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Review Article

Engineering Rubisco to enhance CO_2 utilization

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ABSTRACT

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a pivotal enzyme that mediates the fixation of CO_2. As the most abundant protein on earth, Rubisco has a significant impact on global carbon, water, and nitrogen cycles. However, the significantly low carboxylation activity and competing oxygenase activity of Rubisco greatly impede high carbon fixation efficiency. This review first summarizes the current efforts in directly or indirectly modifying plant Rubisco, which has been challenging due to its high conservation and limitations in chloroplast transformation techniques. However, recent advancements in understanding Rubisco biogenesis with the assistance of chaperones have enabled successful heterologous expression of all Rubisco forms, including plant Rubisco, in microorganisms. This breakthrough facilitates the acquisition and evaluation of modified proteins, streamlining the measurement of their activity. Moreover, the establishment of a screening system in *E. coli* opens up possibilities for obtaining high-performance mutant enzymes through directed evolution. Finally, this review emphasizes the utilization of Rubisco in microorganisms, not only expanding their carbon-fixing capabilities but also holding significant potential for enhancing biotransformation processes.

1. Introduction

The atmospheric CO_2 fixation pathway is a fundamental basis for ecological cycles in nature, connecting the inorganic and organic realms. There are seven natural carbon fixation pathways in nature, including the Calvin-Benson-Bassham (CBB) cycle [1], reductive tricarboxylic acid cycle [2], the Wood−Ljungdahl pathway [3], 3-hydroxypropionate bicycle [4], 3-hydroxypropionate-4-hydroxybutyrate cycle [5], dicarboxylate-4-hydroxybutyrate cycle [6] and reductive glycine pathway [7]. The CBB cycle being the most significant carbon assimilation pathway, accounting for approximately 90 % of total carbon fixation. It is widely present in most autotrophic organisms, including prokaryotes such as cyanobacteria and archaea, as well as eukaryotic organisms such as various algae and higher plants. Rubisco, the key enzyme in the CBB cycle, is a crucial factor in the fixation of atmospheric CO_2 and has a staggering global annual CO_2 fixation capacity of approximately 10^{14} kg [8]. As opposed to its critical importance, Rubisco is a rather inefficient enzyme, with a maximal catalytic rate of only 1–22 CO_2 molecules (in plants of only 1–5 CO_2 molecules) fixed per second per active site [9,10]. This rate is only one-thousandth to one-hundredth of those of most common enzymes, and one to two orders of magnitude below the turnover frequencies of other enzymes in central carbon metabolism, which average around 50-100 s^{-1} [11]. This inefficiency is attributed to its unavoidable oxygenation activity. The ratio of carboxylation efficiency to oxygenation catalytic efficiency reflects Rubisco's specificity for CO_2 and O_2 (Sc/o) [12]. Studies of Rubisco's geological history suggest that it may have evolved from an atmospheric environment rich in CO_2 and devoid of oxygen approximately 3.5 billion years ago [8,13]. Rubisco utilizes ribulose-1,5-bisphosphate (RuBP) as a co-substrate. During carboxylation, it forms two molecules of 3-phosphoglycerate (3-PGA), while during oxygenation, it produces one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG) [14]. The subsequent recycling of the 2-PG back into 3-PGA requires a photorespiration process that releases 25 % of the initially fixed CO_2 and consumes significant energy [15]. Due to Rubisco's poor efficiency, it requires a staggering expression level. The total mass of Rubisco worldwide has been estimated at approximately 7×10^{11} kg [16], accounting for up to 50 % of the total soluble protein in plant leaves or

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microorganisms [17].

Efforts to enhance the carboxylation efficiency of Rubisco have faced great challenges, as there appears to be a trade-off between its maximum turnover rate and its specificity for CO_2/O_2 [18]. This trade-off has hindered successful engineering of Rubisco for improved carboxylation. However, there are alternative viewpoints that highlight the importance of considering the phylogenetic context in these analyses, it is conceivable that the observed correlations may be an artifact of the phylogenetic signal present in Rubisco kinetics and the phylogenetic relationships among the sampled species [18,19]. Another viewpoint suggests that the trade-off between specificity and turnover arises from the competition between protein stability and activity, rather than constraints imposed by the underlying chemistry on rate constants [20]. These perspectives shed light on the complexities of optimizing Rubisco's carboxylation activity and highlight the need for a comprehensive understanding of both the physicochemical and phylogenetic aspects in future research endeavors. An increasing number of findings suggest that the "trade-off" may have been overestimated, giving researchers more confidence to make modifications [10,18–22].

The enhancement of plant Rubisco, particularly in crops, has always remained a paramount objective. Given the extensive distribution of plant Rubisco, especially in crops encompassing an area of 12 million km^2, even a slight improvement in carbon fixation efficiency by a few percentage points could significantly boost agricultural yields [23]. Genetic manipulation in plants presents significant challenges, not only because of their slow growth rate, which leads to inefficiency, but also because plant Rubisco is a unique enzyme controlled by both the nuclear and chloroplast genomes. Its RbcL is encoded by chloroplast DNA and synthesized by chloroplast ribosomes, while its RbcS is encoded by nuclear DNA and synthesized by cytoplasmic ribosomes [24]. The in vitro assembly of active Rubisco is challenging, and the plant-nuclear/chloroplast transformation methods can be energy-costly and time-consuming. Furthermore, the successful transformation of chloroplasts is only feasible in a limited number of species [25,26]. These limitations present obstacles to the rational design of the enzyme.

To overcome these obstacles, the evolution and engineering of Rubisco is better pursued using microorganisms with rapidly replicating and clear genetic backgrounds. Key catalytic sites have been discovered within microorganisms [22,27–30]. Rubisco variants with improved carboxylation efficiency have been engineered through microbial modification [31–33]. These advancements hold the potential to not only enhance carbon fixation efficiency in plants upon reintroduction but also to amplify carbon utilization within microorganisms [34–36].

Considering the critical role of Rubisco in global carbon fixation, any amount of effort to improve Rubisco carboxylation efficiency and specificity is of utmost importance. This review aims to enhance the understanding of the significance of Rubisco and its carbon-fixing ability. It provides a summary of the notable advancements in modifying Rubisco in plants and microbes over the past two decades. Additionally, it explores the practical applications of these modifications in microbial contexts. Moreover, the text delves into the existing bottlenecks encountered in the efforts to improve Rubisco's C1 utilization capability, concurrently exploring potential solutions to address these challenges.

2. Structure and classification of Rubisco

Based on sequence phylogenies, quaternary structures, and functional properties of Rubisco's large subunit, it can be divided into four distinct Form I, II, III and IV (Fig. 1) [37,38].

Form I Rubisco is the most widespread clade, classified based on amino acid sequence homologies, into either "green-type" (Forms IA and IB, primarily found in cyanobacterial, algal, and plant) or "red-type" (Forms IC and ID, predominantly found in phototrophic bacterial and non-green eukaryotic algal) [37,39]. Form I Rubisco is composed of eight large subunits (RbcL, 50–55 kDa) and eight small subunits (RbcS, 12–18 kDa), forming a hexadecameric $(L_2)_4(S_4)_2$ complex. The RbcS are located away from the active center and exhibit greater structural diversity, play a structural role in stabilizing and assembling the large subunits complex [40]. Although the critical role of RbcS in the evolution and impact on catalytic activity of Rubisco has not been fully elucidated, experiments suggest that it regulate overall Rubisco levels and play a crucial role in facilitating the efficient assembly and maximal catalytic activity of Form I Rubiscos [41,42]. However, it appears that not all Form I Rubiscos require RbcS. A novel variant of Form I Rubisco, designated as Form I', has recently been identified. It exhibits a substantial sequence similarity, ranging from 52 % to 61 %, with established Form I Rubiscos. Notably, even in the absence of RbcS, it retains the ability to assemble into a biologically active form [43].

