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Functional Hybrid Rubisco Enzymes with Plant Small Subunits and Algal Large Subunits

ENGINEERED rbcS cDNA FOR EXPRESSION IN CHLAMYDOMONAS[*S◆]

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There has been much interest in the chloroplast-encoded large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) as a target for engineering an increase in net CO_2 fixation in photosynthesis. Improvements in the enzyme would lead to an increase in the production of food, fiber, and renewable energy. Although the large subunit contains the active site, a family of *rbcS* nuclear genes encodes the Rubisco small subunits, which can also influence the carboxylation catalytic efficiency and CO_2/O_2 specificity of the enzyme. To further define the role of the small subunit in Rubisco function, small subunits from spinach, *Arabidopsis*, and sunflower were assembled with algal large subunits by transformation of a *Chlamydomonas reinhardtii* mutant that lacks the *rbcS* gene family. Foreign *rbcS* cDNAs were successfully expressed in *Chlamydomonas* by fusing them to a *Chlamydomonas rbcS* transit peptide sequence engineered to contain *rbcS* introns. Although plant Rubisco generally has greater CO_2/O_2 specificity but a lower carboxylation V_{max} than *Chlamydomonas* Rubisco, the hybrid enzymes have 3–11% increases in CO_2/O_2 specificity and retain near normal V_{max} values. Thus, small subunits may make a significant contribution to the overall catalytic performance of Rubisco. Despite having normal amounts of catalytically proficient Rubisco, the hybrid mutant strains display reduced levels of photosynthetic growth and lack chloroplast pyrenoids. It appears that small subunits contain the structural elements responsible for targeting Rubisco to the algal pyrenoid, which is the site where CO_2 is concentrated for optimal photosynthesis.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco,[2] EC 4.1.1.39) is the key photosynthetic enzyme responsible for CO_2 fixation. In plants and green algae, eight ~16-kDa nuclear encoded small subunits assemble with eight ~55-kDa large subunits in the chloroplast to form the functional holoenzyme (reviewed in Ref. 1). The chloroplast-encoded large subunit contains the α/β-barrel active site at which CO_2 and O_2 compete for substrate ribulose-1,5-bisphosphate (RuBP) (reviewed in Refs. 1–3). Photosynthetic CO_2 fixation depends on the Rubisco V_{max} of carboxylation (V_c) and the K_m for CO_2 (K_c), but net CO_2 fixation is decreased by competitive inhibition from O_2 and the loss of CO_2 in photorespiration, which are defined by the V_{max} of oxygenation (V_o) and the K_m for O_2 (4). The ratio of the catalytic efficiencies of carboxylation (V_c/K_c) to oxygenation (V_o/K_o) defines the CO_2/O_2 specificity factor (Ω) (4, 5). Because Ω is determined by the difference between the free energies of activation for carboxylation and oxygenation at the rate-determining step of catalysis (6), there is much interest in defining the structural basis for variation in this kinetic constant. The catalytic properties of Rubisco vary among divergent species (7–9), indicating that it might be possible to engineer improvements in Rubisco function (1). However, some plant and algal species contain CO_2-concentrating mechanisms (CCMs) (reviewed in Refs. 10–14), which make it difficult to tell whether any Rubisco enzyme in nature is better than any other with respect to the CO_2 and O_2 concentrations (and temperatures) that the enzyme encounters *in vivo* (1, 15). Nonetheless, various mutant substitutions have been identified that can influence Ω (reviewed in Refs. 1 and 3). A deeper understanding of the structure-function relationships of Rubisco may prove useful for developing genetic engineering strategies aimed at either increasing carboxylation (and photosynthetic productivity) or decreasing non-essential oxygenation (and the loss of fixed carbon in photorespiration). An increase in net CO_2 fixation would lead to an increase in agricultural productivity for the production of food, fiber, and renewable energy (1).

Considering that the nuclear encoded small subunits of Rubisco are evolving faster than the large subunits (*i.e.* small subunits have greater sequence diversity), it would seem quite possible that small subunits provide unique structural and functional properties to the Rubisco enzymes of diverse species (reviewed in Ref. 16). Directed mutagenesis of Rubisco from the cyanobacterium *Synechococcus* expressed in *Escherichia coli* and directed mutagenesis and genetic selection *in vivo* in the green alga *Chlamydomonas reinhardtii* have identified several residues of the small subunit that can influence Ω (17–21). When the longer loop between β-strands A and B of the *Chlamydomonas* small subunit was replaced with the shorter loop of *Synechococcus*, a decrease in Ω was observed (15, 22). This small subunit loop from the spinach enzyme, along with five substitutions in the large subunit, increased Ω and imparted land plant catalytic properties to the *Chlamydomonas*

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S The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[2] The abbreviations used are: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; CCM, CO_2-concentrating mechanism; Ω, CO_2/O_2 specificity factor; PIPES, 1,4-piperazinediethanesulfonic acid.

enzyme (15). Alanine-scanning mutagenesis has recently identified potential functional interactions between the most conserved small subunit residues and α-helix 8 of the large subunit α/β-barrel (21). Perhaps there are other small subunit residues and regions that play a role in Rubisco function and that may, in part, contribute to the differences in catalytic properties observed among divergent Rubisco enzymes (15).

One other approach that may be useful for dissecting the role of the small subunit is to create hybrid enzymes composed of subunits from Rubisco enzymes that differ in their catalytic properties. When the large subunit of *Synechococcus* Rubisco ($\Omega = 41$) and the small subunit of the marine diatom *Cylindrotheca* ($\Omega = 107$) were expressed in *E. coli*, a holoenzyme was formed that had a 58% increase in the *Synechococcus* Ω value, but this beneficial increase in Ω occurred at the expense of a 95% decrease in V_c (23). When hybrid enzymes composed of *Synechococcus* large subunits and the small subunits of other higher Ω Rubisco enzymes from prokaryotes, algae, and plants were expressed *in vitro* or in *E. coli*, they had the same or slightly lower Ω values relative to that of *Synechococcus* Rubisco (23–28). It has been difficult to study the contribution of small subunits to the function of eukaryotic Rubisco enzymes because eukaryotic subunits cannot assemble into functional Rubisco when expressed in *E. coli* (29, 30), and plant small subunits are coded by a family of *rbcS* genes in the nucleus that cannot be eliminated and replaced with foreign copies. However, pea small subunits were introduced into *Arabidopsis thaliana* to form a heterologous Rubisco enzyme that had a small decrease in carboxylation activity nearly equal to the percentage of foreign small subunits in the holoenzyme (31). The Rubisco large subunit of sunflower was also used to replace the chloroplast large subunit in tobacco to produce a hybrid enzyme composed of sunflower large subunits and tobacco small subunits (32). Although tobacco ($\Omega = 85–89$) and sunflower ($\Omega = 98–105$) Rubisco were reported to have different Ω values (32, 33), the hybrid enzyme was found to have an Ω value similar to tobacco Rubisco (32), indicating that small subunits may determine the value of Ω. However, the hybrid enzyme tobacco plants had a substantial decrease in Rubisco V_c and an increase in K_c and required an elevated CO_2 concentration for growth (32). A more recent study of similar hybrid Rubisco tobacco plants, with Rubisco composed of tobacco small subunits and sunflower large subunits, found no major difference in Rubisco catalytic properties and concluded that the hybrid enzyme plants required elevated CO_2 for growth because of a deficiency in Rubisco expression (34).

