An improved *Escherichia coli* screen for Rubisco identifies a protein–protein interface that can enhance CO₂-fixation kinetics

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Robert H. Wilson, Elena Martin-Avila, Carly Conlan, and Spencer M. Whitney

From the Research School of Biology, Australian National University, Acton, Australian Capital Territory 2601, Australia

Edited by Joseph Jez

An overarching goal of photosynthesis research is to identify how components of the process can be improved to benefit crop productivity, global food security, and renewable energy storage. Improving carbon fixation has mostly focused on enhancing the CO₂ fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This grand challenge has mostly proved ineffective because of catalytic mechanism constraints and required chaperone complementarity that hinder Rubisco biogenesis in alternative hosts. Here we refashion *Escherichia coli* metabolism by expressing a phosphoribulokinase-neomycin phosphotransferase fusion protein to produce a high-fidelity, high-throughput Rubisco-directed evolution (RDE2) screen that negates false-positive selection. Successive evolution rounds using the plant-like *Te*-Rubisco from the cyanobacterium *Thermosynechococcus elongatus* BP1 identified two large subunit and six small subunit mutations that improved carboxylation rate, efficiency, and specificity. Structural analysis revealed the amino acids clustered in an unexplored subunit interface of the holoenzyme. To study its effect on plant growth, the *Te*-Rubisco was transformed into tobacco by chloroplast transformation. As previously seen for *Synechococcus* PCC6301 Rubisco, the specialized folding and assembly requirements of *Te*-Rubisco hinder its heterologous expression in leaf chloroplasts. Our findings suggest that the ongoing efforts to improve crop photosynthesis by integrating components of a cyanobacteria CO₂-concentrating mechanism will necessitate co-introduction of the ancillary molecular components required for Rubisco biogenesis.

Improving carbon fixation in agriculture has mostly focused on enhancing the activity of the CO₂-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) by modifying the enzyme itself or increasing the CO₂ levels around it (1). Improving the kinetics of Rubisco has proved challenging because of its complex catalytic mechanism of fixing CO₂ to ribulose-1,5-bisphosphate (RuBP) and cleaving it into two 3-phosphoglycerate (3-PGA) molecules (2–4). The carboxylation rate is slow (k_car ≈ 1 to 5 reactions/s in plants) and often confines flux through the Calvin–Benson–Bassham (CBB) cycle, hence limiting the rate of photosynthesis and plant growth. Because of this limitation, large amounts of Rubisco are needed to support adequate CO₂-assimilation rates (5–7). This results in Rubisco being the Earth’s most abundant protein (8). Further encumbering its performance, Rubisco also catalyzes RuBP oxygenation to produce 3-PGA and 2-phosphoglycolate. The recycling of 2-phosphoglycolate back into 3-PGA via photorespiration is considered wasteful because it consumes energy and releases fixed CO₂ (9).

The potential for successfully improving the carboxylation properties of Rubisco is spurred on by findings that crop Rubisco is not the pinnacle of evolution, with kinetically more efficient Rubisco forms found in some red algae (1, 3, 6, 10). Evolutionary adaptation of Rubisco kinetics appears to have been constrained by the complexity of its catalytic chemistry and multiplexed subunit folding and assembly requirements (3, 11–13). The need to retain complementarity with ancillary proteins involved in the biogenesis and metabolic repair of Rubisco appears particularly pertinent for form I Rubisco, which comprises eight RbcL and eight RbcS subunits to form a L₈S₈ complex. Examples of Rubisco-dedicated components include the assembly chaperones BSD2, RbcX, Rubisco accumulation factors 1 and 2 (Raf1, Raf2), and the metabolic repair protein Rubisco activase (14). The protein-folding chaperonins of bacteria (GroEL), chloroplasts (Cpn60), and their protein co-factors GroES (bacteria) and Cpn10/Cpn20 (chloroplasts) are also essential components in Rubisco biogenesis (15). The need for Rubisco to maintain complementarity with these varied chaperone and chaperonin components appears to limit the span of Rubisco isoforms that can be produced in *Escherichia coli*, as well as those that can be bioengineered in the chloroplasts of vascular plants. For example, the chloroplasts of the model *C₅* plant tobacco can produce high levels of the RbcS-lacking bacterial form II *Rhodospirillum rubrum* *L₂* Rubisco and *Methanococcoides bur-...
tonii archaeal L10 Rubisco (10–25 μmol active sites·m−2) because of their simple biogenesis requirements (16, 17). Differences in the biogenesis requirements of L8S8 Rubisco from red algae and monocot plants, however, preclude their assembly in tobacco and E. coli (10, 18). By contrast the assembly requirements of Synechococcus elongatus PCC6301 L8S8 Rubisco (Se-Rubisco) are partially met in chloroplasts (5 μmol active sites·m−2) (19) and in E. coli, with ~98% of the Se-RbcL produced forming insoluble, misfolded aggregates in the bacterium (20).

To avoid the constraints a photosynthetic environment may pose on the catalytic evolution of Rubisco, modern laboratory evolution applications have made particular use of Rubisco-dependent E. coli (RDE) screens (12, 21, 22). An elegant advance has been the extensive rewiring of E. coli metabolism to incorporate a non-native CBB cycle where cell survival can be made dependent on CO2-fixation by Rubisco (23). This contrasts with conventional RDE screens that simply express recombinant phosphoribulokinase (PRK) to produce RuBP, the 5-carbon substrate of Rubisco. As summarized in Fig. 1A, RuBP is unexpectedly toxic to E. coli such that the rate of colony growth in RDE screens is dependent on the level of Rubisco activity. This type of selection has proved useful for evolving M. burtonii L10 Rubisco mutants with desired improvements in carboxylation rate (kcat), CO2 affinity (lower Km for CO2), and specificity for CO2 over O2 (SiciO2) (17). Other attempts to improve the catalysis of L8S8 Rubisco using RDE screens have only identified RbcL mutations that enhance the folding and assembly of the subunit with RbcS into a functional L8S8 complex (i.e. improving Rubisco solubility) (22).

