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Research Report

Bacterial Form II Rubisco can support wild-type growth and productivity in *Solanum tuberosum* cv. Desiree potato under elevated CO_2

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Abstract

The last decade has seen significant advances in the development of approaches for improving both the light harvesting and carbon fixation pathways of photosynthesis by nuclear transformation, many involving multigene synthetic biology approaches. As efforts to replicate these accomplishments from tobacco into crops gather momentum, similar diversification is needed in the range of transgenic options available, including capabilities to modify crop photosynthesis by chloroplast transformation. To address this need, here we describe the first transplastomic modification of photosynthesis in a crop by replacing the native Rubisco in potato with the faster, but lower CO_2-affinity and poorer CO_2/O_2 specificity Rubisco from the bacterium *Rhodospirillum rubrum*. High level production of *R. rubrum* Rubisco in the potRr genotype (8 to 10 μmol catalytic sites m^2) allowed it to attain wild-type levels of productivity, including tuber yield, in air containing 0.5% (v/v) CO_2. Under controlled environment growth at 25°C and 350 μmol photons m^2 PAR, the productivity and leaf biochemistry of wild-type potato at 0.06%, 0.5%, or 1.5% (v/v) CO_2 and potRr at 0.5% or 1.5% (v/v) CO_2 were largely indistinguishable. These findings suggest that increasing the scope for enhancing productivity gains in potato by improving photosynthate production will necessitate improvement to its sink-potential, consistent with current evidence productivity gains by eCO_2 fertilization for this crop hit a ceiling around 560 to 600 ppm CO_2.

Keywords: chloroplast transformation, crop improvement, carbon fixation, metabolic engineering

Significance Statement:

Here, we develop the first photosynthetically modified plastome transformed food crop by substituting the native CO_2-fixing enzyme, Rubisco, in potato with a faster primitive variant from the bacterium *Rhodospirillum rubrum*. Though the poor CO_2-affinity of the structurally simple bacterial Rubisco impaired leaf carbon assimilation rates in air (ambient CO_2), the transplastomic potato lines showed wildtype vegetative growth, leaf nutrient quality, carbohydrate content, and tuber production under elevated CO_2. Our data support observations that the sink strength in potato will allow for up to approximately 30% improvements over current tuber yields via enhancing photosynthate production (source strength).

Introduction

The pressing global concern around food security underpins a multitude of biotechnological and breeding activities focused on improving crop resilience, nutrition, and yield (1). Challenging these efforts are the need to address the increased frequency of extreme weather events, depletion in the quality and availability of arable land, and rapidly changing rainfall patterns (2). Recent years have seen an increased interest and advances in enhancing photosynthesis to improve crop productivity (3). Studies with the model C_3-plant tobacco have identified a range of strategies for improving growth rates in the field. Examples include increasing light harvesting capacity (4), re-engineering photorespiration to increase CO_2 release at the site of Rubisco in the chloroplast (5), and enhancing the regeneration of Rubisco's substrate Ribulose-1,5-bisphosphate (RuBP) under high light (6). In some instances, these strategies have been successfully translated into crop enhancing outcomes with the level of success in yield gain heavily dependent on plant sink potential and resource (water, nitrogen) availability (7, 8).

Improving the carboxylation properties of the photosynthetic enzyme Rubisco has itself been a longstanding target for increasing plant productivity, albeit with no success to date in a crop context (9) except in some species through overexpression of the native Rubisco (10–12). Unfortunately, direct replacement of crop

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Rubisco with the more efficient variants made in some red-algae is untenable as their assembly requirements are not met in leaf chloroplasts (13), precluding their use to potentially improve C_3-plant photosynthesis by up to 30% (14). Nevertheless, directed evolution using *Escherichia coli* selection has proven successful in identifying point mutations in both the large (RbcL) and small (RbcS) subunits that impart step change catalytic improvements to cyanobacteria Form IB Rubisco (15) whose phylogenetic lineage and quaternary $RbcL_8RbcS_8$ (L_8S_8) structure match the plant enzyme (9).

While coupling directed evolution with new capabilities to produce plant Rubisco in *E. coli* (16) provide a promising process to identify mutations that enhance plant Rubisco catalysis (17), integrating the substitutions into crop Rubisco can be problematic. Fortunately, targeted modification of the multiple *RbcS* gene copies in the nucleus now appears tenable by gene editing (18), especially via optimized CRISPR/Cas9 editing systems (19). In contrast, modification of the plastome *rbcL* gene suffers from the limited range of crop species for which chloroplast transformation is feasible (20).

In planta studies on *rbcL* bioengineering have, to date, been limited to tobacco where plastome transformation is routine (21, 22). The efficiency of the plastome repair system in chloroplasts has proven to be a challenge in *rbcL* transforming studies where the efficiency at which the introduced point mutations are maintained is significantly impaired (23–26). To avoid this the tobacco ^{cm}trL genotype [now termed tobRr (27)] was developed. In this markerless (*aadA*-free) *rbcL*-transforming tobacco master line, the native *rbcL* is replaced with a codon modified *rbcM* gene that codes the structurally simple, 100 kDa $RbcL_2$ Rubisco from the bacterium *Rhodospirillum rubrum* (21). The tobRr line has since been used almost exclusively to bioengineer *rbcL* in tobacco, producing a myriad of photosynthetically modified transplastomic lines expressing foreign and mutated Rubisco. These studies have (1) examined the biogenesis compatibility of phylogenetically diverse Rubiscos with the protein folding machinery of tobacco chloroplasts and (2) discovered catalytic enhancing switches in both RbcL and RbcS [(27–29) and reviewed in ref. (30)].

Globally, potato production is the largest of the noncereal crop species making it also a prized target for yield improvement (31). Current evidence from both growth chamber and field enhanced CO_2-fertilization studies imply the sink potential of potato can facilitate up to 30% improvements above current tuber yields under optimal, resource replete growth (32–39). This suggests enhancing the source strength of potato via transgenic approaches will deliver improvements in yield, though not necessarily by enhancing photoprotection (40). Fortunately, potato, like tobacco, is a member of the Solanaceae family and amenable to plastome transformation (41, 42). Despite this genetic capability, metabolic engineering of potato has primarily involved nuclear transformation; targeting traits such as disease resistance and tuber quality (43) and altering hormone signaling and photosynthetic behavior through overexpression of B-Box (BBX) transcription factors (44). To extend plant Rubisco research from tobacco to an economically important species, here we describe the successful replacement of Rubisco in potato with the *R. rubrum* Rubisco. The potRr lines, like the corresponding tobRr genotype, share similar impairments in photosynthetic potential under ambient CO_2 (aCO_2). However, under 0.5% (v/v) CO_2, the productivity of potRr matched wild-type potato, with the growth and leaf biochemistry of both genotypes unaffected by additional CO_2 fertilization (1.5% (v/v) CO_2) congruent with further gains in yield being limited by the sink potential of potato (7).

