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At the time of publication, author Henry Daniell was affiliated with the University of Pennsylvania. Currently, he is a faculty member at the School of Dental Medicine at the University of Pennsylvania.

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Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a key enzyme that converts atmospheric carbon to food and supports life on this planet. Its low catalytic activity and specificity for oxygen leads to photorespiration, severely limiting photosynthesis and crop productivity. Consequently, Rubisco is a primary target for genetic engineering. Separate localization of the genes in the nuclear and chloroplast genomes and a complex assembly process resulting in a very low catalytic activity of hybrid Rubisco enzymes have rendered several earlier attempts of Rubisco engineering unsuccessful. Here we demonstrate that the *Rbc*S gene, when integrated at a transcriptionally active spacer region of the chloroplast genome, in a nuclear *Rbc*S antisense line and expressed under the regulation of heterologous (gene 10) or native (*psb*A) UTRs, results in the assembly of a functional holoenzyme and normal plant growth under ambient CO_2 conditions, fully shortcircuiting nuclear control of gene regulation. There was ≈150-fold more *Rbc*S transcript in chloroplast transgenic lines when compared with the nuclear *Rbc*S antisense line, whereas the wild type has 7-fold more transcript. The small subunit protein levels in the gene 10/*Rbc*S and *psb*A/*Rbc*S plants were 60% and 106%, respectively, of the wild type. Photosynthesis of gene 10/*Rbc*S plants was approximately double that of the antisense plants, whereas that of *psb*A/*Rbc*S plants was restored almost completely to the wild-type rates. These results have opened an avenue for using chloroplast engineering for the evaluation of foreign Rubisco genes *in planta* that eventually can result in achieving efficient photosynthesis and increased crop productivity.

Disciplines

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Comments

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Enhanced translation of a chloroplast-expressed *Rbc*S gene restores small subunit levels and photosynthesis in nuclear *Rbc*S antisense plants

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a key enzyme that converts atmospheric carbon to food and supports life on this planet. Its low catalytic activity and specificity for oxygen leads to photorespiration, severely limiting photosynthesis and crop productivity. Consequently, Rubisco is a primary target for genetic engineering. Separate localization of the genes in the nuclear and chloroplast genomes and a complex assembly process resulting in a very low catalytic activity of hybrid Rubisco enzymes have rendered several earlier attempts of Rubisco engineering unsuccessful. Here we demonstrate that the *Rbc*S gene, when integrated at a transcriptionally active spacer region of the chloroplast genome, in a nuclear *Rbc*S antisense line and expressed under the regulation of heterologous (gene 10) or native (*psb*A) UTRs, results in the assembly of a functional holoenzyme and normal plant growth under ambient CO_2 conditions, fully short-circuiting nuclear control of gene regulation. There was ≈150-fold more *Rbc*S transcript in chloroplast transgenic lines when compared with the nuclear *Rbc*S antisense line, whereas the wild type has 7-fold more transcript. The small subunit protein levels in the gene 10/*Rbc*S and *psb*A/*Rbc*S plants were 60% and 106%, respectively, of the wild type. Photosynthesis of gene 10/*Rbc*S plants was approximately double that of the antisense plants, whereas that of *psb*A/*Rbc*S plants was restored almost completely to the wild-type rates. These results have opened an avenue for using chloroplast engineering for the evaluation of foreign Rubisco genes *in planta* that eventually can result in achieving efficient photosynthesis and increased crop productivity.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most prevalent enzyme on this planet, accounting for 30–50% of total soluble protein in the chloroplast; it fixes carbon dioxide, but oxygenase activity severely limits photosynthesis and crop productivity (1, 2). Rubisco consists of eight large subunits (LSUs) and eight small subunits (SSUs). The SSU is imported from the cytosol, and both subunits undergo several posttranslational modifications before assembly into functional holoenzyme (3).

Genetic manipulation of Rubisco to improve its function has involved both nuclear and plastid genetic engineering, mostly in tobacco. When the plastid *rbc*L gene encoding the LSU was deleted and expressed via the nuclear genome, transgenic plants exhibited a severe Rubisco deficiency (4). When the *rbc*L gene from *Chromatium vinosum* was expressed via the nuclear genome of Rubisco-deficient plants, it was poorly transcribed, and no foreign LSU was detected (5).