A feature of RDE screens that limit their throughput and reliability (i.e. selection fidelity) is the high proportion of false positives selected (24, 25). These arise from the inactivation of PRK function by transposon integration (Fig. 1A and Ref. 22). Here we present the development and implementation of a simple, faster, high-throughput screen called RDE2 that specifically selects for improvements in Rubisco activity, not PRK inactivation mutants. By eliminating false-positive selection, we demonstrate the versatility of the RDE2 screen in selecting for mutants of Thermosynechococcus elongatus BP-1 (Te-) Rubisco that improve RbcL folding and assembly in E. coli (i.e. Te-Rubisco solubility), the enzyme carboxylation kinetics, or both. We subsequently reveal, using chloroplast genome transformation, how the specialized folding and assembly requirements of Te-Rubisco hinder its translational testing in tobacco (Nicotiana tabacum). The outcomes demonstrate that Rubisco biogenesis in E. coli is not a reliable proxy for expression in chloroplasts. As a consequence, deciphering the crucial molecular partnerships required for Rubisco biogenesis is needed to optimize heterologous Rubisco expression in crop chloroplasts, as well as adapting the RDE2 for future evolution of eukaryotic form I Rubisco.

Results

RDE2 screen development

Rubisco-directed evolution studies that have utilized either RDE or photosynthetic mutant screens have almost exclusively...
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selected for one or more amino acid substitutions in RbcL that improve Rubisco biogenesis (solubility), not catalysis (12, 22). Impeding success are the poor transformation efficiencies of photosynthetic hosts and the high proportion of false positives selected in RDE screens. As summarized in Fig. 1A, false-positive selection in RDE systems arises from terminating RuBP production via transposon silencing of PRK expression (22). To circumvent this impediment, a synthetic gene coding a fusion protein comprising *S. elongatus* PCC6301 PRK (Se-PRK) and a C-terminal neomycin phosphotransferase (NPTII) was made (Fig. 1B). The PRK–NPTII fusion protein was found to preserve the kanamycin resistance conferring properties of NPTII to *E. coli* in addition to retaining 70% the PRK activity (\(k_{\text{cat}} = 95 \pm 3 \text{ s}^{-1}\)) of unmodified Se-PRK (134 ± 6 s\(^{-1}\)).

The fidelity of the RDE and RDE2 screens were compared by plating XL-1Blue cells producing Se-PRK or the PRK–NPTII fusion, along with wild-type *Te*-Rubisco, on medium containing 0.2% (w/v) arabinose (high PRK inducing) and no kanamycin. Under these conditions, *Te*-Rubisco cannot support cell growth, meaning any colonies formed are false positives caused by transposon silencing of PRK activity (Fig. 1A and Ref. 22).

More than 500 false-positive colonies/10\(^6\) cells plated grew in the RDE screen, whereas only 28 ± 17 colonies/10\(^6\) cells plated grew using the RDE2 screen. Inclusion of 100 μg/ml kanamycin in the growth medium of replica RDE2 cell platings resulted in no colony growth. This confirmed that the RDE2 screen is immune from generating false positives because transposon silencing of PRK–NPTII activity also confers the cells sensitive to kanamycin (Fig. 1A).

Identifying a suitable Rubisco to evolve

The inability of *E. coli* to meet the folding and assembly requirements of plant and algae Rubisco currently prevents their directed evolution using RDE screens (10, 12). By contrast the biogenesis requirements of cyanobacteria *L. S. Rubisco* can be partially met by *E. coli*. For example, only a small proportion (<2%) of the 52-kDa *Se*-RbcL produced in *E. coli* can assemble with the more soluble 14-kDa *Se*-RbcS into functional *Se*-Rubisco (20). As a consequence *Se*-Rubisco produced in *E. coli* only accounts for 1–2% (w/w) of the cell-soluble protein (CSP). RDE screens have therefore primarily only identified substitutions in RbcL that enhance the biogenesis of *Se*-Rubisco (i.e. improved its solubility) (22). Among these are the F140I, V189A, and F345I substitutions in RbcL (numbering relative to spinach and *Te*-Rubisco) that increase *Se*-Rubisco solubility between 3- and 14-fold in *E. coli* (Fig. 2, A and B, and Table 1). Notably these mutations all impair \(k_{\text{cat}}\), contrary to that proposed previously (11). By comparison the biogenesis requirements of *Te*-Rubisco, that already codes Ile-140, are more readily met in *E. coli* and expressed at ~6% (w/w) CSP (Fig. 2A).

Moreover, the apparent carboxylation efficiency under ambient O\(_2\) (\(k_{\text{cat}}^c/K_c^{21%\text{O}_2}\)) and specificity for CO\(_2\) over O\(_2\) (\(S_{c/o}\)) of *Te*-Rubisco are ~40 and 18% higher, respectively, than *Se*-Rubisco (Table 1). Notably these favorable *Te*-Rubisco kinetics come at the expense of a slower carboxylation rate (\(k_{\text{cat}}^c\)) that is ~40% less than *Se*-Rubisco (Fig. 2, A and B). The high solubility of *Te*-Rubisco in *E. coli* and distinctive kinetics made it a superior target for evolution testing using RDE2.

