Seminars in Cell and Developmental Biology 155 (2024) 37–47

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Perspectives on improving crop Rubisco by directed evolution

Matteo Gionfriddo [a,b,1], Timothy Rhodes [a,2], Spencer M. Whitney [a,3,*]

[a] Plant Science Division, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia
[b] Department of Cellular Biochemistry, Max Planck Institute of Biochemistry, Martinsried 82152, Germany

ARTICLE INFO

Keywords:
CO_2-fixation
Photosynthesis
Chloroplast transformation
Directed evolution
Chaperones
Synthetic biology
Chaperonin
Food security
Calvin cycle

ABSTRACT

Rubisco catalyses the entry of almost all CO_2 into the biosphere and is often the rate-limiting step in plant photosynthesis and growth. Its notoriety as the most abundant protein on Earth stems from the slow and error-prone catalytic properties that require plants, cyanobacteria, algae and photosynthetic bacteria to produce it in high amounts. Efforts to improve the CO_2-fixing properties of plant Rubisco has been spurred on by the discovery of more effective isoforms in some algae with the potential to significantly improve crop productivity. Incompatibilities between the protein folding machinery of leaf and algae chloroplasts have, so far, prevented efforts to transplant these more effective Rubisco variants into plants. There is therefore increasing interest in improving Rubisco catalysis by directed (laboratory) evolution. Here we review the advances being made in, and the ongoing challenges with, improving the solubility and/or carboxylation activity of differing non-plant Rubisco lineages. We provide perspectives on new opportunities for the directed evolution of crop Rubiscos and the existing plant transformation capabilities available to evaluate the extent to which Rubisco activity improvements can benefit agricultural productivity.

1. Introduction

Prime among the many challenges stemming from a rising global population is meeting the escalating food demand. Compounding the problem are waning crop yield improvements achievable through breeding, lack of additional arable farming land and escalating frequencies of drought and extreme growing temperatures [1,2]. Science and agriculture are facing the critical challenge of finding step-change solutions to improve crop yield and quality. Underway are a range of bio-engineering strategies to improve crop pathogen resistance, resource use (water, nutrients), resilience to temperature and salinity, and substantial gains in growth rate, yield and quality [3]. Detailed molecular understanding of these biological processes and discovery of beneficial natural genetic diversity to introgress into domesticated crops are helping to accelerate these activities [4]. Access to new multi-target genetic engineering capabilities – some inconceivable a decade ago - is also proving transformative in the race to provide resilient, sustainable, crop harvests [1,5].

Among the plant metabolic pathways targeted for improvement is photosynthesis, a well understood chloroplast process that underpins life on earth. Photosynthesis includes light harvesting and electron transfer reactions within thylakoid membranes that produce oxygen, ATP and NADPH – with the latter energy products used to fuel chloroplast metabolism, including the Calvin Benson Bassham (CBB) cycle (Fig. 1 A). The 11 enzymes in the CBB cycle are responsible for fixing CO_2 into glyceraldehyde 3-phosphate (GAP) that is required for biomass production, in addition to regenerating ribulose-1,5-bisphosphate (RuBP) to which CO_2 is bound by the enzyme RuBP carboxylase/oxygenase (Rubisco, [6]).

Attention on improving photosynthetic efficiency arises from its pervasive impact on crop energy conversion efficiency, a component of yield potential that has substantial room for improvement [7]. While increases in plant growth rate have been obtained by modifying photosynthetic light harvesting metabolism (reviewed in [5]), reactions within the CO_2-fixing and RuBP regeneration phases of the CBB cycle have long been known to also limit the rate of photosynthetic carbon assimilation [6]. A longstanding target has been to improve the carboxylation properties of Rubisco, an inherently slow enzyme that can

* Corresponding author.
E-mail address: spencer.whitney@anu.edu.au (S.M. Whitney).
[1] 0000-0003-2364-0812
[2] 0000-0002-4146-7913
[3] 0000-0003-2954-2359

https://doi.org/10.1016/j.semcdb.2023.04.003
Received 23 January 2023; Received in revised form 14 April 2023; Accepted 15 April 2023
Available online 20 April 2023

also fix O_2 instead of CO_2 to produce toxic 2-phosphoglycolate (2-PG) whose recycling via photorespiration consumes energy and results in the loss of fixed CO_2 (Fig. 1 A, [8]).

The catalytic inefficiencies of Rubisco have raised interest in the design of alternative 'new-to-nature' CO_2-fixing pathways [9,10]. Functional integration of these enterprising, multi-component synthetic pathways into chloroplasts poses a significant challenge, especially as the CBB cycle constitutes a foundation pathway in central metabolism that not only fuels starch and sucrose production but also services the isoprenoid, shikimate, thiamine and nucleotide biosynthetic pathways (Fig. 1A). Nevertheless, such ambitious plant metabolic redesign aspirations are not new, as evidenced by the long-awaited success in generating a functional C_4-rice prototype using modern combinatorial gene transformation tools [11]. One might question the need for such complicated metabolic redesign strategies if the carboxylation properties of Rubisco itself could be significantly improved. For instance the catalytic properties of Rubisco from some red algae would support higher rates of photosynthesis in crops like rice, wheat, canola and soybean with C_3-physiologies (i.e. those that lack a CO_2-concentrating mechanism, CCM) [12]. This discovery has inspired efforts to emulate

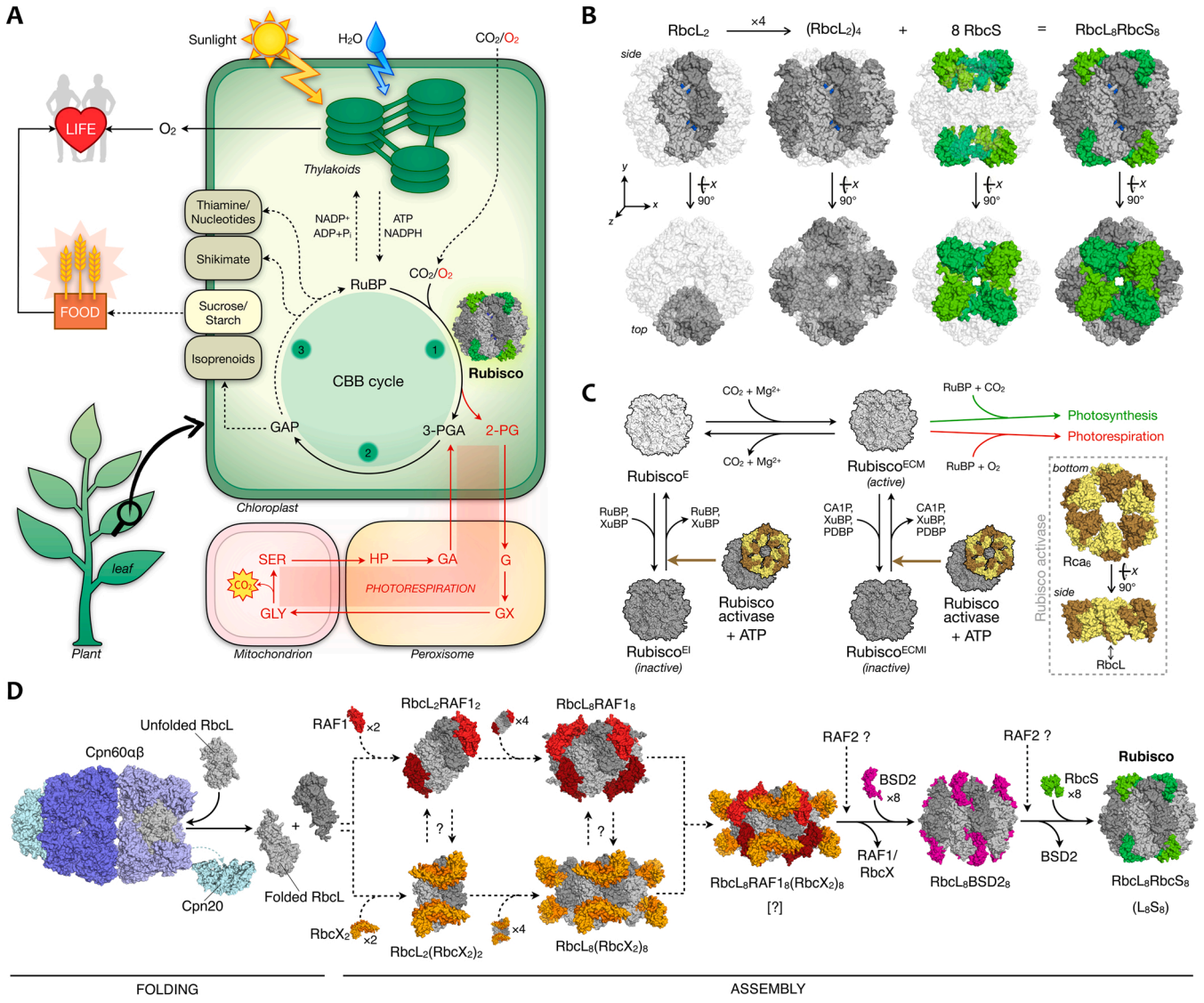

Fig. 1. The role, structure, activation and assembly of Rubisco in plant photosynthesis. (A) Summary of the O_2 and energy producing light harvesting and carbon fixing CBB pathways of photosynthesis that preserve life in the biosphere. The three phases of the CBB cycle comprise (1) ribulose-1,5-bisphosphate (RuBP) carboxylation by Rubisco, (2) use of ATP and NADPH to reduce 3-phosphoglycerate (3-PGA) into glyceraldehyde-3-phosphate (GAP) that is (3) either used to regenerate RuBP or used to produce carbohydrates (sucrose, starch) and isoprenoids. Intermediary CBB metabolites also fuel the thiamine, nucleotide and shikimate biosynthetic pathways. Oxygenation of RuBP by Rubisco produces 2-phosphoglycolate (2-PG) whose recycling by photorespiration (in red) into 3-PGA spans three organelles at the expense of ATP and CO_2 loss. G: glycolate; GX: glyoxylate; GLY: glycine; SER: serine; HP: hydroxypyruvate; GA: glycerate. (B) Side and top views of how the four pairs of RbcL subunits (grey) harbouring the Rubisco catalytic sites arrange as a tetrad core onto which four RbcS (green) arrange at each apex to stabilise and catalytically prime plant Form I $RbcL_8RbcS_8$ Rubisco (PDB: 3AXM). (C) Catalytic priming of Rubisco (E) requires activation with CO_2 and Mg^{2+} (ECM) allowing correct RuBP binding for carboxylation/oxygenation. Inhibitory sugar phosphates (XuBP: Xylulose 1,5-bisphosphate; CA1P: 2-carboxy-D-arabinitol 1-phosphate; PDBP: pentadiulose-1,5-bisphosphate) bound within Rubisco catalytic sites (EI, ECMI) are released via an ATP hydrolytic process facilitated by Rubisco activase (Rca). The primary functional form of Rca is a hexamer (grey dashed box, tobacco Rca structure [PDB: 3ZW6] showing alternately coloured brown and yellow subunits). (D) Proposed pathway of plant Rubisco biogenesis begins with RbcL folding in a chloroplast Cpn60αβ/20 cage prior to forming a variety of possible chaperone bound RbcL-intermediary complexes with RAF1 and/or $RbcX_2$, possibly even a $RbcL_8RAF1_8(RbcX_2)_8$ complex (PDB: 7XSD) before binding BSD2 to form a $RbcL_8BSD2_8$ complex (PDB: 6EKC) to which RbcS binds and dispels BSD2 to form the functional $RbcL_8RbcS_8$ holoenzyme.

this evolutionary feat in other forms of Rubisco using directed evolution ([13]).