Results

Plastid transformation and regeneration of the potRr lines

Following biolistic transformation of *Solanum tuberosum* leaves with pLEVpotRr (Figure 1A), the dissected tissue was successively transferred to fresh selective StM3 medium (for callus induction and shoot regeneration) containing 0.4 $mg.mL^{-1}$ spectinomycin every 4 weeks until the first green shoots appeared after 14 weeks (Figure S1). The shoots were transferred to StM4 media for elongation then hormone free StBasal medium to promote root development (Figure 1B). From the 156 spectinomycin resistant ($spec^R$) shoots obtained from 45 bombardments, the genomic DNA (gDNA) extracted from the first 20 lines identified 13 putative transformants by PCR (Figure S2). After 9 months in tissue culture the first two lines, potRr#1 and #2, were transferred to soil and grown under eCO_2 (air + 1.5% (v/v) CO_2) alongside wild-type potato (Figure 1C). DNA blot analyses corroborated the identity of the potRr#1 and #2 lines and that they were homoplasmic (i.e., no wild-type plastome copies remained, Figure 1D).

The reduced photosynthetic potential of the potRr genotype

Leaf gas exchange measurements showed both potRr lines had impaired photosynthetic rates relative to wild-type (Figure 1E). Over the intercellular CO_2 (C_i) range examined, the CO_2 assimilation rates (A) in the potRr leaves remained Rubisco carboxylase activity (A_c) limited. By contrast, the photosynthetic rate in potato are A_c limited up to ~350 μbar C_i before CO_2 assimilation was limited by the electron transport rate capacity to regenerate RuBP [i.e., A_J limited, (45)]. The A_C-C_i response in both potato and potRr leaves closely matched that simulated from their differing Rubisco contents (potRr producing 70% the catalytic site content of wildtype) and contrasting catalytic properties (Figure 1F) when modeled according to (45). PAGE analyses from the same leaves confirmed both potRr lines were already homoplasmic and only made *R. rubrum* Rubisco with no potato RbcL or RbcS detectable by SDS PAGE (Figure 1G). Similarly, native PAGE analyses confirmed the potRr lines made *R. rubrum* $RbcL_2$ (L_2, ~100 kDa) Rubisco and no ~520 kDa potato L_8S_8 complexes (Figure 1H).

The dependency of potRr viability on eCO_2

Consistent with that previously observed in the tobRr genotype (46), the A_C-C_i response of the potRr leaves showed that the poorer CO_2-affinity under ambient O_2 (K_c^{air}) and ~7-fold lower CO_2/O_2 specificity ($S_{c/o}$) of *R. rubrum* Rubisco (Figure 1F) prevent this genotype from net photosynthetic gains below ~600 μbar CO_2 (Figure 1E). This was tested using vegetative cuttings from the plants analyzed by gas exchange. Replica chamber grown potato (wild-type), potRr#1 and #2 plants cultivated to ~20 cm in height under eCO_2 (air + 1.5% v/v CO_2, Figure S3) were either maintained under eCO_2 or transferred to aCO_2 in a glasshouse. Over 34 days, all three genotypes under eCO_2 showed little difference in their growth rate and phenotype (Figure 2A) or in their tuber production and floral development (Figure 2B). In contrast, only wild-type grew under aCO_2 with the development of both the potRr#1 and #2 lines stalling after a few days. As seen previously with the tobRr genotype (46), under aCO_2 the potRr lines became visibly chlorotic prior to the onset of necrosis after ~4 weeks (Figure 2A). Accompanying this photosynthetic impairment, the potRr lines produced no tubers under aCO_2.