In yet another approach, a mutated *rbc*L gene, engineered into the chloroplast genome of tobacco, resulted in a Rubisco with a specificity factor and carboxylation rate of only 25% of the wild type (6). Also, when the tobacco *rbc*L gene was replaced with a heterologous gene from cyanobacteria or *Helianthus annus*, Rubisco-deficient plants were obtained (7). Engineering the nuclear genome of *Arabidopsis* with a cDNA encoding a pea SSU also resulted in a hybrid Rubisco that was compromised in catalysis (8). Engineering the chloroplast genome with the *rbc*L-*rbc*S operon from a nongreen alga resulted in lack of Rubisco assembly (9). Engineering the L2 form of Rubisco from *Rhodospirillum rubrum* resulted in transgenic plants that were unable to survive at ambient CO_2 levels (10).

Yet another approach for Rubisco manipulation was to engineer the *Rbc*S gene into the chloroplast genome. Whitney and Andrews (11) expressed two copies of a His-tagged *Rbc*S gene in transgenic chloroplasts and obtained less than ≈1% accumulation of the SSU. When a nuclear *Rbc*S antisense tobacco mutant (12–14) was used for engineering the *Rbc*S gene via the chloroplast genome, it resulted in decreased photosynthetic activity and retarded growth (15). This mutant line is an ideal target for Rubisco engineering, because any successful Rubisco assembly would be easily discernible. The *Rbc*S gene was expressed under the regulation of a chloroplast ribosome binding site (RBS) (GGAGG); the chloroplast transgenic plants accumulated abundant plastid *Rbc*S transcript, but it was not translated (15). The failure of several attempts to introduce into the chloroplast genome an *Rbc*S gene that can be expressed properly to produce a fully functional Rubisco cast doubt on the usefulness of this approach for Rubisco engineering.

It is evident that a completely functional eukaryotic Rubisco has never been assembled in any foreign host. Therefore, in this study, *Rbc*S cDNA was engineered into a transcriptionally active spacer region of the chloroplast genome of nuclear *Rbc*S antisense tobacco plant. In addition, two different 5′ UTRs were used to facilitate enhanced translation of the *Rbc*S cDNA. This report demonstrates successful Rubisco expression and assembly from plastid-derived SSUs and LSUs of Rubisco to achieve normal plant growth under ambient CO_2 conditions and photosynthesis.

Materials and Methods

Construction of Chloroplast Transformation Vectors. The *Rbc*S cDNA was amplified by using PCR from the vector pTSA (a kind gift from Xing-Hai Zhang, University of Illinois at Urbana–Champaign, Urbana) incorporating an *Nco*I restriction enzyme site at the 5′ end to facilitate its cloning downstream to gene 10 and *psb*A 5′ UTRs. The gene 10 5′-UTR/*Rbc*S cDNA and *psb*A promoter/5′-UTR/*Rbc*S cDNA cassettes were cloned into the multiple cloning site of the basic chloroplast transformation vector pLD-CtV (16, 17) to yield pLDADg10*Rbc*S and pLDAD*psb*A*Rbc*S, respectively.

Chloroplast Transformation and Regeneration of Transgenic Plants. Seeds from nuclear *Rbc*S antisense tobacco plants (12) were surface-sterilized and germinated on Murashige and Skoog salt

Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; LSU, large subunit; SSU, small subunit; 0D, zero days.

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mixture without organic components (MSO)-kanamycin (50 mg/liter) media (18). All the germinating seedlings were resistant to kanamycin, indicating homozygous status of the transgene. Seedlings were transferred to small jars and maintained at 26°C under a 16-h light/8-h dark photoperiod. Leaves obtained from the nuclear *Rbc*S antisense plants were used for particle bombardment, and these original mother plants were maintained in tissue culture or in soil to be used as controls for subsequent analysis.

Particle bombardment with the chloroplast transformation vectors pLDADg10*Rbc*S and pLDAD*psb*A*Rbc*S was carried out as described (18). Putative chloroplast transgenic shoots were screened for site-specific integration of the transgene cassette by using PCR (detailed below). Positive shoots were subjected to another round of selection, and regenerating shoots were transferred to small jars containing Murashige and Skoog salt mixture without organic components (MSO)-spectinomycin (500 mg/liter) medium. After 3–4 weeks, plants were transferred to soil and maintained at 26°C under a 16-h light/8-h dark photoperiod in a growth chamber.