Chlamydomonas has served as a useful genetic model for the study of Rubisco because host strains have been isolated that lack chloroplast *rbcL* genes and nuclear *rbcS* genes (35, 36) and because Rubisco-deficient mutants can be maintained with acetate as an alternative source of carbon and energy (reviewed in Refs. 1, 5, and 16). Considering the apparent difficulty of eliminating the *rbcS* gene family of plants (31, 37, 38), it seemed reasonable to attempt investigations of hybrid Rubisco enzymes in *Chlamydomonas*. If photosynthesis-deficient mutant strains are recovered that express foreign small subunits, it might be possible to exploit *in vivo* genetic selection to identify small subunit residues or regions responsible for holoenzyme assembly or function (18, 20). However, because *Chlamydomonas* *rbcS* genes contain introns required for small subunit expression, it was first necessary to develop a method for the expression of foreign *rbcS* cDNAs. This problem has now been solved by transferring the introns to the region of the *rbcS* gene that encodes the small subunit transit peptide. Considering that *Chlamydomonas* and plant small subunits share only ~45% sequence identity, it was remarkable to find that small subunits from spinach, *Arabidopsis*, and sunflower were readily recovered in functional hybrid holoenzymes *in vivo*. These hybrid Rubisco enzymes are expressed at wild-type levels and appear to have near normal rates of carboxylation and small increases in Ω. Nonetheless, the hybrid enzyme mutants display reduced levels of photosynthetic growth and lack chloroplast pyrenoids, indicating that the small subunit may be responsible for targeting Rubisco to the algal pyrenoid, where CO_2 concentration is elevated.

EXPERIMENTAL PROCEDURES

Strains, Plants, and Growth Conditions—*C. reinhardtii* strain SS1 serves as the wild type (21). It was created by transforming the *rbcS*Δ-T60-3 deletion strain with a plasmid (pSS1) containing only *rbcS1* (35). Cell wall-less strain *rbcS*Δ-T60-3 was used as the host for *rbcS* nuclear gene transformation. It lacks photosynthesis and requires acetate for growth due to deletion of the 13-kb locus that contains the *rbcS1* and *rbcS2* gene family (35). All *Chlamydomonas* strains are maintained at 25 °C in darkness on medium containing 10 mM acetate and 1.5% Bacto agar (39). For biochemical analysis, *Chlamydomonas* cells were grown in 250–500 ml of liquid acetate medium at 25 °C on a rotary shaker at 120 rpm. *Arabidopsis* (*A. thaliana* cv. Columbia) and sunflower (*Helianthus annuus* cv. Sunspot) were grown in soil at 27 °C and 65% humidity with a photoperiod (900 microeinsteins/m^2/s) of 12 h. *Arabidopsis* was harvested 30 days after germination. Sunflower was harvested between 7 and 15 days after germination. Spinach (*Spinacia oleracea*) was purchased locally.

Construction of Plasmids—To remove the introns from *rbcS1*, plasmid pSS1 was used as the starting material (35) (see Fig. 1). It contains an ~5-kb EcoRI fragment of *Chlamydomonas* nuclear DNA, which contains *rbcS1* (40) (supplemental Fig. S1), cloned at the EcoRI site of pUC19 (41). This EcoRI fragment apparently contains all the necessary sequence elements for *rbcS1* expression (35), none of which were altered during subsequent *rbcS1* engineering. Intron 3 was removed from *rbcS1* by replacing the StuI fragment with the StuI fragment from plasmid pCS2.1, which contains the *Chlamydomonas rbcS2* cDNA sequence (40). The *rbcS1* and *rbcS2* protein-coding sequences are identical in this region. This new plasmid was named pSS1-ΔI3 (see Fig. 1). Using pSS1-ΔI3 as the template, oligonucleotides with overlapping sequences flanking intron 2 were used to synthesize fragments by PCR before and after intron 2. Both fragments were combined and extended into a longer product (42), which was used to replace the *rbcS1* sequence between NcoI and BlpI (see Fig. 1). The resultant plasmid was named pSS1-ΔI23. Intron 1 was deleted from pSS1-ΔI23 in a similar way, replacing the NcoI-BlpI fragment of *rbcS1* (42). The result-

ant plasmid, which lacks all three introns in *rbcS1*, was named pSS1-cDNA (see Fig. 1).

To insert introns 1 and 2 into the *rbcS1* region of pSS1-cDNA that encodes the small subunit transit peptide, plasmid pSS1 was used as the template. Synthetic oligonucleotides with overlapping sequences were used to synthesize intron 1 from pSS1 and two fragments upstream and downstream of the insertion point in the encoded transit peptide region. The three fragments were combined and extended into a longer product, which was digested and cloned between the EagI and BlpI restriction sites of pSS1-cDNA (see Fig. 1). This new plasmid was named pSS1-I1TP. Intron 2 was inserted into the *rbcS1*-encoded transit peptide region of pSS1-I1TP in a similar way by exchanging an NcoI-BlpI restriction fragment (see Fig. 1). This new plasmid was named pSS1-ITP. Sites for the insertion of introns into the encoded transit peptide region were chosen based on a comparison of splicing sites for other *Chlamydomonas* nuclear genes (43). The wild-type sequence and features of the *Chlamydomonas* EcoRI restriction fragment cloned in pSS1 (35) are presented in supplemental Fig. S1. The sequence and features of the EcoRI restriction fragment of pSS1-ITP are provided in supplemental Fig. S2.

Coding sequences for the mature small subunits of spinach (44), *Arabidopsis* (45), and sunflower (46) were synthesized to encode codons common to the *Chlamydomonas rbcS1* mRNA using overlapping synthetic oligonucleotides (47). When necessary, directed mutagenesis was performed with synthetic oligonucleotides and a QuikChange kit from Stratagene (48). The final sequences are presented in supplemental Fig. S3. In each case, the plant *rbcS* sequence was changed to encode the Met-2 common to the *Chlamydomonas* mature small subunit sequence. The foreign *rbcS* coding region sequences along with their exact *Chlamydomonas* flanking sequences were digested with NcoI and BlpI and used to replace the NcoI-BlpI fragment in the *rbcS1* gene of pSS1-ITP (see Fig. 1). These new plasmids were named pSS1-SSSO, pSS1-SSAT, and pSS1-SSHA (see Fig. 1).

Chlamydomonas Transformation—Transformation of cell wall-less strain *rbcS*Δ-T60-3 was performed by electroporation (22, 49), and photosynthesis-competent colonies were selected on minimal medium (without acetate) in the light (80 microeinsteins/m^2/s). The engineered *rbcS1* genes and appropriate flanking regions were PCR-amplified and completely sequenced at the University of Nebraska DNA sequencing facility to verify gene sequence and accuracy of construction. A wild-type strain recovered by transformation with the pSS1-ITP plasmid was named SS1-ITP. The *rbcS1* hybrid mutant strains were named SSSO, SSAT, and SSHA.