First-generation *Te*-Rubisco solubility mutant selection in RDE2

The efficacy of RDE2 to select for increased *Te*-Rubisco activity was initially tested using a library of 2.7 × 10\(^5\) *Te*-rbcL mutants with an average mutation rate of 2.5 nucleotides (1.6 amino acid substitutions) per variant. After 5 days at 25 °C, 18 faster-growing colonies were identified on plates containing 0.1% (w/v) arabinose (defined as moderate PRK–NPTII induction) and found to code either V300A, F345I, F345L, or P415A substitutions in RbcL. The kinetics of the slower growing V300A mutant resembled *Te*-Rubisco, suggesting that it improved RDE2 fitness possibly from the small (<10%) increase in the amount of soluble *L. S. Rubisco* made (Table 1). The faster-growing F345I, F345L, and P415A mutants produced significantly more *Te*-Rubisco, especially the F345I mutation that stimulated *Te*-Rubisco biogenesis to ~14% (w/w) of the CSP (Fig. 2, A and B). When coupled with the P415A mutation, the solubility of the *Te*-F345I/P415A mutant increased to ~17% (w/w) CSP (Fig. 2, A and B). This improvement came at a cost to the kinetics of each mutant that showed reductions in \(k_{\text{cat}}^c\), \(k_{\text{cat}}^c/K_c^{21%\text{O}_2}\), and \(S_{c/o}\) (Table 1).

Sequence comparisons found that both Phe\(^{345}\) and Pro\(^{415}\) are highly conserved among cyanobacteria and higher plant form IB RbcL but are located in separate, unconnected α-helical regions (Fig. 2C). Interestingly, these loci are modified in the form IA Rubisco lineage that has evolved independent of the assembly chaperones Raf1 and RbcX (11, 14).

Second-generation *Te*-Rubisco catalytic mutant selection

Improving the kinetics of *Te*-Rubisco required a second round of RDE2 screening that targeted mutagenesis of both RbcL and RbcS. A 3 × 10\(^9\) member mutagenic library of the full-length *Te*-rbcLS operon coding the first-generation P415A RbcL mutant (abbreviated *Te*-P415A) was used. Unlike the F345I and F345I/P415A mutants, the catalytic properties of *Te*-P415A more closely matched wild-type *Te*-Rubisco (Fig. 2A). Under strong PRK–NPTII selection (i.e. on medium containing 0.2% (w/v) arabinose), the growth of cells producing wild-type (abbreviated *Te*-LS) or *Te*-P415A Rubisco were impeded (Fig. 3A). After 6 days growth at 25 °C in air supplemented with 2% (v/v) CO\(_2\), 15 colonies were isolated. The sequence and biochemistry of these second-generation mutants (abbreviated *Te*-2G Rubisco variants) were found to enhance RDE2 growth either through further improving *Te*-P415A Rubisco solubility and/or significantly enhancing its carboxylation properties (Fig. 3A and Table 1). The fastest growing *Te*-2Ga mutant was independently selected four times and coded a V98M RbcS point mutation that improved Rubisco biogenesis, \(k_{\text{cat}}^c\), \(k_{\text{cat}}^c/K_c^{21%\text{O}_2}\), and \(S_{c/o}\) by ~310, 28, and 43, and 6% relative to *Te*-Rubisco (Fig. 3, B–E). Four additional *Te*-P415A Rubisco mutants with improvements in \(k_{\text{cat}}^c\) and \(k_{\text{cat}}^c/K_c^{21%\text{O}_2}\) and unchanged solubility were also identified. They were found to code for point mutations in either RbcS (A448V, *Te*-2Gb; H37L, *Te*-2Gc; or Y36N/G112D, *Te*-2Gd) or RbcL (L74M/D397N, *Te*-2Gg). The *Te*-2Gg mutant also showed a small but significant increase of ~4% in its *S_{c/o}* relative to native *Te*-Rubisco (Table 1 and Fig. 3E).
Clustering of the catalytic mutations at a RbcL-RbcS interface in Te-Rubisco

Structural analysis revealed that the H37L, Y36N, V98M, L74M, and D397N substitutions all occur at residues that cluster on a surface exposed region of an RbcS–RbcL interface (Fig. 4, A and B). The A48V mutation occurs on the same interface but is oriented toward the inner enzyme surface (Fig. 4, A and C). The common locality of these mutations suggest this region to be a hot spot for Te-Rubisco catalytic modification, despite being located quite distant from the catalytic pockets in adjoining regions of paired RbcL subunits (Fig. 4). In support of this assertion, the RbcS residue Tyr36 is located directly adjacent to Val98 in the Te-Rubisco holoenzyme (Fig. 4B) and can convey comparable improvements in $k_{cat}$ and $k_{cat}/K_{c}$ when mutated to Asn (Y36N mutant Te-2Gd; Fig. 3, C and D). Curiously the selected RbcS mutants comprise residue changes adjacent to highly conserved regions of cyanobacterial and higher plant form IB Rubisco (Fig. S2). This suggests that this RbcL-RbcS interface may pose a hot spot for manipulating the kinetics in related plant Rubisco lineages.

Second-generation Te-Rubisco solubility mutant selection

Of the 10 different Te-P415A Rubisco-derived mutants selected, five arose from improvements in the biogenesis of soluble Te-Rubisco, not catalysis (Fig. 3A). The improved solubility derived either from a R51H substitution in RbcS (or A48V, see above) or from I393M, A398T, or A414T mutations in RbcL (Table 1). Notably none of these RbcL substitutions were
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Table 1

| E. coli Lp8 Rubisco content and catalytic parameters at 25 °C |
|-------------------------------------------------------------|
| The values are the means ± S.E. of typically N = 3 biological samples assayed in duplicate or triplicate (details shown in top row). One-way ANOVA was undertaken with reference to either WT Synechococcus PCC 6301 Rubisco (Se-LS) or WT Te-Rubisco (Te-LS). Symbols show the statistical significance levels relative to WT enzymes: *p < 0.05; **p < 0.01. 1G, first generation RDE2 Te-LS Rubisco mutants selected on media containing 0.1% (w/v) arabinose; 2G, second generation mutants derived from Te-P415A under higher PKR selection on 0.25% (w/v) arabinose medium (3); NA, not applicable; NM, not measured. |