Continuous Illumination. Leaf samples of comparable age and morphology, grown under a normal photoperiod of 16-h light/8-h dark were harvested and frozen in liquid nitrogen for subsequent Northern and Western analyses [zero days (0D)]. Light intensity was maintained at ≈200 $\mu E \cdot m^{-2} \cdot s^{-1}$ [1 Einstein (E) is equal to 1 mol of photons] with fluorescent lights. Afterward, plants were kept under continuous light, and leaf tissue was harvested after 1, 3, or 5 days for Northern and Western analyses. The nuclear *Rbc*S antisense line used for this study was the original mother plant from which the chloroplast transgenic plants were obtained.

PCR Screening and Southern Analysis. Total cellular DNA was isolated from 100 mg of leaf tissue by using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). To investigate homoplasmy, *Sma*I-digested total plant DNA was probed with the radiolabeled DNA fragment P1 (Fig. 1*A*). Prehybridization and hybridization were carried out per manufacturer protocol (Stratagene). The radiolabeled probe was prepared by using Ready-To-Go DNA labeling beads per manufacturer protocol (Amersham Pharmacia Biosciences). To confirm the presence of chloroplast-localized *Rbc*S, total plant DNA from Nt-pLDADg10*Rbc*S was digested with *Xba*I and that from Nt-pLDAD*psb*A*Rbc*S with *Spe*I. A radiolabeled 0.5-kb *Rbc*S cDNA fragment was used as the probe to detect plastid-localized *Rbc*S cDNA.

Northern Analysis. Total cellular RNA was isolated from leaves of similar developmental age by using RNeasy Plant Mini Kit (Qiagen). Northern blot analysis (5 μg of RNA per sample) was carried out as described (19). Northern blots were probed with a radiolabeled 0.5-kb *Rbc*S cDNA fragment to detect relative *Rbc*S transcript levels and a radiolabeled 0.9 *Apa*I-digested fragment (P2) (Fig. 1*A*) representing 16S rRNA-encoding DNA. Relative transcript levels were measured by using spot densitometry (Alphaimager 3300, Alpha Innotech, San Leandro, CA). For the densitometric measurement of chloroplast transcripts, lane 1 (Fig. 2 *A* and *C*) was used, and for determining the antisense and wild-type transcript ratio, lane 3 (Fig. 2 *A* and *C*) was used. The measured values were plotted as a bar chart (Fig. 2 *B* and *D*).

Protein Extraction and Western Analysis. Total soluble protein was extracted from 100 mg (fresh weight) of leaf tissue by using 2× Laemmli buffer. Protein was quantified in three replicates by using the Bio-Rad RC-DC protein quantification kit per manufacturer protocol. An equal volume (10 μl) of the protein extract (qualitative analysis) or equal quantity (10 μg, quantitative analysis) of total soluble protein was subjected to 0.1% SDS/14% PAGE, and Western blot analysis was performed by using antibody against tobacco Rubisco (20). Alkaline phosphatase-linked secondary antibody was used to detect the Rubisco LSUs and SSUs by using Lumi-Phos WB chemiluminescent reagent (Pierce). The SDS/PAGE gels were stained by using GelCode blue stain (Pierce). Relative SSU levels were measured by using spot densitometry (Alphaimager 3300, Alphaimager Corp.). The measured values were plotted as a bar chart (Figs. 3 *B* and *D* and 4 *B* and *D*).