Form II Rubisco is composed of 2–8 RbcL and is primarily found in prokaryotes (mostly in proteobacteria species) and microeukaryotes [44]. Despite sharing a highly conserved amino acid residue in the active site, the homology between the large subunits of Form II and Form I Rubisco is only around 28 % [44,45]. Form III Rubisco is also composed solely of large subunits and has been found in some thermophilic archaea. It is considered a potential precursor of Rubisco. Form III Rubisco possesses carboxylation activity but maybe does not participate in autotrophic CO_2 fixation. Instead, it uses ribonucleotides via the pentose-bisphosphate pathway. However, recent discoveries have revealed the existence of an archaeon that possesses both Form III and the transaldolase variant CBB cycle. This suggests that Form III Rubisco might also play a role in carbon fixation [46]. Form IV Rubisco, also known as Rubisco-like protein (RLP), has been detected exclusively in non-photosynthetic bacteria, certain photosynthetic bacteria that do not

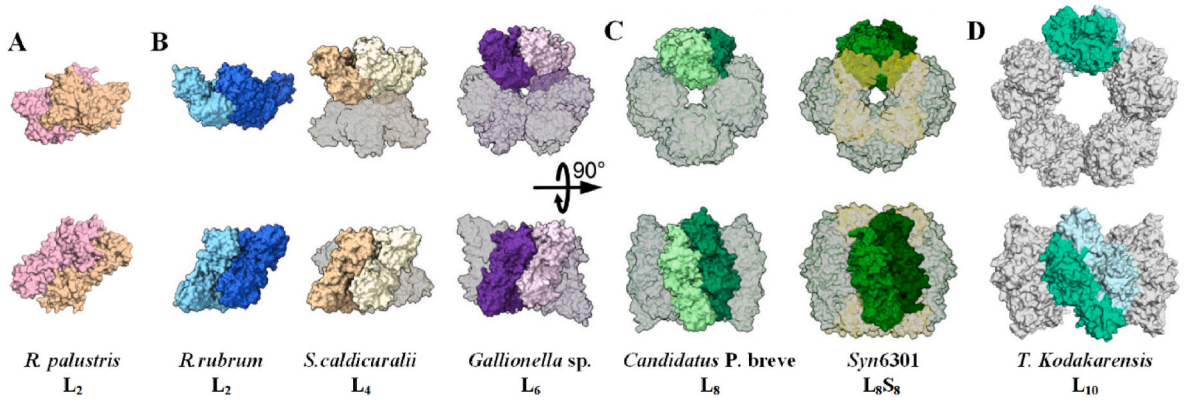

Fig. 1. Representative structures of different forms of Rubisco (A) Form IV dimer. (B) Form II dimer, tetramer, hexamer [38]. (C) Form I′ and Form I hexadecamer [38]. (D) Form III decamer. Protein Data Bank (PDB) codes (left to right): 2QYG, 5RUB, 7T1C, 5C2C, 6URA, 1RBL, and 3A12.

rely on the CBB cycle, and archaea such as green sulfur bacteria. In contrast to Forms I, II, and III, Form IV lacks several conserved amino acid residues in its active sites, rendering it incapable of Rubisco catalysis [39,47]. There are more Rubiscos waiting to be explored, which not only deepen our understanding of the origin and evolution of Rubisco but also enrich our resource pool. By comparing the differences in amino acid sequences and functional variations, it also provides some inspiration for directed modification [46,48,49].

A Rubisco dimer, consisting of two large subunits, represents the smallest functional unit. The carboxyl-terminal region of one large subunit partially covers the amino-terminal region of another large subunit, forming a funnel-shaped active center [50]. To function, Rubisco activation requires the carbamylation of the active-site Lys 201 by CO_2 activator and stabilization of the labile carbamate through Mg^{2+} coordination [51]. The biogenesis of Rubisco requires the assistance of multiple chaperones [52]. The RbcL subunit relies on the chloroplast chaperonin (Cpn60), which is a homolog of *E. coli* GroEL and mitochondrial Hsp60, as well as its cofactors (Cpn20/Cpn10), to facilitate proper folding [53,54]. RbcS aids in its stable aggregation in Form I [55]. Recent studies have revealed that the assembly of the Rubisco holoenzyme from *Arabidopsis thaliana*, when expressed recombinantly in *E. coli*, necessitates the chaperonins Cpn60αβ/Cpn20, Rubisco accumulation factor 1 and 2 (Raf1 and Raf2) [56,57], RbcX [58], and Bundle sheath defective-2 (BSD2) [59]. Plant Rubisco exemplifies the assisted assembly involving multiple auxiliary factors, and comprehending these factors and their functional cooperation will be crucial in optimizing Rubisco biogenesis, thus enhancing carbon fixation.

3. Direct or indirect engineering Rubisco in plants

With the global population on the rise and the growing demand for food production, there is a heightened focus on research aimed at enhancing the carbon fixation capacity of Rubisco in plants, particularly in crop species. Plant Rubisco is the only known enzyme controlled by both nuclear and chloroplast genomes. Its RbcL is encoded by chloroplast DNA and synthesized by chloroplast ribosomes, while its RbcS is encoded by nuclear DNA and synthesized by cytoplasmic ribosomes [24]. The in vitro assembly of the active Rubisco is challenging, and the plant-nuclear/chloroplast transformation methods can be costly and time-consuming. Furthermore, the successful transformation of chloroplasts is only feasible in a limited number of species [25,26]. Some progress have been made through the modification of Rubisco or the enhancement of its expression level [60–69]. Additionally, the rational design of other auxiliary factors associated with Rubisco activity has shown promising and encouraging outcomes (Fig. 2).

3.1. Direct modifications related to plant Rubisco

In recent years, numerous crystal structures of different Rubiscos have been elucidated, leading to a more profound comprehension of their structure and function. The augmented understanding provides invaluable perspectives for potential adjustments. Through methods such as direct manipulation, substitution, or overexpression, a range of modification attempts have been undertaken targeting plant Rubisco (Table 1).

Direct point mutation-based modification of plant Rubisco has been proven to be challenging. The chloroplast transformation method overcomes past challenges in modifying higher-plant Rubisco through

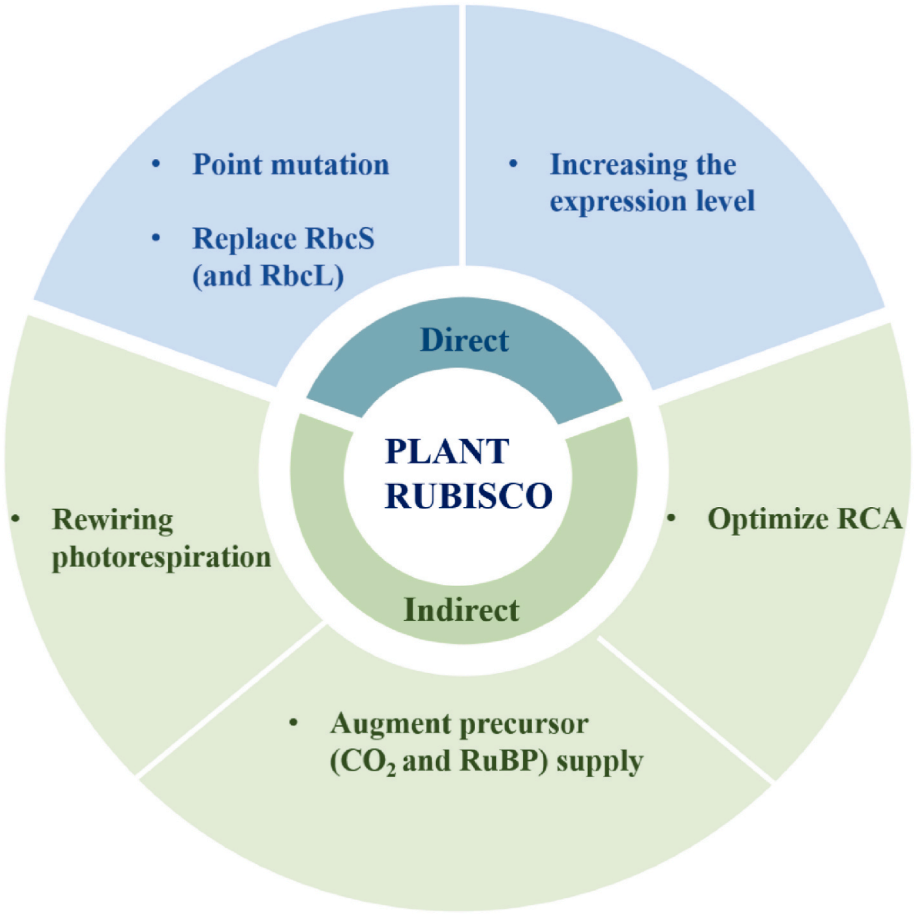

Fig. 2. Strategies for engineering Rubisco in plants.

Table 1
Research achievements in the direct or indirect modification of Rubisco in plants.

