Isoleucine 309 acts as a C₄ catalytic switch that increases ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) carboxylation rate in Flaveria

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Edited by George H. Lorimer, University of Maryland, College Park, MD, and approved July 27, 2011 (received for review June 13, 2011)

Improving global yields of important agricultural crops is a complex challenge. Enhancing yield and resource use by engineering improvements to photosynthetic carbon assimilation is one potential solution. During the last 40 million years C₄ photosynthesis has evolved multiple times, enabling plants to evade the catalytic inadequacies of the CO₂-fixing enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco). Compared with their C₃ ancestors, C₄ plants combine a faster rubisco with a biochemical CO₂-concentrating mechanism, enabling more efficient use of water and nitrogen and enhanced yield. Here we show the versatility of plastome manipulation in tobacco for identifying sequences in C₄-rubisco that can be transplanted into C₃-rubisco to improve carboxylation rate (Vₐ). Using transplastomic tobacco lines expressing native and mutated rubisco large subunits (L-subunits) from Flaveria pringlei (C₃), Flaveria floridana (C₃-C₄), and Flaveria bidentis (C₄), we reveal that Met-309-Ile substitutions in the L-subunit act as a catalytic switch between C₃ (Ile; faster Vₐ, lower CO₂ affinity) and C₄ (Met; slower Vₐ, higher CO₂ affinity) catalysis. Application of this transplastomic system permits further identification of other structural solutions selected by nature that can increase rubisco Vₐ in C₃ crops. Coengineering a catalytically faster C₃ rubisco and a CO₂-concentrating mechanism within C₃ crop species could enhance their efficiency in resource use and yield.

CO₂ assimilation | rbcL mutagenesis | gas exchange | chloroplast transformation

The future uncertainties of global climate change and estimates of unsustainable population growth have increased the urgency of improving crop yields (1). One possible solution is to “supercharge” photosynthesis by improving the C₃ cycle (2, 3). Although a simple idea, this is a complex challenge that involves several possible alternatives. Many of these alternatives focus on enhancing the performance of the CO₂-fixing enzyme ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (rubisco), which catalyses the first step in the synthesis of carbohydrates. Despite its pivotal role, rubisco is a slow catalyst, completing only one to four carboxylation reactions per catalytic site per second in plants (4, 5). Moreover CO₂ not only is fixed through a complex catalytic process but also must compete with O₂. The oxygenation of RuBP produces 2-phosphoglycolate, whose recycling by photorepiration requires energy and results in the futile loss of fixed carbon (~50% of fixed CO₂ in many C₃ plants (6)).

To compensate for rubisco’s catalytic limitations, plants invest as much as 25% of their leaf nitrogen in rubisco (7). This value is much lower in C₄ plants, where a biochemical CO₂-concentrating mechanism (CCM) elevates CO₂ around rubisco. This optimized microenvironment allows rubisco to operate close to its maximal activity, reducing O₂ competition. This CCM has enabled C₄ plants to evolve rubiscos with substantially improved carboxylation rates (Vₐ) relative to their C₃ ancestors, albeit at the expense of reducing CO₂ affinity [i.e., a higher apparent Kₘ for CO₂ (Kₘ)] (8, 9). As a consequence, C₄ plants require less rubisco, thereby enhancing nitrogen use with improvements in Vₐ correlating with improved efficiency in nitrogen use (10). In addition, the high concentration of CO₂ around rubisco allows C₄ plants to operate at lower CO₂ pressures within their leaf air spaces, thereby reducing their stomatal conductance requirements and the associated H₂O loss by transpiration. Indicative of these growth advantages, C₄ photosynthesis has evolved independently several times from multiple C₃ lineages during the last 20–40 million years (11, 12).

Following nature’s example, a number of CO₂ transgenic approaches have been designed to emulate CCM strategies in C₃ plastids and improve rubisco performance (2). These approaches include elevating the CO₂ concentration within chloroplasts using recombinant CO₂/HCO₃⁻ transporters from cyanobacteria or engineering alternative pathways to bypass photorepiration and release CO₂ within the stroma (13, 14). Although each strategy faces continuous challenges in its fine tuning and integration into crops, further improvements in yield and in the efficiency of water and nitrogen use are likely by concurrently “speeding up” rubisco (9).