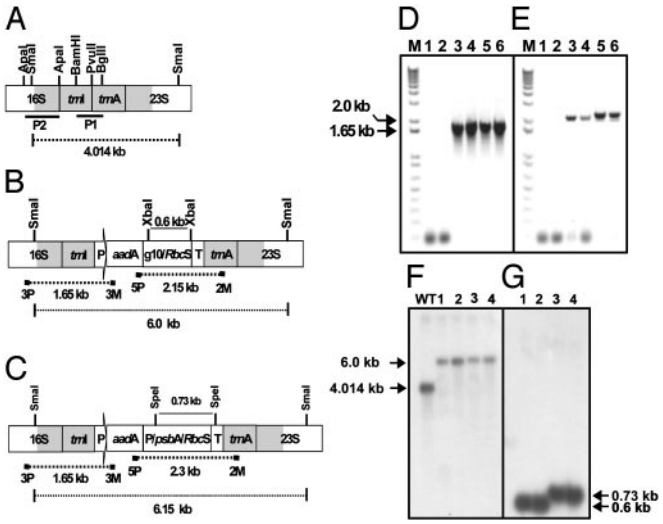

Fig. 1. (*A*) Schematic representation of the 16S-23S region of wild-type tobacco chloroplast genome. The *Bam*HI/*Bgl*II restriction fragment, P1, was used as a probe to investigate homoplasmy. The *Apa*I restriction fragment, P2, was used as a probe to detect 16S rRNA transcript levels in Northern analysis. Also shown are transgenic chloroplast genomes harboring pLDADg10*Rbc*S (*B*) or pLDAD*psb*A*Rbc*S (*C*) as well as the annealing positions of primers 3P/3M and 5P/2M and expected amplicon sizes. (*D*) PCR analysis with primer pair 3P/3M. Lane M, 1-kb DNA ladder; lanes 1 and 2, untransformed; lanes 3 and 4, Nt-pLDADg10*Rbc*S; lanes 5 and 6, Nt-LDAD*psb*A*Rbc*S. (*E*) PCR analysis with primer pair 5P/2M. Lane M, 1-kb DNA ladder; lanes 1 and 2, untransformed; lanes 3 and 4, Nt-pLDADg10*Rbc*S; lanes 5 and 6, Nt-pLDAD*psb*A*Rbc*S. (*F*) Southern blot analysis. Total DNA was digested with *Sma*I and probed with a radiolabeled *Bgl*II/*Bam*HI fragment, P1. WT, untransformed; lanes 1 and 2, Nt-pLDADg10*Rbc*S; lanes 3 and 4, Nt-pLDAD*psb*A*Rbc*S. (*G*) Southern blot analysis. Total DNA was probed with *Rbc*S. Nt-pLDADg10*Rbc*S was digested with *Xba*I (lanes 1 and 2) and Nt-pLDAD*psb*A*Rbc*S was probed with *Spe*I (lanes 3 and 4).

Measurement of Photosynthetic CO_2 Assimilation Rate. The photosynthetic CO_2 assimilation rates were measured with an LI-6400 instrument (Li-Cor, Lincoln, NE). The following parameters were used: 28°C leaf temperature, 21% O_2, 1,200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light, 40% relative humidity, and CO_2 concentration ranging from 0 to 1,200 μbar (1 bar = 100 kPa). Two leaves per plant of similar developmental age (≈50 days old) were assayed.

Results and Discussion

Chloroplast Transformation Vectors. The tobacco chloroplast transformation vector pLD-CtV targets the transgene cassette to the 16S-*trn*I/*trn*A-23S region of the chloroplast genome (Fig. 1*A*; see refs. 16 and 17 for details). To achieve enhanced translation, the tobacco *Rbc*S cDNA along with its transit peptide-coding sequence was cloned into the multiple cloning site of pLD-CtV downstream of two different 5′ UTRs. A *psb*A 3′ UTR is already present in pLD-CtV, which aids in transcript stability (21). The transit peptide sequence was retained for chloroplast expression in both the vectors, because it is probably involved in positively