Modified Methods	Engineering Strategies	Host	Achievements	References
Direct Modification	L335V	tobacco	Overcoming past challenges in the alteration of higher-plant Rubisco through chloroplast transformation approach	[61]
	M309I	flaveria	*Flaveria bidentis*^{309}I, faster V_c, lower CO_2 affinity and *Flaveria pringlei*^{309}M, slower V_c, higher CO_2 affinity	[62]
	Replaced by *R. rubrum* Rubisco	tobacco	This establishes that the activity of a Rubisco from a very different phylogeny can be integrated into chloroplast photosynthetic metabolism without prohibitive problem	[64]
	Replaced by *S. elongatus* PCC7942 Rubisco	tobacco	Supporting autotrophic growth near wild-type growth rates at elevated CO_2.	[65]
	Replaced by *H. neapolitanus* Rubisco	tobacco	These HnRubisco subunits efficiently assemble into functional hexadecamers, enabling transgenic plants to achieve a comparable autotrophic growth rate as wild-type plants when cultivated in air supplemented with 1 % CO_2.	[66]
	Replaced by *R. sphaeroidess* Rubisco or *R. sphaeroidess* Rubisco chimaera	tobacco	Plastome transformation of RsRubisco or RsRubisco Loop 6 mutant into tobacco enhanced photosynthesis and growth up to twofold over tobacco producing wild-type RsRubisco at elevated CO_2.	[71,73]
	Overexpress RbcS	rice	Rubisco content increased by 30 % on a leaf area basis. The ratio of Rubisco-N to leaf-N was also increased by 10–20 %	[67]
	Overexpress RbcS	rice	Rubisco content increased by 90 %–110 %, resulting in an approximately 14 % enhancement in photosynthetic rate under moderate and high nitrogen growth conditions, leading to a yield increase of 20 %–28 % in paddy field experiments	[68]
	Overexpress RbcL and RbcS	maize	Rubisco content increased by 30 %, leading to an approximate 15 % enhancement in CO_2 assimilation rate.	[69]
Indirect Modification	Evolutionarily obtaining thermostable RCA1 variants	*Arabidopsis*	RCA1 variants showed increased photosynthetic rates, enhanced growth patterns, greater biomass, and higher seed yields in a long-term growth test at either constant 26 °C or daily 4 h 30 °C exposure	[80]
	Modified *Arabidopsis* RCA using thermostable tobacco RCA as a model	*Arabidopsis*	After a short exposure to higher temperatures, this activase showed higher rates of photosynthesis, recovered betterunder; Extended exposure to moderately elevated temperature, the transgenic lines had higher biomass and seed yield.	[81]
	Overexpress maize RCA in rice	rice	Rubisco activation and CO_2 assimilation accelerate significantly at all leaf temperatures, with an impact on steady-state photosynthesis at 40 °C.	[82]
	Overexpress *Oryza australiensis* RCA in domesticated rice (*Oryza sativa*)	rice	This overexpression significantly enhanced rice's carbohydrate storage throughout its lifecycle, resulting in remarkable yield improvements following heat exposure during the vegetative phase.	[83]
	Heterologous expression of *Synechococcus* sp. PCC 7942 *ictB* in *Arabidopsis* and tobacco	*Arabidopsis,* tobacco	Under conditions of limited CO_2 concentration or low relative humidity, they exhibited significantly faster photosynthetic rates than their wild-type counterparts.	[85]
	Heterologous expression of *Synechococcus* sp. PCC 7942 ictB and FBP/Sbpase in rice	rice	An additive effect was observed in the combination of ictB + FBP/SBPase. This led to increased mesophyll conductance and net photosynthetic rate, with improvements of around 10.5–36.8 % and 13.5–34.6 %, respectively.	[86]
	Heterologous expression of *Synechococcus* sp. PCC 7942 ictB in soybean	soybean	Transgenic plants exhibited higher photosynthesis, growth, and drought resilience compared to wild type plants in greenhouse, field, and drought mimic conditions	[87]
	Incorporation of BicA and SbtA proteins into the chloroplast of *Arabidopsis*	*Arabidopsis*	The installation of authentic BicA and SbtA to the chloroplast inner envelope membrane has been achieved via the proteolytic cleavage of chimeric proteins	[94]
	Overexpression of CsβCA1, CsβCA4, CsSBP and CsFBA	cucumber	The net photosynthesis biomass yield and photosynthetic rate increased by a maximum of 49 % and 79 % compared to those of the wild type.	[95]
	Introducing carboxysomes from cyanobacteria into the chloroplasts of CCM-free tobacco	tobacco	This minimal gene set generates carboxysomes and facilitates autotrophic growth under elevated CO_2 conditions	[96]
	Introducing partial carboxysomes from cyanobacteria into the chloroplasts of tobacoo	tobacco	The minimal gene set produces carboxysomes supported the autotrophic growth of transgenic lines at 2 % CO_2.	[97]
	Introducing complete carboxysomes from cyanobacteria into the chloroplasts of tobacoo	tobacco	Constructed complete carboxysomes in tobacco chloroplasts that resemble the native carboxysomes, supporting autotrophic growth in 1 % CO_2.	[98]
	Overexpressing *Arabidopsis* sedoheptulose-1,7 bisphosphatase	tobacco	Transgenic tobacco plants have the potential for greater stimulation of photosynthesis and biomass production relative to wild type tobacco when grown at elevated [CO_2] (585 ppm).	[105]
	Overexpressing *Arabidopsis* plastid fructose-1,6-bisphosphate aldolase	tobacco	Under 700 ppm CO_2, the increase reached 2.2-fold relative to wild-type plants.	[106]
	Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase (FBP/SBPase)	tobacco	Under 360 ppm CO_2, compared with wild-type tobacco, final dry matter and photosynthetic CO_2 fixation of the transgenic plants were 1.5-fold and 1.24-fold higher, respectively. Transgenic tobacco also showed a 1.2-fold increase in initial activity of Rubisco compared with wild-type plants.	[107]

(*continued on next page*)

Table 1 (*continued*)

Modified Methods	Engineering Strategies	Host	Achievements	References
	Overexpressing cyanobacterial FBP/SBPase	lettuce	The photosynthetic capacity and productivity were increased 1.3-fold and 1.6-fold, respectively.	[108]
	Overexpression of cyanobacterial FBP/SBPase	soybean	Under ambient (400 ppm) and elevated (600 ppm) CO_2 concentrations [CO_2] and under ambient and elevated temperatures (+2.7 °C during daytime, +3.4 °C at night), FBP/SBPase-expressing (FS) plants had significantly higher carbon assimilation (4–14 %), maximum carboxylation capacity $V_c$max (5–8%), and electron transport rates Jmax (4–8%). Under elevated [CO_2] and elevated temperature, FS plants maintained seed yield levels.	[109]
	Establish synthetic glycolate metabolic pathways and evaluate their performance under both current and elevated temperatures (+5 °C) in agricultural field conditions	tobacco	Tested synthetic pathways enhanced photosynthetic quantum yield by 20 %, and in replicated field trials, multiple homozygous transgenic lines boosted biomass productivity by 19–37 %. Under elevated temperatures, engineered plants showed improved photosynthetic quantum efficiency compared to control plants, resulting in a 26 % increase in total biomass compared to an 11 % increase under normal conditions.	[112,113]

mutagenesis. This approach successfully induces targeted mutation L335V in tobacco chloroplasts, enabling the evaluation of its impact on leaf photosynthesis [61]. The M309I substitutions in RbcL resulted in an increase in the carboxylation rate of *Flaveria bidentis* (C4), a decrease in selectivity, a reduction in the carboxylation rate of *Flaveria pringlei* (C3), but an enhancement in selectivity [62]. The changes brought about by this mutation demonstrate the potential of the points around the active site as potential targets for remodeling, highlighting their complexity as well.

Replacing Rubisco did not yield the desired results as anticipated. It was reported that a transplastomic tobacco whose native tobacco Rubisco was replaced by a highly active *Synechococcus elongatus* PCC7942 or *Rhodospirillum rubrum* Rubisco showed a slow growth rate even at a higher CO_2 concentration but they still signify a significant stride towards enhancing plant photosynthesis [64,65,70]· Recent studies have shown that Form 1A Rubisco from the proteobacterium *Halothiobacillus neapolitanus* can form functional L_8S_8 hexadecamers in tobacco chloroplasts, despite accounting for approximately 40 % of the wild-type tobacco Rubisco content, it can support autotrophic growth rates similar to the wild type. However, it also requires high concentration of CO_2 (1 %) [66]. The Form I red-type (C, D) Rubiscos are distinguished by their higher affinity for the carboxylation reaction compared with plant Form IB Rubiscos, making them an appealing pathway for enhancing photosynthesis. Compared with Form ID *Griffithsia monilis* Rubisco (GmRubisco), Form IC *Rhodobacter sphaeroides* Rubisco (RsRubisco) can readily assemble in tobacco chloroplasts. Coexpressing its cognate Rubisco activase (RCA) and guided by the higher-performance GmRubisco for modifications in the Loop 6 region enhances RsRubisco activity, leading to improved plant photosynthesis and growth [22,71].

Increasing the expression level of endogenous Rubisco can enhance overall plant photosynthetic efficiency. There have been successful experiments where overexpressing rice RbcS led to an increased formation of Rubisco holoenzyme [67]. This, in turn, resulted in higher yields in paddy field experiments when sufficient nitrogen fertilization was provided [68]. Similarly, when the maize Rubisco large and small subunits were overexpressed along with the assembly chaperone Raf1, a remarkable increase of over 30 % in Rubisco content was observed. This enhanced Rubisco content contributed to a 15 % improvement in CO_2 assimilation, enabling maize plants to better withstand chilling stress and facilitating their subsequent recovery [69].