Biochemical Analysis—*Chlamydomonas* cells were sonicated at 0 °C for 3 min in extraction buffer composed of 50 mM Bicine (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, and 1 mM dithiothreitol. Plant leaves (2 g) were ground with a mortar and pestle at 0 °C in 5 ml of 50 mM Bicine (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 2% polyvinylpyrrolidone 40 (Sigma-Aldrich). Cell debris was removed from extracts by centrifugation at 30,000 × *g* for 15 min, and the amount of protein in the supernatant was quantified (50). The cell extract was subjected to SDS-PAGE and Western blotting (51, 52) or fractionated on 10–30% sucrose gradients prepared in the same extraction buffer (without polyvinylpyrrolidone) to isolate pure Rubisco holoenzyme (53). Thermal stability of purified Rubisco was determined as described previously (19, 54).

The kinetic constants of purified and activated Rubisco were measured by the incorporation of acid-stable ^{14}C from $NaH^{14}CO_3$ (55). The value of Ω (V_cK_o/V_oK_c) was determined as the ratio of the rates of carboxylase (v_c) and oxygenase (v_o) activities measured simultaneously with 88 μM [1-^{3}H]RuBP (15.8 Ci/mol) and 2 mM $NaH^{14}CO_3$ (0.5 Ci/mol) in 30-min reactions at 25 °C according to the formula $\Omega = v_c/v_o \cdot [CO_2]/[O_2]$ (56, 57). The [1-^{3}H]RuBP and phosphoglycolate phosphatase used in the assays were synthesized/purified by standard methods (56, 58).

Electron Microscopy—*Chlamydomonas* cells were fixed for 3 h at 4 °C with 2% glutaraldehyde in 0.1 M PIPES (pH 7.4). Samples were then fixed and osmicated for 1 h in 1% OsO_4, 1.5% $K_3Fe(CN)_3$, and 2 mM $CaCl_2$. They were stained for 1 h in 2% uranyl acetate and dehydrated progressively in 75, 95, and 100% ethanol followed by two washes in 100% acetonitrile. The samples were embedded in epoxy resin containing 34% Quetol 651, 44% nonenyl succinic anhydride, 20% methyl-5-norbornene-2,3-dicarboxylic anhydride, and 2% catalyst dimethylbenzylamine (Agar Scientific, Essex, UK). Samples were degassed and cured at 60 °C for 24 h. Sections (50 nm) were obtained with a Leica Ultracut UCT (Leica Microsystems, Milton Keynes, UK), mounted on 300 mesh copper grids, counterstained with uranyl acetate followed by lead citrate, and examined with a transmission electron microscope at 80 kV (Philips CM100, Eindhoven, The Netherlands).

RESULTS

Expression of Chlamydomonas rbcS cDNA—To investigate the influence of foreign small subunits on Rubisco holoenzyme function, it seemed sensible to use cDNA sequences rather than contend with potential problems that may arise from the processing of divergent introns. However, numerous attempts to transform the *rbcS*Δ-T60-3 deletion mutant with *Chlamydomonas rbcS1* cDNA were unsuccessful. Of the three introns present in *rbcS1* (Fig. 1), only intron 3 is not required for expression (22). In a previous study of *Chlamydomonas* gene expression (59), engineering *Chlamydomonas rbcS2* intron 1 into either the flanking region or the coding region of a heterologous selectable marker gene increased transformation frequency and expression. Furthermore, when transit peptides were first discovered (60, 61), it was shown that the transit peptide of the Rubisco small subunit may have species specificity for *Chlamydomonas* chloroplast targeting (61, 62). Therefore, we decided to move *rbcS1* introns 1 and 2 from the mature protein-coding region into the transit peptide-coding region (Fig. 1). In this way, the introns would still be present to facilitate gene expression, and the *Chlamydomonas* transit peptide would allow targeting of foreign small subunits to the *Chlamydomonas* chloroplast. When this *rbcS1*-ITP gene was created (in plasmid pSS1-ITP; Fig. 1) and transformed into the photosynthesis-deficient *rbcS*Δ-T60-3 strain, photosynthesis-competent colonies were recovered at the same frequency as when transformed with the wild-type *rbcS1* gene (~5 × 10^{-6}/cell). One of these

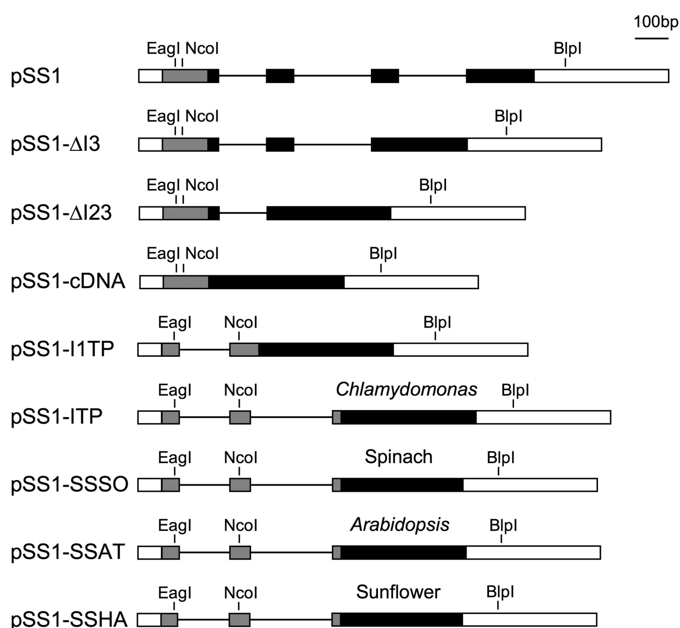

FIGURE 1. **Engineered plasmids containing the wild-type *Chlamydomonas rbcS1* gene (pSS1), *rbcS1* with various introns deleted (pSS1-ΔI3, pSS1-ΔI23, pSS1-cDNA), *rbcS1* with introns inserted into the transit peptide-encoding region (pSS1-I1TP, pSS1-ITP), and *Chlamydomonas rbcS1* with the mature Rubisco small subunit-encoding sequence replaced with plant *rbcS* cDNA sequences (pSS1-SSSO, pSS1-SSAT, pSS1-SSHA).** Only the transcript-encoding regions of *rbcS1* are displayed (40). Regions encoding the transit peptide and mature small subunit are shown as *gray* and *black boxes*, respectively. Introns are denoted by *black lines*. DNA sequences and features are presented in supplemental Figs. S1–S3.