Here we review the advances that have been achieved using directed evolution to improve the CO_2-fixing speed (k^c_{cat}), affinity (K_c) and CO_2/O_2 specificity ($S_{c/o}$) of different non-plant Rubisco isoforms. We focus on the few genuine examples where the catalytic prowess has been improved and explore how the approach might be extended to evolve plant Rubisco. A core requirement of such endeavours is being able to test by genetic transformation the translational impact an evolved Rubisco has on plant photosynthesis and growth. We therefore appraise the plant transformation capabilities available for bioengineering Rubisco that are currently constrained by the range of species amenable to chloroplast transformation [14].

2. Enhancing Rubisco activity – a target for improving photosynthesis

Under ambient CO_2 and saturating illumination, the rate of photosynthesis in C_3-plants is predominantly Rubisco activity (A_c) limited. As evident from the productivity gains experienced in CO_2-fertilisation field studies, C_3-plant photosynthesis and growth will continue to experience Rubisco activity productivity limitations under the elevated CO_2 (eCO_2) levels envisaged over the next century [15]. Enhancing Rubisco activity in C_3-crops has thus been a focal point for improving photosynthesis, growth and yield under both current and future eCO_2 [14,16].

As evident from the biochemical models of photosynthesis in C_3-plant leaves [17], improving the rate of CO_2-assimilation when A_c limited could be achieved by increasing the k^c_{cat} and $S_{c/o}$ of Rubisco and enhancing its affinity for CO_2 (decreasing K_c) and reducing its affinity for O_2 (increasing K_o) (Fig. 2A). While a preference would be to achieve concurrent improvements in all four catalytic parameters (as idealistically portrayed in Fig. 2B), it is not essential as long as the net kinetic gains in combination increase the initial slope under A_c-limited growth [14]. This could be achieved by improving the Rubisco carboxylation efficiency (defined as $k^c_{cat} / K_c(1 + [O_2]/K_o)$ while maintaining a k^c_{cat} that is at least wild-type equivalent (Fig. 2). Photosynthetic gains by improving $S_{c/o}$ are also envisaged to arise from enhancing electron transport rate limited photosynthetic rates (A_j) due to declines in 2-PG production and costly recycling by photorespiration. Improving $S_{c/o}$ would also lower the CO_2 compensation point (Γ) under A_c-limited growth (Fig. 2B). As Rubisco is already inherently produced in high abundance in plants (up to 50% w/w of the leaf protein in C_3-species, [18]), improving its catalysis may provide opportunities to improve plant nitrogen use efficiency by reducing the Rubisco levels required [14,16]. Reciprocal Rubisco overexpression studies have however shown productivity increases in field grown rice [19] and chamber grown maize [20]. In both instances the gains were achieved under carefully managed resource replete conditions, an economic cost that might constrain their practical utility in some agricultural settings.

A variety of bioengineering strategies aimed at enhancing Rubisco activity indirectly in C_3-crops are also being pursued. These include introducing components of the CCM from cyanobacteria or algae into leaf chloroplasts to allow Rubisco to work under saturating CO_2 [21,22]. While the genetic complexity of introducing these CCM's into plants pose significant bioengineering hurdles, the ongoing efforts have dramatically improved our CCM mechanistic understanding [23,24]. The predicted crop productivity gains of up to 60% afforded by introducing a heterologous CCM are however modelled on producing their cognate high k^c_{cat} Rubiscos in equivalent abundance to native plant Rubisco [22]. However, the biogenesis requirements of cyanobacteria Rubisco (summarised in Figure S1) and algae Rubisco [12,25] are either

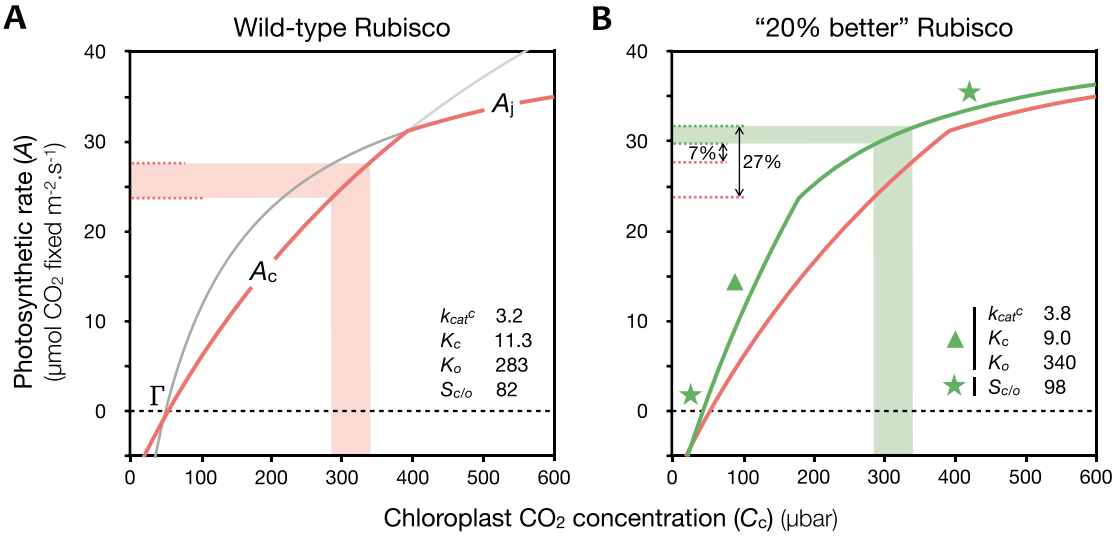

Fig. 2. The impact of improving Rubisco carboxylation on C_3-photosynthesis?. The biphasic responses of photosynthetic rate (A, red line) to varying chloroplast CO_2 pressures (C_c) at 25 °C in a tobacco leaf expressing (A) wild-type Rubisco (intersecting red lines) and (B) a Rubisco with 20% improvements in k^c_{cat} (s^{-1}), K_c (μM), K_o (μM) and $S_{c/o}$ (green lines). Rubisco activity limited rates of photosynthesis (A_c) using the shown kinetic values were fitted to the equation: $A_c = \frac{m.k_{cat}{}^c\left(C_c.s_c - 0.5O_c/S_{\frac{c}{o}}\right)}{(C_c.s_c + K_c(1+O_c/K_o))} - R_d$, and the electron transport limited photosynthetic rate (A_j) fitted according to: $A_j = \frac{\left(C_c.s_c - 0.5O_c/S_{\frac{c}{o}}\right)J_{max}}{4\left(C_c.s_c + O_c/S_{\frac{c}{o}}\right)} - R_d$, as defined by [17]. Solid lines indicate the minimal rates of CO_2-assimilation from the intersecting A_c and A_j modelled rates. Triangles indicate the kinetic parameters impacting the increase in A_c, stars indicate the improvement in A_j and lower CO_2 compensation point (Γ) arising from the $S_{c/o}$ increase. O_c, chloroplast O_2 concentration (modelled at 252 μbar); s_c, CO_2 solubility constant (0.0334 M bar^{-1}); s_o, O_2 solubility constant (0.00126 M bar^{-1}); m, Rubisco content (30 μmol catalytic sites.m^{-2}), J_{max}, electron transport rate (180 μmol.$m^{-2}.s^{-1}$), R_d, mitochondrial respiration rate (1 μmol CO_2 released.$m^{-2}.s^{-1}$). The pink/green shading represent the extrapolated span in CO_2-assimilation rates (A) under the C_c pressures in tobacco leaves grown under ambient CO_2 (aCO_2, 410 ppm) with the "better" Rubisco simulated to improve A by 7–27%. Tobacco Rubisco kinetic values from [26].

poorly met, or not met at all, by leaf chloroplasts. Producing a heterologous CCM in leaf chloroplasts that can support faster rates of photosynthesis may therefore necessitate additional genetic engineering to bolster cognate Rubisco activity levels by co-transplanting in compatible assembly and metabolic repair chaperones (for example Rubisco activase, [26]) from the cyanobacteria or algae.

Alternative strategies for improving Rubisco activity have focused on altering chloroplast metabolism to limit flux through the resource costly photorespiratory cycle (reviewed in [27]). For example, introducing synthetic CO_2-emmiting glycolate metabolizing pathways in the stroma and slowing chloroplast glycolate and glycerate transport can improve the rates of photosynthesis and growth of tobacco, though only in some lines producing each recombinant component in optimal quantities [28].

3. Plant Rubisco structure, regulation and biogenesis

Plant Rubisco constitutes a Form I structure of ~520 kDa that comprises eight ~50 kDa large (RbcL) subunits coded by the *rbcL* gene in the chloroplast genome (plastome) and eight ~15 kDa small (RbcS) subunits coded by multiple *RbcS* genes in the nucleus [29]. The RbcL's bind head-to-tail into dimers that house two catalytic sites at their interface (Fig. 1B). Four dimers then arrange to form an octameric $(RbcL_2)_4$ core to which eight RbcS bind to form a highly stable, catalytically primed, $RbcL_8RbcS_8$ holoenzyme [30].

3.1. The need for activation and regulation

The RbcL amino acids that form the catalytic site are conserved in nature, as is the activation process wherein a molecule of CO_2 reacts with lysine-201 to form a carbamate that is stabilised by Mg^{2+} binding (Fig. 1C). In this active 'ECM' form the catalytic site can correctly bind RuBP for binding substrate CO_2 to form a β–keto acid that is cleaved into two molecules of 3-phosphoglycerate (3-PGA, [14,29,31]). Competing oxygenation of RuBP can also occur, in C_3-plants occurring at a frequency that leads to ~30% of the fixed CO_2 being lost during photorespiratory recycling of 2-PG back into 3-PGA (Fig. 1A)[8].