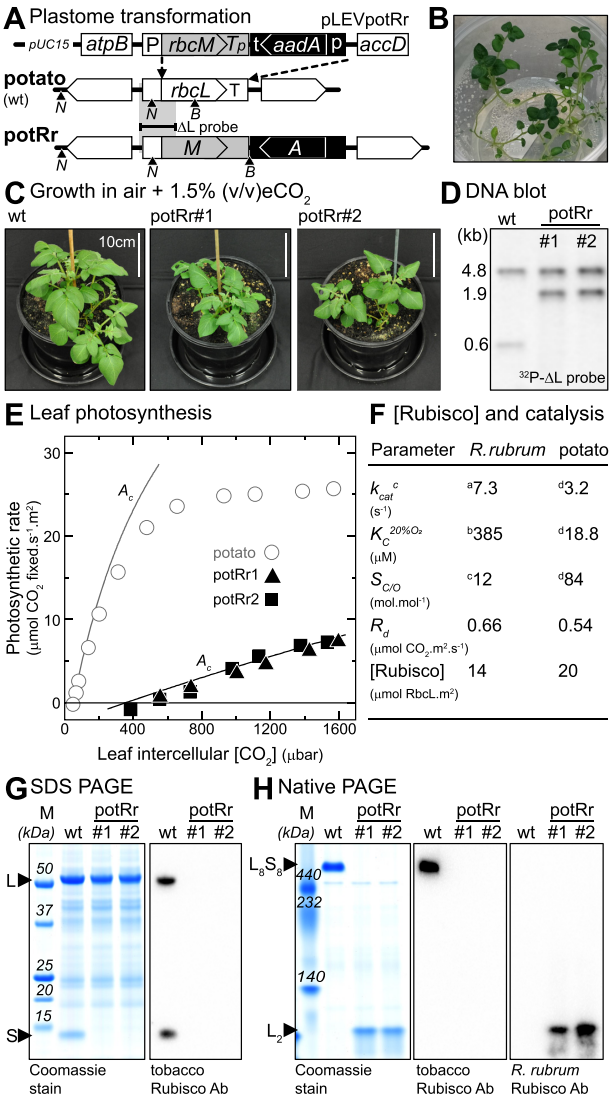

Fig. 1. Generation of homoplasmic potRr lines with altered CO_2-assimilation biochemistries. (A) The transforming plasmid pLEVpotRr contains 1102-bp and 866-bp of flanking plastome sequence spanning part of the *atpB* (chloroplast ATP synthase β subunit) and *accD* (β-subunit acetyl-CoA carboxylase) genes, respectively, to replace the *rbcL* gene and 3′UTR (T) (dashed arrow lines) in *S. tuberosum* (potato) with a *rbcM-aadA* (*M*, *A*) operon via homologous recombination. Gene regulatory elements in the transplastomic potRr lines include the *S. tuberosum rbcL* promoter + 5′UTR (P), *psbA* 3′UTR (*Tp*), and the tobacco 16S rDNA *rrn* promoter (p) and *rps* 3′UTR (t). Annealing position of the ΔL-DNA probe and the *NcoI* (*N*) and *BamHI* (*B*) restriction sites are shown. (B) Spectinomycin resistant lines propagated 9 months in tissue culture were (C) transferred to soil, grown under elevated CO_2 (eCO_2), and (D) DNA blots of *NcoI/BamHI* cut leaf gDNA probed with a ^{32}P-ΔL-DNA probe. No wild-type 1.91 kb fragment was detected in the potRr samples indicating they were homoplasmic. (E) Leaf gas exchange measures at 25°C of CO_2-assimilation rates under varying intercellular CO_2 pressures (C_i) made at 1200 μmol photons m^{-2} s^{-1} illumination. Rubisco activity limited assimilation rates (A_c) were modeled as described in Figure S4 using (F) published kinetic properties and the measured potato Rubisco (gray line) and *R. rubrum* Rubisco (black line) contents. R_d, mitochondria respiration rate, k_{cat}^c Rubisco CO_2-fixation rate: K_c^{air}, K_m for CO_2 under ambient O_2; $S_{c/o}$, specificity for CO_2 over O_2. See supplemental Figure S4 for more details. (G) SDS and (H) native PAGE analyses of the leaf soluble protein confirmed the potRr lines only produced the RbcS (S) lacking 100 kDa *R. rubrum* L_2 Rubisco that is immunologically distinct from the 520 kDa L_8S_8 potato Rubisco. Kinetic values from [a](61), [b](62), [c](63), [d](27).

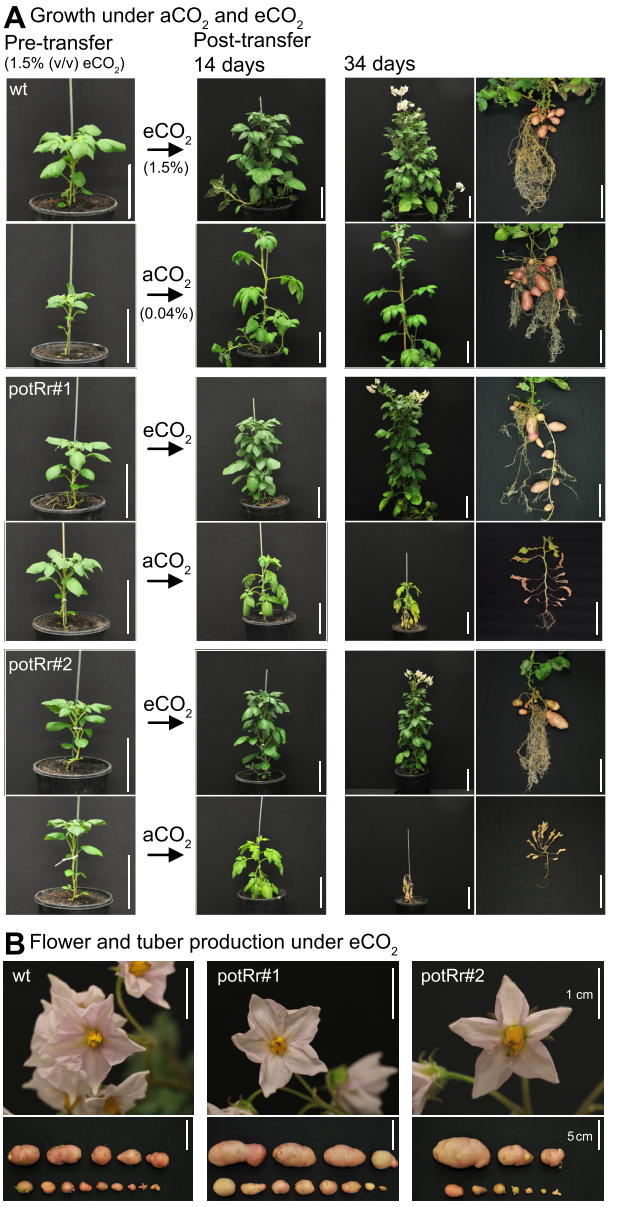

Fig. 2. The growth and productivity of potRr in soil requires elevated CO_2 (eCO_2). (A) Plants generated from vegetative tissue cuttings from the potato (wt) and potRr lines #1 and #2 shown in Figure 1C were propagated in chambers under eCO_2 [air + 1.5% (v/v) CO_2] to a comparable juvenile stage and then either maintained under eCO_2 or grown under identical chamber conditions under ambient CO_2 [aCO_2; 0.06% (v/v)]. Vegetative growth and tuber production by the potRr lines was supported under eCO_2, but not aCO_2. (B) The floral phenotype and tuber production in the eCO_2 grown potRr plants matched that of eCO_2 grown wild-type.

The wild-type like growth and leaf biochemistry of potRr under eCO_2

Simulations of the wild-type and potRr A_C-C_i responses under elevated CO_2 indicated that the *R. rubrum* Rubisco could support wild-type levels of photosynthesis in air containing 0.5% (v/v) CO_2 when CO_2-assimilation rates in both wild-type and potRr would be predicted to be primarily light (A_J) limited (Figure S4). To test this, adventitious shoots arising from the wild-type and potRr#1 tubers (Figure 2B) were grown under matching chamber conditions in air + 0.5% or 1.5% (v/v) CO_2 with wild-type also grown under aCO_2 (maintained at 590 ± 15 ppm, not 400 ppm). For