Identifying the natural changes that result in the faster C₄ rubiscos is far from simple, given the complex structure and biogenesis pathway of the hexadecameric rubisco (L₄S₈) in vascular plants, whose assembly requirements cannot be met by conventional bacterial or in vitro expression systems (15). The catalytic core of L₄S₈ rubisco comprises four 52-kDa large (L)-subunit tetramers that provide structural stability and inactivation catalysis (16, 17). Although supplementing rice rubisco with S-subunits from the C₄ plant sorghum was found to improve Vₐ of the heterologous L₄S₈ enzyme (16), crosses between C₃ and C₄ Flaveria and Atriplex species showed C₄ catalysis to be maternally inherited (18, 19) and hence defined by the chloroplast-encoded L-subunit gene (rbcL). Therefore, changes in both Rubisco L- and S-subunits can influence catalysis.

Although phylogenetic studies have identified potential L-subunit residues involved in the transition from C₃-like to C₄-like rubisco, it is uncertain which residues are catalytically determinate (11, 20). Here we undertake a transplastomic approach to identify such residues in vivo. By manipulating the rbcL gene in tobacco to produce hybrid L₄S₈ rubiscos (containing variant Flaveria L- and tobacco S-subunits), we demonstrate that Met-309-Ile substitutions in the L-subunit act as
rubisco synthesis probably is perturbed posttranscriptionally. As lines (Fig. 1). In Flaveria rbcL genes, nonsilent nucleotide changes occur only at residues 149, 265, and 309 (20). The Flaveria L-subunits show >95% identity with the tobacco L-subunit, with 22–24 amino acid differences in addition to a highly charged TDKDDKKKR extension at the C terminus (Fig. S1). The Flaveria rbcL genes were cloned into the plastome-transforming plasmid pLEV4, where the expression of the transgenes is regulated by the native tobacco rbcL gene regulatory sequences [i.e., its promoter, 5′-, and 3′-untranslated sequences (22)]. The transforming plasmids, including the control pLEV4, were introduced biolistically into 5·3·tobL, a tailor-made tobacco master line for integrating rbcL transgenes into the tobacco chloroplast by homologous recombination (Fig. 1B) (23). Two independent transplastomic lines for each rbcL transgene were grown to maturity in soil in air supplemented with 0.5% (vol/vol) CO2. The transformed tobacco lines that incorporated the F. floridana, F. bidentis, and F. pringlei rbcL genes were called tobtop, tobPd, and tobpring, respectively.

Nondenaturing PAGE (ndPAGE) analysis of the soluble leaf protein was used to confirm the production of the hybrid L8S9L9 rubisco (comprising Flaveria L-subunits and tobacco S-subunits) and to assess the homoplasy of the tobtop, tobPd, and tobpring lines (Fig. 1C). Each of the transformed lines examined was deemed to be homoplasmic, because no L2 Rhodospirillum rubrum rubisco from the parental cmrL line was detected; homoplasy was further confirmed by DNA blot analysis (Fig. S2).

Differential Expression of the Hybrid L8S9L9 Rubiscos. Differences in the intensities of the L8S9L9 bands in ndPAGE indicated that the amounts of rubisco produced in the T0 lines varied (Fig. 1C). This variation was confirmed by quantitative [14C]2-carboxyarabinitol-1,5-bisphosphate (CABP) binding. The amount of L8S9L9 produced in the tobtop, tobPd, and tobpring lines was reduced by approximately 50%, 65%, and 75% relative to wild-type, respectively (Fig. 1C). In contrast, rubisco content in the tobLEV4 control transformants matched that in wild-type, indicating that the additional genome changes around rbcL were not the cause of the reduced L8S9L9 expression. SDS PAGE-immunoblot analysis showed no unassembled Flaveria L-subunits accumulated as insoluble aggregates.