| Rubisco Mutant library | Amino acid mutation(s) in Rubisco | Expression (% (w/w) sol E. coli protein) | $K_{s}^{A}$ | $K_{m}^{A}$ | $K_{m}^{A}$/$K_{m}^{A}$ | $S_{c/O}$ | $T_{m}$ |
|--------------------------|----------------------------------|------------------------------------------|--------------|-------------|-------------------|-----------|---------|
| n = biological samples, technical replicates | | | | | | | |
| Se-LS (WT) | NA | None | None | 1.3 ± 0.2 | 12.6 ± 0.1 | 251 ± 4 | 50 ± 1 | 45.9 ± 0.7 | 60.2 ± 0.3 |
| Se-F345I | Refs. 11 and 20 | F345I | None | 14.4 ± 0.9** | 10.3 ± 0.4** | 285 ± 9* | 36 ± 2** | 41.8 ± 1.1** | NM |
| Se-F140I | Ref. 11 | F137I | None | 12.1 ± 1** | 11.1 ± 0.5** | 231 ± 5* | 53 ± 3 | 46.0 ± 0.7 | NM |
| Se-V186I | Ref. 11 | V189I | None | 6.3 ± 0.7 | 10.6 ± 0.2** | 186 ± 7* | 57 ± 2** | 39.3 ± 0.7** | NM |
| Te-LS (WT) | NA | None | None | 6.6 ± 0.7 | 7.4 ± 0.1 | 107 ± 2 | 70 ± 2 | 53.3 ± 0.5 | 72.8 ± 0.2 |

First generation Te-rbcL−RDE2 mutants

| Te-F345I | 1G | F345I | None | 14.6 ± 0.7** | 4.8 ± 0.3** | 101 ± 1 | 47 ± 2** | 48.0 ± 0.5** | 72.3 ± 0.3 |
| Te-P415A | 1G | P415A | None | 14.0 ± 0.8** | 6.9 ± 0.2 | 112 ± 1 | 62 ± 2* | 52.6 ± 0.5 | 73.7 ± 0.1** |
| Te-V300A | 1G | V300A | None | 7.1 ± 0.2 | 6.6 ± 0.2 | 135 ± 9** | 49 ± 6** | 51.9 ± 0.6 | 71.4 ± 0.6 |
| Te-FIPA | 1G chimer | F345I, P415A | None | 17.5 ± 1.2 | 4.8 ± 0.4** | 102 ± 5 | 47 ± 6** | 49.2 ± 0.4** | 75.9 ± 0.1** |

Second generation Te-rbcLS−RDE2 mutants

| Te-2Ga (1) | 2G | P415A | V98M | 20.4 ± 3.3** | 9.5 ± 0.3** | 93 ± 3** | 100 ± 3** | 56.3 ± 0.3** | NM |
| Te-2Gb (1) | 2G | P415A | A48 | 9.7 ± 1.2 | 8.8 ± 0.2** | 105 ± 3 | 84 ± 3** | 48.0 ± 0.8** | NM |
| Te-2Ge (1) | 2G | P415A | H37L | 11.1 ± 0.2 | 8.4 ± 0.3 | 100 ± 2* | 84 ± 4** | 49.7 ± 0.3** | NM |
| Te-2Gd (5) | 2G | P415A | Y36N, G112D | 9.7 ± 0.8 | 9.3 ± 0.2** | 94 ± 0.4** | 99 ± 2* | 47.7 ± 0.1** | NM |
| Te-2Ge (8) | 2G | P415A | Y36N, R51H | 13.6 ± 1.6** | 8.0 ± 0.4 | 120 ± 8 | 68 ± 2** | 51.8 ± 0.2** | NM |
| Te-2Gg (10) | 2G | A398T, P415A | A48V | 13.3 ± 3.2 | 8.1 ± 0.1* | 105 ± 3 | 75 ± 5 | 45.5 ± 0.1** | NM |
| Te-2Gg (11) | 2G | L74M, D397N, | None | 12.5 ± 0.8** | 8.2 ± 0.5 | 92 ± 2.3** | 90 ± 6** | 55.4 ± 0.3** | NM |
| Te-2Gh (12) | 2G | A414T, P415A | None | 13.9 ± 3.0* | 8.0 ± 0.1 | 102 ± 2 | 78 ± 1 | 55.8 ± 0.5** | NM |
| Te-2Gh (13) | 2G | I395M, P415A | None | 16.6 ± 2.4** | 7.8 ± 0.4 | 90 ± 2.2** | 87 ± 1** | 48.1 ± 0.4** | NM |
| Te-2Gh (15) | 2G | A398T, P415A | None | 16.6 ± 3.6** | 6.4 ± 0.2** | 110 ± 4 | 59 ± 3 | 50.5 ± 0.5** | NM |

* Measurement of thermal stability ($T_{m}$) were made by circular dichroism spectroscopy as described (38). The $K_{m}$ for RuBP of Se-LS and Te-LS Rubisco are 44 ± 3 μM (20) and 36 ± 2 μM (this study), respectively; −10-fold less than the 0.4 mM RuBP concentration used in the $^{14}$CO$_2$-fixation assays. Rubisco expression levels were confirmed by native PAGE (Fig. S1).

selected in the first-generation RDE2 screen, suggesting that they complement the P415A mutation. Structural analyses revealed, however, that the residues associated with increasing Te-Rubisco solubility show no apparent relation to one another and are located quite distant from Pro415 within the quaternary Rubisco structure (Fig. S3). Consistent with that postulated previously (11), this suggests these residues pose important determinants of RbcL subunit folding by GroEL or provide structural stability to avoid their misfolding and aggregation in the absence of ancillary molecular assembly components.