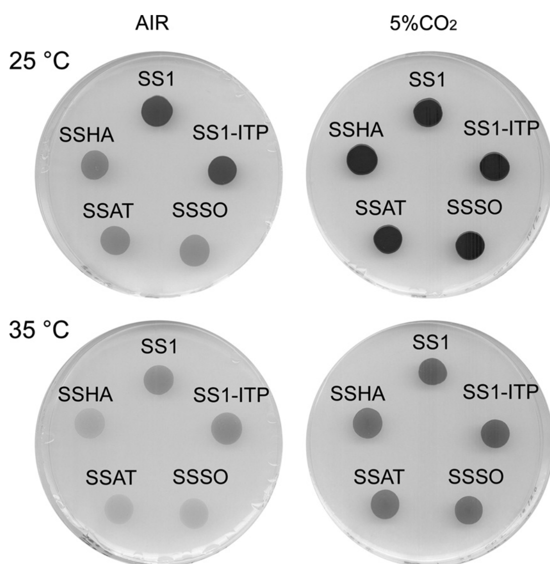

FIGURE 2. **Photosynthetic growth phenotypes of *Chlamydomonas* wild-type SS1, wild-type SS1-ITP, and hybrid Rubisco mutants containing the mature small subunits of spinach (*SSSO*), *Arabidopsis* (*SSAT*), and sunflower (*SSHA*).** Spot tests were performed by plating equal numbers of dark-grown cells on minimal medium in the light (80 microeinsteins/m^2/s) at either the normal growth temperature of 25 °C or the elevated temperature of 35 °C (39). The wild-type SS1 and SS1-ITP control strains were created by transforming the *rbcS*Δ-T60-3 *rbcS* deletion mutant with the wild-type *rbcS1* gene or the *rbcS1* gene with introns engineered into the transit peptide-encoding region, respectively.

transformants, named SS1-ITP, was analyzed further. As shown in Fig. 2, the SS1-ITP strain has a photosynthetic growth phenotype nearly identical to the SS1 wild-type strain, which

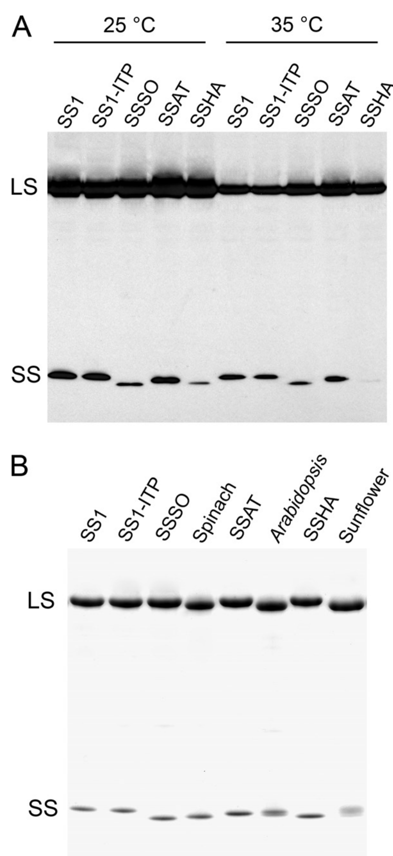

FIGURE 3. **Western blot analysis of total soluble proteins (*A*) and SDS-PAGE of purified Rubisco (*B*) isolated from *Chlamydomonas* wild-type strains SS1 and SS1-ITP, hybrid Rubisco mutants containing the mature small subunits of spinach (*SSSO*), *Arabidopsis* (*SSAT*), and sunflower (*SSHA*), and various plant species.** For Western analysis, protein extracts (60 μg/lane) of cells grown at either 25 °C or 35 °C in darkness were fractionated by gradient gel (7.5–15% polyacrylamide) electrophoresis (51), blotted to nitrocellulose, probed with rabbit anti-*Chlamydomonas* Rubisco immunoglobulin G (0.5 μg/ml) (22), and detected by enhanced chemiluminescence (Amersham Biosciences) (52). For SDS-PAGE, purified Rubisco (6 μg/lane) was fractionated by gradient gel (7.5–15% polyacrylamide) electrophoresis and stained with Coomassie Brilliant Blue (Sigma-Aldrich) (51). The Rubisco 55-kDa large subunit (*LS*) and 16-kDa small subunit (*SS*) are indicated.

was recovered by transformation with the wild-type *rbcS1* gene (21). Furthermore, SS1-ITP expresses the same level of Rubisco holoenzyme as wild-type SS1 (Fig. 3). Thus, transferring introns 1 and 2 to the transit peptide-encoding region of *rbcS1* has little if any negative effect on the expression of the Rubisco small subunit, and pSS1-ITP appears to be a suitable vector for ultimately expressing foreign *rbcS* cDNAs in *Chlamydomonas*.

Expression of Foreign Rubisco Small Subunits in Chlamydomonas—Because small subunits are coded by families of *rbcS* genes (16), decisions had to be made about which sequences would be used for genetic engineering. *Chlamydomonas* contains two *rbcS* genes that encode mature small subunit proteins that differ by four amino acid residues. The *rbcS1* gene is routinely used for experiments solely because it was observed to give a higher frequency of transformation than *rbcS2* (35). Although *Chlamydomonas* cells containing only one *rbcS* gene have about half as much Rubisco as wild-type cells (35), Rubisco enzymes containing either small subunit 1 or small subunit 2 do not differ with respect to level of expression, catalytic properties, or thermal stability (19, 20, 35). Only one *rbcS* sequence is

known for sunflower (32, 46). Four *rbcS* genes are present in *Arabidopsis* that differ from each other by as many as nine amino acids in the encoded mature proteins (45). We chose *rbcS1B* because it is neither the most conserved nor the most divergent among the four homologs. Spinach small subunit sequences have been determined by x-ray crystallography (63, 64) and direct protein sequencing (65). However, in both cases, the sequences likely represent a mixture of different small subunits. Therefore, we used a sequence deduced from a cloned spinach *rbcS* gene (44), which differs from the high resolution crystal structure of spinach Rubisco (63) by only seven residues near the small subunit protein termini.

The *Chlamydomonas rbcS1* sequence that encodes the mature small subunit in plasmid pSS1-ITP was first replaced exactly with the codon-optimized *rbcS* sequence for the spinach small subunit. After numerous attempts to transform the *rbcS*Δ-T60-3 strain with this engineered spinach *rbcS* gene, only one slow growing, photosynthesis-competent colony was recovered. When Rubisco was purified from this strain and analyzed by SDS-PAGE, the small subunit was found to be about 2 kDa larger than the native spinach small subunit (data not shown). This observation was quite similar to that previously observed when small subunit transit peptide processing was blocked by directed mutant substitutions flanking the transit peptide-processing site (66). Because transit peptide processing in *Chlamydomonas* occurs in a two-step fashion, only 21 residues of the 45-residue transit peptide remained attached to the amino terminus of the mature small subunit sequence (66). Furthermore, transit peptide processing in *Chlamydomonas* may depend on residues next to those at the primary processing site (67). Taking these observations into account, we wondered whether the nature of residue 2 in the mature small subunit sequence might influence transit peptide processing. This residue is Met in the *Chlamydomonas* small subunit but Lys in the spinach small subunit. When directed mutagenesis was used to change the Lys residue to Met, the resultant plasmid, named pSS1-SSSO, yielded photosynthesis-competent transformant colonies at the same frequency as the wild-type pSS1-ITP plasmid. *Arabidopsis* and sunflower *rbcS* genes were synthesized to also encode Met-2 (supplemental Fig. S3). These plasmids, named pSS1-SSAT and pSS1-SSHA, also yielded photosynthesis-competent colonies at wild-type frequencies when transformed into the *rbcS*Δ-T60-3 strain. Growth phenotypes of the transformants were compared, and one representative strain obtained from each transforming plasmid was designated for further study. These hybrid Rubisco *rbcS1* mutant strains were named SSSO, SSAT, and SSHA.