Contrary to the reduced L8S9L9 content in the T0 leaves, there was little or no difference in the Flaveria rbcL mRNA levels in the same leaves relative to wild type (Fig. 1D), indicating that L8S9L9 synthesis probably is perturbed posttranscriptionally. As shown previously (23–25), a less abundant rbcL-aadA dicistronic mRNA (~10% that of the rbcL mRNA) was produced in all transformants as a result of inefficient transcription termination by the tobacco rbcL 3′UTR. The stages that hinder L8S9L9 expression during rubisco biogenesis or degradation remain to be identified fully.

Plant Growth and Leaf Photosynthesis. The disparity in L8S9L9 levels in leaves of the tobtop, tobPd, and tobpring lines persisted to the T3 progeny and correlated with their relative differences in photosynthesis and growth rates. For the tobtop and tobPd plants, the higher L8S9L9 levels produced were sufficient for them to survive through to maturity in air (without CO2 enrichment), although they grew more slowly than wild-type plants (Fig. 2A). In contrast, the tobpring transformants grew poorly in air. As seen previously in tobaccoRub lines producing hybrid L8S9L9 rubisco (comprising sunflower L- and tobacco S-subunits) (24), the juvenile tobpring plants displayed a pale green leaf phenotype with marginal curling and dimpling (Fig. 2A, Right). This phenotype is...
likely a consequence of the very low \( L_{8}^{8}S_{8}^{8} \) content during early vegetative growth.\(^{3}\) Measurements of the ratio of variable fluorescence to maximal fluorescence \( (F_{v}/F_{m}) \) in wild-type leaves \((0.82 \pm 0.01)\) were identical to those in the three \( \text{tob}^{\text{Flaveria}} \) genotypes \((0.83 \pm 0.01)\), indicating no difference in photochemical efficiency under the growth conditions.

### Catalysis by Each \( L_{8}^{8}S_{8}^{8} \) Rubisco Matches the Source \( \text{Flaveria} \) Enzyme.

The catalytic properties of the recombinant \( L_{8}^{8}S_{8}^{8} \) were compared with the source \( L_{8}^{8}S_{8}^{8} \) enzymes from the corresponding \( \text{Flaveria} \) species (Fig. 3). As seen previously for the \( L_{8}^{8}S_{8}^{8} \) rubisco produced in tobacco,\(^{24}\) the \( L_{8}^{8}S_{8}^{8} \) and equivalent \( \text{Flaveria} \) \( L_{8}^{8}S_{8}^{8} \) enzymes were catalytically comparable with respect to their carboxylation \( (V_{C}) \) and oxygenation \( (V_{O}) \) rates, their apparent Michaelis constants \( (K_{m}) \) for \( \text{CO}_{2} \) \( (K_{C}) \) and \( \text{O}_{2} \) \( (K_{O}) \), and \( \text{CO}_{2}/\text{O}_{2} \) specificities \( (S_{\text{CO}_{2}/\text{O}_{2}}) \). Of particular interest was the faster carboxylation rate (\( \approx -35\% \) higher \( V_{C} \)) relative to the \( C_{3} \) rubiscos and lower \( \text{CO}_{2} \) affinity (\( \approx -50\% \) higher \( K_{C} \)) of the hybrid \( L_{8}^{8}S_{8}^{8} \) rubisco from \( \text{tob}^{\text{Flaveria}} \) that matched the \( F. \text{bidentis} \) \( L_{8}^{8}S_{8}^{8} \) enzyme (Fig. 3). As expected, when the catalytic properties and content of each \( L_{8}^{8}S_{8}^{8} \) hybrid enzyme were used to model \( \text{CO}_{2} \) assimilation rates according to Farquhar et al \((26)\), the final values closely matched those measured by whole-leaf gas exchange (Fig. 2C). These results provide confidence both in the accuracy of the measured catalytic properties of the \( L_{8}^{8}S_{8}^{8} \) hybrid enzymes and the rubisco-limited \( \text{CO}_{2} \)-assimilation model.