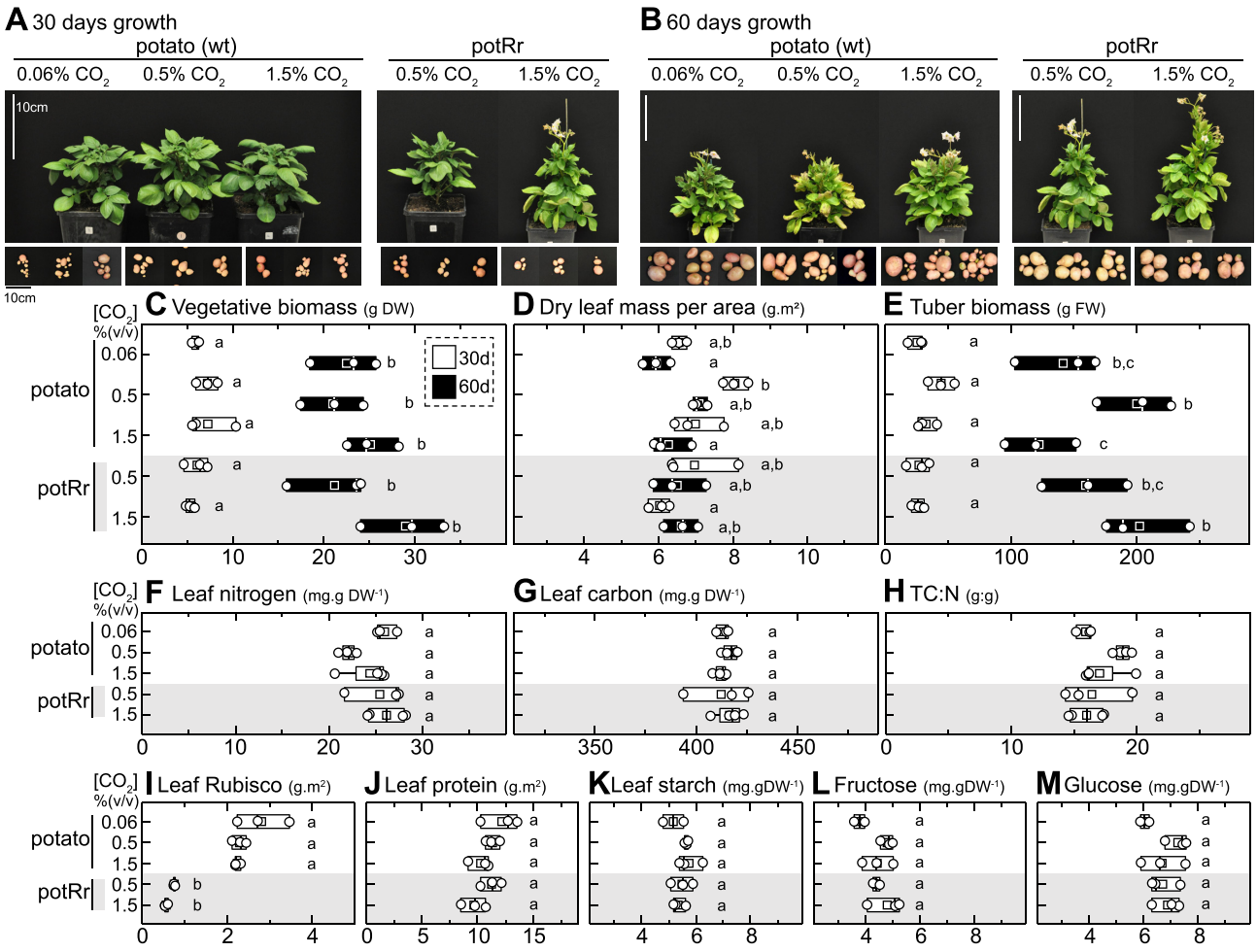

Fig. 3. Comparative growth, tuber production, and leaf biochemistry in wild-type potato and potRr under eCO_2 growth. Comparative growth and tuber production between vegetative propagated wild-type (wt) potato and potRr plants grown for (A) 30 or (B) 60 days in replica growth chambers under either ambient CO_2 (0.06%) or eCO_2 [air + 0.5% or 1.5% (v/v) CO_2]. (C) Total above ground dry weight, (D) LMA, and (E) tuber fresh weight of $n = 3$ (SE) plants 30-day (white shading) and 60-day (black shading). Prior to harvest samples from young, healthy, fully expanded leaves of the 30-day plants were analyzed for (F) nitrogen, (G) carbon, (H) the C:N ratio ($n = 3$–4 ± SE), (I) Rubisco abundance, (J) soluble protein, and carbohydrate content [(K) starch; (L) fructose; and (M) glucose]. Lowercase letters indicate statistical differences at the 5% level ($P < 0.05$).

each treatment, replica plants ($n = 3$) selected from those whose vegetative emergence occurred in synchrony were photographed, leaves sampled, and the vegetation and tubers harvested after 30 days (Figure 3A) and 60 days (Figure 3B). While vegetative dry biomass between 30- and 60-day-old plants differed by 3 to 4-fold, there was no significant difference between each genotype and growth CO_2 at either harvest date (Figure 3C). The dry leaf mass per area (LMA) remained relatively constant over time, between genotype and CO_2 treatment with only the 0.5% eCO_2 wild-type leaves having a significantly higher LMA than the aCO_2, 1.5% eCO_2 60-day wild-type plants, and 1.5% eCO_2 30-day potRr plants (Figure 3D). Tuber production visualized (Figure 3A) and quantified (Figure 3E) after 30 days showed no significant difference between both genotypes or CO_2 treatment. Only after 60 days did the higher tuber production in wild-type under 0.5% eCO_2 differ from those grown under 1.5% eCO_2, while tuber production in potRr remained equivalently high under both eCO_2 growth conditions. Taken together, these findings indicate that despite their lower photosynthetic potential (Figure 1E), the chamber grown potRr share equivalent rates of vegetative and tuber productivity at both 0.5% and 1.5% v/v eCO_2 that mimic those of aCO_2 and eCO_2 grown wild-type.

Closer examination of leaf biochemistry was undertaken on samples from 30-day-old plants where, unlike the phenology of leaves at 60-day, showed no onset of senescence (Compare Figure 3A and B). No significant difference in the total nitrogen (Figure 3F), carbon (Figure 3G), or C:N ratio (Figure 3H) was found between the wild-type and potRr leaves or between CO_2 treatment. Growth CO_2 also had no significant impact on the L_8S_8 Rubisco content in wild-type potato whose average level across all three CO_2 treatments (~2.4 g m^2 leaf, or 36 μmol catalytic sites m^2) exceeded the amount of *R. rubrum* L_2 Rubisco produced in potRr under both 0.5% and 1.5% eCO_2 by ~3.5-fold (~0.7 g m^2 leaf, or 14 μmol catalytic sites m^2; Figure 3I). Despite this variation in the wild-type and potRr Rubisco contents, both genotypes shared equivalent leaf soluble protein contents between the aCO_2 and eCO_2 treatments (Figure 3J). Likewise, the leaves of both genotypes accumulated comparable levels of starch (Figure 3K), fructose (Figure 3L), and glucose (Figure 3M) at all CO_2 levels. Together these finding indicate that despite producing less Rubisco, the photosynthetic rate in the potRr lines is comparable under both 0.5% and 1.5% eCO_2 and thus both are likely A_J limited. Accordingly, potRr growth, productivity, and leaf biochemistry was equivalent to wild-type under the growth chamber conditions

trialed (14 h photoperiod at 25°C and 350 ± 80 μmol photons m^2 s^{-1} leaf surface illumination), where photosynthesis appears to have been A_J limited under all three CO_2 treatments.