Inhibition of plant Rubisco can occur when a catalytic site, both before and after CO_2-Mg^{2+} activation, bind sugar phosphate compounds produced either as catalytic misfire products or as natural regulators (Fig. 1C). Displacement of these compounds from a Rubisco catalytic site is facilitated by Rubisco activase (Rca), a AAA+ (ATPase Associated Activity) metabolic repair protein where the hexamer is the most active oligomeric conformation [29,32]. There is increasing attention to better understanding the mechanistic properties of Rca as it can crucially impact the temperature response of photosynthesis. That is, unlike the thermal stability of plant Rubisco (denaturation requiring temperatures >60 °C), the structural and functional integrity of Rca in plants is compromised under moderate heat stress (between ~35 – 40 °C) making enhancing the thermostability of Rca a target for improving crop productivity under warmer temperatures [32,33].

3.2. The multi-chaperone dependency of plant Rubisco biogenesis

A decade of discoveries into the multi-chaperone Rubisco biogenesis process in plant chloroplasts led to the long-awaited, and heralded, achievement of producing Arabidopsis and tobacco Rubisco in *E. coli* [30]. As summarised in Fig. 1D, the biogenesis pathway involves at least 7 chloroplast components that include 3 elements of the chaperonin protein folding cages (Cpn60α, Cpn60β, Cpn20) and four assembly chaperones (RAF1, RAF2, RbcX, BSD2). Both RAF1 and BSD2 are specific to plant Rubisco assembly with RAF2 serving an unknown, but essential, role in $RbcL_8RbcS_8$ biogenesis – possibly facilitating RbcS binding in the complex [34]. While RbcX is not essential for plant Rubisco synthesis in *E. coli*, its inclusion enhances production [30]. RAF1 and RbcX can function independently to augment intermediary $RbcL_8$-chaperone complex formation, and perhaps even form heterologous structures containing both chaperones ([35], Fig. 1D). More detailed understanding of plant Rubisco biogenesis is still needed, in particular understanding the sequence complementarity requirements between plant RbcL and its auxiliary chloroplast chaperones which can impair, sometimes prevent, the biogenesis of heterologous plant Rubiscos in both *E. coli* and plant chloroplasts [36].

4. Directed evolution of Rubisco

Natural evolution of proteins entails iterated mutation and selection over many generations of adaptive changes that benefit function. A comparable approach employed by scientists is directed evolution that uses laboratory methods to artificially select for alterations in the activity of individual biomolecules [37]. A foremost requirement of directed evolution is the generation of suitably diverse mutant gene libraries. The second core component is a sensitive, high throughput screen capable of selecting for the desired biochemical trait. Screens utilising fast growing, high transformation efficiency hosts like *E. coli* and yeast (*Saccharomyces cerevisiae*) are the most popular. As such, the development of Rubisco Dependent *E. coli* (RDE) screens (Fig. 3A) has underpinned the successful directed evolution of some Rubisco isoforms to identify mutations that enhance catalysis and/or holoenzyme biogenesis in the bacterium.

4.1. Historic summary of RDE screen development

The discovery spinach phosphoribulokinase (PRK) expression was toxic to *E. coli* metabolism (Fig. 3B) due to the accumulation of RuBP [38] led to the design of a theoretical RDE screen incorporating a glycolysis pathway deletion complemented with a PRK-Rubisco metabolic bypass [39]. This led to the production of the *E. coli* Δ*gapA* mutant MM1 strain where glyceraldehyde-3-phosphate dehydrogenase production was deleted (Fig. 3C) [40]. MM1 RDE screens were undertaken on minimal media containing a carbon source, such as glycerol, upstream of the glycolytic lesion so that cell growth was dependent on PRK and Rubisco expression. Directed evolution experiments using MM1 were successful in selecting catalysis changing mutations in the bacterial *Rhodospirillum rubrum* L_2 Form II *Rr*Rubisco [41] and archaeal *Methanococcoides burtonii* L_{10} Form III *Mb*Rubisco (Fig. 4A, [42]). The metabolic re-wiring in MM1 however impaired its cellular viability that reduced its transformation efficiency and marred Rubisco mutant selection efficiency [40]. Other RDE screens using wild-type *E. coli* showed the selection of higher activity *Synechococcus elongatus Se*Rubisco mutants was possible without need for a glycolytic bypass (Fig. 3D), however the RbcL mutants selected improved the biogenesis (solubility) of the cyanobacterial L_8S_8 holoenzyme, not its k^c_{cat}, K_c or $S_{c/o}$ properties (reviewed in [13]).

A limitation of wild-type based RDE screens is the extremely high prevalence of false positives selected where ~0.5% of the colony forming units screened grow under non-permissive PRK selection despite coding unmutated Rubisco. Genetic analyses showed these 'escape mutants' mostly arise from *prk* transgene transposon insertions that silence PRK production [43]. To avoid escape mutant selection subsequent RDE screens have utilised a PRK::NPTII fusion protein linking kanamycin resistance to PRK production to inhibit cell growth on kanamycin if PRK::NPTII production is genetically silenced (Fig. 3D, [44]). Also limiting existing RDE screens is their exclusive use of solidified agar plate-based selection. Upscaling to continuous liquid culture RDE screens have been marred by the faster growth of PRK toxicity escape mutants that, even if arising infrequently, encumber the ability of higher Rubisco activity mutants to dominate growth [40,41]. Metabolically rewired *E. coli* strains derived by combining rational metabolic design and chemostat-based directed evolution however now exist that incorporate a life preserving, Rubisco dependent, CBB pathway where carbon assimilation is solely supported from CO_2-fixation [9,10].

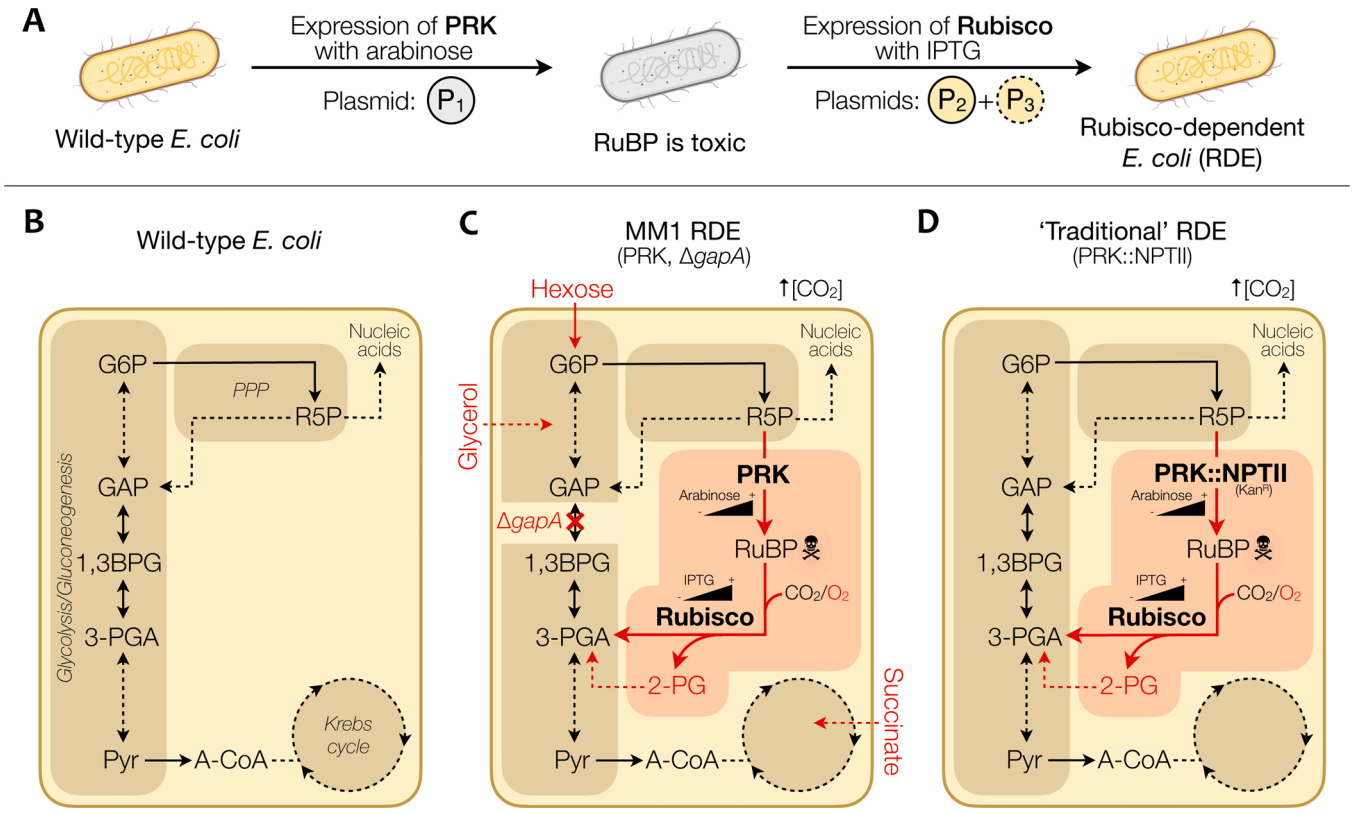

Fig. 3. Rubisco Dependent *E. coli* – a directed evolution screening tool. A variety of Rubisco dependent *E. coli* (RDE) screens have been utilized in the directed evolution of Rubisco. (A) Common to all RDE screens is the expression of phosphoribulokinase (PRK) to convert ribulose 5-phosphate (R5P) produced in the pentose phosphate pathway into RuBP, a non-native and inexplicably toxic metabolite to *E. coli*. Cell survival is thus dependent on Rubisco activity to alleviate this toxicity. Current directed evolution RDE screens rewire (B) wild-type *E. coli* metabolism by incorporating a PRK-Rubisco shunt (red arrows and shading) into strains coding (C) a glycolytic mutation (e.g. the Δ*gapA* deletion MM1 strain [41]) or (D) no mutation. RDE screens are performed under elevated CO_2 to promote RuBP carboxylation by Rubisco to produce 3-phosphoglycerate (3-PGA), a compatible glycolytic intermediate [13,40]. *E. coli* can also metabolise 2-PG produced via Rubisco RuBP oxygenation[41]. The rate of RuBP metabolism, and thus cell viability, is thus linked to the level of cellular Rubisco activity. More efficient RDE screens use a PRK::NPTII fusion protein and undertake selection on kanamycin to avoid false positive ('escape mutant') selection [44]. P_1, plasmid coding P_{BAD} promoter arabinose inducible PRK::NPTII; P_2, plasmid coding IPTG inducible T7 promoter regulated Rubisco gene(s); P_3, compatible plasmid to P_2 coding T7 regulated auxiliary chaperones required for plant Rubisco biogenesis (see [36] and Fig. 5, step 4 *). G6P: glucose 6-phosphate; GAP: glyceraldehyde 3-phosphate; 1, 3BPG: 1,3-bisphosphoglycerate; 3-PGA: 3-phosphoglycerate; Pyr: pyruvate; A-CoA: Acetyl-CoA; R5P: ribose 5-phosphate; RuBP: ribulose 1,5-bisphosphate; PRK: phosphoribulokinase; PPP: Pentose Phosphate Pathway.