Comparative analysis of Te-Rubisco biogenesis in chloroplasts

The varied biogenesis requirements within the Rubisco superfamily limit not only which isoforms that can be produced in E. coli but also those that can be assembled within leaf chloroplasts (10, 14, 18). The availability of efficient plastome transformation capabilities in tobacco has made it the model plant for Rubisco bioengineering (13, 16). Consistent with leaf photosynthesis modeling (Fig. 5A), the growth of tobacco producing Se-Rubisco necessitate high levels of CO$_2$ for growth in soil to compensate for the enzyme’s poor kinetics and limited biogenesis potential in chloroplasts (19). Comparable modeling using Te-Rubisco kinetics show it would support higher rates of CO$_2$ assimilation in a C$_3$ plant over all intracellular CO$_2$ concentrations, more so in plants producing the Te-2Ga Rubisco mutant selected in this study using the RDE2 screen (Fig. 5A).

To test the correlative potential between Te-Rubisco biogenesis in E. coli and leaf chloroplasts, a synthetic Te-rbcLS operon coding for either Te-Rubisco or the high-solubility F345I/P415A mutant was transformed in the tobacco chloroplast genome (plasmate) in place of the native tobacco rbcL gene (Fig. 5B and Fig. S4). To optimize translation, the codon use of the transformed genes matched the native tobacco rbcL, and 12 amino acids of the native tobacco RbcL N terminus were retained (Fig. S4a). Independently transformed lines for each tobacco genotype (called tobTeLS and tobTeLS-FIPA; Fig. 5B) were obtained and grown in tissue culture until homoplasmic (Fig. 5C). Plastome sequencing confirmed correct integration of the transgenes, and RNA blots confirmed high Te-rbcL−rbcS and Te-rbcL−rbcS-aadA mRNA levels (Fig. S4b). Nevertheless the tobTeLS and tobTeLS-FIPA plants shared a pale green phenotype and could only survive in tissue culture (Fig. 5C). CABP binding (Fig. 5D) and native PAGE analysis (Fig. 5F) confirmed the assembly of functional $Lp_{8}$ Te-Rubisco in tobTeLS and slightly more of the F345I/P415A mutated Te-Rubisco in tobTeLS-FIPA. However, the amount of Te-Rubisco produced were more than 1000-fold lower than Rubisco production in the wild-type tobacco controls (Fig. 5D). Reciprocal mutagenic tests showed introducing the F345I, P415A, or dual F345I/P415A mutations into the N. tabacam RbcL did not permit the assembly of tobacco Rubisco in E. coli (Fig. S5).

Discussion

Following successive evolution rounds, this study reveals a novel region in the subunit interface of the plant-like Rubisco from the T. elongatus BP1 that offers multiple solutions for improving carboxylation rate, efficiency, and specificity. Key to this success is the unwavering fidelity, faster screening, and higher throughput of the new RDE2 screening platform. To date, the RDE screen designed around $\Delta GAP$ E. coli mutant (a...
strains (MM1) (26) has proven the most effective in Rubisco-directed applications because of the low number of false positives selected (12). Offsetting this improved fidelity is the slow growth rate of the MM1 strain and its need for special media that prolongs the screening time frame to 3–5 days at the optimal growth temperature of 25 °C (11, 17, 20, 26). Comparatively, the RDE2 screen is immune to false-positive selection and uses LB growth medium, allowing the screens to be completed in 3–5 days at 25 °C. The feasibility of adapting the new CO2-dependent sugar synthesizing CBB–E. coli strains (23) as an alternative RDE screen for selecting Rubisco catalytic mutants remains to be demonstrated (21).

There has been long-standing interest in modifying Rubisco catalysis via mutagenesis of RbcL (3, 5–7, 10). More recent RbcS mutagenic studies have demonstrated how it can pervasively influence plant Rubisco kinetics (27), a finding supported by structure–function surveys that indicate a role by the RbcS in the kinetic adaptation of Rubisco to environmental cues (18). X-ray crystallography comparisons show that the quaternary RbcS structure remains highly conserved among the differing L5S4 lineages, especially among the plant and cyanobacteria Form I B isoforms (Fig. 6A). This suggests that RbcS-mediated changes to catalysis can be strongly influenced by subtle changes in electron density within key areas. One particular area, as demonstrated in this study, is located between the antiparallel β-sheet pairs around Val198, Tyr35, and His37 in Te-RbcS that share structural similarity to Cys122, Trp38, and Val39 in tobacco RbcS (Fig. 6B). Generally, the Met36, Asn36, or Leu37 substitutions differentially improved Te-Rubisco catalysis by modification toward more plant-like structures (Fig. 6C). Notably, these substitutions did not arise from limitations in codon redundancy at these residues in Te-RbcS. This suggests that further improvements may be possible by additional rounds of laboratory evolution, or possibly even by rational design guided by the vast array of crystal structure information already available for plant and algae Rubisco (4).