Discussion

The discovery two decades ago that the high turn-over rate Form II *R. rubrum* Rubisco could support photosynthesis and growth in tobRr lines (21) initiated a new era in bioengineering photosynthetic CO_2-assimilation in tobacco, the model chloroplast transformation species (20, 30). Subsequent Rubisco replacement activities in tobacco have included the transplastomic insertion of *rbcL-rbcS* operons also coding fast, but low CO_2-affinity and specificity, Form IB Rubiscos from cyanobacteria [*Se*Rubisco (47, 48), *Cy*Rubisco (49)] and the "red-type" Form IC enzyme from *Rhodobacter sphaeroides* [*Rs*Rubisco, (29)]. In this study, we now broaden the capacity for bioengineering Rubisco in plants by producing the first photosynthetically modified transplastomic crop genotype potRr. As expected from its carboxylation properties, the *R. rubrum* Rubisco was unable to support net gains in CO_2-assimilation rate in the potRr lines under ambient CO_2 (Figure 1E). Accordingly, autotrophic growth of potRr in soil necessitated CO_2 fertilization (Figure 2A), a requirement shared by transplastomic tobacco lines producing any prokaryotic Rubisco (29, 47–49). Fortunately, the simple assembly properties of *R. rubrum* RbcL facilitated efficient L_2 enzyme production in potRr leaves (~14 μmol catalytic sites m^2) in amounts equivalent to that made in tobRr expressing the same codon modified *rbcM* gene [~16 μmol μmol catalytic sites m^2, (50)]. Our discovery that these *R. rubrum* Rubisco levels in potRr can support wild-type growth under 0.5% (v/v) CO_2 raises the question whether this is also emulated by tobRr, a growth analysis as yet untested.

Taking into account the content and kinetics of *R. rubrum* Rubisco and the moderate leaf level illumination intensity (350 ± 80 μmol photons m^2 s^{-1}), our simulations of photosynthetic capacity (Figure S4) forecast the photosynthetic rate in potRr would transition from being Rubisco activity limited (A_c) to being light limited (A_J) in air containing 0.5% (v/v) CO_2. In support of this, the comparable growth, productivity, and leaf biochemistry of both potRr and wild-type under both eCO_2 treatments (Figure 3) suggested both genotypes were similarly A_J limited. This finding contrasts with the slower growth under these, and higher, super-elevated eCO_2 levels by tobacco genotypes producing *Se*Rubisco, *Cy*Rubisco, or *Rs*Rubisco. Unlike the *R. rubrum* L_2 enzyme, the folding and assembly requirements of cyanobacteria Rubisco are poorly met in leaf chloroplasts such that L_8S_8 biogenesis is limited to, at best, 5 μmol catalytic sites m^2 of leaf (47–49). As a result, photosynthesis in these plants remains A_c limited, even under 2% (v/v) CO_2, thus slowing their growth relative to wild-type. By comparison, the assembly requirements of L_8S_8 *Rs*Rubisco are more readily met in leaf chloroplasts (8 to 10 μmol catalytic sites m^2); however, maintaining its functionality requires co-expression of its cognate Rubisco activase (CbbX) to remove inhibitory sugar phosphates from its catalytic pockets [i.e., "metabolic repair," (29)]. Even when co-expressed with CbbX however, their photosynthetic rates in tobacco producing *Rs*Rubisco remained A_c limited under 1% (v/v) CO_2 and thus grew slower than wild-type. Taken together, these findings emphasize the need to consider the kinetics, biogenesis requirements, and metabolic repair needs when transplanting heterologous Rubisco isoforms into plant chloroplasts so as to optimize the level of functional enzyme produced and thus the photosynthetic rate. This is particularly relevant in the context of efforts to introduce the CO_2-concentrating mechanisms from cyanobacteria and algae into leaf chloroplasts (51) where the genetically complex multicomponent transplantation challenge may also need to consider co-transplanting a cognate activase and Rubisco biogenesis chaperones.

Our findings suggest that super-elevated CO_2 levels had little impact on the above and below ground biomass of potato cv. Desiree plants relative to those grown under ~600 ppm CO_2 (the "ambient" growth chamber concentration, Figure 3A to E). This finding accords with observations potato cv. Denali plants cultivated in control environments at 250 to 300 μmol photons m^2 s^{-1} showed no differences in vegetative and tuber biomass when grown at 0.1%, 0.5%, or 1.0% (v/v) CO_2 (32, 33) with the contention tuber yields were modestly improved under 0.1% CO_2 growth unsubstantiated statistically. Free air CO_2-enrichment (FACE) field studies with potato subsequently showed no difference in vegetative and tuber production when grown at 560 or 660/680 ppm CO_2, though tuber yields were improved up to 30% above the potatoes grown at ambient (380 to 440 ppm) CO_2 (34, 35). Together these findings imply that there is a limitation in the sink potential of potato where additional gains in photosynthetic capacity above ~560 ppm CO_2 are not translatable to improvements in vegetative growth or tuber production. In accordance with this, our data also suggest that the carbon, nitrogen, protein, and carbohydrate biochemistries in young, healthy potato leaves (Figure 3A) are negligibly impacted by additional CO_2 fertilization above ~560 (Figure 3F to M); an eCO_2 ceiling that coincided with the aCO_2 treatment in this study. Of particular note are that these leaf biochemical properties are mostly sustained in potRr grown at 0.5% and 1.5% (v/v), excluding the 3.5-fold reduction in leaf Rubisco content (Figure 3I). As observed in many eCO_2 studies, reductions in Rubisco content are often not accompanied by correlative reductions in leaf soluble protein contents (52), as seen here between potRr and wild-type (Figure 3J). These findings support the need to improve our appreciation of the source-sink regulatory mechanisms in crops (35), especially considering there are cultivar dependent differences in sink potential that, for example, differentially impacted the eCO_2-growth response among tobacco species (53). Understanding how differences in triose phosphate utilization capacity and triose-6-phosphate signaling impact the sink strength in potato is therefore critical if improvements in photosynthetic productivity, either through improving Rubisco catalysis or as accomplished by other photosynthetic engineering approaches in tobacco [see reviews (1, 3)], are to be fully realized as gains in vegetative and tuber yields of this globally important crop (8, 31, 39).