Adapting these strains for use in an RDE bioreactor screen poses an enticing experimental prospect in developing higher throughput directed evolution tools for Rubisco bioengineering.

4.2. *Improving bacterial and archaea Rubisco catalysis by directed evolution*

Until now the directed evolution of Rubisco using RDE selection has been limited to isoforms from bacteria and Archaea whose holoenzyme biogenesis needs can be met, either partially or fully, in *E. coli*. To date, mutations that improve the carboxylation properties of *Mb*Rubisco (Fig. 4A, [42]), *ThermoSynechococcus elongatus* BP1 Form IB *Te*Rubisco (Fig. 4B, [44]) and *Rhodobacter sphaeroides* Form IC *Rs*Rubisco (Fig. 4C, [13]) have been identified by using RDE selection, in most cases the mutations also improving the amount (solubility) of holoenzyme produced. Where in some cases the amino acid substitutions are localised nearby the catalytic sites (e.g. K332E in *Mb*Rubisco and Y345F in *Rs*Rubisco), other activity enhancing mutations are located more remotely. For example, directed evolution of *Te*Rubisco identified an array of catalysis enhancing mutations that all clustered at a remote, previously unconsidered, RbcL-RbcS interface (Fig. 4B). Other remote sequence changes in plant Rubisco with inexplicable catalytic impact have been identified [45,46], congruent with a growing database of other enzymes whose activities have been dramatically impacted by mutations located remotely from catalytic/substrate binding sites [47]. Discovering how remote residue substitutions might impact Rubisco catalysis using *in silico* rational and computational protein design methods currently appear particularly challenged by the multi-step catalytic chemistry and structural complexity of Rubisco [48]. Indeed, only ancestral sequence reconstruction has, to date, provided biochemical evidence of substitutions in RbcL and RbcS that enhance the carboxylation efficiency tobacco Rubisco, with corresponding impacts on $S_{c/o}$ and plant growth not yet tested [49].

Key outcomes of the Rubisco directed evolution research summarised in Fig. 4 are the demonstrated feasibility of the technology to improve Rubisco catalysis and that both k^c_{cat} and $S_{c/o}$ can be improved simultaneously. This finding supports the discovery that the antiquated k^c_{cat}-$S_{c/o}$ trade-off hypothesis was overestimated from a phylogenetic bias in the kinetic data analysed [50]. Of additional interest is the favourable outcomes of the directed evolution pilot study with Form IC *Rs*Rubisco ([13], Fig. 4C) that not only shares a phylogenetic lineage with the high $S_{c/o}$ Rubiscos of red algae, but whose biogenesis needs are met in leaf chloroplasts [26]. Together, these properties provide an enticing possibility for using *Rs*Rubisco as a proxy to explore the catalytic enhancing sequence space of red algae Rubisco towards bioengineering *Rs*Rubisco mutants that might be capable of improving the rates of leaf photosynthesis, plant growth and yield.

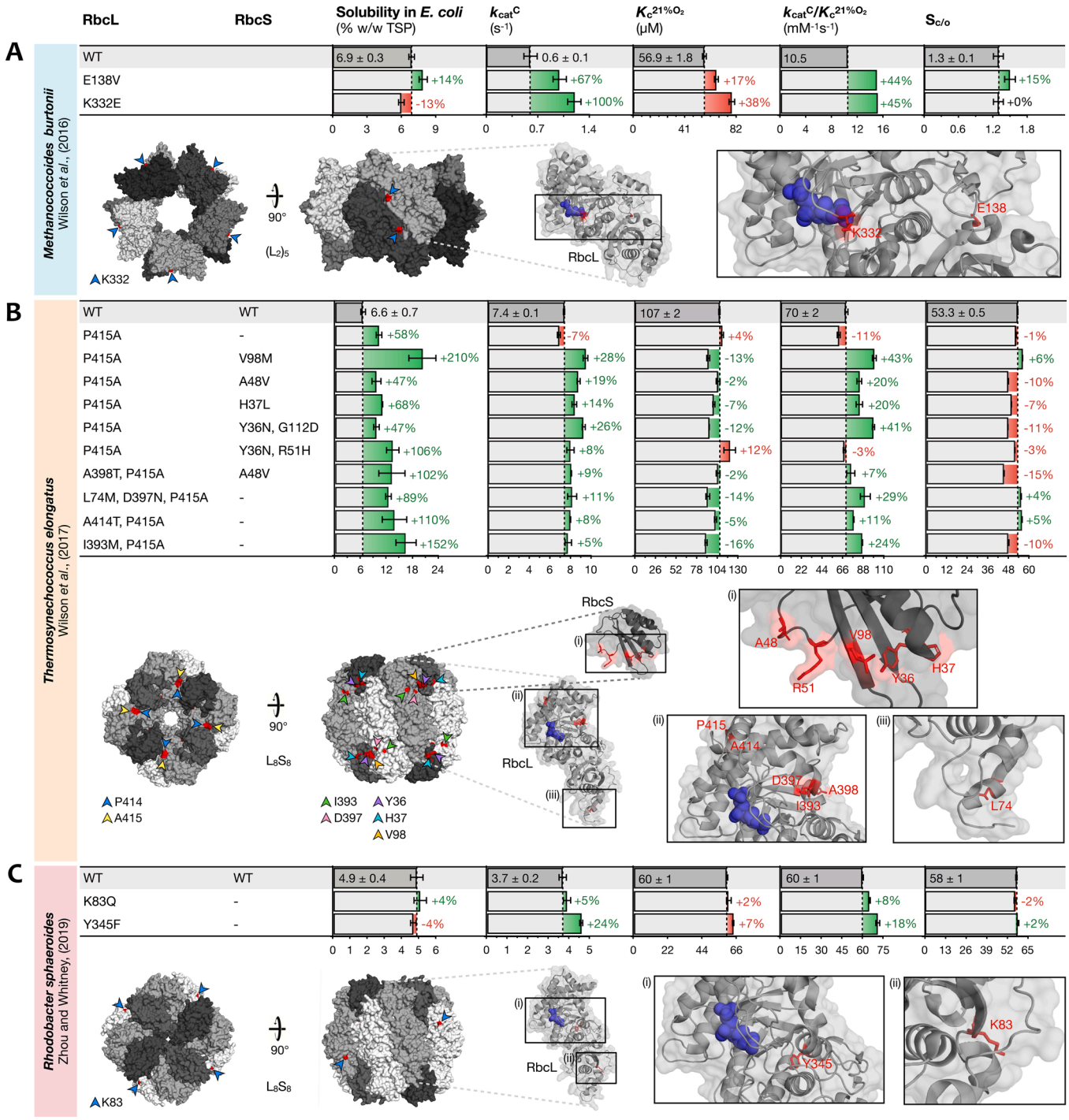

Fig. 4. Successful directed evolution of Rubisco catalysis by RDE screening. Summary of the biochemical properties of (A) *M. burtonii Mb*Rubisco [42], (B) *T. elongatus Te*Rubisco [44] and (C) *R. sphaeroides Rs*Rubisco [13] mutants selected by RDE screening. Mutants with improved (green) or impaired (red) holoenzyme biogenesis (solubility) or kinetics are indicated, including the % change relative to wild-type (WT, grey bars). Residues numbering for *Mb*Rubisco and *Te*Rubisco are relative to the parental enzyme with *Rs*Rubisco numbered relative to tobacco Rubisco (see RbcL and RbcS alignments in Fig. S2). Shown are the crystal structures of the L_{10} *Mb*Rubisco (PDB: 5MAC), the L_8S_8 *Te*Rubisco (PDB: 3ZXW) and *Rs*Rubisco (PDB: 5NV3) and the location of the amino acid mutations (in red) relative to the catalytic sites (indicated by bound sugar phosphate in blue) in both the unassembled RbcL and RbcS and in the assembled quaternary structure. Surface mutations are indicated with differing colored triangles. TSP, total soluble protein.

4.3. *Developing a new RDE screen for enhancing plant Rubisco catalysis*

Biochemical surveys among C_3-plants and C_4-plants have revealed significant variation in the carboxylation properties of their Rubisco [51, 52]. While C_3-photosynthesis simulations indicate examples where this variability might benefit leaf CO_2-assimilation rates [14], questions remain as to whether the improvement levels are sufficient to afford productivity gains in a crop context [53]. While the feasibility of achieving catalytic gains equivalent to a "20% better" plant enzyme (as modelled in Fig. 2B) are contentious, being able to now express plant Rubisco in *E. coli* provides an unprecedented opportunity to more fully explore the sequence space of plant Rubisco for improvements in

catalysis [30,54]. Accelerating such efforts requires the *E. coli* expression technology be adapted for directed evolution. As shown for *Te*Rubisco (Fig. 4B), identifying structural changes in crop Rubisco that provide a step improvement in carboxylation (that is, at a scale of transformative impact to plant productivity) may only be feasible by directed evolution.

Systems for plant Rubisco expression in *E. coli* have been developed in several laboratories [30,36,49]. Recent success in condensing the expression system to two, rather than three, plasmids and co-ordinating expression of all 9 transgenes with IPTG inducible T7 promoter provides the genetic circuitry needed for RDE development [54]. As summarised in Fig. 5 (step 4 *), this golden gate cloning based tobacco chloroplast compatible tcc*E. coli* expression system codes the Rubisco genes in pET16 (ampicillin resistance; plasmid P_2) and the 7 tobacco chloroplast chaperones required for Rubisco biogenesis (see Fig. 1D) in pCDF (spectinomycin resistance; plasmid P_3). Adapting this expression system for RDE screening should therefore be feasible by transforming in pACYC$^{prk:nptII}$ (chloramphenicol resistance, plasmid P_1) where PRK:: NPTII production is regulated by the arabinose inducible BAD promoter (Fig. 3D).

5. Plant Rubisco directed evolution, new possibilities and in planta testing

The traditional directed evolution pipeline using RDE screens encompass generating and transforming IPTG inducible mutant Rubisco libraries (typically produced by error-prone PCR) into *E. coli* cells (MM1, XL1-Blue, JM109(DE3) or BL21(DE3)) already transformed with an arabinose inducible PRK or PRK::NPTII expressing plasmid (Fig. 5, steps 1–4). The libraries are then plated on selective media containing suitable concentrations of IPTG and arabinose that only allow Rubisco mutants with 'above wild-type' activity to grow (Fig. 5, step 7). Re-screening is undertaken to compare and confirm the capacity of each Rubisco mutant to improve RDE fitness before sequencing and analysing the biochemical properties of each novel mutant (Fig. 5, steps 6–9) to identify suitable Rubisco mutants for additional rounds of directed evolution.

