Evolving *Methanococcoides burtonii* archaeal Rubisco for improved photosynthesis and plant growth

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In photosynthesis Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the often rate limiting CO\(_2\)-fixation step in the Calvin cycle. This makes Rubisco both the gatekeeper for carbon entry into the biosphere and a target for functional improvement to enhance photosynthesis and plant growth. Encumbering the catalytic performance of Rubisco is its highly conserved, complex catalytic chemistry. Accordingly, traditional efforts to enhance Rubisco catalysis using protracted "trial and error" protein engineering approaches have met with limited success. Here we demonstrate the versatility of high throughput directed (laboratory) protein evolution for improving the carboxylation properties of a non-photosynthetic Rubisco from the archaea *Methanococcoides burtonii*. Using chloroplast transformation in the model plant *Nicotiana tabacum* (tobacco) we confirm the improved forms of *M. burtonii* Rubisco increased photosynthesis and growth relative to tobacco controls producing wild-type *M. burtonii* Rubisco. Our findings indicate continued directed evolution of archaeal Rubisco offers new potential for enhancing leaf photosynthesis and plant growth.

Improving the performance of the CO\(_2\)-fixing enzyme Rubisco has the potential to significantly enhance photosynthetic efficiency and yield\(^1\). Strategies to achieve this goal involve either modifying the biochemistry and ultrastructure of leaf chloroplasts to concentrate CO\(_2\) around Rubisco, or directly improving Rubisco catalysis itself by genetic crossing or transgenic modification\(^2\). While both approaches face significant technical challenges, suggestions that Rubisco in plants is already operating at or near physiological optimum poses uncertainty as to the level of improvement possible\(^3,4\). Somewhat overlooked in these small data set analyses is that plant Rubisco is not the pinnacle of evolution - as the superior Rubisco from some red algae have the potential to benefit C\(_3\)-plant productivity by as much as 30%\(^2\). Unfortunately, replacing plant Rubisco with red algal Rubisco appears untenable due to chaperone incompatibilities that preclude assembly of algal Rubisco large (L-) and small (S-) subunits into functional L\(_8\)S\(_8\) hexadecamer complexes in leaf chloroplasts\(^6\). In recent years there has been significant advances in understanding the complex and specialised ancillary chaperones for the biogenesis of cyanobacteria and plant L\(_8\)S\(_8\) Rubisco\(^6–9\), however homologs for many of these chaperones in red algae are not readily identifiable.

Despite five decades of research, a dramatic amplification in computational power and more than 25 X-ray structures for different Rubisco isoforms\(^10\) we remain unable to improve Rubisco catalysis by rational design\(^11–13\). This limitation has led to the development of directed (in vitro or laboratory) protein evolution approaches tailored to select for Rubisco mutants with improved function\(^12\). In general, directed protein evolution involves the identification of proteins with desired properties from a mutant library comprising sufficient genetic diversity\(^14\). Advances in directed protein evolution technologies have spurred its success in identifying mutations that improve, or alter, the catalysis and/or solubility of a diverse array of enzymes\(^14–16\). A key benefit of directed evolution is it can reveal novel fitness solutions that would likely otherwise go unexplored during natural evolution\(^14,17\).

Directed evolution of Rubisco has primarily used low throughput photosynthetic selection systems (e.g. *Rhodobacter capsulatus*) or high throughput Rubisco dependent *E. coli* (RDE) selection systems that vary dramatically in efficiency\(^12,18,19\). Common to RDE selection systems is the ectopic expression of phosphoribulokinase (PRK) whose product, the 5-carbon substrate of Rubisco ribulose-1,5-bisphosphate (RuBP) is fortuitously toxic to bacteria. A refined MM1-prk RDE selection has been genetically tailored to use a 'PRK-Rubisco shunt' to bridge a gap\(^1\)-introduced break in glycolysis (Fig. 1\(^a\)\(^20,21\)). The low frequency of false positives obtained using the MM1-prk RDE system contrasts with the striking inefficiency of other RDE systems\(^12,22\). As a result, the MM1-prk RDE system has identified mutations that negatively influence the CO\(_2\)/O\(_2\) specificity (S\(_{CO2}\)) of...
Figure 1. Selecting for improvements in MbR catalysis using Rubisco Dependent E. coli (RDE).