The conservation of plant Rubisco makes all modifications targeting its active sites fail. Moreover, there are no promising approaches to identify alternative sites for modification, and high-throughput screening methods used in enzyme engineering cannot be readily applied. Adding to the challenge, the transformation of chloroplasts is also highly complex and limits the possibilities for successful modifications. As a result, for several decades, the success in enhancing plant Rubisco efficiency remains limited.

3.2. Modifying auxiliary factors to indirectly enhance plant Rubisco's carbon-fixing capability

Beyond direct modifications to Rubisco, other factors related to Rubisco have also been investigated and researched. For instance, strategies like enhancing Rubisco activase (RCA) to improve photosynthesis, introducing CO_2 concentration mechanism (CCM) to enhance precursor supply, and optimizing the expression of CBB cycle enzymes have been explored. Moreover, there have been efforts to rewire photorespiration to minimize carbon losses (Table 1). Some comprehensive reviews have already been conducted on these topics [15, 72–76].

The ancillary enzyme RCA plays a facilitating role in the activation process by aiding in the release of inhibitory sugar phosphates from Rubisco's catalytic sites [77]. In the biochemical limitations of photosynthesis under dynamic light and elevated temperatures, RCA is one of the targets for genetic engineering to improve plant Rubisco carboxylation efficiency [72,78,79]. Possibly due to RCA limitation, overexpression of maize Rubisco resulted in a 23 % decrease in its activation state [69]. Perhaps further improvements in photosynthetic potential can be achieved by modifying RCA. As global temperatures rise, the inhibitory effect of high temperatures on RCA catalytic activity further decreases Rubisco carboxylation activity. Increasing the content of RCA or screening and constructing RCA variants with improved heat stability can significantly enhance Rubisco activation and catalytic efficiency, thereby improving overall plant photosynthetic efficiency [80–83]. In addition, RCA proteins CbbQ and CbbO could serve as structural components of reconstituted α-carboxysomes, enhances the CO_2-fixation activities within the CCM [84].

In cyanobacteria, CCM is primarily achieved through inorganic carbon transporters, carbonic anhydrases, and carboxysomes. In contrast, in plants, CCM is accomplished through the C4 pathway or crassulacean acid metabolism (CAM) pathway, both of which increase the concentration of CO_2 around Rubisco. Researchers have reported that overexpressing the putative inorganic carbon transporter B (ictB) from cyanobacteria or carbonic anhydrase, in *Arabidopsis*, tobacco, rice, and soybean led to an increase in both photosynthetic rate and biomass [85–87]. However, it appears that, for unclear reasons, only this particular transporter is effective in eliciting such effects. The incorporation of HCO_3^- transporters, bicarbonate uptake A (BicA) and sodium-bicarbonate transporter A (SbtA), has the potential to efficiently accumulate HCO_3^- within the chloroplast stroma, facilitating carboxysome function [88–90]. Recent strides in comprehending the structure, function, and engineering of BicA and SbtA/B HCO_3^- transporters

[91–93], combined with the successful incorporation of authentic BicA and SbtA proteins into the chloroplast envelope membrane via the proteolytic cleavage of chimeric proteins in *Arabidopsis* [94], represent significant advancements.

Carbonic anhydrase (CA), a zinc-containing metal enzyme, catalyzes the reversible hydration reaction of CO_2. In higher plant photosynthetic carbon metabolism, CA accelerates the diffusion of CO_2 to the active site of Rubisco, thereby enhancing the rate of CO_2 fixation. As the only CA that responds to low levels of CO_2, the significance of βCA in enhancing plant photosynthesis has been substantiated through the overexpression of β-carbonic anhydrase 1 (CsβCA1), β-carbonic anhydrase 4 (CsβCA4) in cucumber [95]. Introducing carboxysomes from cyanobacteria into the chloroplasts of CCM-free crops is predicted to improve yields by up to 60 % under hot and dry conditions [88]. Encapsulation of two key α-carboxysome structural proteins with cyanobacterial Rubisco in tobacco chloroplasts has been successfully achieved, enabling autotrophic growth at elevated CO_2 levels [96]. Furthermore, by integrating nine carboxysome genetic components from a proteobacterium, fully functional α-carboxysome components were engineered into tobacco chloroplasts [97]. These achievements make the step-by-step installation of an entire functional CCM in chloroplasts more feasible and mature, with the aim of enhancing crop photosynthesis and yields.

On the other hand, the successful reconstruction of CCM in *E. coli* [98] and the study of mechanisms governing the assembly and functional maintenance of carboxysomes in bacteria [99] have significantly advanced our understanding of the molecular processes underlying CCM, as well as the structure, assembly, biogenesis, and physiology of carboxysomes, thus laying the foundation for further research and engineering support of cellular biology for CO_2 assimilation in different organisms.

Around 80 % of agricultural land is covered by C3 crops that lack the CCM found in C4 crops [100]. Introducing the C4 photosynthetic

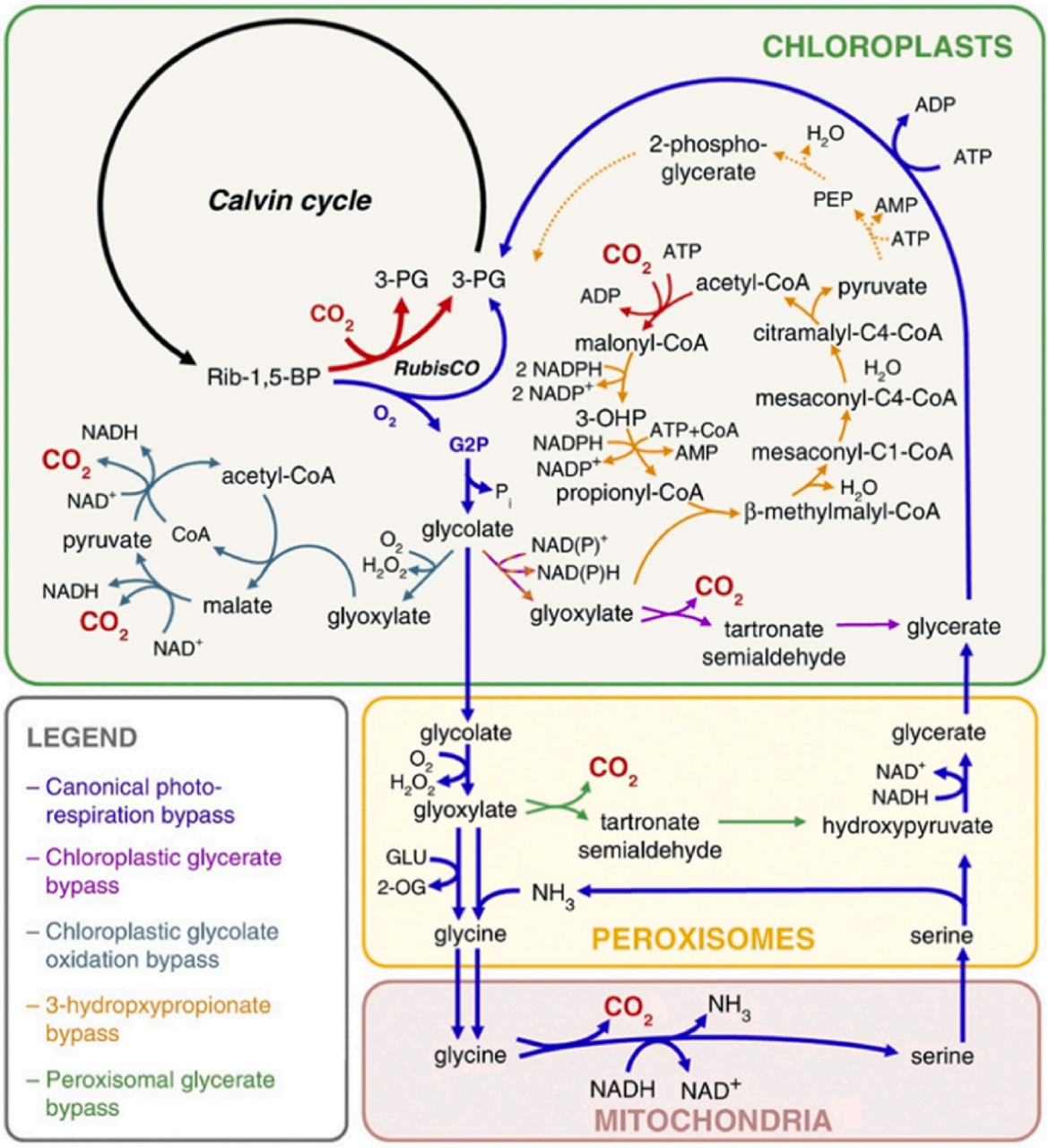

Fig. 3. Natural and synthetic plant photorespiration bypasses [114]. 3-PG, 3-phosphoglycerate; G2P, 2-phosphoglycerate; GLU, glutamate; 2-OG, 2-oxoglutarate; PEP, phosphoenolpyruvate.

pathway into C3 plants, such as engineering C4 rice, has become a prominent research focus [101,102]. Compared to C4 metabolism, the CAM pathway might be an easier target for engineering or manipulation because its necessary components already exist in C3 plants [103]. Utilizing CCM to elevate CO_2 concentration around Rubisco can directly reduce the occurrence of oxygenation side reactions and enhance yields [75,104]. Similarly, augmenting the supply of co-substrate RuBP can also moderately enhance plant carbon fixation efficiency. Under high CO_2 concentrations, the overexpression of sedoheptulose-1, 7-bisphosphatase (SBP) or fructose-1,6-bisphosphate aldolase (FBA) leads to increased carbon fixation efficiency and biomass in tobacco plants [105,106]. Even under ambient CO_2 concentrations, the overexpression of SBP and FBA has been shown to improve the growth performance of both tobacco, lettuce and soybeans [107–109].