### Interchanging \( C_{2}-C_{4} \) Catalysis via \( 306\text{Met}^{149}\text{Ile} \) Substitutions in \( \text{Flaveria} \) Rubisco.

The catalytic similarity between native \( F. \text{bidentis} \) rubisco and the hybrid \( \text{tob}^{\text{Flaveria}} L_{8}^{8}S_{8}^{8} \) enzyme indicated that the introduced \( L \)-subunit determined the catalytic phenotype. Because the \( F. \text{bidentis} \) and \( F. \text{floridana} \) \( L \)-subunits differ only at residues 149 and 309 but show significant differences in \( V_{C} \) and \( K_{C} \) (Fig. 3), domain swapping of their \( rbcL \) was used to identify which residue(s) imparted the \( C_{4} \) catalysis of \( F. \text{bidentis} \) rubisco. The chimeric \( rbcL \) gene in the transforming plasmid \( \text{pLEV}^{\text{fIe}} \) introduced a \( C_{4} \)-like substitution into the \( F. \text{floridana} \) \( rbcL \) gene, whereas \( \text{pLEV}^{4999} \) the chimeric \( rbcL \) gene coded an Ile-309-Met substitution in the \( F. \text{bidentis} \) \( rbcL \) gene (Fig. 1B). Both plasmids were transformed into \( \text{mut}^{\text{trL}} \), and

\[ \text{Fig. 2.} \text{ Measurements of growth and leaf gas exchange in the transformants producing the variant} L_{8}^{8}S_{8}^{8} \text{rubiscos.} \text{(A)} \text{ (Left)} \text{ Comparative slower growth in air of the} \text{tob}^{\text{Flaveria}} \text{transformants as a function of plant height relative to wild-type.} \text{(Right)} \text{ The} \text{tob}^{\text{Flaveria}} \text{lines grew extremely poorly in air.} \text{pce, partial assimilation rate at} 25 \degree \text{C under varying chloroplast CO}_{2} \text{ pressures (C)} \text{ at growth illumination (400} \text{ mol quanta m}^{-2}\text{s}^{-1}). \text{Measurements were made on young mature leaves located at similar canopy positions (fifth leaf from the apical meristem) of physiologically comparable mature plants analogous to those shown in} \text{B.} \text{Leaf rubisco contents were 25.0} \text{ and 30.5; 12.4 and 11.9; 10.1 and 10.8; 4.1 and 4.3 mol rubisco sites} m^{-2} \text{ in the independent wild-type (circles),} \text{tob}^{\text{Flaveria}} \text{ (squares; line 1 white, line 2 black),} \text{tob}^{\text{Flaveria}} \text{ (triangles; line 1 white, line 2 black), and} \text{tob}^{\text{Flaveria}} \text{ (diamonds; line 1 white, line 2 black) plants analyzed, respectively.} \text{The lines show the rubisco limited CO}_{2} \text{ assimilation rates for wild-type (••••),} \text{tob}^{\text{Flaveria}} \text{ (– – ••••),} \text{tob}^{\text{Flaveria}} \text{ (– – – –), and} \text{tob}^{\text{Flaveria}} \text{ (– • – • –) modeled according to ref.} 26 \text{ using the catalytic parameters for the respective hybrid} L_{8}^{8}S_{8}^{8} \text{rubiscos in} \text{Fig. 3 and assuming rubisco was fully activated and a value of} 0.3 \text{ mol m}^{-2}\text{s}^{-1} \text{bar}^{-1} \text{ for mesophyll conductance.}