5.1. Employing next generation genomic tools to accelerate discovery

As summarised in Fig. 5, adapting the 'traditional' RDE screen for plant Rubisco directed evolution may simply entail adopting the 3-plasmid expression system described in Section 4.3 (step 4 *). Changes to the analytical pipeline might also consider using Deep Mutational Scanning (DMS) to sequence the combined Rubisco plasmid pool from surviving colonies (Fig. 5, steps 6 * and 7 *) in place of individual colony picking, rescreening and Rubisco plasmid sequencing (Fig. 5, steps 6–8). Of potential use is the rapid, low cost, mutational screening capabilities of Oxford Nanopore MinION long-read sequencing that make it an appealing DMS tool in protein analytics [55,56]. Of particular merit, DMS would both identify the selection frequency of fitness enhancing amino acid mutations in Rubisco as well as identify in what residue combinations they might occur. Problems with false positive colony selection is also avoided as only novel (non-wild-type) amino acid changes are mined for. Subsequent testing the impact these residue changes have on RDE selection fitness and carboxylation kinetics (Fig. 5, steps 7 * and 8 *) would require additional mutant gene generation, a bottleneck of diminishing significance with the rapid improvement in the speed and economics of single reaction restriction-ligation technologies and gene synthesis [36,57]. Once biochemically characterised, Rubisco mutant(s) modelled to improve photosynthesis (as simulated in Fig. 2) would be transformed into plants to test their translational impact on photosynthesis, growth and productivity (Fig. 5, steps 9 * and 10 *). This *in planta* translational testing is important as Rubisco biogenesis in *E. coli* poses an unreliable proxy for production in chloroplasts [36].

The choice of epPCR to generate lower mutation rate Rubisco libraries has been traditionally favoured over techniques incorporating larger domain swaps or saturation mutagenesis due the increased likelihood the latter methods introduce structurally destabilizing changes that impact holoenzyme assembly [37]. While the validity of such caution is tenuous, there remain new directed evolution technologies with the potential to accelerate progress in optimizing Rubisco performance. Prime among these is the process of continuous evolution where gene(s) of interest are subject to repeated cycles of hypermutation and selection in vivo [58]. The approach is advantage by the capacity to successively accumulate functionally neutral mutations over generation cycles before acquiring fitness-enhancing epistatic mutations. The approach thus provides a robust, faster method for progressively selecting better-performing variants at each round of mutation and selection. While continuous evolution systems such as Phage-assisted continuous evolution (PACE), PRANCE, EvolvR, OrthoRep, MutaT7 have been used to evolve a wide range of proteins [59,60], their utility in the directed evolution of Rubisco remains hypothetical (e.g. as an alternative to step 4 * in Fig. 5). Possibly a suitable "low experimental effort" PACE system might encompass coupling Rubisco catalysed 3-PGA production to the synthesis of the protein pIII required for M13 phage replication in *E. coli*, a selection screen successful in evolving T7 RNA Polymerase [61]. As with current plate based RDE selection systems, the continuous Rubisco evolution system design will need to ensure its efficiency is not compromised by escape mutant selection.

New gene library synthesis technologies with potential to more fully explore the sequence space of proteins include single-site variant libraries (SSVLs). Unlike epPCR that only survey approximately one-third of the possible amino acid diversity due to the redundancy in the genetic code [62], SSVLs provide user-defined control over the range of amino acid substitutions incorporated at a desired residue within a protein. Exploiting the capacity of SSVLs to access Rubisco residue substitutions unobtainable by epPCR is highly enticing, though challenging in deciding on what residues to focus. Fortunately, there exists a rising catalogue of specific amino acids in RbcL and RbcS that have been identified by directed evolution (e.g. Fig. 4 and S2), RbcL phylogeny-catalysis mapping [63,64] and catalytic site mutagenic profiling [65] as functionally important. As with any new technology however, the high cost of SSVL's pose a significant constraint on their utility. A more economical alternative might therefore be to consider trialing other genetic recombinant methods used in protein evolution [37] to generate Rubisco mutant libraries that can explore greater sequence space than epPCR with a focus on regions currently identified as functionally important (step 2, Fig. 5) [62].

5.2. Current and future tools for in planta testing of evolved plant Rubiscos

Confirming a modified biomolecule has the desired impact in an applied context is the core aim of directed evolution endeavours. In the context of bioengineering Rubisco to improving photosynthesis, ideally this entails transforming any 'improved' variants back into a photosynthetic host to validate their 'translational' capacity to enhance productivity and growth [14]. The last decade has seen rapid improvements in the efficiency, economics, species diversity and genetic precision of plant transformation technologies [66]. Inefficiencies associated with traditional Agrobacterium-mediated nuclear transformation approaches are slowly being superseded with alternate, multiplexed gene-targeting DNA delivery systems that offer improvements in transformation cost, efficiency and diversification in the spectrum of crop species amenable to genetic modification [67]. The advances are providing researchers opportunities to genetically modify crops directly and thus circumvent the traditional practice of first undertaking transformation tests in model plants like Arabidopsis.

Translational testing how changes to Rubisco catalysis impact plant photosynthetic and growth face two core challenges. Firstly, introducing changes to RbcL require the availability of chloroplast transformation tools to modify the *rbcL* gene in the plastome (Fig. 6A). Unfortunately,

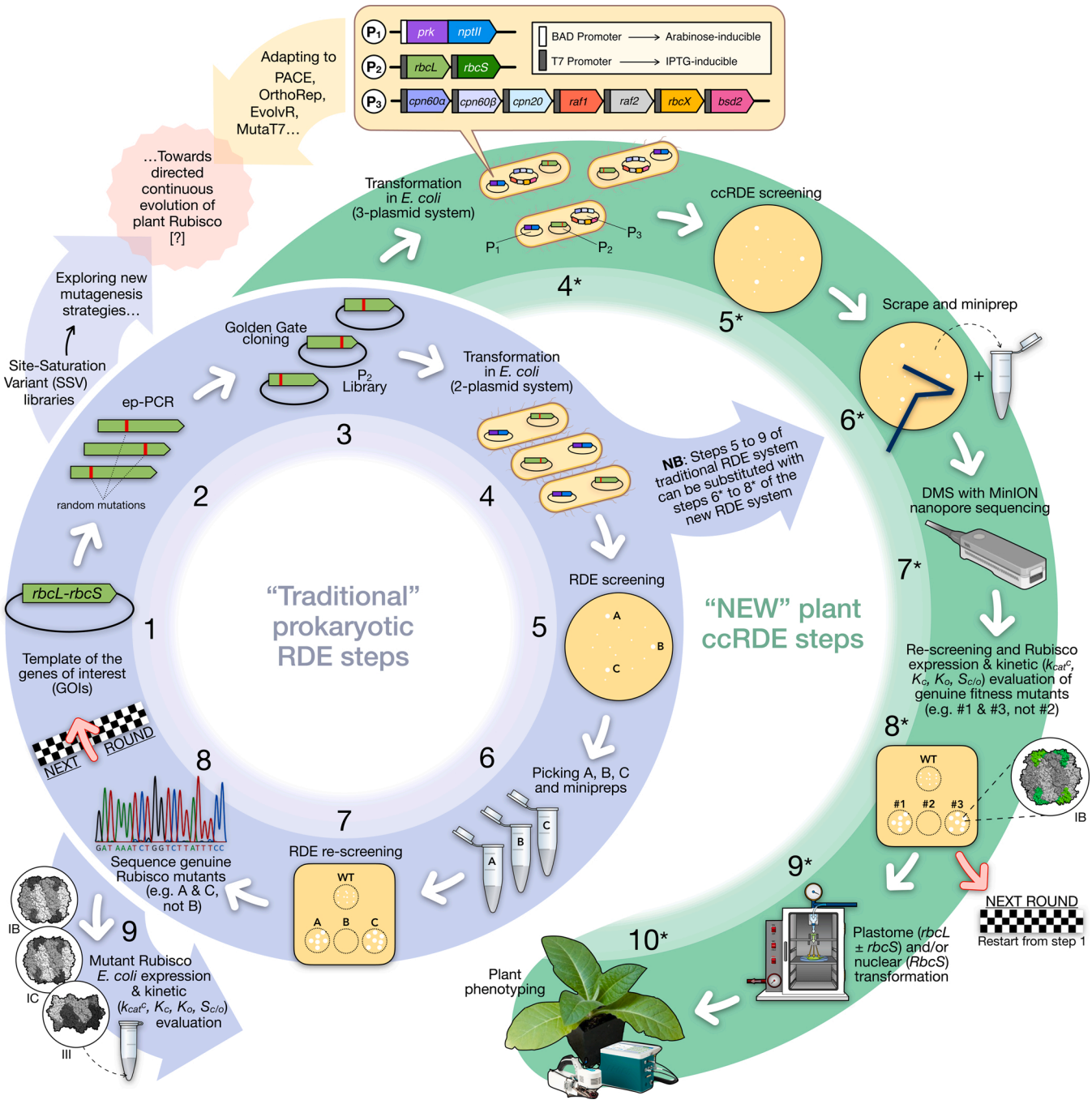

Fig. 5. Next-generation directed evolution and sequencing tools to improve Rubisco. Summary of the traditional mutate-screen-test iterative cycles in the directed evolution of Rubisco using RDE selection (blue circle) and new possibilities to (i) evolve plant Rubisco (green), (ii) speed-up winner mutant detection using Deep Mutational Sequencing (DMS), (iii) expand the accessible amino acid sequence space for screening (SSV library) and (iv) adapt continuous evolution platforms (e.g. PACE, OrthoRep, EvolvR, MutaT7, [60]) for Rubisco activity selection. The cycle steps include: (1) a template including *rbcL* (± *rbcS*) gene(s), (2) mutagenesis (epPCR, SSV library), (3) expression vector library construction (e.g. Golden Gate cloning) and (4) transforming into *E. coli* containing plasmid P_1 coding *PRK::NPTII* for (5) RDE screening using appropriate conditions of IPTG and arabinose for selection (Fig. 3). (6) Colonies with 'better-than-wild type' growth fitness are selected, their Rubisco plasmid purified and (7) re-screened by RDE to confirm improved growth fitness before (8) *rbcL-rbcS* sequencing and (9) expressing in *E. coli* and assessing how the mutations impact Rubisco biogenesis (solubility) and catalysis [13]. (4 *) A plant Rubisco RDE screen could entail 3 plasmids that builds on the 2-plasmid based chloroplast compatible *E. coli* (cc*E. coli*) plant Rubisco expression system of [36] (plasmid P_2 coding plant Rubisco genes and plasmid P_3 its 7 auxiliary chloroplast folding/assembly chaperones, see Fig. 1D). For quicker mutant screening, (5 *) the faster growing ccRDE winner mutants on the entire plate are (6 *) co-harvested by scraping and (7 *) the Rubisco amino acid mutations and frequency identified by *rbcL-rbcS* deep mutation screening (e.g. Oxford Nanopore MinION sequencing) before (8 *) re-testing the impact of high frequency mutations on ccRDE fitness and Rubisco biochemistry (solubility/kinetics). (9 *) 'Better' Rubisco mutants are introduced by chloroplast transformation into tobacco to assess (10 *) their translational impact on plants photosynthesis and growth (Fig. 6). Successive rounds of directed evolution are undertaken on higher activity ('winner') Rubisco mutants detected at steps (9) and (8 *).