(a) Simplified schematic of the RDE selection system that uses a glycolysis/gluconeogenesis interrupted glyceraldehyde-3-phosphate dehydrogenase deletion (gapA−) strain of E. coli (MM1). Ectopic expression of phosphoribulokinase (PRK) and Rubisco in MM1 acts as a bypass shunt for glycolysis to enable carbon flow from hexose carbon to the TCA cycle for energy and growth. PRK catalyses the conversion of ribulose-5-phosphate (rib-5-P) produced by the pentose phosphate pathway (PPP) to RuBP, which is toxic to cell growth.

(b) Wild type MbR

(b) MbR mutant

(c) MbR mutant # % (w/v) arabinose in growth media MbR mutation (Locus selection frequency)

| MbR mutant # | 0.05% | 0.10% | 0.15% | MbR mutation | (Locus selection frequency) |
|--------------|-------|-------|-------|--------------|-----------------------------|
| WT           | +     | -     | -     | nil          | -                           |
| 1            | ++++  | ++    | +     | K332E        | (7)                         |
| 10A          | ++++  | ++++  | +     | E138V        | (1)                         |
| 63           | ++++  | ++    | +     | T421A        | (3)                         |
| 55           | ++++  | ++    | +     | M423V        | (5)                         |
| 45           | ++    | ++    | +     | H379R        | (3)                         |
| 14           | ++++  | ++    | -     | Y471N        | (1)                         |
| 23           | ++++  | +     | -     | G327S        | (3)                         |
Rubisco from *Rhodospirillum rubrum* as well as mutations that significantly enhance the assembly (solubility) of cyanobacteria L₈S₈ Rubisco and, in one instance, marginally improved all catalytic parameters²¹,²². More recently, the MM1-prk RDE identified a *Synechocystis* PCC6803 L₈S₈ Rubisco mutant with 3-fold improvement in carboxylation efficiency that improved photosynthesis rates by >50% when re-integrated into the high CO₂, carboxysome compartment within the cyanobacterium¹¹. In contrast, it is unlikely that these improvements would be of benefit in plant leaves as the high CO₂ levels needed to account for the low CO₂ affinity and poor S_C/O of cyanobacteria Rubisco are not met by chloroplasts²⁵.

The non-photosynthetic role of the ancient L₈/₁₀ Rubisco isoforms in archaea implies they have likely undergone alternative selection pressures to photosynthetic Rubisco during evolution. For example, Rubisco from archaea have a high affinity for RuBP and high thermostability but low carboxylation rates (*kₐₗ*) and S_C/O²⁶–²⁸. This catalytic distinctiveness arises from the alternative biological role of archaean Rubisco in the pentose bisphosphate pathway or the non-photosynthetic function, archaean Rubisco can still support plant photosynthesis and growth. For example, the *Methanococccoides burtonii* L₁₀ Rubisco (MbR) is highly expressed in leaf chloroplasts and shown to support the growth to fertile maturity of tobacco under the high CO₂ levels needed to accommodate the low *kₐₗ* and S_C/O of MbR²⁶.

The significantly poorer carboxylase properties and alternative function of Rubisco in archaea suggest this form of the enzyme has adapted to alternative evolutionary pressures compared with Rubisco in photosynthetic organisms. This questions whether the carboxylation properties of the archaenal L₁₀ Rubisco might be more amenable for improvement towards those required for enhanced photosynthetic potential. To address this question we used the MM1-prk RDE system (Fig. 1a) to select evolved MbR mutants with improvements in catalytic properties that are required to enhance C₄-plant photosynthesis²⁹. These properties include increasing *kₐₗ*, carboxylation efficiency (*kₐₗc*) divided by *kₐₗ*²⁰-²², the *kₐₗ* for CO₂ under ambient O₂ and S_C/O. Using chloroplast genome (plastome) transformation we introduce mbR genes into tobacco to demonstrate successful translation of improved MbR properties selected in *E. coli* into leaf chloroplasts. The enhanced photosynthesis and growth of the transformed plants producing improved MbR mutants relative to control lines producing non-mutated MbR provides novel proof of concept on the utility of improving Rubisco catalysis by directed evolution in *E. coli* to improve the CO₂-assimilation rate in leaves.