Phenotypes of Hybrid Rubisco Mutants—Although the SSSO, SSAT, and SSHA hybrid enzyme mutants grew as well as the wild-type SS1 and SS1-ITP strains in darkness (data not shown), they displayed reduced levels of photosynthetic growth on minimal medium at either 25 °C or 35 °C (Fig. 2). Growth is routinely checked at the restrictive temperature of 35 °C because temperature-conditional mutants may be useful for selecting second site suppressor substitutions (18, 20, 68). When photosynthetic growth was examined at a saturating level of CO_2 (5% in air), it was somewhat surprising to find that the hybrid enzyme mutants grew as well as the wild-type SS1 and SS1-ITP strains at both 25 °C and 35 °C (Fig. 2). If photosynthetic growth at air levels of CO_2 (.03%) was decreased due to a decreased amount of Rubisco holoenzyme, one would not expect to see a wild-type level of growth at elevated CO_2.

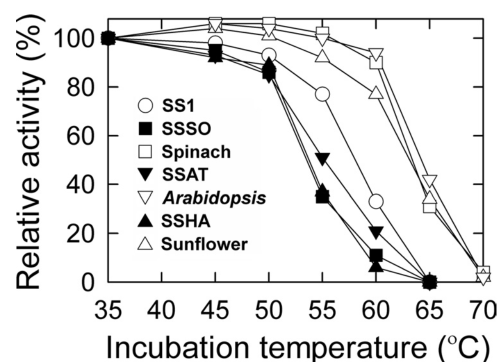

FIGURE 4. **Thermal inactivation of Rubisco purified from *Chlamydomonas* wild-type SS1, hybrid Rubisco mutants containing the mature small subunits of spinach (*SSSO*), *Arabidopsis* (*SSAT*), and sunflower (*SSHA*), and various plant species (19, 54).** Rubisco (5 μg) in 0.5 ml of 50 mM Bicine (pH 8.0), 10 mM $NaH^{14}CO_3$ (58 Ci/mol), 10 mM $MgCl_2$, and 1 mM dithiothreitol was incubated at each temperature for 10 min. The samples were then cooled on ice for 5 min, and carboxylase activity was initiated at 25 °C by adding 20 μl of 10 mM RuBP. Reactions were terminated after 1 min with 0.5 ml of 3 M formic acid in methanol. Activities were normalized to the specific activities measured after the 35 °C incubation (wild-type SS1, 1.5 μmol/min/mg; mutant SSSO, 1.5 μmol/min/mg; mutant SSAT, 1.1 μmol/min/mg; mutant SSHA, 1.4 μmol/min/mg; spinach, 1.0 μmol/min/mg; *Arabidopsis*, 1.0 μmol/min/mg; sunflower, 1.2 μmol/min/mg).

Protein Levels, Processing, and Stability—When extracts of cells grown at 25 and 35 °C in darkness were subjected to SDS-PAGE and Western analysis (Fig. 3*A*), the SSSO, SSAT, and SSHA hybrid enzyme mutants were found to have levels of Rubisco large subunits equal to or greater than those of the wild-type SS1 and SS1-ITP strains. Small subunit bands varied in intensity because the antibody used for detection was affinity-purified with *Chlamydomonas* Rubisco (22), and small subunits are less conserved in sequence than are large subunits (16). Furthermore, when Rubisco was purified from 25 °C-grown mutant strains on sucrose gradients, the amount of Rubisco in the hybrid enzyme mutants per total cell protein was equal to that in the SS1 and SS1-ITP strains (data not shown).

When purified Rubisco was separated by SDS-PAGE and detected with Coomassie Blue staining (Fig. 3*B*), the stoichiometry of large and small subunits was found to be the same among all the samples. The observed molecular masses of the small subunits in the SSSO, SSAT, and SSHA hybrid enzyme mutants closely matched those of the small subunits in Rubisco holoenzymes purified from spinach, *Arabidopsis*, and sunflower, respectively (Fig. 3*B*). However, it is apparent that there are multiple small subunits in *Arabidopsis* and sunflower that differ in size or charge. To ensure that transit peptides were processed correctly for the SSSO, SSAT, and SSHA hybrid enzyme small subunits, small subunits were excised from SDS-polyacrylamide gels, digested with trypsin, and analyzed by mass spectrometry in the University of Nebraska Center for Proteomics. All of the small subunits begin with *N*-methyl-Met (data not shown) as expected for the mature small subunits of plants and green algae (69, 70).

To determine whether the hybrid Rubisco enzymes may have an associated structural instability, thermal inactivation experiments were performed (19, 54). As shown in Fig. 4, the SSSO,

TABLE 1

Kinetic properties of Rubisco purified from spinach, *Arabidopsis*, sunflower, and *Chlamydomonas* wild-type SS1 and *Chlamydomonas* hybrid Rubisco mutants containing the small subunits of spinach (mutant SSSO), *Arabidopsis* (mutant SSAT), and sunflower (mutant SSHA)

Enzyme	Ω $(V_cK_o/V_oK_c)^a$	$V_c{}^a$	$K_c{}^a$	$K_o{}^a$	$V_c/K_c{}^b$	$K_o/K_c{}^b$	$V_c/V_o{}^b$
		μmol/h/mg	*μM CO₂*	*μM O₂*			
Spinach	80 ± 1	79 ± 4	23 ± 1	520 ± 7	3.4	23	3.5
Arabidopsis	77 ± 1	72 ± 2	22 ± 1	474 ± 20	3.3	22	3.6
Sunflower	77 ± 3	69 ± 8	19 ± 1	640 ± 46	3.6	34	2.3
Chlamydomonas SS1	61 ± 1	112 ± 10	31 ± 1	498 ± 30	3.6	16	3.8
Mutant SSSO	65 ± 1	97 ± 12	34 ± 2	539 ± 43	2.9	16	4.1
Mutant SSAT	68 ± 2	105 ± 10	30 ± 1	547 ± 26	3.5	18	3.7
Mutant SSHA	63 ± 1	114 ± 8	31 ± 2	557 ± 57	3.7	18	3.5

[a] Values are the means ± S.D. ($n - 1$) of three separate enzyme preparations.
[b] Calculated values.

SSAT, and SSHA hybrid enzymes were somewhat more sensitive to thermal inactivation than wild-type SS1 Rubisco. In contrast, plant Rubisco enzymes were more thermally stable than the *Chlamydomonas* enzyme (Fig. 4). Nonetheless, because thermal inactivation occurs at temperatures far above growth or enzyme assay temperatures, any minor instability of the mutant holoenzymes would not be expected to influence the accuracy of catalytic constant measurements.