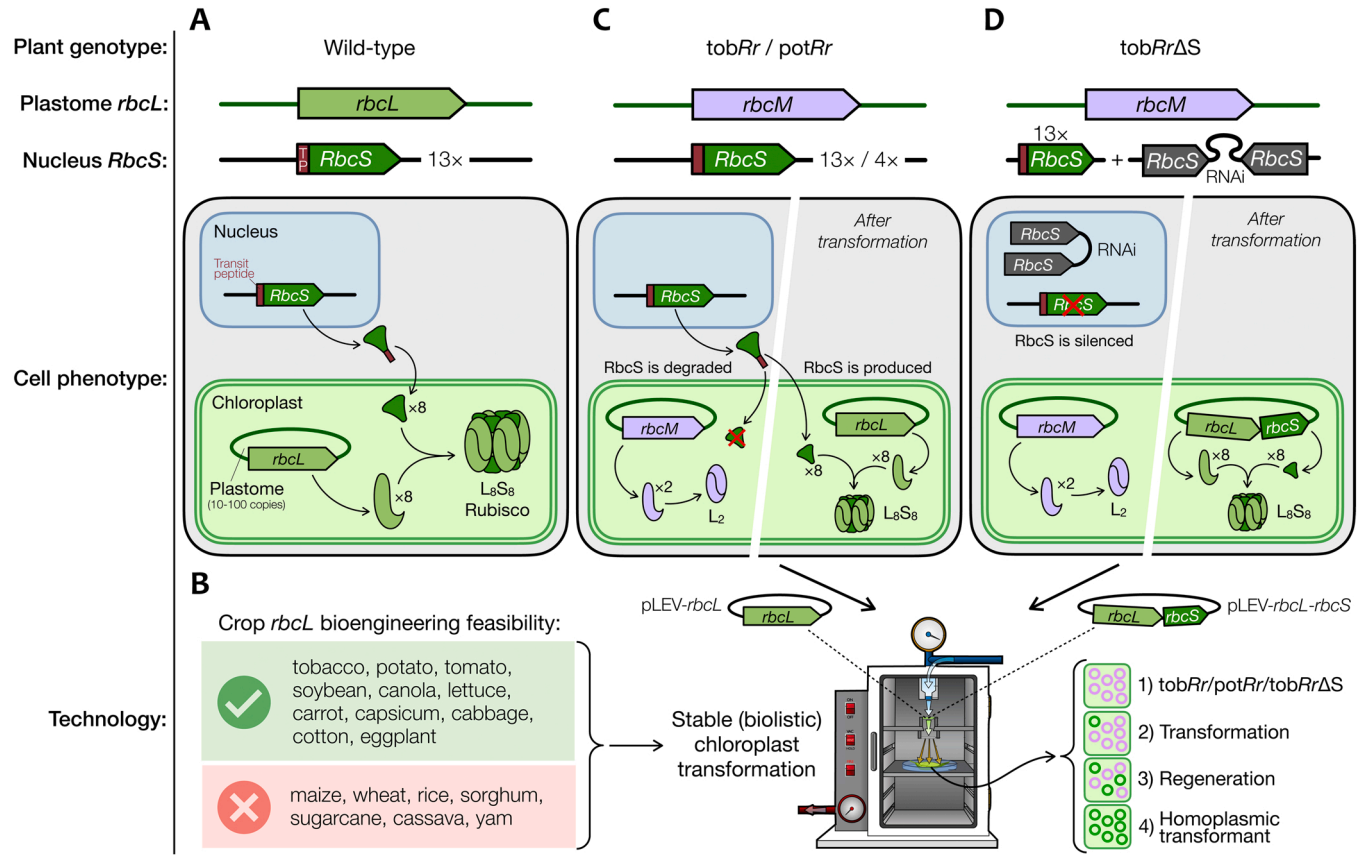

Fig. 6. The translational toolbox for *in planta* testing of directed evolution Rubisco mutants. (A) The high efficiency of plastome transformation in wild-type tobacco (*Nicotiana tabacum*) makes it the favoured model plant in bioengineering the plastome *rbcL* gene [14]. (B) Equivalent *rbcL* engineering efforts have been limited by the limited number of crops where plastome bioengineering is established (that does not include 7 of the 10 primary global crops, [68]). (C) Tobacco (tob*Rr*, [74]) and potato (pot*Rr*, [77]) lines where *rbcL* had been replaced with the *rbcM* gene coding the form II *R. rubrum* L_2 Rubisco have been produced, with the tob*Rr* master-line used for introducing foreign or mutated tobacco *rbcL* genes [14]. Subsequent silencing RbcS production in tob*Rr* by RNAi produced (D) tob*Rr*ΔS, a genotype allowing the simultaneous bioengineering of *rbcL* and *rbcS* by plastome transformation [45]. Transforming higher activity Rubisco mutants selected by RDE into either the tob*Rr* (when mutations are localised to *rbcL*) or tob*Rr*ΔS (when *rbcS* mutations require testing) provides the means to evaluate their chloroplast biogenesis compatibility and comparative impact on plant photosynthesis and growth relative to wild-type. *N. tabacum* and *Solanum tuberosum* (pot) code 13 × and 4 × nuclear *RbcS* copies, respectively [45,71].

methods for generating fertile, fully homoplasmic chloroplast transformed progeny (that is, where all plastome copies contain the genetic modification, Fig. 6) is limited to a small range of crop species that does not include monocot plants where there remains no suitable plastid transformed tissue regeneration systems (Fig. 6B) [68,69]. The second translational testing challenge in plants is the location of the multiple *RbcS* copies in the nucleus (Fig. 6A). Fortuitously the last 5 years has seen rapid advances in multiplex plant gene editing technologies to undertake multi-*RbcS* targeted mutagenesis [66,70], with preliminary gene editing efforts already successful in silencing targeted *RbcS* copies in Arabidopsis and tobacco [71,72].

Improving Rubisco catalysis can arise from substitutions in RbcL and/or in RbcS (see *Te*Rubisco for example, Fig. 4B). *In planta* Rubisco bioengineering activities have therefore employed plastome *rbcL* engineering in tobacco as well as modifying RbcS expression in tobacco, rice and Arabidopsis by either nuclear or chloroplast transformation [14,73]. Most RbcL bioengineering studies have been undertaken in tob*Rr,* a tobacco genotype where the *rbcL* gene is replaced with the *rbcM* gene coding L_2 *R. rubrum Rr*Rubisco (Fig. 6 C) [74]. Although *Rr*Rubisco is faster than tobacco Rubisco (with a ~4-fold higher k^c_{cat} of ~12 s^{-1} [41, 75]) and highly expressed in chloroplasts (Supplementary Figure S2), tob*Rr* growth in soil requires eCO_2 due to the poor CO_2-affinity and low $S_{c/o}$ of *Rr*Rubisco. Nevertheless, the high transformation efficiency and ease of recombinant Rubisco detection by native PAGE makes the tob*Rr* a preferred plastome transformation master-line [76] and inspired the generation of a comparative pot*Rr* line for Rubisco bioengineering in potato [77] (Fig. 6 C). Plastome engineering *rbcL* in wild-type tobacco is however feasible, although less efficient and error prone [78]. Future plant Rubisco bioengineering tools might also consider the recent advances made in utilizing plastid targeted transcription activator-like effector nucleases and cytidine deaminases to edit organelle genomes [79,80]. Perceivably these nuclear transformation tools might alleviate the *rbcL* transformation bottleneck currently impeding Rubisco mutagenesis in most major crops (Fig. 6B).

Recent advances in plant RbcS engineering include the generation of rice, tobacco and Arabidopsis lines where the endogenous RbcS genes have been fully, or selectively, silenced by RNA interference (RNAi) or CRISPR-Cas9 gene editing (reviewed in [73]). Successful examples include the production of RbcS silenced rice lines (edited using CRISPR) that only express an RbcS from sorghum (a C_4-plant) [81]. Though the resulting rice RbcL-sorghum RbcS hybrid enzyme showed an increase in k^c_{cat}, the accompanying impairments in K_c and $S_{c/o}$ impaired leaf CO_2-assimilation rates, as anticipated by the C_3-photosynthesis models (Fig. 2). RbcS production was also successfully silenced in tob*Rr* by RNAi-*RbcS* to produce the tob*Rr*ΔS genotype (Fig. 6D) where both *rbcL* and a synthetic *rbcS* can be co-engineered by plastome engineering [45]. Using potato Rubisco, this study generated four transplastomic lines producing potato RbcL and each of its four different RbcS. Kinetic comparisons identified two amino acid differences among its 3-mesophyll cell expressed RbcS variants that influence catalysis, and that

the trichome made RbcS indeed enhanced k^c_{cat} (as initially shown [82]), but at a corresponding cost to K_c and $S_{c/o}$ that impaired leaf photosynthesis [45].

6. Conclusion

Among the handful of directed evolution applications so far envisaged in plant science [83,84], most attention has been on evolving Rubisco. As outlined in this review, new genetic technologies for the directed evolution of plant Rubisco bioengineering in *E. coli* now exist. The advances include the development of next-generation plant directed evolution platforms and DMS analytics that can provide unprecedented throughput and opportunities to survey sequence space currently inaccessible within photosynthetic hosts and existing RDE screens. Improving the fidelity of plant Rubisco RDE screens for catalytic mutant selection will require genetic fine tuning to optimise the expression of each chaperone, RbcL, RbcS for maximal Rubisco production and possibly including Rca expression to maintain full Rubisco activity. Optimising plant Rubisco production in *E. coli* may also provide expression consistency that provides a reliable proxy for its production in chloroplasts. Coupled with this is the need to develop appropriate genetic tools for transforming into crops, while considering responsible innovation principles around plant transformation [3]. Ideally the redesign of Rubisco fitness should arise via minimal changes to the plastome *rbcL* gene and/or nuclear *RbcS* copies within target crops via gene editing. The potential to engineer step-change improvements to crop Rubisco performance by directed evolution and test their translational impact on agricultural productivity now appear close at hand.

Declarations of interest

None.

Acknowledgements and funding

This research was supported by the Australian Government through the Australian Research Council project DP210101757.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.semcdb.2023.04.003.