\[ \text{Fig. 3.} \text{ Comparative catalysis at 25} \degree \text{C of the wild-type and} \text{O}_{2}^{149} \text{A} \text{–} \text{V}^{2499} \text{Ile} \text{mutated tobacco} L_{8}^{8}S_{8}^{8} \text{rubisco, the source} \text{Flaveria} L_{8}^{8}S_{8}^{8} \text{rubisco, the hybrid} \text{L}_{8}^{8}S_{8}^{8} \text{comprising} \text{Flaveria} \text{L- and tobacco S-subunits, and chimeric} \text{L}_{8}^{8}S_{8}^{8} \text{comprising chimeric Flaveria L- and tobacco S-subunits rubisco variants produced in the plastomastic tobacco plants.} \text{The L-subunit amino acid residues at codons 149, 265, and 309 in each rubisco type are shown.} \text{Values shown are the average ± SD of independent assays (n = 4–24; see Materials and Methods for details).} \text{The maximal oxygenation rate} V_{O} \text{ was calculated using the equation} S_{\text{CO}_{2}/\text{O}_{2}} = \frac{V_{O}/K_{O}}{V_{C}/K_{C}}. \text{Whitney et al.} \text{14690} \text{www.pnas.org/cgi/doi/10.1073/pnas.1109503108} \text{Whitney et al.}
higher plant L-subunits shows that Met-309 is highly conserved in most C_3-plant rubiscos, the tobacco L-subunit encodes 309Ile (Fig. 4).

**Amino Acid 149 Is Catalytically Neutral but Can Influence Rubisco Expression.** The matching C_3-like catalysis of rubisco from tobo^{bld}- and tobo^{bld}-like catalysis of the tob^{bld}-, tob^{flo} and tob^{ring} rubiscos suggests that changes to amino acid 149 in *Flaveria* rubisco are catalytically neutral and possibly account for the amino acid heterogeneity at this position (Fig. 1A) (20). Likewise, conservation of 309Ile in *F. flordiana* and *F. bidentis* rubisco indicates that this residue also is catalytically neutral. The influence of Gln-149-Ala and Val-265-Ile L-subunit substitutions (to match those in *F. bidentis* rubisco; Fig. 1B) on tobacco rubisco were tested by transforming *tobacco* with the pLEV^{149A,265I} construct by rubisco in the tob^{149A,265I} lines matched that of the wild-type enzyme, demonstrating that both substitutions are catalytically neutral and are not able to impart C_3-like catalysis on tobacco rubisco (Fig. 3).

Despite the apparent neutrality of amino acid 149 on catalysis, changes at this position affected the level of the hybrid L_{S}S_{b} expression. In young mature leaves of both tobo^{bld} and tobo^{bld}, whose L-subunits share 149Asp (and 265Ile), the rubisco levels were comparable (10–13 μmol sites m^{-2}s^{-1}). In contrast, the rubisco content in equivalent leaves from tobo^{bld} and tobo^{flo} (whose L-subunits code 149Ala and 265Ile) were lower (6–8 μmol sites m^{-2}s^{-1}) (Fig. S3). These results suggest that changes to the amino acid (or its mRNA sequence) at residue 149 might be responsible for the variations in hybrid rubisco expression. However, this did not appear to be the case for tobacco Rubisco as the leaf Rubisco levels in the tob^{149A,265I} lines matched that in the wild-type leaves (Fig. 1C). How changes at residue 149 in the *Flaveria* L-subunit might differentially influence its translation, folding, and/or assembly with tobacco S-subunits or the stability of L_{S}S_{b} complexes remains to be examined.

**Discussion**

Using transgenic tobacco lines expressing hybrid rubiscos containing *Flaveria* L- and tobacco S-subunits (L_{S}S_{b}), we have identified 309Ile as the key residue that imparts C_3-like catalytic properties to *Flaveria* rubisco. The determinant role of this residue supports observations from prior crossing studies that showed C_3 catalysis to be maternally inherited in *Flaveria* (19). Linkages between catalysis and sequence phylogenies of different *Flaveria* rubisco L- and S-subunits indicated that C_{3} catalysis was associated with two positively selected L-subunit amino acid substitutions: Asp-149-Ala and Val-265-Ile (20). Here we show that amino acid differences at position 149 in *Flaveria* rubisco probably are catalytically silent, because interchanging 149Ala with 149Asp in the L-subunit from either *F. flordiana* or *F. bidentis* rubisco had no influence on catalysis (Fig. 3). Similarly, tobacco rubisco catalysis was unaffected by Q149A and V265I substitutions (Fig. 3).