An obvious extension of our study is to test how amino acid changes around this region in RbcS influence plant Rubisco catalysis. Such efforts are hindered by the inadequate transgenic methods for replacing or modifying all the multi-copy gene copies in plants. The low throughput of modern nucleus gene editing approaches limit their usefulness in mutagenic screening, despite their capacity to introduce multiplexed nucleotide changes with relatively high accuracy (28). Although...
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**Figure 5. Te-Rubisco expression in tobacco.** A, simulated effect of Se-Rubisco (SelS), Te-Rubisco (TeLS), and the catalytically improved Te2Ga·Rubisco (Te2Ga) on CO₂ assimilation rate (A) in tobacco (a model C₃ plant) at 25 °C in response to varying chloroplast CO₂ partial pressures (Cₚ). A was calculated as the minimum of the Rubisco carboxylase-limited (DCCABP binding (directed integration of codon optimized assuming a leaf Rubisco content of 30 mol active sites m⁻² as observed in tob-SeLS (19)). B, the tobacco plastome was transformed with plasmids pLEV·TelS and pLEV·TelS·FIPA containing homologous plastome flanking sequences (indicated by dashed lines; numbering relative to *N. tabacum* (WT) plastome; GenBank™ accession no. Z00044) that directed integration of codon optimized rbcLS operon (GenBank™ accession no. MG000149); coding WT or FIPA Te-Rubisco to produce the tobacco genotypes tob²⁶⁶LS and tob²⁶⁶LS·FIPA (see Fig. 5 for further detail). C-E, the tob²⁶⁶LS and tob²⁶⁶LS·FIPA plants could be grown in tissue culture (C) but not in soil because both produced finite levels of L₅⁸S·Te-Rubisco as quantified by [(¹⁴)C]·CABP binding (D) and confirmed by native PAGE (E). Loading controls included tobacco Rubisco (tob), known amounts of Te-Rubisco (pTrc²⁶⁶LS·TeS) expressed in *E. coli*, and an empty vector (Trc) negative control. No L₅⁸S·Te-Rubisco band was evident by Coomassie staining in the tob²⁶⁶LS and tob²⁶⁶LS·FIPA leaf protein samples but detected by immunoblotting using a Se-Rubisco antibody. *, non-Rubisco tobacco protein that separates at the same location as Te-Rubisco by native PAGE.

**Table 5. **

| Tobacco (wt) chloroplast genome (plastome) |
|-------------------------------------------|
| alpB  | prk  |
| P  | Trc   |
| T  | accD  |
| tob²⁶⁶LS  |  |
| tob²⁶⁶LS·FIPA  |  |

**Plant phenotype**

| tob²⁶⁶LS·FIPA | wt |
|--------------|----|
| 3 cm  | 17 cm |

**Leaf Rubisco content**

| tob²⁶⁶LS·FIPA | wt |
|--------------|----|
| 0.02 mol active sites m⁻² | 0.02 mol active sites m⁻² |

Rubisco in leaf chloroplasts (13). Understanding these requirements is critical to enabling heterologous Rubisco expression potential in cyanobacteria, *E. coli*, and chloroplasts. Despite the common prokaryotic connections of these hosts, our work demonstrates that *E. coli* cannot be used as a proxy for chloroplast Rubisco assembly. This underpins the importance of understanding the specific chaperonin (Cpn60α, 60β), co-chaperonin (Cpn10, 20, and 21), and assembly chaperone requirements of plant and algal Rubisco or strategies to circumvent their necessity for successful production in a non-native host. The last decade has seen significant advances into understanding the mechanisms for many of these components (14), but they have not yet been incorporated into a screen such as RDE2. Conceivably such an approach would provide a high-throughput screen of plant (or algae) Rubisco activity that would be envisaged to function equivalently chloroplasts (29).

Efforts to introduce a cyanobacterial CO₂-concentration mechanism (CCM) into tobacco plastids are also likely to require the inclusion of Rubisco biogenesis components. In this study we show that *Te*-Rubisco has the highest CO₂ affinity and CO₂/O₂ specificity of known cyanobacteria Rubisco. These kinetic features make *Te*-Rubisco better able to support *C₃* photosynthesis, relative to the more commonly studied *Se*-Rubisco. The reverse would be the case if the extensive requirements for building a functional CCM could be met in plastids to support the higher *kᵩ*value of *Se*-Rubisco (30, 31). The limited biogenesis capacity of *Te*-Rubisco (Fig. 5) and *Se*-Rubisco (19, 30, 32) in tobacco suggest that building a CCM in chloroplasts will require not only introducing the multiple carboxysome components, inorganic carbon membrane transporters, and modulating CA levels, but also incorporating the necessary chaperonin/chaperone components for assembling cyanobacteria Rubisco.

**Conclusion**

Much of the effort to identify superior Rubiscos to date has focused on measuring the natural variation in Rubisco kinetics. This approach is slowed by the complexity of the catalytic assay methods and the vast spectrum of natural diversity available. Here we show how directed evolution using RDE2 poses a potentially faster alternative. The challenge is to extend past the prior success of RDE screens in investigating prokaryotic and Archaea Rubisco sources and adapt RDE2 for the directed evolution of plant and algae Rubisco. As with Rubisco engineering in plant chloroplasts, our data advocate that this will necessitate the co-expression of the complimentary ancillary proteins needed for the biogenesis and metabolic repair of the target Rubisco.

**Experimental procedures**

**Expression, purification, and assay of PRK and PRK:NPTII**

The *prkA* gene from *Synechococcus* PCC6301 was synthesized (Genscript) and cloned downstream of the arabinose inducible BAD promoter in pACBAD (26) to generate pACYCPRE. Sequence coding a synthetic nptII gene was cloned in frame to the 3’ of *prkA* to produce the *prk-nptII* gene coding a PRK–NPTII fusion protein (GenBank™ accession no. MG000147). The *prkA* and *prk-nptII* genes were cloned in frame to a N-terminal His₆–ubiquitin fusion protein using the expression our RDE2 screen poses a potential remedy in terms of screening throughput, our data highlight its versatility for evolving vascular plant Rubisco will require incorporation of requisite Rubisco assembly components from leaf chloroplasts. This observation accords with the underpinning need for ancillary protein complementarity for folding and assembly of form I Rubisco.
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plasmid pHue, expressed in BL21(DE3) E. coli, purified by immobilized metal affinity chromatography and the 10-kDa His6-ubiquitin sequence removed as previously described (33). PRK activity ($k_{cat}$) was measured using a NADH enzyme-linked assay as described (34).