Among the numerous aspects of photosynthesis targeted for improvement, most strategies seek to improve the performance of Rubisco by either directly improving the carboxylation kinetics of the enzyme or by elevating CO_2 levels around the enzyme (1, 3). As indicated above, aims to introduce the CCM's of algae and cyanobacteria are genetically complex (51, 54), as is incorporating photorespiratory by-pass pathways and other aims to amalgamate synthetic CO_2-fixing pathways into plant metabolism (55). While enhancing plant Rubisco catalysis itself has so far proven difficult (30), new capabilities for expressing plant Rubisco in *E. coli* (16) have led to the discovery of mutations in RbcL and RbcS that may improve the carboxylation properties of Solanaceae Rubiscos (56). Such discoveries complement the advances being made in developing crop genetic tools for introducing nuclear *rbcS* substitutions coding RbcS mutations that can pervasively impact Rubisco catalysis [reviewed in ref. (57)]. These efforts complement the traditional focus on engineering the catalytic site containing RbcL via plastome transformation. Extending on the successful

use of tobRr as a master-line for RbcL bioengineering Rubisco in tobacco (21), generating a comparable master-line by removing the *aadA* selectable marker gene from potRr will allow for efficient engineering of foreign or mutated native *rbcL* genes in potato, a venture we are now pursuing. As fortuitously revealed in this study, generating homoplasmic potato lines without the need for repeated rounds of regeneration can be achieved by extending the period of callus culture on selective media, a process also successfully used to more quickly obtain homoplasmy in cauliflower (58) and Arabidopsis (59).

Materials and methods

Potato plastome transformation and tissue culture

The potato plastome transforming plasmid pLEVpotRr (GenBank Accession ON217534) was assembled by amplifying nucleotides 55,429 to 59,096 of the potato plastome (Genbank Accession DQ386163.2) and replacing the *rbcL* and 3′UTR (nucleotides 56,531 to 58,229) with a synthetic codon modified *rbcM* gene similar to that used by (21) (GenBank AY827488) and an inversely orientated *Prrn-aadA-Trps16* genetic cassette comparable to that used in pRV112A (22). Purified pLEVpotRr (Qiagen) was transformed into tissue culture grown *S. tuberosum* cv. Desiree leaves by biolistic bombardment and spectinomycin resistant plantlets selected as previously described (41) and summarized in Figure S1. Callus selection, shoot induction, and elongation under spectinomycin (0.4 mg ml^{-1}) selection pressure was extended for up to 6 months before two independent lines, denoted potRr #1 and #2, were transferred to soil and maintained for analysis (Figure S1).

Plant growth, vegetative, and tuber biomass

Shoot cultures of tissue culture grown potRr#1, #2 lines and wild-type (wt) potato were transferred into 2 l pots of commercial potting soil mix (Green Wizard, Debco) and grown in a 3 × 3 × 5 m (L × W × H) phoenix walk-in growth chamber at 25/22°C, 14/10 h, L/D cycle under eCO_2 [air + 1.5% (v/v) CO_2], and a upper leaf surface illumination of 350 ± 80 μmol photons m^2 s^{-1} (4 × 1000 W Red Sunrise Grow lamps, Sunmaster). Vegetative cuttings were grown in soil under eCO_2 until 20 cm in height (Figure S3) and then either maintained under eCO_2 or grown in a replica chamber under ambient CO_2 (aCO_2; 600 ± 10 ppm [0.06% (v/v)] CO_2). Tubers produced from these plants were stored in darkness for 2 months for vegetative propagation. Twenty wild-type and potRr#1 nodal auxillary buds (sprouts) and accompanying sections of tuber were grown separately in 2 l of soil under eCO_2. At the appearance of emerging shoots, replica plants of each potato genotype were moved to chambers under aCO_2 (wild-type only) and either 0.5% or 1.5% (v/v) eCO_2. After 30 and 60 days, 0.5 cm^2 discs from healthy, upper-canopy leaves from replica plants ($n \geq 3$) at aCO_2 and eCO_2 were harvested with a Cork borer 6 h into the light cycle and immediately N_2 frozen and stored at −80°C for biochemical analysis (see below). Ten leaves of known area (LI-3000C Portable Leaf area mater, LI-COR) and the remaining vegetative tissue were collected separately and dried at 80°C to determine LMA and total above ground dry biomass, respectively. The tubers were harvested for photography and their fresh weight.

Leaf protein, Rubisco, elemental, and carbohydrate

Quantification of leaf protein (against BSA) and Rubisco content (by ^{14}C-CABP binding) in the −80°C stored leaf discs was performed according to (60). The dried leaf material used to measure LMA was used to measure the total N and C content using an elemental analyzer (model AEA 1110; Carlo Erb Instruments, Milan). The starch, glucose, and fructose content in additional leaf discs samples were measured using a Megazyme kit (Boronia, Australia).

PAGE, western, and DNA blot analyses

Samples of the leaf soluble protein were prepared and separated by native PAGE (4% to 12% Tris-glycine) and SDS-PAGE (4% to 12% NuPAGE Bis-Tris) gels (ThermoFisher) as described (46). The gels were either Coomassie stained or blotted onto nitrocellulose and probed with antisera to tobacco or *R. rubrum* Rubisco according to (21). Total leaf DNA was purified, digested with *NcoI* and *BamHI*, separated by 0.8% (w/v) agarose gel electrophoresis, transferred onto nylon membrane, hybridised with a ^{32}P-labeled L-probe and the densitometry signal visualized as previously described (46).

Leaf gas exchange

Gas exchange was performed on healthy upper canopy leaves of plants of similar physiological age (Figure 1C) using a Li-COR 6400–02B fitted with a red/blue (10%) LED light source. CO_2 assimilation rate measurements under varying intercellular CO_2 concentrations (C_i) (i.e., A-C_i response) were undertaken at a set leaf temperature of 25°C, 1200 μmol photons m^{-2} s^{-1} illumination and ambient O_2. The simulated photosynthesis rates when Rubisco carboxylation rate limited (A_c) were modeled using the equation

$$A_c = \frac{m.k_{cat^c}\,(C_c.s_c - 0.5O_c/S_{c/o})}{(C_c.s_c + K_c^{air})} - R_d,$$

where C_c and O_c are the CO_2 and O_2 concentrations in the chloroplast (assuming $C_c \approx C_i$, wherein there is infinite conductance between the intercellular and chloroplast pCO_2) and solubility constants for CO_2 ($s_c = 0.0334$ M bar^{-1}) and O_2 ($s_o = 0.00{,}126$ M bar^{-1}) (45). Values fitted to the equation are listed in Figure 1F, where m denotes Rubisco content.