**Results**

**Directed evolution of *M. burtonii* Rubisco (MbR) in *E. coli*.** The native *mbiI* gene coding *M. burtonii* Rubisco is efficiently translated in *E. coli* and assembled into abundantly expressed (>6% (w/w) of soluble cell protein) as functional L₁ Rubisco (MbR)²⁶. In the presence of substrate RuBP (or structurally comparable sugar phosphate ligand) the L₁ units assemble into a stable L₁₀ MbR complex. Random mutations were introduced into *mbiI* using error prone PCR (averaging 2 mutations per kb) and the mutant genes ligated into a lac inducible vector pTrcHisB²⁹.

Three *mbiI* libraries (each comprising ~180k variants) were transformed into MM1-prk cells (Fig. 1a) and grown at 23 °C as described²⁰. The initial selection was performed under high-Rubisco inducing (0.5 mM IPTG) and low-PRK inducing conditions (0.05% (w/v) arabinose) in air supplemented with 2.5% (v/v) CO₂. After 9–16 days 80 colonies showing improved growth relative to MM1-prk producing wild-type MbR were identified (Supplementary Table 1). The Rubisco-containing plasmid from each colony was sequenced revealing substituions in 78 of the 474 amino acids (Supplementary Table 1). Each mbii mutant was cloned back into pTrcHisB and re-transformed into MM1-prk RDE cells and separately grown under higher Rubisco activity selection (i.e. on media containing 0.1% (w/v) arabinose to elevate PRK expression; Fig. 1b). Colony growth was scored relative to MM1-prk cells expressing wild-type MbR that could not grow on media containing 0.1% (w/v) arabinose (Fig. 1b). Seven *mbiI* mutant genes were found to convey a distinct selective advantage to MM1-prk *E. coli* growth (Fig. 1c).

The seven mutant and wild-type pTrc- *mbiI* genes were expressed in XL1-Blue *E. coli* without co-expressing PRK. This resulted in only L₁ MbR oligomers being formed as the cells made no RuBP that is required for the formation of L₁₀ MbR complexes²⁶. The cellular content and catalytic properties of each L₁ MbR enzyme was measured (Fig. 2a). Maximal rates of CO₂ fixation (*kₐₗ*) at 25 °C were determined under ambient O₂ levels (~252 kM O₂) and at pH 7.2 due to the low pH favoured by MbR catalysis²⁶. Under these conditions MbR mutant isolates #1 (MbR-K332E), #10A (MbR-E138V) and #63 (MbR-T421A) showed significant 40% to 90% improvements in *kₐₗc* and corresponding 10% to 25% increases in S_C/O (Fig. 2a). Quantification of MbR expression in *E. coli* by ¹⁴C-CABP binding and confirmation by SDS PAGE (Fig. 2b) showed that most mutations had little effect on the level of MbR expression. The MbR-T421A mutant (#63) showed a modest, but significant, increase in expression while the mutations in MbR mutants #14, #23 and #45 significantly impeded MbR production (Fig. 2b).
Expression of improved MbR in tobacco chloroplasts. The tobacco genotype “cmtrL” has been genetically tailored for Rubisco engineering using chloroplast transformation. In the plastome of cmtrL chloroplasts, the wild type rbcL gene has been replaced with a synthetic, codon modified version of the R. rubrum bacterial rbcM gene (cmrbcM) that codes for Form II L2 Rubisco in place of tobacco L8S8 Rubisco (Fig. 3a). The increased O2 sensitivity of R. rubrum L2 Rubisco reduces both SC/O and carboxylation efficiency under ambient O2 (kcatC/KC21%O2) by ~7-fold relative to tobacco L8S8 Rubisco (Table 1). These poorer catalytic properties result in the cmtrL genotype requiring high CO2 for growth in soil. As shown by Alonso et al. (2009) the catalytic properties of MbR (both in L2 and L10 complexes) are even more impeded than R. rubrum L2 Rubisco, especially with increasing alkaline pH. Despite this impairment, transplastomic replacement of the cmrbcM gene in cmtrL with the wild-type mbiiL gene generated the L10 MbR producing tobacco genotype tobmbiiL that could survive under elevated CO2 (2.5% v/v) in soil. The tobmbiiL lines took more than 300 days to reach fertile maturity compared with ~30 days for wild type tobacco and ~32 days for cmtrL under the same growth conditions.