Additionally, optimizing the expression of photorespiratory enzymes and reconstructing the photorespiratory bypass pathway offer another approach [110]. Photorespiration can lead to a decrease in the photosynthetic efficiency of C3 crops by 20–50 % [76]. Even in the face of predicted future climate change with atmospheric CO_2 levels reaching their highest projected levels, reducing plant photorespiration is expected to increase photosynthetic efficiency by 12 %–55 % [111]. For example, establishing synthetic glycolate metabolic pathway has resulted in a 19 %–37 % increase in biomass for tobacco plants in field conditions [112]. Furthermore, the introduction of the glycolate metabolic has increased the thermotolerance of net photosynthesis and alleviated end-of-season biomass loss in tobacco cultivated under elevated temperatures in agriculturally relevant conditions [113]. Previously, there have been several reviews that provided detailed and thorough summaries of bypass introductions (Fig. 3) [15,76,114].

Compared to direct modifications targeting Rubisco, the alterations of these related factors are not only easier but also yield better results. The exact mechanism by which RCA remodels Rubisco's structure remains unclear. The complex structure or mechanisms of CCM, along with the insufficient understanding of the molecular basis of structure formation, and the challenges posed by the lengthy metabolic pathways in constructing multi-gene homozygous expression strains, have hindered the development of its applications.

With a deeper understanding of RCA and CCM functions, there is potential for more effective rational design and engineering of the protein organelles to enhance CO_2 fixation and introduce new functionalities through this approach.

4. Utilizing microorganisms to engineer Rubisco

In microorganisms, the RbcL and RbcS of Rubisco are generally located on the same operon, and the structure is similar to that of higher plants' Rubisco, making gene manipulation easier [1,115]. This is advantageous for constructing a random mutagenesis library, developing a rational screening system, and accomplishing genetic engineering tasks. In addition, it is beneficial for breaking the bottleneck of energy supply in natural autotrophic microorganisms, utilizing efficient energy sources such as formate, methanol, or pyruvate, achieving high-density cultivation (Table 3).

4.1. Rational design for the modification of Rubisco in microorganisms

Research on Rubisco has spanned over 50 years, starting from the initial discovery of the relationship between photorespiration and Rubisco in 1971 [116]. In 1984, *Rhodospirillum rubrum* Rubisco was targeted for point mutations for the first time. Regarding D198E mutation near the active-site L201, it was established to some extent that D198 contributes to the formation of the binding site for divalent metal ions [27]. The functional expression of Rubisco in bacteria makes rational engineering and evaluation of these selected potential sites efficient and easy through sequence alignment. To illustrate, the Form III Rubisco derived from the hyperthermophilic archaeon *Thermococcus kodakarensis* (Tk-Rubisco) at 100 °C shows approximately twenty times the activity of spinach Rubisco at 25 °C, but only one-eighth the activity at ambient temperature. To enhance the activity of Tk-Rubisco at ambient temperature, novel Tk-Rubisco mutants were designed based on its three-dimensional structure and a sequence comparison of thermophilic and mesophilic plant Rubiscos. As a result, T289D mutant was successfully constructed in *E. coli*, demonstrating a 25 % increase in carboxylase activities compared to the wild-type enzyme [28].

Through sequence and structure alignment, rational design studies can be conducted on previously unlocatable modification regions distant from the active center. Through structural analysis of GmRubisco and RsRubisco, sites located away from the active center were identified, including RbcL Loop 6, RbcL C terminus, and the RbcS C-terminal βE-βF domains. Kinetic analysis of 11 RsRubisco chimaeras, incorporating various combinations of these domains from GmRubisco, revealed that the C329A and A332V changes could simultaneously enhance the carboxylation rate and specificity of RsRubisco [22]. By aligning the amino acid sequences of RbcL from the green alga *Chlamydomonas reinhardtii* and land plants, researchers identified specific residues located at the bottom of the large-subunit α/β-barrel active site. In *Chlamydomonas reinhardtii* Rubisco, the residues V221 and V235 were changed to C221 and I235, respectively, mimicking the corresponding residues found in land-plant Rubisco. These substitutions complemented the original changes and restored the enzyme's specificity to normal levels. Moreover, introducing an additional substitution with the shorter βA-βB loop from the spinach small subunit resulted in a significant 12–17 % increase in specificity [30].

The discovery of highly efficient high-temperature Rubisco, along with diverse Rubisco variants sourced from various organisms, has revealed a valuable collection of biological assets. Utilizing machine learning to predict the Rubisco kinetics based on the large subunit sequence may uncover more high-performance Rubisco through higher-throughput pre-screening [117]. These variants demonstrate considerable specificity and variations in carboxylation activity, adding to the wealth of available resources. Similarly, the exploration of unique forms of Rubisco, such as Form I', Form II/III, has enhanced our comprehension of their structure and function, potentially guiding rational modifications. With the development of sequencing technology and Metagenomics, genomic information from uncultivable microorganisms in extreme environments has been explored. Rubisco from Asgard archaea is considered as Form IV, but our research findings indicate that the sequence alignment results suggest, it has more active sites compared to other Form IV Rubiscos and is more conserved (Table 2). Considering the unique evolutionary position, studying Asgard Rubisco may provide valuable insights into the critical active sites. Such insights could potentially revolutionize the previously deemed challenging research on modifying these active sites.

4.2. Constructing an efficient screening platform to engineer Rubisco

Due to limited understanding of the catalytic mechanism, it is challenging to precisely identify the restricted sites for modifications. Although obtaining engineered enzymes through heterologous expression in *E. coli* has become relatively straightforward, the lack of high-throughput evaluation methods remains a limitation. The use of ^{14}C-based methods for individual mutation evaluation is inefficient and costly. However, by coupling Rubisco activity with growth and employing directed evolution, it becomes possible to achieve a relatively high-throughput screening. This approach holds the potential to expedite the discovery of high-performance enzymes.

The initial report on the directed evolution of Rubisco was obtained in *Rhodobacter capsulatus*, where mutant substitutions F342V and A375V were found to confer "positive" growth phenotypes to *Synechococcus* PCC 6301 [31,32]. Subsequently, *E. coli,* as the extensively studied model microorganism, was adopted as a screening system for directed evolution of Rubisco [118,119]. Heterologous expression of

Table 2
Different Rubisco active site residue. The PDB IDs are pre-annotated before the organism name, and for Asgard Rubisco, their GenBank IDs are provided at the beginning of the organism information. White-shaded positions indicate conservation, blue indicates a semi-conservative substitution, and grey indicates a non-conservative substitution.

