the overall solar energy conversion efficiency and production are enhanced. However, it has been reported that the TLA did not enhance *Synechocystis* PCC 6803 productivity under low light (<200 μmol photons m^{-2} s^{-1}) and did not enhance cell growth in the early stage (OD_{735} < 1).[112] In contrast, a strain of *Chlamydomonas reinhardtii* with a truncated antenna showed enhanced oxygen evolution and a higher light saturation point, and strongly enhanced biomass productivities under greenhouse conditions with about 1500 μmol photons m^{-2} s^{-1}.[109] Enhanced growth was also reported in *Synechocystis* PCC 6803 when 200 μmol photons m^{-2} s^{-1} light intensity was applied.[113] Besides, the truncated antenna benefits cell growth and biomass accumulation in *Synechocystis* PCC 6803 only under ambient CO_2 and high light (1000 μmol photons m^{-2} s^{-1}) conditions.[114] Furthermore, another interesting strategy to improve light utilization is to introduce pigments that allow cells to absorb light of longer, red-shifted wavelengths.[115]

In order to convert the output compound(s) of carbon fixation cycles, like PGA in the CBB cycle, to compounds interesting for production, further enzymatic steps are required. Traditional engineering methods, such as introducing genes with known functions from another organism, and new synthetic biology methods like assembling new pathways[116] and computational modelling[41,117] are available to optimize this process. In addition to genetic engineering strategies, growth conditions, like nitrogen starvation, medium pH adjustment, and salt stress, may also have major effects on product yields. Continuously extracting the desired product out of the cells during cultivation may also benefit photosynthesis or/and carbon fixation.[6,7]

5. Conclusions

As the only prokaryotic oxygenic photoautotrophic organisms, cyanobacteria are promising green cell factories to produce sustainable bioproducts. During the whole process, from carbon fixation to extraction of products, optimization at every step may contribute to enhancing growth and the product yield (Fig. 3).

In the photochemical phase, RuBisCO, FBA, FBP/SBPase and TK are four enzymes investigated in cyanobacteria, algae and plants as targets of engineering to enhance photosynthesis, growth and/or product formation. Since RuBisCO has a low efficiency and since protein engineering has not resulted in significant improvement in RuBisCO performance, increasing the CO_2 concentration near RuBisCO is an alternative strategy. Research is also directed towards finding more efficient carbon fixation cycles, natural or artificial, which could be implemented in engineered cells. With the development of new technologies, like cyanobacterial computational modelling and gene editing technologies, scientists have a better and better understanding of the biosynthetic phase, which will give clues to future studies.

Conflicts of interest

The authors declare no competing financial interests.

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