Comparing the selection stringency of RDE and RDE2

The Te-Rubisco expressing plasmid $pTrc^{remL}$ (Fig. 1B) was produced by separately amplifying the $rbcL$ and $rbcS$ genes from genomic DNA isolated from *T. elongatus* BP1 (gifted by Dr. Warwick Hillier, Australian National University) and cloning into $pTrcHisB$ as a Ncol-SalI fragment (GenBank™ accession no. MG000148). E. coli XL1-Blue cells containing $pTrc^{remL}$ were transformed with either $pACYCPRK$ (RDE) or $pACYCPRK-Kan$ (RDE2; Fig. 1B) and grown at 25 °C for 6 days in air with 2% (v/v) CO2 on LB-agar containing 0.5 mM IPTG (Te-Rubisco induction), 0.2% (w/v) arabinose (high PRK/PRK–NPTII induction) and with/without 0.2 mg ml$^{-1}$ kanamycin.

Library construction and Rubisco selection using the RDE2 screen

The $rbcL$ operon in $pTrc^{remL}$ (first-generation library) or $pTrc^{repA}$ (second-generation library) were amplified from 10 ng of plasmid DNA by error-prone PCR using the primers Trc55 (5’-GAGTTATATTTAATTGTATCG-3’) and Trc33 (5’-ATCTTTCTCTCATCCGCA-3’) and the Genomorph II random mutagenesis kit (Agilent), as per the manufacturer’s recommendations. Libraries were transformed into CaCl2-compotent RDE2 cells and plated onto LB medium containing 32 μg/ml chloramphenicol, 200 μg/ml ampicillin, 100 μg/ml kanamycin, 0.5 mM IPTG, and 0.05 to 0.25% (w/v) arabinose. After growing at 25 °C for 3–7 days in air containing 2% (v/v) CO2, the faster-growing colonies were replated, and then their $pTrc^{remL}$ plasmids were purified, sequenced, and retransformed into RDE2, and the screen was repeated to confirm their selective advantage.

Rubisco content and catalysis

Cyanobacteria Rubisco expression was induced in XL-1Blue E. coli transformed with $pTrc^{remL}$, $pTrcSynLS$ (20), or their mutant plasmid derivatives with 1 mM IPTG at 28 °C. After 6 h the cells were harvested by centrifugation (5 min at 4 °C, 6200 $	imes$ g), and the cell pellets were N2-frozen and stored at −80 °C. Their soluble protein was isolated following lysis using a French pressure cell (140 MPa) in ice-cold extraction buffer (100 mM EPES-NaOH, pH 8.05, 15 mM MgCl2, 0.5 mM EDTA, 1 mM PMSF, 2.5 mM DTT). After centrifugation (3 min, 2 °C, 15,000 × g), the supernatant was mixed with an equal volume of reaction buffer (100 mM EPES-NaOH, pH 8.05, 15 mM MgCl2, 0.5 mM EDTA) containing 40 mM NaH14CO3 and incubated at 25 °C for 8 or 12 min (technical repeats) to activate Rubisco. The 14CO2 fixation assays (0.5 ml of total volume) were performed in 7.7 ml of septum-capped scintillation vials in reaction buffer containing 10 μg ml$^{-1}$ carbonic anhydrase and saturating (0.4 mM) RuBP synthesized and purified according to Kane et al. (36). All assay components were equilibrated in CO2-free air (i.e. 20.9% (v/v) O2 in N2) prior to adding a series of five differing amounts of NaH14CO3. The final 14CO2 concentrations were 0–240 μM or 0–350 μM when assaying the wild-type and mutant variants of Te-Rubisco or Se-Rubisco, respectively. The assays were initiated by addition of 20 μl of 14CO2-activated E. coli soluble protein. RuBP-independent 14CO2 fixation control assays were run for each protein sample and contained H2O in place of RuBP. The assays were terminated after 1 min by rapid mixing with 0.2 ml of 20% (v/v) formic acid and then dried at 80 °C before adding 0.5 ml of H2O and mixing with 1 ml of Ultima-Gold scintillant (PerkinElmer). The fixed 14C was measured in a Tri-carb 4910TR scintillation counter with the carboxylase activity between the technical repeats varying by <2%, confirming the extracted Rubisco was fully activated and stable.

The specific activity of each NaH14CO3 stock was determined in assays (n = 4) containing the highest 14CO2 concentration and 5 nmol of pure RuBP. These assays were allowed to react for 30 or 90 min to ensure full RuBP fixation. After acid treatment and drying, the scintillation counter 14C values were divided by 5 to derive the specific activity value, which varied between 1500 and 1800 cpm/nmol CO2 fixed.

The CO2 levels in the assays were calculated using the Henderson–Hasselbalch–derived equation,

\[
[\text{CO}_2] = \frac{1}{1 + \frac{V}{vqRT}} \left( 10^{pH-pK_1} + 10^{2pH-pK_1-pK_2} \right)
\]

(Eq. 1)
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where \( V/v \) is the ratio of reaction vial headspace (\( V \)) to assay volume (\( v \)); \( C_i \) is the total NaHCO\(_3\) concentration (including the 0.4 \( \mu \)mol of NaH\(^{14}\)CO\(_3\) in the \( E. coli \) soluble protein); \( q \) is the CO\(_2\) solubility at 1 atm (0.0329 mol/liter/atm at 25 °C); \( R \) is the universal gas constant (0.082057 liter/atm/K/mol); \( T \) is the assay temperature (298 K); and dissociation constants are 6.251 (\( pK_1 \)) and 10.329 (\( pK_2 \)). The \( ^{14}\)C data were fitted to the Michaelis–Menten equation to derive the apparent Michaelis–Menten constant (\( K_m \)) for CO\(_2\) under air levels of O\(_2\) (\( K_m^{21\%O_2} \)) and the maximal rate of carboxylation (\( V_c^{\text{max}} \)) from at least three independent \( E. coli \) soluble protein preparations. The carboxylation rate for each Rubisco (\( k_{\text{cat}}^{\text{Rubisco}} \)) was standardized by dividing \( V_c^{\text{max}} \) by the number of Rubisco catalytic sites in the \( E. coli \) protein quantified by \([^{14}\text{C]}-2\)-CABP binding and native PAGE (20, 35). The soluble protein concentration in each \( E. coli \) extract was quantified using a Coomassie dye binding assay against BSA. Assays to measure the \( K_m \) for RubBP of \( Te\)-Rubisco (see legend to Table 1) were undertaken in reactions containing 20 mM NaH\(^{14}\)CO\(_3\) and a series of six RubBP concentrations (0–150 \( \mu \)M).