A structural/functional explanation for how 309Ile increases V_{C} in *Flaveria* rubisco is unclear. The similar positioning of conserved catalytic site residues in the crystal structures of catalytically different rubiscos makes it difficult to rationalize how distant changes influence catalysis (4, 20). The structure for spinach L_{S}S_{b} rubisco (Fig. 4B) shows 309Ile located at the L-interface (i.e., between the L-subunits in each L_{2} dimer) more than 17 Å away from the Mg^{2+} bound to each catalytic site and at least 13 Å away from the nearest conserved active site residue, 295Arg (Fig. 4C). In contrast, 149Gln is located further away from the active sites and close to the interface of the adjoining L_{2} dimers that form the L_{S} core (Fig. 4B). In the absence of a crystal structure for a *Flaveria* rubisco, it is difficult to explain how in-
sion of a more hydrophobic Ile this far from the active site might influence $V_C$ (20).

Despite the improvements in $V_C$ imparted by Ile in hybrid L$_4$ S$_8$ rubisco, the accompanying reductions in CO$_2$ affinity (i.e., increased K$_C$) precluded gains in carboxylation efficiency. At 25 °C under ambient oxygen levels, the carboxylation efficiency of tobacco (i.e., $V_{C/EC}$) of the C$_4$-like Ile-containing rubiscos in tob$^{bblo}$ and tob$^{bo-}$ (145 mM$^{-1}$s$^{-1}$) were poorer than the C$_4$-like Met-L$_4$/S$_8$ enzymes in tob$^{prigg}$ and tob$^{lio}$ (150 and 161 mM$^{-1}$s$^{-1}$, respectively). Thus, because of the low CO$_2$ levels within (unstressed) C$_4$ chloroplasts (<10 μM), the faster C$_4$-like enzymes probably provide no advantage to plant growth within a C$_3$ plant (at least at 25 °C), as shown recently in rice (16). As modeled recently, optimal CO$_2$ concentrations required for C$_4$ rubisco are substantially higher (~80 μM) (8), indicating that taking full advantage of a faster CO$_2$ rubisco in a C$_3$ plant will require the combinatorial effect of a suitable CCM, for which a number of strategies are being pursued (2). Some of these approaches already have demonstrated that elevating CO$_2$ pressures within C$_3$ plastids can improve the capacity for CO$_2$ assimilation by reducing the energy costs of photorespiration (15, 14).

Our results suggest that the carboxylation rate of rubisco in a C$_3$ plant might be increased either by direct replacement with L-subunits sourced from C$_4$ plants (as in the tob$^{bo-}$ plants; Fig. 3) or by tailoring the appropriate sequence mutations into related C$_3$ rubisco L-subunits (as in the tob$^{bo-}$ plants; Fig. 3). Although the first approach suffers from our inability to predict a priori the assembly properties of foreign rubiscos within the chloroplast of the recipient transplastomic line, the second approach would require knowing the catalytic/structural effect of every possible mutation within the context of a particular rubisco enzyme, an understanding that we are still far from achieving. Indeed, the finding that tobacco rubisco encodes Ile shows C$_4$ catalysis but suggests C$_3$ catalysis highlights the complex natural variation in the sequence–structure–function relationships among plant L-subunits. Even the sequence diversity at position 309 among C$_3$ rubiscos (Fig. 4A) indicates that this residue is not the only one that can impart C$_4$ catalysis. This result is consistent with the polyphyletic evolution of C$_4$ photosynthesis (12) and with predictions that at least eight L-subunit residues (including residue 309 but not residue 149) have been selected for positivity by C$_4$ catalysis (11). Experimentally testing these predictions, identifying other catalytically determinate L-subunit residues, and exploring which particular rubiscos are affected by these changes have been hampered by the preferential location of rbcL in the plastome (27) and the small range of species whose plastomes can be transformed stably (28).