References

[1] J. Bailey-Serres, J.E. Parker, E.A. Ainsworth, G.E.D. Oldroyd, J.I. Schroeder, Genetic strategies for improving crop yields, Nature 575 (7781) (2019) 109–118.
[2] M.J. Paul, Improving photosynthetic metabolism for crop yields: what is going to work? Front Plant Sci. 12 (2021), 743862.
[3] A.F. Bowerman, C.S. Byrt, S.J. Roy, S.M. Whitney, J.C. Mortimer, R.A. Ankeny, M. Gilliham, D. Zhang, A.A. Millar, G.J. Rebetzke, B.J. Pogson, Potential abiotic stress targets for modern genetic manipulation, Plant Cell 35 (1) (2022) 139–161.
[4] G.N. Atlin, J.E. Cairns, B. Das, Rapid breeding and varietal replacement are critical to adaptation of cropping systems in the developing world to climate change, Glob. Food Secur. 12 (2017) 31–37.
[5] A.J. Simkin, P.E. López-Calcagno, C.A. Raines, Feeding the world: improving photosynthetic efficiency for sustainable crop production, J. Exp. Bot. 70 (4) (2019) 1119–1140.
[6] C.A. Raines, Improving plant productivity by re-tuning the regeneration of RuBP in the Calvin–Benson–Bassham cycle, N. Phytol. 236 (2) (2022) 350–356.
[7] R.T. Furbank, W.P. Quick, X.R.R. Sirault, Improving photosynthesis and yield potential in cereal crops by targeted genetic manipulation: Prospects, progress and challenges, Field Crops Res. 182 (2015) 19–29.
[8] F.A. Busch, Photorespiration in the context of Rubisco biochemistry, CO_2 diffusion and metabolism, Plant J. 101 (4) (2020) 919–939.
[9] N. Antonovsky, S. Gleizer, E. Noor, Y. Zohar, E. Herz, U. Barenholz, L. Zelcbuch, S. Amram, A. Wides, N. Tepper, D. Davidi, Y. Bar-On, T. Bareia, G. David, I. Wernick, S. Shani, G. Malitsky, A. Jona, R. Bar-Even, Milo, Sugar synthesis from CO_2 in Escherichia coli, Cell 166 (1) (2016) 115–125.
[10] S. Gleizer, R. Ben-Nissan, Y.M. Bar-On, N. Antonovsky, E. Noor, Y. Zohar, G. Jona, E. Krieger, M. Shamshoum, A. Bar-Even, R. Milo, Conversion of *Escherichia coli* to generate all biomass carbon from CO_2, Cell 179 (6) (2019) 1255–1263, e12.
[11] M. Ermakova, S. Arrivault, R. Giuliani, F. Danila, H. Alonso-Cantabrana, D. Vlad, H. Ishihara, R. Feil, M. Guenther, G.L. Borghi, S. Covshoff, M. Ludwig, A. B. Cousins, J.A. Langdale, S. Kelly, J.E. Lunn, M. Stitt, S. von Caemmerer, R. T. Furbank, Installation of C4 photosynthetic pathway enzymes in rice using a single construct, Plant Biotech. J. 19 (3) (2021) 575–588.
[12] S.M. Whitney, P. Baldet, G.S. Hudson, T.J. Andrews, Form I Rubiscos from non-green algae are expressed abundantly but not assembled in tobacco chloroplasts, Plant J. 26 (5) (2001) 535–547.
[13] Y. Zhou, S. Whitney, Directed evolution of an improved Rubisco; *In vitro* analyses to decipher fact from fiction, Int J. Mol. Sci. 20 (20) (2019) 5019.
[14] R.E. Sharwood, Engineering chloroplasts to improve Rubisco catalysis: prospects for translating improvements into food and fiber crops, N. Phytol. 213 (2) (2017) 494–510.
[15] M.E. Dusenge, A.G. Duarte, D.A. Way, Plant carbon metabolism and climate change: elevated CO_2 and temperature impacts on photosynthesis, photorespiration and respiration, N. Phytol. 221 (1) (2019) 32–49.
[16] C. Iñiguez, P. Aguiló-Nicolau, J. Galmés, Improving photosynthesis through the enhancement of Rubisco carboxylation capacity, Biochem. Soc. Trans. (2021).
[17] G.D. Farquhar, S. von Caemmerer, J.A. Berry, A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species, Planta 149 (1980) 78–90.
[18] J.R. Evans, V.C. Clarke, The nitrogen cost of photosynthesis, J. Exp. Bot. 70 (1) (2019) 7–15.
[19] D.-K. Yoon, K. Ishiyama, M. Suganami, Y. Tazoe, M. Watanabe, S. Imaruoka, M. Ogura, H. Ishida, Y. Suzuki, M. Obara, T. Mae, A. Makino, Transgenic rice overproducing Rubisco exhibits increased yields with improved nitrogen-use efficiency in an experimental paddy field, Nature, Food 1 (2) (2020) 134–139.
[20] C.E. Salesse-Smith, R.E. Sharwood, F.A. Busch, J. Kromdijk, V. Bardal, D.B. Stern, Overexpression of Rubisco subunits with RAF1 increases Rubisco content in maize, Nature, Plants 4 (10) (2018) 802–810.
[21] C. Fei, A.T. Wilson, N.M. Mangan, N.S. Wingreen, M.C. Jonikas, Modelling the pyrenoid-based CO_2-concentrating mechanism provides insights into its operating principles and a roadmap for its engineering into crops, Nature, Plants 8 (5) (2022) 583–595.
[22] J.M. McGrath, S.P. Long, Can the cyanobacterial carbon-concentrating mechanism increase photosynthesis in crop species? A theoretical analysis, Plant Physiol. 164 (4) (2014) 2247–2261.
[23] L. Adler, A. Díaz-Ramos, Y. Mao, K.R. Pukacz, C. Fei, A.J. McCormick, New horizons for building pyrenoid-based CO_2-concentrating mechanisms in plants to improve yields, Plant Physiol. 190 (3) (2022) 1609–1627.
[24] H. Wang, X. Yan, H. Aigner, A. Bracher, N.D. Nguyen, W.Y. Hee, B.M. Long, G. D. Price, F.U. Hartl, M. Hayer-Hartl, Rubisco condensate formation by CcmM in β-carboxysome biogenesis, Nature 566 (7742) (2019) 131–135.
[25] M.T. Lin, M.R. Hanson, Red algal Rubisco fails to accumulate in transplastomic tobacco expressing Griffithsia monilis RbcL and RbcS genes, Plant Direct 2 (2) (2018), e00045.
[26] L.H. Gunn, E. Martin Avila, R. Birch, S.M. Whitney, The dependency of red Rubisco on its cognate activase for enhancing plant photosynthesis and growth, Proc. Nat. Acad. Sci. 117 (41) (2020) 25890–25896.
[27] M. Eisenhut, M.-S. Roell, A.P.M. Weber, Mechanistic understanding of photorespiration paves the way to a new green revolution 223 (4) (2019) 1762–1769.
[28] P.F. South, A.P. Cavanagh, H.W. Liu, D.R. Ort, Synth. glycolate Metab. Pathw. Stimul. Crop Growth Product. Field 363 (6422) (2019) eaat9077.
[29] A. Bracher, S.M. Whitney, F.U. Hartl, M. Hayer-Hartl, Biogenesis and metabolic maintenance of Rubisco, Ann. Rev. Plant Biol. 68 (1) (2017) 29–60.
[30] H. Aigner, R.H. Wilson, A. Bracher, L. Calisse, J.Y. Bhat, F.U. Hartl, M. Hayer-Hartl, Plant Rubisco assembly in *E. coli* with five chloroplast chaperones including BSD2, Science 358 (6368) (2017) 1272–1278.
[31] T.D. Sharkey, The discovery of Rubisco, J. Exp. Bot. 74 (2) (2022) 510–519.
[32] Y. Qu, O. Mueller-Cajar, W. Yamori, Improving plant heat tolerance through modification of Rubisco activase in C3 plants to secure crop yield and food security in a future warming world, J. Exp. Bot. 74 (2) (2022) 591–599.
[33] A.P. Scafaro, N. Bautsoens, B. den Boer, J. Van Rie, A. Galle, A conserved sequence from heat-adapted species improves Rubisco activase thermostability in wheat, Plant Physiol. (2019) pp.00425.2019.
[34] L. Feiz, R. Williams-Carrier, S. Belcher, M. Montano, A. Barkan, D.B. Stern, A protein with an inactive pterin-4a-carbinolamine dehydratase domain is required for Rubisco biogenesis in plants, Plant J. 80 (5) (2014) 862–869.
[35] Q. Li, Y.-L. Jiang, L.-Y. Xia, Y. Chen, C.-Z. Zhou, Structural insights into cyanobacterial RuBisCO assembly coordinated by two chaperones Raf1 and RbcX, Cell Discov. 8 (1) (2022) 93.
[36] S. Buck, T. Rhodes, M. Gionfriddo, T. Skinner, D. Yuan, R. Birch, M.V. Kapralov, S. M. Whitney, *Escherichia coli* expressing chloroplast chaperones as a proxy to test heterologous Rubisco production in leaves, J. Exp. Bot. 74 (2) (2023) 664–676.
[37] Y. Wang, P. Xue, M. Cao, T. Yu, S.T. Lane, H. Zhao, Directed Evolution: Methodologies and Applications, Chem. Rev. 121 (20) (2021) 12384–12444.
[38] G. Hudson, M. Morell, Y. Arvidsson, T. Andrews, Synthesis of spinach phosphoribulokinase and Ribulose 1,5-bisphosphate in *Escherichia coli*, Funct. Plant Biol. 19 (3) (1992) 213–221.
[39] M. Morell, K. Paul, H. Kane, T. Andrews, Rubisco: maladapted or misunderstood, Funct. Plant Biol. 40 (5) (1992) 431–441.
[40] O. Mueller-Cajar, S.M. Whitney, Directing the evolution of Rubisco and Rubisco activase: first impressions of a new tool for photosynthesis research, Photosynth Res 98 (1–3) (2008), 667-75.