Statistics

Statistical analysis was conducted in OriginPro (OrginLab Corporation) using one-way analysis of variance followed by a Tukey's post hoc test.

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Supplementary Material

Supplementary material is available at *PNAS Nexus* online.

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Authors' Contributions

S.W., G.N., and T.S. conceived the project; T.M. generated the potato transgenics, undertook experimental procedures, interpreted data, and drafted the manuscript; R.B. performed the growth experiments and assisted S.M. and T.M. with biochemical analyses.

Data Availability

All data that support the findings of this study are available within its Supplementary Materials. Plasmids and potRr tubers generated in this study are available from the corresponding author.

References

1. Bailey-Serres J, Parker JE, Ainsworth EA, Oldroyd GED, Schroeder JI. 2019. Genetic strategies for improving crop yields. Nature. 575(7781):109–118.
2. Jägermeyr J, *et al.* 2021. Climate impacts on global agriculture emerge earlier in new generation of climate and crop models. Nat Food. 2(11):873–885.
3. Simkin AJ, López-Calcagno PE, Raines CA. 2019. Feeding the world: improving photosynthetic efficiency for sustainable crop production. J Exp Bot. 70(4):1119–1140.
4. Kromdijk J, *et al.* 2016. Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. Science. 354(6314):857–861.
5. South PF, Cavanagh AP, Liu HW, Ort DR. 2019. Synthetic glycolate metabolism pathways stimulate crop growth and productivity in the field. Science. 363(6422):eaat9077.
6. Rosenthal DM, *et al.* 2011. Over-expressing the C_3 photosynthesis cycle enzyme Sedoheptulose-1-7 Bisphosphatase improves photosynthetic carbon gain and yield under fully open air CO_2fumigation (FACE). BMC Plant Biol. 11(1):123.
7. Dingkuhn M, *et al.* 2020. The case for improving crop carbon sink strength or plasticity for a CO_2-rich future. Curr Opin Plant Biol. 56:259–272.
8. Paul MJ. 2021. Improving photosynthetic metabolism for crop yields: what is going to work? Front Plant Sci. 12:743862.
9. Iñiguez C, Aguiló-Nicolau P, Galmés J. 2021. Improving photosynthesis through the enhancement of Rubisco carboxylation capacity. Biochem Soc Trans. 49:2007–2019.
10. Salesse-Smith CE, *et al.* 2018. Overexpression of Rubisco subunits with RAF1 increases Rubisco content in maize. Nat Plants. 4(10):802–810.
11. Tanaka M, *et al.* 2022. Photosynthetic enhancement, lifespan extension, and leaf area enlargement in flag leaves increased the yield of transgenic rice plants overproducing Rubisco under sufficient N fertilization. Rice. 15(1):10.
12. Yoon D-K, *et al.* 2020. Transgenic rice overproducing Rubisco exhibits increased yields with improved nitrogen-use efficiency in an experimental paddy field. Nat Food. 1(2):134–139.
13. Whitney SM, Baldet P, Hudson GS, Andrews TJ. 2001. Form I Rubiscos from non-green algae are expressed abundantly but not assembled in tobacco chloroplasts. Plant J. 26(5):535–547.
14. Long Stephen P, Marshall-Colon A, Zhu X-G. 2015. Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. Cell. 161(1):56–66.
15. Wilson RH, Martin-Avila E, Conlan C, Whitney SM. 2018. An improved *Escherichia coli* screen for Rubisco identifies a protein–protein interface that can enhance CO_2-fixation kinetics. J Biol Chem. 293(1):18–27.
16. Aigner H, *et al.* 2017. Plant Rubisco assembly in *E. coli* with five chloroplast chaperones including BSD2. Science. 358(6368):1272–1278.
17. Conlan B, Whitney S. 2018. Preparing Rubisco for a tune up. Nat Plants. 4(1):12–13.
18. Matsumura H, *et al.* 2020. Hybrid Rubisco with complete replacement of rice Rubisco small subunits by sorghum counterparts confers C_4 plant-like high catalytic activity. Mol Plant. 13:1570–1581,
19. Stuttmann J, *et al.* 2021. Highly efficient multiplex editing: one-shot generation of 8× *Nicotiana benthamiana* and 12× arabidopsis mutants. Plant J. 106(1):8–22.
20. Bock R. 2015. Engineering plastid genomes: methods, tools, and applications in basic research and biotechnology. Annu Rev Plant Biol. 66(1):211–241.
21. Whitney SM, Sharwood RE. 2008. Construction of a tobacco master line to improve Rubisco engineering in chloroplasts. J Exp Bot. 59(7):1909–1921.
22. Zoubenko OV, Allison LA, Svab Z, Maliga P. 1994. Efficient targeting of foreign genes into the tobacco plastid genome. Nucleic Acids Res. 22:3819–3824.
23. Kanevski I, Maliga P. 1994. Relocation of the plastid *rbcL* gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts. Proc Natl Acad Sci. 91(5):1969–1973.
24. Lin MT, *et al.* 2021. A procedure to introduce point mutations into the Rubisco large subunit gene in wild-type plants. Plant J. 106(3):876–887.
25. Sinagawa-García SR, Tungsuchat-Huang T, Paredes-López O, Maliga P. 2009. Next generation synthetic vectors for transformation of the plastid genome of higher plants. Plant Mol Biol. 70(5):487–498.
26. Whitney SM, von Caemmerer S, Hudson GS, Andrews TJ. 1999. Directed mutation of the Rubisco large subunit of tobacco influences photorespiration and growth. Plant Physiol. 121(2):579–588.
27. Martin-Avila E, *et al.* 2020. Modifying plant photosynthesis and growth via simultaneous chloroplast transformation of Rubisco large and small subunits. Plant Cell. 32(9):2898–2916.
28. Conlan B, *et al.* 2019. BSD2 is a Rubisco-specific assembly chaperone, forms intermediary hetero-oligomeric complexes, and is nonlimiting to growth in tobacco. Plant Cell Environ. 42(4):1287–1301.
29. Gunn LH, Martin Avila E, Birch R, Whitney SM. 2020. The dependency of red Rubisco on its cognate activase for enhancing plant photosynthesis and growth. Proc Natl Acad Sci. 117(41):25890–25896.
30. Sharwood RE. 2017. Engineering chloroplasts to improve Rubisco catalysis: prospects for translating improvements into food and fiber crops. New Phytol. 213(2):494–510.
31. Jennings SA, *et al.* 2020. Global potato yields increase under climate change with adaptation and CO2 fertilisation. Front Sustain Food Syst. 4:519324.
32. Wheeler RM, Mackowiak CL, Sager JC, Knott WM. 1994. Growth of soybean and potato at high CO_2 partial pressures. Adv Space Res. 14(11):251–255.
33. Mackowiak CL, Wheeler RM. 1996. Growth and stomatal behavior of hydroponically cultured potato (*Solanum tuberosum* L.) at elevated and super-elevated CO_2. J Plant Physiol. 149(1):205–210.
34. Miglietta F, *et al.* 1998. Free air CO_2 enrichment of potato (*Solanum tuberosum* L.): development, growth and yield. Global Change Biol. 4(2):163–172.