To test whether the evolved MbR enzymes translated to improved tobacco photosynthesis and growth, synthetic mbR genes were made that incorporated the codon use of the tobacco rbcL gene (Fig. 3a). In addition, the native MbR N-terminal MSLIYEDLV sequence was replaced with the MSPQTETKASVGF sequence of the tobacco L-subunit that undergoes a range of post-translational modifications that tentatively provide protection from proteolysis. Three mbR genes coding wild-type MbR, MbR-K332E and MbR-E138V were cloned into the pLEV4 plastome transforming plasmid and transformed into cmtrL leaves. Transplastomic tobacco lines producing L10 MbR were identified by native PAGE (Fig. 3b). At least two independent lines for each of the tobMbR, tobMbRE138V and tobMbRK332E genotypes were continuously propagated on spectinomycin-containing media until homoplasmic (i.e. no longer producing L2 R. rubrum Rubisco) before growing the T0 plants to maturity in soil in air supplemented with 2.5% [v/v] CO2.

Only L10 MbR was detected in leaves (Fig. 3b) due to the continuous production of RuBP under illumination and the relative stability of the decameric complex. While the T0 tobMbRE138V and tobMbRK332E plants grew substantially quicker than tobMbR, little difference was detected in the L10 MbR content in comparable upper canopy leaves of the juvenile (~21 cm tall) T0 plants (Fig. 3c). When at ~60 cm in height, 3–6-fold higher levels of L10 MbR were measured in the newly emerging upper canopy leaves with significantly higher amounts detected in the faster growing, healthier looking, tobMbRE138V and tobMbRK332E T0 plants (Fig. 3c). At both development stages, the...
Leaf MbR levels were generally 3–4-fold lower than the L2 and L8S8 Rubisco content in the cmtrL and wild-type tobacco controls growing alongside.

Figure 3. Transformation and expression of the mutated MbR enzymes in tobacco leaves. The varying cmmbR genes coding wild type and mutant MbR were integrated into the rbcL region of the tobacco plastome by chloroplast transformation. (a) Comparison of the plastome sequence and types of Rubisco made in the varying tobacco genotypes examined. The cmmbR and selectable marker aadA gene in the pLEVmbR, pLEVmbR-E138V and pLEVmbR-K332E transforming plasmids were transformed into the plastome of the cmtrL tobacco genotype to replace the cmrbcM (that codes R. rubrum L2 Rubisco31) by homologous recombination of the flanking plastome sequence (located between the dashed lines, numbering relevant to Genbank sequence Z00044). P, 292-bp rbcL promoter/5′ UTR; T, 288-bp rbcL 3′ UTR; t, 147-bp rps16 3′ UTR. Alignment position for primers LsD and LsE32 and the 221-bp 5UTR probe8 are shown. (b) native PAGE analysis of the L8S8, L2 and L10 Rubisco isoforms produced, respectively, in leaves from tobacco, cmtrL and the three tobMbR genotypes. *non-Rubisco protein. (c) 14C-CABP quantification of Rubisco active site content in comparable young upper canopy leaves of each genotype during early (~20 cm in height, colored bars) and late (~60 cm in height, black bars) exponential growth.

| Rubisco type | S_{CO2} (mol.mol^{-1}) | K_{C} (μM) | k_{oxygen} (s^{-1}) | K_{o} (μM) | k_{oxygen}/K_{O} (s^{-1}) | k_{oxygen}/K_{C} (μM.s^{-1}) | K_{m^{O_{2}}} (μM) |
|-------------|------------------------|------------|---------------------|------------|---------------------------|-----------------------------|-------------------|
| MbR         | 1.3 ± 0.1              | 56.9 ± 1.8 | 0.6 ± 0.1           | 11.2 ± 1.4 | 0.09                      | 10.5                        | 0.5               |
| MbR-E138V   | 1.5 ± 0.1              | 66.3 ± 2.1 | 1.0 ± 0.1*          | 35.1 ± 6.1 | 0.35                      | 15.1                        | 1.8*              |
| MbR-K332E   | 1.3 ± 0.1              | 78.7 ± 2.4 | 1.2 ± 0.1*          | 24.4 ± 4.0 | 0.30                      | 15.2                        | 1.4*              |
| R. rubrum [1] | (12)                   | (149)      | (9.0)               | (159)      | (0.8)                     | (60.4)                      | 23.3              |
| N. tabacum [2] | (81)                   | (11)       | (3.4)               | (259)      | (0.8)                     | (309.1)                     | 171               |