Enzyme Kinetics—Previous studies had indicated that sunflower Rubisco might have a higher Ω value than other plant Rubisco enzymes (32, 33). However, when the Rubisco enzymes of spinach, *Arabidopsis*, and sunflower were analyzed with the highly accurate, dual labeling assay (56, 57), their Ω values were found to be quite similar (Table 1). In fact, the Ω value of spinach Rubisco may be 4% greater than the Ω values of *Arabidopsis* and sunflower Rubisco. Further analysis of kinetic constants indicated that sunflower Rubisco does have a higher K_o/K_c value than that of spinach and *Arabidopsis* Rubisco (Table 1). This beneficial increase results from both an increase in K_o and a decrease in K_c. However, the value of Ω is not increased relative to the Ω values of spinach and *Arabidopsis* Rubisco because the increase in K_o/K_c would be offset by a decrease in V_c/V_o (Table 1).

When the hybrid mutant enzymes were analyzed with respect to catalytic properties, all were found to have small increases in Ω relative to *Chlamydomonas* wild-type SS1 Rubisco (Table 1). The greatest increase was observed for mutant SSAT Rubisco, which contains *Arabidopsis* small subunits. The Ω value is 11% greater than the Ω value of *Chlamydomonas* Rubisco but 12% lower than the Ω value of *Arabidopsis* Rubisco (Table 1). It is difficult to decide with confidence whether any of the other kinetic constants differ between *Chlamydomonas* SS1 Rubisco and the hybrid Rubisco enzymes, but small increases in K_o could account for the increases in Ω (Table 1). With an increase in Ω and no substantial decrease in V_c (Table 1), the hybrid enzymes may be somewhat better than the wild-type *Chlamydomonas* enzyme with regard to net CO_2 fixation (1, 4). However, although the SSSO, SSAT, and SSHA hybrid enzymes are no worse than wild-type SS1 Rubisco with regard to catalytic properties (Table 1) or levels of expression (Fig. 3*A*), the mutant cells do not grow as well as wild-type SS1 under photosynthetic conditions at air levels of CO_2 (Fig. 2). One might consider that interactions between Rubisco and Rubisco activase have been altered in the hybrid enzyme mutants (1). However, a mutant of *Chlamydomonas* that lacks a functional activase gene grows as well as wild type at air levels of CO_2 (71), and the species specificity of Rubisco activase interactions has been shown to occur via the Rubisco large subunit (72, 73).

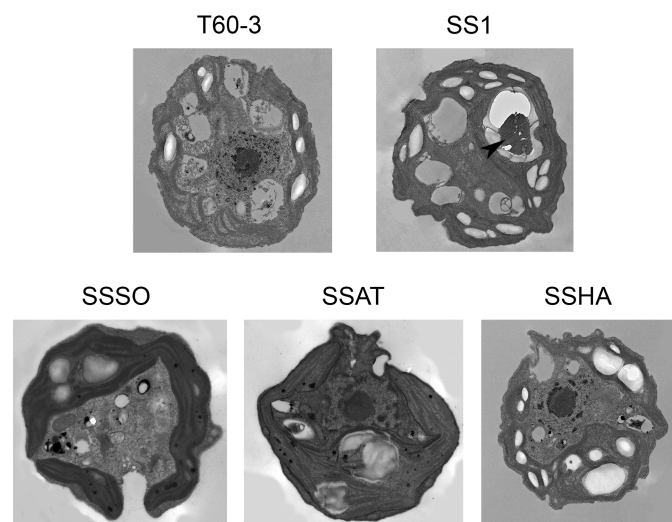

FIGURE 5. **Electron microscopic images of the *Chlamydomonas rbcS* deletion mutant T60-3 complemented with *Chlamydomonas* (*SS1*), spinach (*SSSO*), *Arabidopsis* (*SSAT*), and sunflower (*SSHA*) *rbcS* genes.** The pyrenoid in the *Chlamydomonas* wild-type SS1 strain is marked with an *arrow*. Photographs were taken at a magnification of ×4,900.

Analysis of the Chloroplast Pyrenoid—We wondered whether the poor photosynthetic growth of the SSSO, SSAT, and SSHA hybrid enzyme mutants may result from a loss of the CCM (10). *Chlamydomonas* mutants that lack the CCM grow poorly, if at all, at air levels of CO_2 but are indistinguishable from wild type with regard to photosynthetic growth at elevated CO_2, which is a condition when the CCM is normally repressed (74, 75). The hybrid enzyme mutants have a similar CO_2-requiring phenotype (Fig. 2). Furthermore, it was shown previously in cyanobacteria that mistargeting of carboxysomal carbonic anhydrase to the cytosol blocks or eliminates the CCM and produces a CO_2-requiring phenotype (76, 77). Because Rubisco is normally located in, and required for the formation of, the chloroplast pyrenoid in *Chlamydomonas* (78, 79) and because the pyrenoid is an integral component of the CCM (10), it seemed possible that the hybrid Rubisco enzymes might not be correctly targeted for pyrenoid formation. Light microscopic observation of the SSSO, SSAT, and SSHA hybrid enzyme mutants indicated that they lacked the pyrenoid. This was confirmed by electron microscopy (Fig. 5). Because the probability of observing a pyrenoid depends on the plane of section, not

every wild-type cell was expected to display a pyrenoid (78). In single sections of ~3,000 cells of each strain observed at low magnification (×1,200), pyrenoids were unambiguously identified in 66% of the wild-type SS1 cells. No pyrenoids were observed in the *rbcS*Δ-T60-3 strain or in any of the three hybrid enzyme mutant strains (Fig. 5). Thus, despite normal levels of catalytically proficient Rubisco (Fig. 3 and Table 1), the loss of the pyrenoid and the CCM likely accounts for the poor photosynthetic growth of the SSSO, SSAT, and SSHA hybrid enzyme mutants in air (Fig. 2).

DISCUSSION

Our desire to express foreign *rbcS* cDNAs in *Chlamydomonas* led us to consider moving introns 1 and 2 of *Chlamydomonas rbcS1* into the sequence encoding the transit peptide (59) (Fig. 1). This solved the problem of *Chlamydomonas rbcS* cDNA expression, and no difference was observed in the levels of Rubisco holoenzyme when either the *Chlamydomonas rbcS1* cDNA (pSS1-ITP) or the genomic (pSS1) coding sequences were transformed into the *rbcS*Δ-T60-3 deletion strain (Figs. 2 and 3). However, similar levels of hybrid holoenzyme expression were achieved only after changing residue 2 of the spinach, *Arabidopsis*, and sunflower mature small subunits to the Met residue present in *Chlamydomonas* small subunits (Fig. 3). Based on previous studies with the *Chlamydomonas* small subunit (66, 67), it is likely that the nature of residue 2 plays a role in efficient transit peptide processing. In the x-ray crystal structures of *Chlamydomonas* and spinach Rubisco (63, 64, 70), residue 2 is in contact with and shields large subunit residues Trp-411, Pro-415, Pro-453, and Glu-454 from solvent, but there are no notable differences between the two structures in this region. Thus, it seems unlikely that the identity of residue 2 would influence Rubisco function. The engineered *rbcS1* transit peptide region containing introns 1 and 2 may be useful for expressing and targeting other cDNA-encoded proteins to the chloroplast of *Chlamydomonas*. However, because there are multiple transit peptide-processing peptidases in *Chlamydomonas* (62), additional engineering of the amino terminus of the mature protein may be necessary. Nonetheless, the idea of moving introns to transit peptide-encoding regions may serve as a common strategy for cDNA expression in *Chlamydomonas*.