[41] O. Mueller-Cajar, M. Morell, S.M. Whitney, Directed evolution of Rubisco in *Escherichia coli* reveals a specificity-determining hydrogen bond in the form II enzyme, Biochem 46 (49) (2007), 14067-74.
[42] R.H. Wilson, H. Alonso, S.M. Whitney, Evolving *Methanococcoides burtonii* archaeal Rubisco for improved photosynthesis and plant growth, Sceintific Rep. 6 (2016) 22284.
[43] R.H. Wilson, S.M. Whitney, Improving CO_2 fixation by enhancing Rubisco performance, in: M. Alcalde (Ed.), Directed Enzyme Evolution: Advances and Applications, Springer International Publishing, Cham, 2017, pp. 101–126.
[44] R.H. Wilson, E. Martin-Avila, C. Conlan, S.M. Whitney, An improved Escherichia coli screen for Rubisco identifies a protein–protein interface that can enhance CO2-fixation kinetics, J Biol Chem (2018) 18–27.
[45] E. Martin-Avila, Y.-L. Lim, R. Birch, L.M.A. Dirk, S. Buck, T. Rhodes, R. E. Sharwood, M.V. Kapralov, S.M. Whitney, Modifying plant photosynthesis and growth via simultaneous chloroplast transformation of rubisco large and small subunits, Plant Cell 32 (9) (2020) 2898–2916.
[46] S.M. Whitney, R.E. Sharwood, D. Orr, S.J. White, H. Alonso, J. Galmés, Isoleucine 309 acts as a C_4 catalytic switch that increases ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate in *Flaveria*, Proc. Nat. Acad. Sci. 108 (35) (2011), 14688-93.
[47] M. Wilding, N. Hong, M. Spence, A.M. Buckle, C.J. Jackson, Protein engineering: the potential of remote mutations, Biochem Soc. Trans. 47 (2) (2019) 701–711.
[48] P.L. Cummins, B. Kannappan, J.E. Gready, Revised mechanism of carboxylation of ribulose-1,5-biphosphate by rubisco from large scale quantum chemical calculations, J. Comput. Chem. 39 (21) (2018) 1656–1665.
[49] M.T. Lin, H. Salihovic, F.K. Clark, M.R. Hanson, Improving the efficiency of Rubisco by resurrecting its ancestors in the family Solanaceae, ScienceAdvances 8 (15) (2022) abm6871.
[50] J.W. Bouvier, D.M. Emms, T. Rhodes, J.S. Bolton, A. Brasnett, A. Eddershaw, J. R. Nielsen, A. Unitt, S.M. Whitney, S. Kelly, Rubisco adaptation is more limited by phylogenetic constraint than by catalytic trade-off, Mol. Biol. Evol. (2021).
[51] D.J. Orr, A. Alcântara, M.V. Kapralov, P.J. Andralojc, E. Carmo-Silva, M.A.J. Parry, Surveying Rubisco diversity and temperature response to improve crop photosynthetic efficiency, Plant Physiol. 172 (2) (2016) 707–717.
[52] R.E. Sharwood, O. Ghannoum, M.V. Kapralov, L.H. Gunn, S.M. Whitney, Temperature responses of Rubisco from Paniceae grasses provide opportunities for improving C_3 photosynthesis, Nat. Plants 2 (2016) 16186.
[53] A. Wu, J. Brider, F.A. Busch, M. Chen, K. Chenu, V.C. Clarke, B. Collins, M. Ermakova, J.R. Evans, G.D. Farquhar, B. Forster, R.T. Furbank, M. Groszmann, M.A. Hernandez-Prieto, B.M. Long, G. Mclean, A. Potgieter, G.D. Price, R. E. Sharwood, M. Stower, E. van Oosterom, S. von Caemmerer, S.M. Whitney, G. L. Hammer, A cross-scale analysis to understand and quantify the effects of photosynthetic enhancement on crop growth and yield across environments, Plant Cell Environ. 46 (1) (2023) 23–44.
[54] B. Conlan, S. Whitney, Preparing Rubisco for a tune up, Nat. Plants 4 (1) (2018) 12–13.
[55] N. Cho, B. Hwang, J.K. Yoon, S. Park, J. Lee, H.N. Seo, J. Lee, S. Huh, J. Chung, D. Bang, De novo assembly and next-generation sequencing to analyse full-length gene variants from codon-barcoded libraries, Nat. Commun. 6 (2015) 8351.
[56] D.M. Fowler, S. Fields, Deep mutational scanning: a new style of protein science, Nat. Methods 11 (8) (2014), 801-7.
[57] S. Marillonnet, R. Grützner, Synthetic DNA assembly using golden gate cloning and the hierarchical modular cloning pipeline, Curr. Protoc. Mol. Biol. 130 (1) (2020), e115.
[58] M.S. Morrison, C.J. Podracky, D.R. Liu, The developing toolkit of continuous directed evolution, Nat. Chem. Biol. 16 (6) (2020) 610–619.
[59] E.A. DeBenedictis, E.J. Chory, D.W. Gretton, B. Wang, S. Golas, K.M. Esvelt, Systematic molecular evolution enables robust biomolecule discovery, Nat. Methods 19 (1) (2022) 55–64.
[60] R.S. Molina, G. Rix, A.A. Mengiste, B. Álvarez, D. Seo, H. Chen, J.E. Hurtado, Q. Zhang, J.D. García-García, Z.J. Heins, P.J. Almhjell, F.H. Arnold, A.S. Khalil, A. D. Hanson, J.E. Dueber, D.V. Schaffer, F. Chen, S. Kim, L.Á. Fernández, M. D. Shoulders, C.C. Liu, In vivo hypermutation and continuous evolution, Nat. Rev. Methods Prim. 2 (1) (2022) 36.
[61] A.H. Badran, D.R. Liu, In vivo continuous directed evolution, Curr. Opin. Chem. Biol. 24 (2015) 1–10.
[62] L. Alejaldre, J.N. Pelletier, D. Quaglia, Methods for enzyme library creation: which one will you choose? BioEssays 43 (8) (2021) 2100052.
[63] J. Galmés, M.V. Kapralov, P.J. Andralojc, M. Conesa, A.J. Keys, M.A. Parry, J. Flexas, Expanding knowledge of the Rubisco kinetics variability in plant species: environmental and evolutionary trends, Plant Cell Environ. 37 (9) (2014) 1989–2001.
[64] C. Hermida-Carrera, M.A. Fares, M. Font-Carrascosa, M.V. Kapralov, M.A. Koch, A. Mir, A. Molins, M. Ribas-Carbó, J. Rocha, J. Galmés, Exploring molecular evolution of Rubisco in C3 and CAM Orchidaceae and Bromeliaceae, BMC Evolut. Biol. 20 (1) (2020) 11.
[65] E.A. Kellogg, N.D. Juliano, The structure and function of RuBisCO and their implications for systematic studies, Am. J. Bot. 84 (3) (1997) 413–428.
[66] Z. Chen, J.M. Debernardi, J. Dubcovsky, A. Gallavotti, Recent advances in crop transformation technologies, Nature, Plants 8 (12) (2022) 1343–1351.
[67] R.B. Anjanappa, W. Gruissem, Current progress and challenges in crop genetic transformation, J. Plant Physiol. 261 (2021), 153411.
[68] R. Bock, Engineering plastid genomes: methods, tools, and applications in basic research and biotechnology, Ann. Rev. Plant Biol. 66 (1) (2015) 211–241.
[69] S.M. Rozov, Y.V. Sidorchuk, E.V. Deineko, Transplastomic plants: problems of production and their solution, Russ. J. Plant Physiol. 69 (2) (2022) 20.
[70] Y. Zhang, A.A. Malzahn, S. Sretenovic, Y. Qi, The emerging and uncultivated potential of CRISPR technology in plant science, Nature, Plants 5 (8) (2019) 778–794.
[71] S. Donovan, Y. Mao, D.J. Orr, E. Carmo-Silva, A.J. McCormick, CRISPR-Cas9-mediated mutagenesis of the Rubisco small subunit family in *Nicotiana tabacum*, Front. Genome Ed. 2 (28) (2020).
[72] P. Khumsupan, M.A. Kozlowska, D.J. Orr, A.I. Andreou, N. Nakayama, N. Patron, E. Carmo-Silva, A.J. McCormick, Generating and characterizing single- and multigene mutants of the Rubisco small subunit family in Arabidopsis, J. Exp. Bot. 71 (19) (2020) 5963–5975.
[73] Y. Mao, E. Catherall, A. Díaz-Ramos, G.R.L. Greiff, S. Azinas, L. Gunn, A. J. McCormick, The small subunit of Rubisco and its potential as an engineering target, J. Exp. Bot. 74 (2) (2023) 543–561.
[74] S.M. Whitney, R.E. Sharwood, Construction of a tobacco master line to improve Rubisco engineering in chloroplasts, J. Exp. Bot. 59 (7) (2008), 1909-21.
[75] B.J. Gomez-Fernandez, E. Garcia-Ruiz, J. Martin-Diaz, P. Gomez de Santos, P. Santos-Moriano, F.J. Plou, A. Ballesteros, M. Garcia, M. Rodriguez, V.A. Risso, J. M. Sanchez-Ruiz, S.M. Whitney, M. Alcalde, Directed -in vitro- evolution of Precambrian and extant Rubiscos, Sci. Rep. 8 (1) (2018) 5532.
[76] S.M. Whitney, R.E. Sharwood, Rubisco engineering by plastid transformation and protocols for assessing expression, Methods Mol. Biol. 2317 (2021) 195–214.
[77] T. Manning, R. Birch, T. Stevenson, G. Nugent, S. Whitney, Bacterial Form II Rubisco can support wild-type growth and productivity in Solanum tuberosum cv. Desiree (potato) under elevated CO_2, PNAS Nexus 2 (2) (2023).
[78] M.T. Lin, D.J. Orr, D. Worrall, M.A.J. Parry, E. Carmo-Silva, M.R. Hanson, A procedure to introduce point mutations into the Rubisco large subunit gene in wild-type plants, Plant J. 106 (3) (2021) 876–887.
[79] B.-C. Kang, S.-J. Bae, S. Lee, J.S. Lee, A. Kim, H. Lee, G. Baek, H. Seo, J. Kim, J.-S. Kim, Chloroplast and mitochondrial DNA editing in plants, Nature, Plants 7 (7) (2021) 899–905.
[80] P. Maliga, Engineering the plastid and mitochondrial genomes of flowering plants, Nature, Plants 8 (9) (2022) 996–1006.
[81] H. Matsumura, K. Shiomi, A. Yamamoto, Y. Taketani, N. Kobayashi, T. Yoshizawa, H. S.-i. Tanaka, M. Yoshikawa, H. Endo, Fukayama, Hybrid Rubisco with complete replacement of rice rubisco small subunits by sorghum counterparts confers C4 plant-like high catalytic activity, Mol. Plant (2020).
[82] R. Laterre, M. Pottier, C. Remacle, M. Boutry, Photosynthetic trichomes contain a specific Rubisco with a modified pH-dependent activity, Plant Physiol. 173 (4) (2017) 2110–2120.
[83] M. Gionfriddo, L. De Gara, F. Loreto, Directed evolution of plant processes: towards a green (r)evolution? Trends Plant Sci. 24 (11) (2019) 999–1007.
[84] M.K.M. Engqvist, K.S. Rabe, Applications of protein engineering and directed evolution in plant research, Plant Physiol. 179 (3) (2019) 907–917.