Table 1. Rubisco catalysis measurements. L2 MbR catalytic properties at pH 8.0, 25 °C relative to R. rubrum L2 and tobacco L8S8 Rubisco. k_{oxygen}, maximal oxygenation rate calculated from S_{CO2} = (k_{oxygen}/K_{C})(k_{oxygen}/K_{O}). K_{C} under ambient atmospheric O2 levels (Q = 252 μM O2 in air saturated H2O) calculated as K_{C}(1 + O/K_{C}). Values in parenthesis are those measured previously by [1]20 and [2]5. Significance variation relative to wild type MbR at pH 8.0 (*p < 0.001) determined by T-test.

leaf MbR levels were generally 3–4-fold lower than the L2 and L8S8 Rubisco content in the cmtrL and wild-type tobacco controls growing alongside.
Limitations in the steady state \( mb \)R mRNA levels in each genotype contributed to the deficiency in MbR (Supplementary Fig. 1). As indicated in Fig. 3a, both a monocistronic \( mb \)R and a (50–70% less abundant) discistronic \( mb \)R-\( aad \)A transcript were made in each \( T_0 \) tob\( MbR \) genotype. In the young upper leaves of \( T_0 \) plants at \(~21\) cm in height the total \( mb \)R mRNA abundance was 30–70% lower in abundance than the \( rbcL \) mRNA levels in wild-type (Supplementary Fig. 1). As seen previously in Rubisco-modified tobacco genotypes with reduced photosynthetic potential\(^8,26,30,32\), these reduced mRNA levels correlate with the impaired viability of the thinner, smaller sized, pale green leaves of each transplastomic genotype (see below).

The evolved MbR have improved carboxylase activity. The catalytic properties of the wild type and mutant \( L_{10} \) MbR isoforms produced in the \( T_0 \) progenies were measured at \( pH\) 8 (the approximate \( pH\) of the chloroplast stroma, Table 1). While the \( S_{CO} \) values matched those measured for the \( L_{2} \) enzymes produced in \( E. coli \) (Fig. 2b), the \( k_{cat}^C \) rates were lower than those measured at \( pH 7.2 \) due to the increased activity of MbR at low \( pH\)\(^26\). Nevertheless, even at \( pH\) 8 both \( k_{cat}^C \) and the carboxylation efficiencies (\( k_{cat}^C/R_{21%O2}^C \)) of the MbR-E138V and MbR-K332E enzymes were between 2 and 3.4-fold higher than MbR, with an accompanying ~15% increase in \( S_{CO} \) for the MbR-E138V enzyme (Table 1). Importantly, these improvements in \( CO_2 \) affinity, specificity and fixation speed came without expense to the natural high affinity of MbR for RuBP (i.e. a low \( K_m \)\( RuBP \), Table 1).

Enhancing MbR catalysis improves tobacco photosynthesis and growth. The improved growth and healthier phenotype of the \( tob^{MbRK332E} \) and \( tob^{MbRE138V} \) genotypes relative to the \( tob^{MbR} \) lines was evident in the \( T_1 \) progeny. In tissue culture germination trials all the \( T_1 \) progeny emerged as green cotyledons on spectinomycin media after 1 week confirming all were transplastomic (Fig. 4). After 5 weeks it was evident that addition of sucrose to the tissue culture media was required for the germinated \( tob^{MbR} \) and \( tob^{MbRE138V} \) lines to survive under elevated (2.5% \( v/v \)) \( CO_2 \) (Fig. 4). In contrast, the \( tob^{MbRK332E} \) plants survived under high \( CO_2 \) without sucrose supplementation and grew quicker under all tissue culture conditions tested.
As shown by Alonso et al., (2009), air enriched with >2% (v/v) CO$_2$ was needed for each MbR producing tobacco line generated to grow to fertile maturity in soil. Consistent with the improved catalysis of the transplanted MbR-E138V and MbR-K332E enzymes (Table 1), the tobMbRE138V and tobMbRK332E genotypes supported faster leaf photosynthetic CO$_2$ assimilation rates relative to the tobMbR lines (Fig. 5a). To compensate for the lower leaf levels of MbR and the poorer catalytic properties of the L10 MbR relative to tobacco L8S8 Rubisco (Fig. 3c and Table 1), measurements of photosynthetic CO$_2$-assimilation rates in all the MbR transformed leaves were performed under 1% (v/v) O$_2$. Even under these low O$_2$ pressures, photosynthesis remained limited by MbR-activity over the full range of intercellular leaf CO$_2$ pressures (C$_i$) tested (Fig. 5a) with the highest assimilation rate of 2.4 μmol CO$_2$ fixed.m$^{-2}$.s$^{-1}$ measured in tobMbRK332E leaves under the highest leaf gas exchange C$_i$ of 2000 μbar CO$_2$ (Fig. 4b). As this rate is more than 10-fold slower than the 26–30 μmol CO$_2$ fixed.m$^{-2}$.s$^{-1}$ rates measured in high CO$_2$ grown wild type leaves the tobMbRK332E grew ~5-fold slower than wild-type under high CO$_2$ (Fig. 5b,c).