Family	Organism	Active Site Residues																	
		C	R	C	C	C	CATALYTIC MODIFY						C	R	R	C	R	R	R	R
Form I	8RUC|*Spinacia oleracea*	E	T	N	K	K	G	D	F	K	D	E	H	R	H	K	S	G	G	G
	1RBL|*Synechococcus elongatus* PCC 6301	E	T	N	K	K	G	D	F	K	D	E	H	R	H	K	S	G	G	G
Form I'	6URA|*Promineofilum breve*	E	T	N	K	K	G	D	F	K	D	E	H	R	H	K	S	G	G	G
Form II	4LF1|*Rhodopseudomonas palustris*	E	T	N	K	K	G	D	F	K	D	E	H	R	H	K	S	G	G	G
	9RUB|*Rhodospirillum rubrum*	E	T	N	K	K	G	D	F	K	D	E	H	R	H	K	S	G	G	G
Form II/III	5MAC|*Methanococcoides burtonii* DSM	E	T	N	K	K	G	D	F	K	D	E	H	R	H	K	S	G	G	S
Form III	3WQP|*Thermococcus kodakarensis*	E	T	N	K	K	G	D	Y	K	D	E	H	R	H	K	S	G	G	G
	2D69|*Pyrococcus horikoshii*	E	T	N	K	K	G	D	L	K	D	E	H	R	H	K	S	G	G	G
	6HUN|*Hyperthermus butylicus* DSM 5456	E	T	N	K	K	G	D	L	K	D	E	H	R	H	K	S	G	G	G
Form III Asgard Rubisco	KKK43062.1[*Lokiarchaeum* sp. GC14_75]	E	T	N	K	K	G	D	L	K	D	E	H	R	H	K	S	G	G	G
	RLI62811.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	K	G	D	L	K	D	E	H	R	H	K	S	G	G	G
	OLS31478.1[*Candidatus Heimdallarchaeota archaeon* AB_125]	E	T	N	K	K	G	D	F	K	D	E	H	R	H	K	S	G	G	G
	PWI48176.1[*Candidatus Heimdallarchaeota archaeon* B3_Heim]	E	T	N	K	K	G	D	L	K	D	E	H	R	H	K	S	G	G	G
	OLS27808.1[*Candidatus Heimdallarchaeota archaeon* LC_2]	E	T	N	K	K	G	T	W	K	D	E	H	R	H	N	S	G	G	G
	OLS23198.1[*Candidatus Heimdallarchaeota archaeon* LC_3]	E	T	N	K	K	G	D	V	K	D	E	H	R	H	K	S	G	G	G
	WEU40092.1[*Candidatus Odinarchaeota archaeon* LCB_4]	E	T	N	K	K	G	T	S	K	D	E	H	R	H	K	S	G	G	G
	QEE15426.1[*Candidatus Prometheoarchaeum syntrophicum*]	E	T	N	K	K	G	D	V	K	D	E	H	R	H	K	S	G	G	G
Form IV	1YKW|*Chlorobaculum tepidum*	E	Q	E	K	N	G	D	I	K	D	E	H	F	I	R	G	G	G	R
	4NAS|*Alicyclobacillus acidocaldarius*	G	T	K	K	C	G	D	L	K	D	E	H	P	I	S	S	A	G	G
	3NWR|*Burkholderia fungorum*	E	T	N	K	N	G	D	F	K	D	E	H	R	H	K	S	S	G	G
	1TEL|*Chlorobium tepidum TLS*	E	Q	E	K	N	G	D	I	K	D	E	H	F	I	R	G	G	G	R
	3FK4|*Bacillus cereus* ATCC 14579	E	S	K	K	M	G	D	I	K	D	E	H	P	L	S	S	A	G	G
	3QFW|*Rhodopseudomonas palustris*	E	C	N	K	.	G	D	L	K	D	H	H	P	V	R	A	G	G	G
	2OEJ|*Geobacillus kaustophilus*	G	T	K	K	C	G	D	L	K	D	E	H	P	L	S	S	A	G	G
	2ZVI|*Bacillus subtilis*	G	S	K	K	V	G	D	L	K	D	E	H	P	L	S	S	A	G	G
	2QYG|*Rhodopseudomonas palustris*	E	Q	E	K	N	G	D	I	K	D	E	H	F	I	R	G	G	G	R
Form IV Asgard Rubisco	KKK45122.1[*Lokiarchaeum* sp. GC14_75]	E	T	N	K	C	G	D	V	K	D	E	H	M	V	K	S	G	G	G
	KKK44923.1[*Lokiarchaeum* sp. GC14_75]	E	T	N	K	C	G	D	V	K	D	E	H	M	V	K	S	G	G	G
	RDE16801.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	A
	RDE12223.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	G
	RDE17141.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	G
	RLI54124.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	A
	KXH74397.1[*Candidatus Thorarchaeota archaeon* SMTZ1-45]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	A
	KXH71633.1[*Candidatus Thorarchaeota archaeon* SMTZ1-83]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	A
	KXH73673.1[*Candidatus Thorarchaeota archaeon* SMTZ-45]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	G
	TFG08318.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	G
	TFG13607.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	G
	TFG27408.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	A
	TFG99348.1[*Candidatus Thorarchaeota archaeon*]	E	T	N	K	C	Y	D	I	K	D	E	H	M	V	K	S	G	G	A
	QEE17139.1[*Candidatus Prometheoarchaeum syntrophicum*]	E	T	N	K	C	G	D	V	K	D	E	H	M	V	K	S	G	G	G
C=catalytic	R=Rubp binding	C	R	C	C	C	CATALYTIC MODIFY						C	R	R	C	R	R	R	R

cyanobacterial Rubisco in microorganisms such as *E. coli*, yeast or *Ralstonia eutropha* is relatively easier using GroEL-GroES and RbcX [33,120–122]. Introducing phosphoribulokinase (PRK) into *E. coli* generates RuBP, which is toxic to bacteria. Rubisco detoxifies by using RuBP as a substrate, coupling bacterial growth with Rubisco activity. Utilizing this Rubisco-dependent *E. coli* (RDE) screens for the evolution screening of *Synechococcus* sp. PCC7002 Rubisco mutant with E49V and D82G substitutions in the small subunit has shown an 85 % increase in specific carboxylation activity and a 45 % improvement in catalytic efficiency towards CO_2 [33]. Utilizing RDE in a similar manner, the acquired F140I mutation in the RbcL of *Synechococcus* PCC 6301 led to a remarkable threefold enhancement in carboxylation efficiency without CO_2 specificity. This alteration also led to a substantial approximately 55 % rise in the photosynthesis rate of *Synechocystis* sp. PCC 6803 [123]. Due to natural transposon-mediated silencing of PRK can also restore *E. coli* growth, it might result in a large number of false positives. A good solution is to express a fusion protein of PRK and neomycin phosphotransferase to include the additional selection pressure of antibiotic resistance [124]. Using this strategy, an enhancement in the specificity and catalytic efficiency of *Thermosynechococcus elongatus* BP1 Rubisco has been achieved [124]. Furthermore, it is worth noting that this is based on high protein expression levels. The achievement of beneficial mutations in cyanobacteria can be attributed to the augmented Rubisco solubility facilitated by the overexpression of GroEL-ES, consequently broadening the spectrum of viable mutation possibilities [123]. In fact, the evolution of Rubisco in *E. coli* may lead to mutations that tend to increase expression levels rather than improving carboxylation activity or selectivity [60,125]. High initial heterologous expression levels can be advantageous for directed evolution. Codon optimization has shown potential in improving expression levels, but there is a need for more

Table 3
Research achievements pertaining to the effective modification of Rubisco in microorganisms.

Modified Methods	Engineering Strategies	Host	Achievements	References
Rational Design	D198E	*R. rubrum*	The mutant enzyme displayed slight changes in kinetic properties, with a subtle shift in the metal ion's surrounding environment.	[27]
	T. kodakarensis T289D	*E. coli*	25 % increase in carboxylase activities compared to the wild-type	[28]
	R. sphaeroides Rubisco C329A and A332V	*E. coli*	Increased the carboxylation rate by 60 %, the carboxylation efficiency in air by 22 % and the CO_2/O_2 specificity by 7 %.	[22]
	V221C, V235I; Substitution with the βA-βB loop of the spinach small subunit	*C. reinhardtii*	Restored the Rubisco's specificity to normal levels; Enhanced by 12–17 % in specificity	[30]
Direct Evolution	*Synechococcus* PCC6301 Rubisco F342V	*R. capsulatus*	The specific activity increased by 50 %, and the doubling time shortened by 68 %, enabling photoautotrophic growth under 5 % CO_2 conditions	[31]
	Synechococcus PCC6301 Rubisco A375V	*R. capsulatus*	A375V was able to suppress the negative phenotype caused by a D103V substitution by decreasing oxygen sensitivity	[32]
	Synechococcus sp. PCC7002 Rubisco E49V and D82G	*E. coli*	85 % increase in specific carboxylation activity and a 45 % improvement in catalytic efficiency towards CO_2	[33]
	F140I	*E. coli, Synechocystis* sp. PCC 6803	The F140I mutation in the large Rubisco subunit of *Synechococcus* PCC 6301 tripled carboxylation efficiency while maintaining CO_2 specificity, leading to a 55 % enhancement in photosynthesis rate in *Synechocystis* PCC 6803	[123]
	Thermosynechococcus elongatus BP1 Rubisco V98 M, A48V, H37L, Y36 N and G112D (R51H), F345I, P415A	*E. coli*	Improved carboxylation rate, efficiency and specificity	[124]
	Synechococcus PCC6301 Rubisco I174V, Q212L, M262T, F345L or F345I	*E. coli*	The Rubisco large subunit substitutions I174V, Q212L, M262T, F345L or F345I were repeatedly selected and shown to increase functional Rubisco expression 4–7 fold in the RDE and 5–17 fold when expressed in XL1-Blue *E. coli.*	[125]
	Rhodobacter sphaeroides Rubisco K83Q, V11I, A252L, Y345F	*E. coli*	Enhanced carboxylation rate by 27 %, elevated carboxylation efficiency by 17 %, maintained CO_2/O_2 specificity, reduced holoenzyme biogenesis capacity by 40 %	[60]
	Methanococcoides burtonii Rubisco E138V, K332E, T421A	*E. coli*	K332E, E138V, T421A showed significant 40 %–90 % improvements in k^c_{cat} and corresponding 10 %–25 % increases in $S_{c/o}$. T421A mutant showed a modest, but significant, increase in expression	[127]

refined optimization strategies. Our recent research indicates that partial codon optimization strategies can enhance the solubility of Rubisco compared to complete codon optimization. Moreover, to accurately quantify Rubisco content and kinetics and avoid misinterpreting directed evolution outcomes, an analytical pipeline employs a novel T7-promoter regulated RDE screen. This method effectively identifies the mutant of *Rhodobacter sphaeroides* Rubisco with enhanced carboxylation capabilities while maintaining an unchanged specificity [60].