Rubisco \( S_{\text{C/O}} \) measurements were made as described by Kane et al. (36) using recombinant Rubisco purified from \( E. coli \) by anion exchange chromatography and then Superdex 200 (GE Life Sciences) size exclusion column chromatography (35). Each purified Rubisco was equilibrated with a gas mixture of 0.05% (v/v) CO\(_2\) and 99.95% (v/v) O\(_2\) (accurately mixed using Wostoff gas mixing pumps) at 25 °C in a replica septum seal 20-ml glass vial assays comprising 1 ml of specificity buffer (30 mM triethanolamine, pH 8.1, 10 mM MgSO\(_4\), 10% (v/v) glycerol). After 1 h the reactions were initiated by the addition of 1 nmol [1-\(^{14}\)H]-RubBP (10 MBq/nmol), and then 10 units of alkaline phosphatase (Sigma) was added 30–60 min later. The resulting \(^{14}\)H-glycerate and \(^{14}\)H-glycolate products were then separated by HPLC and measured by scintillation counting as described (36). The \( S_{\text{C/O}} \) factor was calculated using the following equation,

\[
S_{\text{C/O}} = \left( \frac{R_{\text{glycerate/glycolate}} \times \frac{M_{\text{O}_2}}{M_{\text{CO}_2}}} \right) \times 0.037 \quad \text{(Eq. 2)}
\]

where \( R \) is the ratio of \(^{14}\)H-glycerate to \(^{14}\)H-glycolate; \( M_{\text{O}_2} \) and \( M_{\text{CO}_2} \) are the mole fractions of O\(_2\) and CO\(_2\), respectively, in the assay; and 0.037 is the ratio between the aqueous solubility of O\(_2\) and CO\(_2\) at 25 °C.

PAGE analyses

The leaf and \( E. coli \) soluble protein extracts were prepared and analyzed by SDS-PAGE, native PAGE, and immunoblot analysis as described (35).

Tobacco chloroplast transformation

The plasmids pLEV\( Te\)LS (GenBank\(^\text{TM} \) accession no. MG000149) and pLEV\( Te\)LS-FIPA were biolistically transformed into the plastome of the \( c^\text{mt}l \) tobacco genotype as previously described (16). The resulting transplastomic genotypes tob\( Te\)LS and tob\( ^{35}\)el-S-FIPA coded a synthetic \( rbc\)LS operon and the \( aadA \) marker gene (coding spectinomycin resistance) in place of the tobacco \( rbc\)L gene (Fig. 5A); see also Fig. S4 for additional transformation and RNA blotting detail). Correct transgene insertion was confirmed by fully sequencing the PCR product amplified from total leaf genomic DNA isolated using the DNeasy\textsuperscript{®} plant mini kit and primers LSD (5′-CAGGGAAATTGGTGC-GAGTAG-3′) and LSZ (5′-ATCCTTCTTTATTTTCTTG-3′).

Leaf photosynthesis simulations

Photosynthetic CO\(_2\) assimilation rates (\( A \) in tobacco at 25 °C under varying chloroplast CO\(_2\) partial pressures (\( C_{\text{C}} \)) were simulated as the minimum of the Rubisco carboxylase-limited (\( A_c \)) and light-limited (\( A_l \)) CO\(_2\)-assimilation rates modeled according to Ref. 37,

\[
A_c = \frac{m \cdot k_{\text{cat}} (C_c \cdot S_c - 0.5 O_2/S_{\text{O}_2})}{(C_c \cdot S_c + K^{21\%O_2})} - R_d \quad \text{(Eq. 3)}
\]

\[
A_l = \frac{(C_c \cdot S_c - 0.5 O_2/S_{\text{O}_2})M_{\text{max}}}{4(C_c \cdot S_c + O_2/S_{\text{O}_2})} - R_d \quad \text{(Eq. 4)}
\]

using the Rubisco kinetic values listed in Table S1 and a leaf Rubisco content (\( m \)) of 30 \( \mu \)mol active sites \( m^{-2} \) (or 5 \( \mu \)mol active sites \( m^{-2} \) for the assembly impaired \( Se\)-Rubisco producing tobacco line tob-\( Se\)LS, (19)); a maximal RubBP regeneration rate (\( M_{\text{max}} \)) of 160 \( \mu \)mol \( m^{-2} \) s\(^{-1} \); a mitochondrial respiration rate (\( R_d \)) of 1 \( \mu \)mol \( m^{-2} \) s\(^{-1} \) and using the solubility constants 0.0334 \( m \) bar\(^{-1} \) (\( s_c \)) and 0.00126 \( m \) bar\(^{-1} \) (\( s_o \)) to calculate the CO\(_2\) (\( C_c \)) and O\(_2\) (\( O_2 \)) concentrations in the chloroplast.

Author contributions—R. H. W. and S. M. W. conceived the study. R. H. W. designed RDE2, performed the directed evolution research, and generated the tobacco transgenics that were maintained and analyzed by C. C., E. M.-A., and S. M. W. R. H. W. and S. M. W. performed the Rubisco biochemical analyses and wrote the manuscript.

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Note added in proof—Fig. 2D was removed during redaction.

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