The relative differences in the leaf CO$_2$-assimilation rates of each transplastomic genotype (Fig. 5a) correlated with their growth rate (Fig. 5b). The tobMbRE138V plants grew faster and reached fertile maturity before the tobMbRK332E lines while the growth of the tobMbR controls were substantially impaired (Fig. 5b). This discrepancy can be attributed to the ~50% lower levels of MbR-E138V produced in tobMbRE138V leaves relative to MbR levels produced in comparable leaves from both the tobMbR and tobMbRK332E genotypes (Fig. 5a). Identifying if the E138V mutation impedes the translation, biogenesis or/and stability of MbR remains to be tested.

The structural location of the E138V and K332E mutations in MbR. Phylogenetic analysis of MbR reveals it shares closer sequence homology with *R. rubrum* Form II Rubisco than other archaeal Rubisco (Supplemental Fig. 2). These alignments showed that E138 and K332 in MbR align with A134 and E331 in *R. rubrum* Rubisco and R134 and E324 in the *T. kodakarensis* archaeal L10 Rubisco (Fig. 6a). In both *R. rubrum* and *T. kodakarensis* Rubisco, the corresponding E331 and E324 residues are located near the hinge of the conserved flexible loop 6 structure of the C-terminal α/β-barrel (Fig. 6b). A glutamate at this position in loop 6 is highly conserved among photosynthetic L$_8$S$_8$ Rubisco isoforms (e.g. E336 in plants like tobacco, E339 in red algae such as *Griffithsia monilis*) and is in close vicinity to the strictly conserved K334 catalytic residue (tobacco Rubisco numbering) whose side-chain interactions with RuBP and gaseous substrate are critical determinants of catalytic efficiency (*i.e.* $k_{cat,C}$ and $S_{CO_2}$). The increased catalytic turnover rate and
Carboxylation efficiency of MbR-K332E (Table 1) imply a glutamate at this position in loop 6 may benefit Rubisco catalysis in photosynthetic organisms but pose no benefit for the non-photosynthetic role of archaea Rubisco.

Discussion

We uniquely demonstrate the potential of directed evolution using RDE selection to successfully deliver more efficient forms of the non-photosynthetic *M. burtonii* archaeal Rubisco (MbR). The derived improvements in CO₂-fixation speed, CO₂-affinity and specificity for CO₂ of the evolved MbR-E138V and MbR-K332E mutant enzymes translated to supporting faster rates of CO₂ assimilation and growth in tobacco relative to the control tobMbR genotype producing wild-type MbR. This finding provides the first proof of concept that directed evolution of non-photosynthetic Rubisco in *E. coli* can deliver mutants with improvements in all the catalytic parameters needed to stimulate photosynthesis in leaf chloroplasts. This contrasts with prior success in evolving improved catalytic mutants of cyanobacterial Rubisco that either show only marginal (<5%) overall improvements in catalysis or a significant enhancement (>50%) in carboxylation efficiency that came at the expense of an unwanted parallel increase in inhibitory oxygenation efficiency. An additional challenge with cyanobacterial

Figure 6. Structural analysis of the catalysis enhancing mutations in MbR. (a) Alignment of MbR, *R. rubrum* (Rr) and *T. kodakarensis* (Tk) Rubisco large subunit sequences adjoining the E138 and K332 mutation sites in MbR. Only amino acids differing from MbR are shown. Secondary structure information is relative to that in Tk L₁₀ Rubisco. Only amino acids differing from MbR are shown. Secondary structure information is relative to that in Tk L₁₀ Rubisco. (b) Location of the mutated residues in *R. rubrum* L₂ Rubisco (A134 and E321: PDB 9RUB) and Tk L₁₀ Rubisco (R134 and E324: PDB 3A12) are highlighted in cyan. Rr L-subunits are shaded red and green, active site bound RuBP or CABP in yellow. In the Tk structure R134 is located at the L₂-L₃ interface (differentially coloured red and blue). E321 and E324 are located within the flexible loop 6 region in both Rr and Tk Rubisco respectively. Diagrams constructed using PyMOL.
L₈S₈ Rubisco is its limited biogenesis potential in tobacco chloroplasts (~10% of wild-type) compared with MbR L₁₀ which is produced at ~25–50% of wild-type tobacco Rubisco (Fig. 3c). The high solubility and overall success with evolving MbR catalysis inspires continued effort to evolve properties along evolutionary trajectories that further enhance its photosynthetic potential.