However, as shown in this and previous studies (24, 29, 30), these experimental limitations may be circumvented by expressing hybrid rubiscos in tobacco plastids. The generality of this system for examining sequence–performance relationships within otherwise inaccusable, catalytically diverse foreign L-subunits remains to be explored fully.

Although this study demonstrates the pervasive role of the L-subunit in shaping catalysis in plant rubisco, the important role of the S-subunits on catalysis cannot be overlooked. The apparent catalytic neutrality of the tobacco S-subunit when assembled with heterologous L-subunits (Fig. 3) (24, 29, 30) contrasts with the recent success in shaping rice rubisco toward C$_4$-like catalysis using heterologous S-subunits from C$_4$ sorghum (16). Similarly, structural changes to the S-subunit have improved Chlamydomonas rubisco catalysis (17). As highlighted recently (20), differences in rubisco S-subunit sequence also may account for the catalytic deviation of Flaveria palmeri rubisco, whose L-subunit sequence matches that of tob$^{bo-}$ (coding 309Ile) but shows C$_3$ catalysis.

The similar $S_{CO_2}$ values determined for rubisco from F. bidentis, F. floridae, and F. pringlei (Fig. 3) in this study contrast with the slightly varying values determined previously (S$_{CO_2}$ = 76 ± 1, 84 ± 1, and 81 ± 1 respectively) (21). The reason for this variation is unknown but may lie in alterations in the catalytic competence as a result of different purification processes (ion exchange chromatography versus ammonium sulfate fractionation), the final enzyme purity, and the length of ultra-cold storage (24). By using fresh rubisco rapidly purified to >95% homogeneity by ion exchange chromatography, our measured $S_{CO_2}$ values were highly reproducible between preparations from independent biological replicates.

Here we present an $rbcL$ engineering approach involving hybrid rubisco production in tobacco plastids to unravel the sequence and catalytic diversity of related C$_3$ and C$_4$ rubiscos from Flaveria. Future applications of this experimental system are focused on identifying sequence changes that account for the natural diversity of rubisco performance and testing the feasibility of transplanting these catalytic improvements into the rubisco L-subunits of agriculturally relevant crops. In particular, when coengineered with biotechnological strategies to elevate CO$_2$ around rubisco in C$_3$ plants, a faster rubisco may translate into improved efficiency in water and nitrogen use and the enhanced yields currently enjoyed by C$_4$ plants.

**Materials and Methods**

RubP and [14C]2-CABP were synthesized as described (31, 32). Protein content was measured using a dye-binding assay (Pierce) and BSA as a protein standard.

**Tobacco Plastome Transformation and Growth.** The transforming plasmid pLEV4 directs the insertion of an rbcL gene and a promoterless adaA gene (coding spectinomycin resistance) into the tobacco plastome in place of the L$_3$ Rhodospirillum rubrum rubisco coding $rbcM$ gene in the plastome of the tobacco master line “vtl” (Fig. 1B) (23). The rbcL gene from F. bidentis, F. pringlei, and F. floridae was PCR amplified from leaf genomic DNA [isolated using the DNeasy Plant Mini Kit (Qiagen)] with the primers 5’ NheIRbcL (5’-AGTACGCGTTGATCAAGTGGTGTT3’- the NheI site that spans the rbcL codons 9 (Ala) and 10 (Ser) is shown in italics) and 3’ SalIRbcL (3’-TGGTACGCTTATTTACCTTCCATT3’- the reverse complement of the rbcL stop codon is shown in bold, and the SalI site is shown in italics). The 1,439-bp NheI–SalI rbcL products were cloned into pLEV4 to give pLEV4 to give the transforming plasmids pLEV4$^{rbcl}$, pLEV4$^{rbcl}$, and pLEV4$^{rbcl}$. The plasmids pLEV4$^{rbcl}$ and pLEV4$^{rbcl}$ were modified by introducing the 569-bp SphI–SalI fragments of the F. bidentis and F. floridae rbcL genes (Fig. 1B). Mutations coding substitutions Gln-149-Ala and Val-265-Ile from leaf genomic DNA (isolated using the DNeasy Plant Mini Kit (Qiagen)) were cloned into pLEV4 to give the transforming plasmids pLEV4$^{rbcl}$, pLEV4$^{rbcl}$, and pLEV4$^{rbcl}$. All plasmids were sequenced using BigDye terminator sequencing at the Biomolecular Resource Facility, Australian National University (Canberra, Australia).