35. Donnelly A, Craigon J, Black CR, Colls JJ, Landon G. 2001. Elevated CO_2 increases biomass and tuber yield in potato even at high ozone concentrations. New Phytol. 149(2):265–274.
36. Sicher RC, Bunce JA. 2001. Adjustments of net photosynthesis in *Solanum tuberosum* in response to reciprocal changes in ambient and elevated growth CO_2 partial pressures. Physiol Plant. 112(1):55–61.
37. Chen C-T, Setter TL. 2012. Response of potato dry matter assimilation and partitioning to elevated CO_2 at various stages of tuber initiation and growth. Environ Exp Bot. 80:27–34.
38. Kumari S, Agrawal M. 2014. Growth, yield and quality attributes of a tropical potato variety (*Solanum tuberosum* L. cv. Kufri chandramukhi) under ambient and elevated carbon dioxide and ozone and their interactions. Ecotoxicol Environ Saf. 101:146–156.
39. Katoh A, Ashida H, Kasajima I, Shigeoka S, Yokota A. 2015. Potato yield enhancement through intensification of sink and source performances. Breed Sci 65(1):77–84.
40. Lehretz GG, Schneider A, Leister D, Sonnewald U. 2022. High non-photochemical quenching of VPZ transgenic potato plants limits CO_2 assimilation under high light conditions and reduces tuber yield under fluctuating light. J Integr Plant Biol. 64:1821–1832.
41. Valkov VT, Gargano D, Cardi T, Scotti N. 2021. Plastid transformation in potato: an important source of nutrition and industrial materials. In: Maliga P, editor. Chloroplast biotechnology: methods and protocols. New York (NY): Springer US. p. 247–256.
42. Valkov VT, *et al.* 2011. High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5′ and 3′ regulatory sequences. Transgenic Res. 20(1):137–151.
43. Halterman D, Guenthner J, Collinge S, Butler N, Douches D. 2016. Biotech potatoes in the 21st century: 20 years since the first biotech potato. Am J Potato Res. 93(1):1–20.
44. Gómez-Ocampo G, Ploschuk EL, Mantese A, Crocco CD, Botto JF. 2021. BBX21 reduces abscisic acid sensitivity, mesophyll conductance and chloroplast electron transport capacity to increase photosynthesis and water use efficiency in potato plants cultivated under moderated drought. Plant J. 108(4): 1131–1144.
45. Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta. 149:78–90.
46. Whitney SM, Andrews TJ. 2001. Plastome-encoded bacterial Rubisco supports photosynthesis and growth in tobacco. Proc Natl Acad Sci. 98(25):14738–14743.
47. Lin MT, Occhialini A, Andralojc PJ, Parry MA, Hanson MR. 2014. A faster Rubisco with potential to increase photosynthesis in crops. Nature. 513(7519):547–550.
48. Occhialini A, Lin MT, Andralojc PJ, Hanson MR, Parry MAJ. 2016. Transgenic tobacco plants with improved cyanobacterial Rubisco expression but no extra assembly factors grow at near wild-type rates if provided with elevated CO_2. Plant J. 85(1):148–160.
49. Long BM, *et al.* 2018. Carboxysome encapsulation of the CO_2-fixing enzyme Rubisco in tobacco chloroplasts. Nat Commun. 9:3570.
50. Whitney SM, *et al.* 2011. Isoleucine 309 acts as a C_4 catalytic switch that increases ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate in *Flaveria*. Proc Natl Acad Sci. 108(35):14688–14693.
51. Borden JS, Savage DF. 2021. New discoveries expand possibilities for carboxysome engineering. Curr Opin Microbiol. 61:58–66.
52. Evans JR, Clarke VC. 2018. The nitrogen cost of photosynthesis. J Exp Bot. 70:7–15.
53. Ruiz-Vera UM, De Souza AP, Long SP, Ort DR. 2017. The role of sink strength and nitrogen availability in the down-regulation of photosynthetic capacity in field-grown *Nicotiana tabacum* L. at elevated CO_2 concentration. Front Plant Sci. 8:998.
54. Adler L, *et al.* 2022. New horizons for building pyrenoid-based CO_2-concentrating mechanisms in plants to improve yields. Plant Physiol. 190(3):1609–1627.
55. Schwander T, Schada von Borzyskowski L, Burgener S, Cortina NS, Erb TJ. 2016. A synthetic pathway for the fixation of carbon dioxide in vitro. Science. 354(6314):900–904.
56. Lin MT, Salihovic H, Clark FK, Hanson MR. 2022. Improving the efficiency of Rubisco by resurrecting its ancestors in the family Solanaceae. Sci Adv. 8(15):abm6871.
57. Mao Y, *et al.* 2022. The small subunit of Rubisco and its potential as an engineering target. J Exp Bot. 74(2):543–561.
58. Nugent GD, Coyne S, Nguyen TT, Kavanagh TA, Dix PJ. 2006. Nuclear and plastid transformation of *Brassica oleracea* var. *botrytis* (cauliflower) using PEG-mediated uptake of DNA into protoplasts. Plant Sci. 170(1):135–142.
59. Ruf S, *et al.* 2019. High-efficiency generation of fertile transplastomic Arabidopsis plants. Nat Plants. 5(3):282–289.
60. Whitney SM, Sharwood RE. 2021. Rubisco engineering by plastid transformation and protocols for assessing expression. Methods Mol Biol. 2317:195–214.
61. Morell MK, Kane HJ, Andrews TJ. 1990. Carboxylterminal deletion mutants of ribulosebisphosphate carboxylase from *Rhodospirillum rubrum*. FEBS Lett. 265:41–45.
62. Mueller-Cajar O, Morell M, Whitney SM. 2007. Directed evolution of Rubisco in *Escherichia coli* reveals a specificity-determining hydrogen bond in the form II enzyme. Biochemistry. 46(49):14067–14074.
63. Kane HJ, *et al.* 1994. An improved method for measuring the CO_2/O_2 specificity of ribulosebisphosphate carboxylase-oxygenase. Aust J Plant Physiol. 21:449–461.