Exploration of Rubisco sequence space towards mutations that improve its efficiency in crop plants is an ongoing challenge. Our continued inability after 50 years to rationally predict what sequence changes can improve Rubisco function steered our attention towards the potential of directed evolution to explore Rubisco sequence space for improved catalysis. A common requirement of successful directed evolution studies is identifying a suitable starting point for mutagenesis and appropriate selection system. The ease by which the carboxylase activities of MbR could be enhanced by single amino acid changes (Table 1) likely stems from it having undergone specialisation to an alternative metabolic role during its non-photosynthetic evolution. This implies that archaeal Rubisco may occupy an alternative position to photosynthetic Rubisco within the evolutionary landscape of sequence space diversity in relation to catalysis. Consistent with this, archaeal Rubisco catalysis is typically distinct relative to contemporary (photosynthetic) Rubisco. Archaeal Rubisco can sustain functionality at extreme temperatures, under which thermotolerant archaea grow, and exhibit the heightened affinity for RuBP typical of Rubisco from thermophiles. The adaptation of Rubisco cleavage activities of MbR and ability to avoid or circumvent beneficial mutations that might produce destabilizing effects on structure and function. Such uncertainties are common to directed protein evolution studies. Forecasting the extent to which mutations (via direct or long distance amino acid interactions) influence artificial evolutionary trajectories remains unpredictable.

A significant hurdle is the relatively low selection fidelity and throughput of the MM1-prk selection system. The frequency of success in directed evolution applications depends on the library selection throughput and sensitivity of the selection system to detect a desired trait. The reliance and throughput of existing RDE strains suffer from high frequencies of false positives that typically arise through transposon associated PRK escape mutations. While relatively immune to false positives, the MM1-prk RDE selection throughput is impeded by a low growth temperature requirement (25 °C), poor transformation efficiency, and reduced cell viability as a result of the gapX mutation. Improving the selection fidelity of RDE systems is therefore critical to further evolving MbR, and other Rubisco isoforms, with improved photosynthetic properties. One solution might be to tether PRK with an antibiotic resistance protein in an RDE strain thus avoiding selection of “PRK-silenced” false positives as such mutations would also relinquish antibiotic resistance.

Adaptive evolution of archaeal Rubisco in vitro towards one that is more efficient than crop plant L₈S₈ enzymes is undeniably a significant, long term challenge. Unlike L₈S₈ Rubisco from plants and algae, the folding and assembly requirements of archaeal Rubisco, like MbR, are met in E. coli (Fig. 1c). This property strengthens the suitability of archaeal Rubisco for identifying catalysis enhancing mutants using RDE strains as it curtails selection of mutations that enhance solubility, an outcome that has dominated directed evolution studies with cyanobacteria L₈S₈ Rubisco. The amenability of archaeal Rubisco to mutational testing in E. coli has already proven useful to demonstrate how incorporating spinach Rubisco sequence into T. kodakarenensis L₁₀ Rubisco can improve kcat. Improving archaeal Rubisco catalysis by rational design or directed evolution or a combination of both therefore poses viable future pathways to pursue, particularly given our finding that these benefits can directly translate to improving leaf photosynthesis.

As indicated in Fig. 6, the primary sequences of archaeal Rubisco are highly diverse and their oligomer structures as L₁₀ or L₁₀ appears variable. While mass spectrometry analysis infers a mature L₁₀ quaternary structure for MbR it is uncertain if it forms a comparable toroidal structure to T. kodakarenensis archaeal Rubisco (PDB: 1GEH), in particular since they only share 36% amino acid homology and MbR contains a novel 11 amino acid insertion in its C-domain. Ongoing efforts are focused on solving the crystal structures for both L₈ and L₁₀ MbR to better understand the structural diversity among archaeal Rubisco as well as help interpret how mutations, such as E138V and K332E, functionally impart changes to catalysis.