The primary objective of our study was to investigate the influence of foreign small subunits on Rubisco function in *Chlamydomonas*. Previous studies had indicated that hybrid Rubisco enzymes expressed in *Arabidopsis* and tobacco had decreases in Rubisco carboxylation or expression (31, 32, 34), but because it is difficult to eliminate and replace the entire *rbcS* gene family in plants (37, 38), combinations of subunits for analysis have been limited. When the *Chlamydomonas* small subunit was replaced with the small subunit of spinach, *Arabidopsis*, or sunflower, it was surprising to find that the hybrid Rubisco enzymes were expressed at normal levels (Fig. 3*A*) and had near normal catalytic properties (Table 1). As shown in Fig. 6, the *Chlamydomonas* small subunit shares only 44–47% sequence identity with the plant small subunits, whereas plant small subunits share 71–76% identity with each other (44–46, 80–82). Despite this greater sequence identity, heterologous

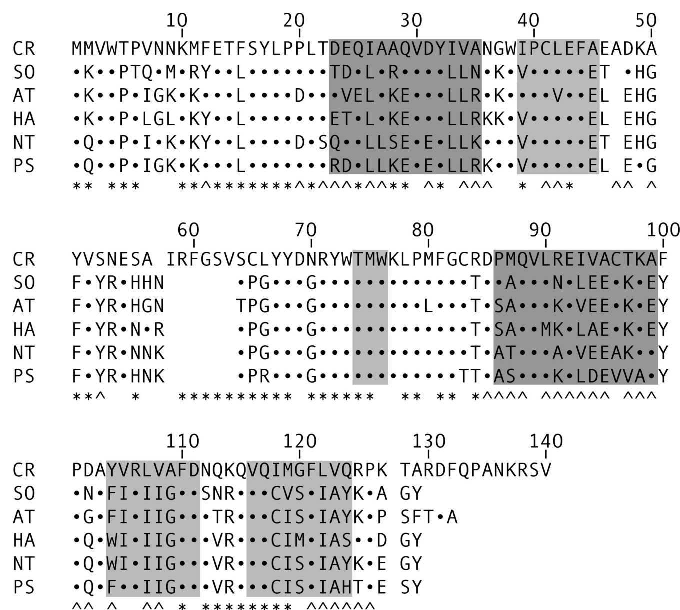

FIGURE 6. **Small subunit sequences aligned according to the x-ray crystal structures of *Chlamydomonas* and spinach Rubisco (63, 70).** The sequences are from *Chlamydomonas rbcS1* (*CR*), spinach (*SO*), *Arabidopsis rbcS1B* (*AT*), sunflower (*HA*), tobacco (*NT*), and pea (*PS*) (44–46, 80, 81). Residues that comprise α-helixes A and B are colored *dark gray*, and those that comprise β-strands A through D are colored *light gray*. Residues identical to those in the *Chlamydomonas* small subunit are denoted as *dots*. Residues in the *Chlamydomonas* small subunit that are in contact with Rubisco large subunit residues are marked with *asterisks* below the sequences. Residues in the *Chlamydomonas* small subunit with the greatest solvent accessibility (82) are marked with *carets* below the sequences.

holoenzymes containing approximately one pea small subunit per *Arabidopsis* holoenzyme had only 85–88% of normal carboxylation activity, apparently due to the loss of one functional active site per holoenzyme (31). *Arabidopsis* plants containing higher levels of pea small subunits in their holoenzymes were not viable (31). Perhaps unfavorable interactions between divergent small subunits in the holoenzyme are more detrimental to function than are altered interactions between foreign small subunits and large subunits. However, hybrid holoenzymes composed entirely of tobacco small subunits and sunflower large subunits were reported to have a greater than 70% decrease in V_c (32), and the hybrid enzyme tobacco plants required an elevated level of CO_2 for growth. In a more recent study, the tobacco-sunflower hybrid enzymes were reported to have normal catalytic properties when assayed in leaf extracts, and the hybrid enzyme plants appeared to require elevated CO_2 for growth because of a decrease in holoenzyme expression (34). Unresolved problems associated with the genetic engineering of Rubisco in plants may contribute to errors in the biochemical analysis of mutant enzymes. Problems of this nature related to expression of plant small subunits in *Chlamydomonas* have apparently been solved.

In comparison with *Chlamydomonas* Rubisco, the plant Rubisco enzymes have lower V_c values, but higher Ω values due to lower K_c and higher K_o/K_c values (Table 1). The hybrid enzymes, composed of plant small subunits and *Chlamydomonas* large subunits, have small increases in Ω relative to wild-type *Chlamydomonas* Rubisco. These increases, ranging from 3 to 11%, may arise from an increase in K_o, but the SSSO spinach

hybrid enzyme may also have an increase in V_c/V_o (Table 1). The V_c and K_c values of the hybrid enzymes remain more like those of *Chlamydomonas* Rubisco than like plant Rubisco (Table 1). Thus, the small subunits can influence the catalytic properties of Rubisco, but the active site large subunit must also contribute to the differences in catalysis observed between the plant and algal enzymes. In a previous study, replacing the longer loop between β-strands A and B of the *Chlamydomonas* small subunit with the shorter loop from the spinach small subunit (Fig. 6) caused a 26% decrease in K_c (22). However, due to decreases in V_c and K_o, the value of Ω was not altered. There must be other regions outside the small subunit βA-βB loop that are responsible for the higher Ω values of the hybrid enzymes, and these regions or residues must be different from those of the *Chlamydomonas* small subunit. As shown in Fig. 6, there are a few residues clustered in the amino-terminal region and in the loop between β-strands C and D that are in van der Waals contact with the large subunit and that differ between *Chlamydomonas* and plant small subunits. Perhaps these residues are responsible for the higher Ω values of the hybrid enzymes. In another study with *Chlamydomonas* Rubisco, when the spinach βA-βB loop was combined with five large subunit direct mutant substitutions, there was a 12–17% increase in Ω, and the other catalytic constants of the mutant enzyme were indistinguishable from those of spinach Rubisco (15). The small subunit βA-βB loop does, in fact, play a role in increasing the value of Ω, but as indicated by the analysis of the hybrid enzymes, there may be additional small subunit regions that can further increase Ω. Because several residues in the amino-terminal region of the small subunit interact with the βA-βB loop (22), this region may deserve further attention for defining the role of the small subunit in Rubisco function.