The difficulty of heterologous expression of plant Rubisco in *E. coli* has limited the use of RDE screens. However, with a deeper understanding of chaperones, it has become feasible [52]. By utilizing coexpression of five plant-derived chaperones (Cpn60αβ/Cpn20, Raf1, Raf2, RbcX, and BSD2), successful assembly of active *Arabidopsis* Rubisco in *E. coli* has been achieved [59]. Further investigations have demonstrated that this strategy is comparably efficacious for Rubisco sourced from potatoes, carrots, strawberries, and tobacco. While assembly is achievable within *E. coli*, the corresponding enzymatic activity has not yet been reported [126]. A robust Golden Gate cloning *E. coli* expression system has been developed, which can effectively predict high-level Rubisco production in chloroplasts. Despite its limitations in accurately predicting the biogenesis potential of isoforms with impaired production in planta, this study also contributes to the development of a truly efficient and low-cost RDE screening tool that can accurately emulate chloroplast expression [126].

Compared to Form I, Forms II and III have simpler structures and are more easily expressed in heterologous systems, requiring only the assistance of GroEL and GroES which belong to *E. coli* native chaperone network. Moreover, Forms II and III demonstrate high levels of protein expression in *E. coli*. Through directed evolution of *M. burtonii* Rubisco in *E. coli*, it has been confirmed that the evolved E138V and K332E mutant enzymes exhibit significantly improved CO_2-fixation speed, CO_2-affinity, and specificity for CO_2 [127]. As more highly active Forms II and III of Rubisco have been discovered [10,28,128], it suggests that these forms, facing different evolutionary pressures from Form I, may be less conservative and hold greater evolutionary potential. Consequently, the ongoing directed evolution of Forms II and III Rubisco opens up new possibilities for enhancing leaf photosynthesis and promoting plant growth. In addition to *E. coli*, *Ralstonia eutropha* has also been genetically modified to enable in vivo screening of Rubisco variants, under robust aerobic growth conditions [129].

Compared to plants, expressing, assembling, and purifying Rubisco in microorganisms is simpler, forming the basis for direct evolution of Rubisco. However, not all Rubiscos are suitable, especially the ones of paramount importance, such as those from wheat, rice and maize. Therefore, further research is needed on plant Rubisco biogenesis and the mechanism of interacting chaperones. Considering that both belong to Form I, cyanobacterial Rubisco shares high sequential and structural identities with plant Rubisco in the large subunit, and their active site residues are completely conserved (Table 2). However, the assembly of cyanobacterial Rubisco in bacteria is much simpler, requiring only the co-expression of RbcX or Raf1 [58,130]. Therefore, important amino acid residues or sequences related to assembly could potentially be identified through structural and sequence alignments.

As efforts are being made to evolve Rubisco through the establishment of an *E. coli* screening platform, parallel advancements should also be made in establishing chloroplast transformation techniques in more plant. This is particularly important when considering the ultimate goal of utilizing these techniques to enhance plant photosynthesis and efficiency.

5. Engineering Rubisco in microorganisms for carbon fixation

With the ease of heterologous expression of Rubisco in microorganisms, the utilization of Rubisco for carbon fixation has garnered increasing attention. Simply expressing PRK and Rubisco heterologously allows for the construction of the CBB cycle in *E. coli* or yeast, enabling the synthesis of sugars and biomass production using CO_2 from the existing metabolic pathways. For production strains, the recycling of carbon in metabolic processes using Rubisco could lead to higher economic benefits.

5.1. Introducing Rubisco-based carbon fixation pathway into heterotrophic microorganisms

Quantitative analysis has revealed that the CO_2-fixation rate of *E. coli* is comparable to that of the autotrophic cyanobacteria and algae, demonstrating great potential of heterotrophic CO_2 fixation [36]. Due to the ease of achieving high cell density in fermenters under well-controlled conditions, heterotrophic microorganisms may possess greater carbon fixation potential [36]. Furthermore, genetic engineering of plants and autotrophic organisms is challenging, complicating efforts to improve natural carbon fixation efficiency. With the development of synthetic biology and extensive research on natural autotrophic microbial carbon fixation pathways, there is increasing interest in transforming heterotrophic microorganisms into carbon-fixing strains using efficient energy sources like formate or glyoxylate. In recent years, significant progress has been made in constructing hemiautotrophic or autotrophic *E. coli* and yeast (Table 4).

Deletion of the phosphoglycerate mutase genes disrupts carbon flow in the glycolytic/gluconeogenic, resulting in the division of central carbon metabolism into two modules: the carbon fixation module and the energy module. By using pyruvate as an energy donor and combining rational design with laboratory adaptive evolution under xylose concentration stress, a semi-autotrophic *E. coli* strain was obtained [131]. Further research, formate instead of an organic carbon source was used as an electron donor that cannot support *E. coli* growth. The strain was modified to inhibit heterotrophic growth metabolism flux allocation and create conditions for directed evolution. Eventually, through long-term evolution, the desired strain was obtained, and $^{13}CO_2$ labeling confirmed that biomass was derived entirely from CO_2, achieving a true autotrophic phenotype in *E. coli* [132].

Adopting a similar research approach as with autotrophic *E. coli*, researchers sought to engineer a self-sustaining strain of *Methylorubrum extorquens* AM1 capable of meeting its energy and biomass needs exclusively from a single carbon source, methanol. Methanol metabolism supplied the necessary energy and reducing power, while the carbon fixation module was established by overexpressing *Rhodospirillum rubrum* S1 Rubisco and spinach PRK. The strain's optical density was increased in methanol-limited media, and ^{13}C-tracer analysis verified the functionality of the CBB cycle. Comparative proteomics analysis elucidated the metabolic remodeling responses of the host [133].

Pichia pastoris, a commonly employed industrial yeast, finds extensive applications in biopharmaceutical and enzyme production. It exhibits a methylotrophic lifestyle, relying solely on the C1 compound methanol as its energy and carbon source. The peroxisomal methanol assimilation pathway in *P. pastoris* was modified to mimic the CO_2 fixation pathway of the CBB cycle. Through adaptive laboratory evolution, the self-autotrophic growth rate was enhanced to 0.018 h^{-1} [134]. Furthermore, the autotrophic *P. pastoris* was further engineered into a platform for producing value-added chemicals using CO_2 as a feedstock. This was achieved through the integration of heterologous genes responsible for the synthesis of lactic and itaconic acids (Fig. 4). The production levels reached 600 mg L^{-1} for lactic acid and 2 g L^{-1} for itaconic acid [135].

These studies have demonstrated the significance of heterologous expression of Rubisco and the tremendous potential of central metabolic pathways, laying the foundation for industrial-scale production of fuels and organic products utilizing CO_2. Furthermore, by utilizing Rubisco to fix CO_2 for the construction of autotrophic bacterial strains, the growth of bacteria is entirely coupled to Rubisco activity. This coupling enables a more targeted directed evolution of Rubisco using RDE screening.

The utilization of Rubisco in constructing artificial autotrophic microorganisms is groundbreaking, although their growth performance is currently limited by insufficient energy supply. Introducing additional energy modules, such as utilizing polyphosphate kinase for ATP production or modifying *E. coli* to utilize electrical energy, presents new avenues for exploration.

Table 4
Transforming microorganisms into autotrophs using Rubisco modification.

Host	Carbon-fixation enzyme	Carbon source	Energy source and reducing power	References
E. coli	*R. rubrum* ATCC 11170 Rubisco	CO_2	pyruvate	[131]
E. coli	*R. rubrum* ATCC 11170 Rubisco	CO_2, formate	formate	[132]
M. extorquens AM1	*R. rubrum* S1 Rubisco	CO_2, methanol	methanol	[133]
P. pastoris	*T. denitrificans* Rubisco	CO_2, methanol	methanol	[134,135]

5.2. Rubisco serves as a valuable tool for converting CO_2 into chemicals

By selectively overexpressing key components of the CBB pathway, particularly Rubisco, in heterotrophic production strains, the process effectively captures and utilizes carbon that would otherwise be lost as CO_2 during product synthesis. This innovative strategy holds great promise in substantially augmenting product yield or productivity. This strategy has already been implemented in various microorganisms (Fig. 4).