Materials and Methods

Evolution, expression and purification of MbR in E. coli. The mbiiL gene from pHUE-mbiiL was cloned into pTrcHisB using NcoI/HindIII and the resulting pTrcMbR plasmid used as template to randomly mutate the mbiiL by error-prone PCR (epPCR) as described. The PCR products were cloned into pTrcHisB and the diversity of the mbiiL mutant library calculated using PEDEL-AA. The library was transformed into the Rubisco Dependent E. coli (RDE) strain MM1-prk and grown under varying selective conditions according to. The mutant mbiiL genes from faster growing colonies were cloned into the 6xhistidine-tagged ubiquitin expression plasmid pHUE and each MbR isoform affinity purified by immobilised metal affinity chromatography (IMAC) as described.
Tobacco plastome transformation and growth. A synthetic gene, mbR, coding for M. burtonii Rubisco both with and without mutations coding E138 V or K332E substitutions was synthesised by GenScript. The codon use of mbR matched the tobacco rbcL gene and replaced the native N-terminal coding sequence (MSLIYEDVLV) with that for the native tobacco Rubisco large (L-) subunit (MSQIYETKASVGF). The 1,418-bp mbR gene fragments were cloned into NheI/Sall cut pLEV32 to produce the plastome transforming plasmids pLEVmbR, pLEVmbR-K332E and pLEVmbR-E138 V and transformed into ten line leaves by biolistic bombardment. Independent positively transformed lines producing Lop MbR were identified by non-denaturing PAGE (native PAGE)26 and two independent lines for each MbR genotype were grown to maturity in soil in a growth chamber with the air supplemented with 2.5% (v/v) CO2. The flowers of the fertile T1 plants were fertilised with wild type pollen and the seed germinated in tissue culture on RMOP media supplemented with 0% to 3% (w/v) sucrose. The germinated T1 progeny were carefully transferred to soil and the leaf gas exchange and cell biochemistry of near fully expanded leaves at comparable positions in the upper canopy analysed when the plants were 20–25 cm in height.

DNA, protein and PAGE analyses. Total leaf genomic DNA was isolated using the DNeasy® Plant Mini Kit and primers LSH and LSE (Fig. 3a) used to PCR amplify and sequence the plastome region transformed in each tobacco genotype as described. The preparation, quantification (against BSA) of soluble leaf protein and analysis by SDS-PAGE, native PAGE and immunoblot analysis was performed as described.

Rubisco content and catalysis. Rates of Rubisco 14CO2 fixation were made using soluble protein extracts isolated from bacteria or leaf protein in 50 mM HEPES-NaOH (pH 7.2 or 8.0) containing extraction buffer as described. Protein extract (20 μL) was used to initiate activity in 0.5 mL assays performed in 7 mL septum-capped scintillation vials. Each sample was measured in duplicate under varying concentrations of NaH14CO3 (0–67 μM) and O2 (0, 2, and 5% (v/v)) to calculate the maximal rate of carboxylation (Vc) and the Michaelis constants (Km) for CO2 (Ko2) and O2 (Ko2). The carboxylation turnover rate (kcat) was calculated by dividing Vc by the Rubisco active sites content quantified by [14C]-2-CABP binding. Rubisco CO2/O2 specificity (SCO2) and the Km for RuBP were quantified as described using MbR purified from E. coli by immobilised metal affinity chromatography or from tobacco leaves by ion exchange.

Growth and photosynthesis analysis. All plants were grown at 25°C in a growth chamber as described under 200 ± 50 μmol quanta m−2 s−1 in air containing 2.5% (v/v) CO2. Once approximately 21 cm in height the leaf photosynthesis rates (A) in the 5th upper canopy leaf were measured using a LI-6400 XT gas exchange system (LI-COR) at varying atmospheric CO2 partial pressures (CP; 50–2000 ppm) at a constant leaf temperate of 25°C and 1000 μmol quanta m−2 s−1. The A-Ci measurements (Ci; leaf intercellular CO2 levels) were performed at low O2 partial pressures (1% (v/v) O2 in N2) to obtain suitable measures of A.

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Author Contributions
R.H.W. and S.M.W. designed the experiments. R.H.W. and H.A. undertook the directed evolution studies with R.H.W. producing and analysing the transformed tobacco lines. R.H.W., H.A. and S.M.W. performed the biochemical analyses and wrote the manuscript.

Additional Information
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