Each of the pLEV-derived plasmids was introduced biologically into three leaves of “Trl1” as described (23), and three to seven independent spectinomycin-resistant plants were obtained for each mutant. Two independent plastome-transformed lines for each introduced rbcL gene were grown to maturity in soil in a growth atmosphere supplemented with 0.5% (vol/vol) CO$_2$ as described (24). At each generation the plants were fertilized artificially with wild-type pollen.

**PAGE and Nucleotide Blot Analyses.** The preparation and analysis of soluble leaf protein by SDS/PAGE, nondenaturing PAGE, and immunoblot analysis was performed as described (33). Total leaf genomic DNA was isolated using the DNeasy Plant Mini Kit and used to PCR amplify and sequence the transformed plastome region using primers 5’-CTATGGATCTGACCTGAATTGACTG3’ (LSH) and 5’-GAGGCTGTGAATTCGTTGATTG3’ (LSF) (Fig. 1B) (24). DNA blot analysis of the genomic DNA was used to confirm homoplasy (Fig. 51). Total RNA was extracted from 0.5 cm$^2$ of leaf in 0.8 mL TRizol (Invitrogen). Six per cent or 12% of the RNA was separated on denaturing formaldehyde gels (34). The RNA was blotted onto Hybond-N non-tellosclenus membrane (GE Healthcare) and probed with a 16S-labeled 5’ $\Delta$bcL probe (Fig. 1B) as described (24).

**Rubisco Content and Catalytic Assessments.** Rates of rubisco $^{14}$CO$_2$ fixation using soluble leaf protein extract were measured in 7-mL septum-capped scintillation vials in reaction buffer [50 mM Mepes-NaOH (pH 7.8), 10 mM MgCl$_2$, 0.5 mM RuBP] containing varying concentrations of NaH$^{14}$CO$_3$ (0–67 μM)
and O₂ (0–25%) (vol/vol), accurately mixed with nitrogen using Wostoff gas-mixing pumps as described (24, 33). Assays (0.5 mL total volume) were started by the addition of activated leaf protein, and the Michaelis constants (Kₘ) for CO₂ (Kₐ) and O₂ (K₉) were determined from the initial data. Replicate measurements (n = 4–8) were made using protein preparations from two to four different leaves of independently transformed lines. For each sample the maximal rate of carboxylation (Vₜ) was extrapolated from the Michaels– Menten fit and then normalized by dividing the rate by the number of rubisco-active sites quantified by [¹⁴C]2-CABP binding (35, 36). Rubisco CO₂/O₂ specificity (S_CO₂) was measured as described (37), using freshly extracted rubisco, quickly purified by ion exchange chromatography (24), from at least two separate plants for each independently transformed line.


Growth and Photosynthesis Analysis. Wild-type (Nicotiana tabacum L. Petit Havana) and transplastomic tobacco lines were grown in growth chambers at 25 °C and 400 μmol photons m⁻² s⁻¹ while being exposed to 4% CO₂-enriched air. Plant height from the soil surface to the apical meristem was measured until the first floral apertures emerged. Leaf photosynthesis and dark respiration rates in plants of comparable physiological development (45–50 cm in height; 14 or 15 leaves) were made in the growth chamber using an LI-6400 gas-exchange system (LI-COR) (24). The maximum quantum efficiency of PSII in dark-adapted leaves [variable fluorescence (F_v)/maximum fluorescence (F_m)] was measured in the same leaves using an LI-6400–40 Leaf Chamber Fluorometer.

ACKNOWLEDGMENTS. This research was supported by Australian Research Council Discovery Grant FT0991407 (to S.M.W.) and by project AGL2009-07999 (Plan Nacional, Spain) (to J.G.).