The SSSO, SSAT, and SSHA hybrid enzyme mutants have wild-type levels of functional Rubisco (Fig. 3 and Table 1), but they require an increased concentration of CO_2 to achieve wild-type levels of photosynthetic growth (Figs. 2 and 3). This phenotype appears to result from the loss of the chloroplast pyrenoid (Fig. 5), which is associated with the algal CCM (10, 11, 78, 79). The mutants grow better in air than other *Chlamydomonas* CCM mutants (74, 75), perhaps indicating that some components of the CCM are still functional but Rubisco is in a CO_2-limited environment. In a previous study, sequence homology was observed between the *Synechococcus* small subunit and a carboxysomal CCM protein (83), indicating that small subunits may have evolved from a structural protein of the cyanobacterial carboxysome (16). If the eukaryotic small subunit plays a primary role in targeting Rubisco for pyrenoid formation (78), residues that differ between the small subunits of plants (which do not have pyrenoids) and *Chlamydomonas* may be most important for this function. As shown in Fig. 6, many of the *Chlamydomonas* small subunit residues that show the greatest level of solvent accessibility (as analyzed with the Protein Interfaces, Surfaces and Assemblies service at the European Bioinformatics Institute (82)) differ from the residues common to plant small subunits. Further dissection of foreign small subunits in *Chlamydomonas* may identify regions that are essential for the targeting of Rubisco to the algal pyrenoid.

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SUPPLEMENTAL DATA

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AATTCGCAACTTGCGCTACTGACTGCCTACTCTCGTGCCGCCTGCAAGCCCACTCCGCCTTCCGCTCTGGTCTACGTACGCATTAGTTCC
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CTCATGCCCTGCCGTTGCCAATTGTGTGCCATCTGTTGGGTTCTGGGAATCAGCTGCACCCTACGGGACTTCACCAACCTGCCTTACCAC
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GCAGGTAAGTAAAATACTGG
```

SUPPLEMENTAL FIGURE 1. **Sequence of the *Chlamydomonas* EcoRI fragment from plasmid pSS1.** This sequence contains the wild-type *rbcS1* gene. Features of the sequence include the transit peptide (3012-3146), mature small subunit (3147-3175, 3314-3393, 3622-3707, 3912-4139), mRNA (2965-4534), intron 1 (3176-3313), intron 2 (3394-3621), and intron 3 (3708-3911).

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AATTCGCAACTTGCGCTACTGACTGCCTACTCTCGTGCCGCCTGCAAGCCCACTCCGCCTTCCGCTCTGGTCTACGTACGCATTAGTTCC
GCAACACTCGTCAATGACTGGTGCAGGCTATAAGCAATAGTGGTTGCACGGAGTCCCCTGTCCCGCGCAGCCGAGGAGCGCAGCCGAAGA
GCGCAGCCGAGGAGAGCTGTACAAGGCATTGGTCGTAGCTAGCACACGTAGCGTGGCTTCTGCTTTTGCTGCCGCTCAGGGCTCGGGCTC
TGAGCGCATGAAGCCATGAACTGCGTCAGCAGGTCCCTCTTGTAACCAATGCAATCTGAGCGCAGGGATGGCCGCGGCTTCTTCGCCTTT
GGTCACGGTCTAGGCCGCTTAGAGCTTGGGTGCGGCTAGGCTCGCGGGGTCTGTGGCGGAATGCGTGCCAACTGCCCCCACCCGCAGCTT
CGTAATCGGGCAGCCAATACCGCAGGCCGCGGCTCCATCGCAGGTGCACACGCAGGCTGCATTGCTCCAGGGTGAGCCCTGGGACTGATC
CTTGACTTGCGTGCACCAAGGGATACCGAGTACTGAAGATGGCGGCATGCAGCTGCTGGAGTAATTGTCTAGCATCGGGACTTACATTCC
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CTCTTTCACACGGTCATGCCAGCAGCAATCCAGCTGTGTGTGCAAGCGCGGTTCAAGGCGGGCCACGGGTTGCACCCTCCTACCGTAGCA
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GTGGCCAAGGACTAGGGGATGCTTTAGGGTGTGCGAGGGCGGCAAGGATTCGGAACATGGGAACGCTACCGAGCTGGACCGGAATGCGCT
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CCCTACGGGACTTCACCAACCTGCCTTACAACTGTCCCATCGCACACCCAGCCGGACCGTTGACCAGCAGGTAAGTAAAATACTGG
```

SUPPLEMENTAL FIGURE 2. **Sequence of the engineered *Chlamydomonas* EcoRI fragment from plasmid pSS1-ITP.** Within the *rbcS1* gene, intron 3 was deleted and introns 1 and 2 were moved to the transit-peptide region. Features of the sequence include the transit peptide (3012-3061, 3200-3259, 3488-3512), mature small subunit (3513-3935), mRNA (2965-4330), intron 1 (3062-3199), and intron 2 (3260-3487).

Spinach *rbcS* sequence in plasmid pSS1-SSSO

```
ATGATGGTGTGGCCCACCCAGAACATGAAGCGCTACGAGACCCTGTCCTACCTGCCCCCCCTGACCACCGACCAGCTGGCCCGCCAGGTG
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TACTACGACGGCCGCTACTGGACCATGTGGAAGCTGCCCATGTTCGGCTGCACCGACCCCGCCCAGGTGCTGAACGAGCTGGAGGAGTGC
AAGAAGGAGTACCCCAACGCCTTCATCCGCATCATCGGCTTCGACTCCAACCGCCAGGTGCAGTGCGTGTCCTTCATCGCCTACAAGCCC
GCCGGCTACTAA
```

Arabidopsis rbcS sequence in plasmid pSS1-SSAT

```
ATGATGGTGTGGCCCCCCATCGGCAAGAAGAAGTTCGAGACCCTGTCCTACCTGCCCGACCTGACCGACGTCGAGCTGGCCAAGGAGGTC
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TACTACGACGGCCGCTACTGGACCATGTGGAAGCTGCCCCTGTTCGGCTGCACCGACTCCGCCCAGGTGCTGAAGGAGGTGGAGGAGTGC
AAGAAGGAGTACCCCGGCGCCTTCATCCGCATCATCGGCTTCGACAACACCCGCCAGGTCCAGTGCATCTCCTTCATCGCCTACAAGCCC
CCCTCCTTCACCGACGCCTAA
```

Sunflower *rbcS* sequence in plasmid pSS1-SSHA

```
ATGATGGTGTGGCCCCCCCTGGGCCTGAAGAAGTACGAGACCCTGTCCTACCTGCCCCCCCTGACCGAGACCCAGCTGGCCAAGGAGGTG
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TACTACGACGGCCGCTACTGGACCATGTGGAAGCTGCCCATGTTCGGCTGCACCGACTCCGCCCAGGTGATGAAGGAGCTGGCCGAGTGC
AAGAAGGAGTACCCCCAGGCCTGGATCCGCATCATCGGCTTCGACAACGTGCGCCAGGTGCAGTGCATCATGTTCATCGCCTCCCGCCCC
GACGGCTACTAA
```

SUPPLEMENTAL FIGURE 3. **Codon-optimized coding sequences for the mature small subunits of spinach, *Arabidopsis*, and sunflower Rubisco.** These sequences were used to exactly replace the *Chlamydomonas rbcS1* mature-protein coding sequence of pSS1-ITP (supplemental Fig. S2) to generate plasmids pSS1-SSSO, pSS1-SSAT, and pSS1-SSHA. The sequences were engineered to replace plant Lys-2 with *Chlamydomonas* Met in the small subunits.