Functional expression of *Spinacia oleracea* PRK and *Hydrogenovibrio marinus* Rubisco in *Saccharomyces cerevisiae* led to a 90 % reduction of the by-product glycerol and a 10 % increase in ethanol production in sugar-limited chemostat cultures on a mixture of glucose and galactose [136]. A similar strategy, combined with continuous optimization, Kai Wang et al. achieved an astounding 33.7-fold enhancement in the ultimate titer of free fatty acids [122].

The PRK and Rubisco from *Synechocystis* sp. PCC 6803 were introduced into *E. coli*. By balancing the ATP-generating phosphoenolpyruvate carboxykinase pathway with the ATP-consuming Rubisco, the CO_2-fixing rate of the engineered *E. coli* increased by 870 %, and the malate production reached 387 mM [137]. In a similar manner, the introduction of the pyruvate to ethanol conversion pathway and the CBB pathway achieves energy balance for mixotrophic growth, with an average CO_2 consumption rate of 55.3 mg L^{-1} h^{-1} and an average ethanol productivity of 144.8 mg L^{-1} h^{-1} [138]. Introducing *Synechococcus* sp. PCC 7002 Rubisco into *R. eutropha* H16 enhanced its CO_2 utilization capacity, resulting in a 93.4 % increase in polyhydroxybutyrate production compared to the wild-type [121]. Several recent reviews have provided detailed summaries on this topic [34,139].

In addition to the mentioned products, theoretically, various other products, such as amino acids and organic acids, can also utilize Rubisco for CO_2 recycling during the production process. Although the utilization of Rubisco for carbon sequestration has achieved certain results, it is essential to acknowledge that Rubisco, being a large protein, can impose a burden on production, especially when co-expressed with chaperones. Additionally, fixing one molecule of CO_2 consumes one extra molecule of ATP, and this cost must be considered, particularly for strains with insufficient energy supply. If it does not impact bacterial growth, the application of Form II or Form III Rubisco, with higher solubility and activity, could also be considered. Rubisco's carbon sequestration may play a crucial role in the construction of artificial biological carbon fixation and new industrial raw material routes in the future. However, the carbon fixation capacity is limited by Rubisco's specificity and carboxylation efficiency, making the exploration or engineering of high-performance Rubisco the primary focus.

6. Conclusion

As the most crucial carbon-fixing enzyme in the nature, enhancing the carbon-fixing efficiency of Rubisco is crucial for global food security

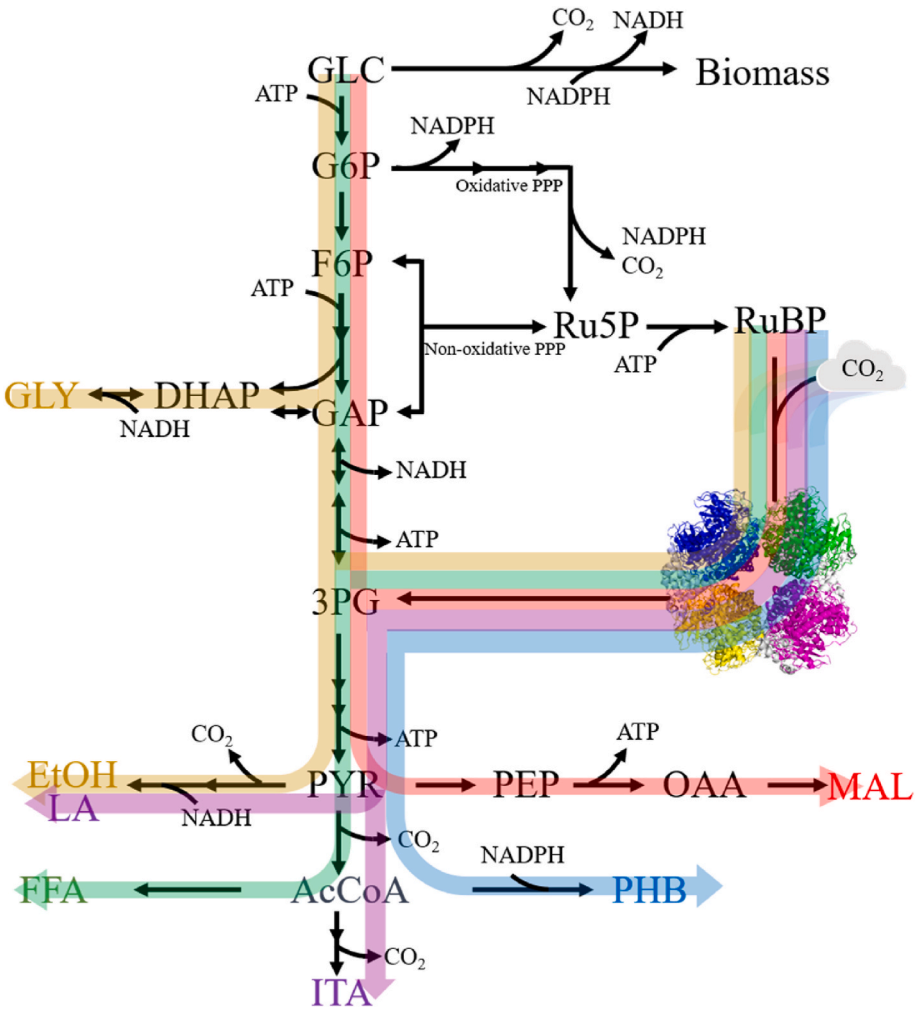

Fig. 4. Rubisco serves as a valuable tool for converting CO_2 into chemicals. The yellow arrow indicates EtOH production; the green arrow represents FFA production; the blue arrow denotes PHB generation; the red arrow stands for MAL production; The purple arrow represents the generation of LA and ITA. Abbreviations: GLC, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; GLY, glycerol; DHAP, dihydroxyacetone phosphate; 3PG, 3-phosphoglycerate; PYR, pyruvate; EtOH, ethanol; FFA, free fatty acid; ITA, itaconic acid; LA, lactic acid; AcCoA, acetyl coenzyme A; Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetic acid; MAL, malate; PHB, polyhydroxybutyrate. *Synechococcus elongatus* PCC 7942 Rubisco structure, PDB ID 6SMH.

and climate change. In recent years, the progress in plant-chloroplast transformation has made it increasingly feasible to manipulate the Rubisco large subunit in plants. A better understanding of Rubisco biogenesis and regulation has provided guidance for strategies such as overexpression, substitution, or modification of the RbcL or RbcS. In contrast, advancements in engineering auxiliary factors like RCA, precursor supply, along with modifications to the photorespiration pathway, have shown to yield better results in enhancing Rubisco enzymatic activity and elevating overall plant photosynthetic efficiency, particularly under high CO_2 concentrations. The successful co-expression of five plant-derived chaperones has enabled assembly of *Arabidopsis*, potatoes, carrots, strawberries, and tobacco Rubisco in *E. coli*, making high-throughput mutagenesis and screening of all forms of Rubisco, including plant Rubisco, feasible. Moreover, by rapidly identifying crucial mutations that enhance Rubisco activity in *E. coli*, there is hope to transfer these mutation sites to plants and thus enhance the carbon fixation ability of plants. The utilization of Rubisco in production strains allows for the recovery of CO_2 generated during metabolic processes or enhances the carbon fixation pathways, thereby increasing the yield or productivity of chemicals. Furthermore, the transformation of heterotrophic microorganisms into autotrophic ones using Rubisco has broadened the scope of C1 utilization. This encompasses not only autotrophic carbon fixation but also the utilization of formate or methanol as viable energy sources for harnessing C1 compounds. With the maturation of renewable photoelectrochemical CO_2-to-formate and CO_2-to-methanol conversion technologies, this approach holds vast potential for diverse applications. In conclusion, the engineering of Rubisco to enhance its carbon fixation ability holds immense significance in addressing global climate change, ensuring global food security, and advancing the C1 industrial biotransformation industry. While there has been a degree of advancement in recent years, the accomplishments remain relatively limited. Achieving further successful transformations hinge on the continuous refinement of chloroplast transformation methods across diverse plant species. A more profound comprehension of Rubisco's structure and functionality is essential, particularly concerning selectivity, where knowledge gaps still exist. Moreover, a holistic grasp of Rubisco biogenesis within both plants and microorganisms is crucial for advancing this field.

Declaration of competing interest

Authors declare that they have no conflict of interest.

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