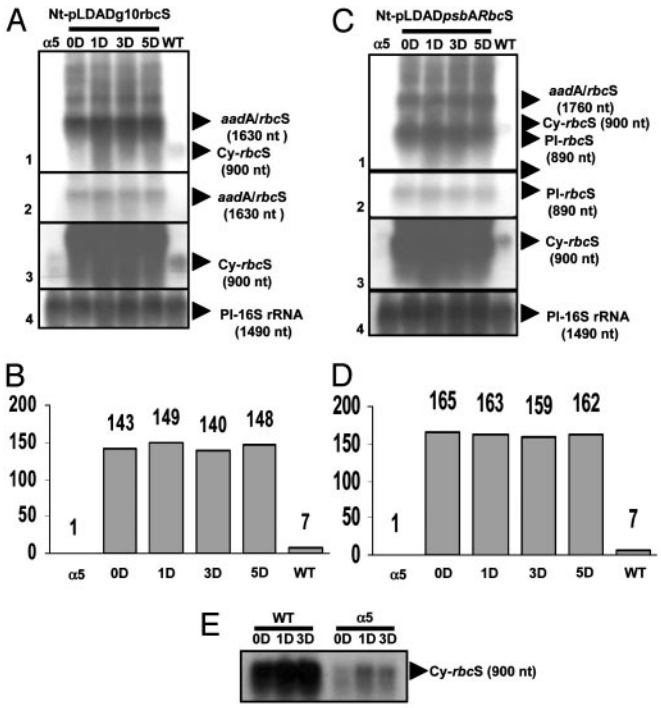

Fig. 2. Analysis of transcript levels under normal photoperiod (0D) or continuous light for 1, 3, and 5 days. (*A*) Nt-pLDADg10*Rbc*S. (*B*) Densitometry analysis of the Northern blot shown in *A*. (*C*) Nt-pLDAD*psb*A*Rbc*S. (*D*) Densitometry analysis of the Northern blot shown in *C*. Lanes 1–3 represent the Northern blot exposed for different durations. Cy-*Rbc*S, cytosolic *Rbc*S mRNA (900 nt), plastid (Pl) 16S rRNA (1,490 nt) was used as a control for equal loading (lane 4). (*E*) Relative *Rbc*S transcript levels in wild-type and *Rbc*S antisense plant (α5). An equal amount of RNA (5 μg) was loaded per sample.

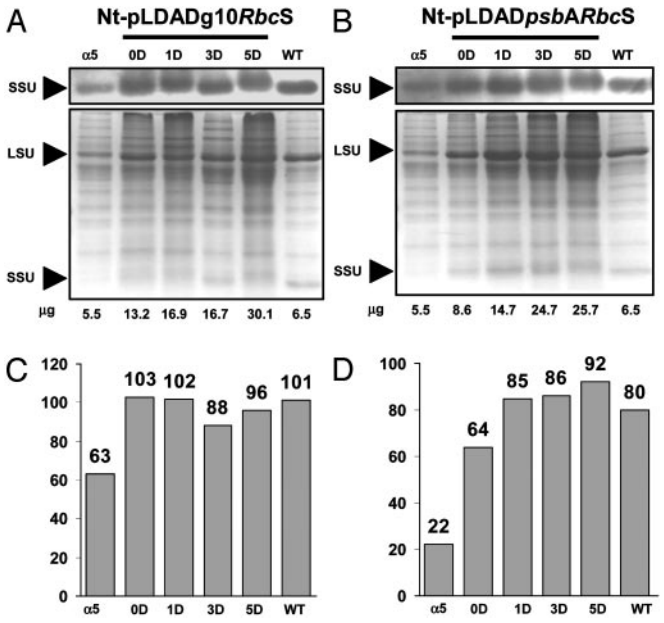

Fig. 3. Qualitative comparison of SSU protein levels in 100 mg (fresh weight) of leaf tissue and an equal volume (10 μl of each) examined on a 14% SDS/PAGE gel. (*A* and *C*, *Upper*) Immunoblot probed with anti-Rubisco antibody. (*A* and *C*, *Lower*) GelCode blue-stained SDS/PAGE gel. Numbers below the stained gel indicate the amount of protein loaded (in μg). Shown are Nt-pLDADg10*Rbc*S (*A*) or Nt-pLDAD*psb*A*Rbc*S (*C*) grown under normal photoperiod (0D) or under continuous light for 1, 3, and 5 days. (*B* and *D*) Densitometry analysis of the immunoblots shown in *A* and *C*, respectively.

affecting *Rbc*S mRNA abundance and transcript stability in chloroplasts (11, 15).

In the chloroplast transformation vector pLDADg10*Rbc*S, expression of the *Rbc*S gene is regulated by a gene 10 5′ UTR (Fig. 1*B*). The gene 10 5′ UTR is derived from bacteriophage and is known to enhance translation in chloroplasts considerably when fused to endogenous promoters (22). In addition, it is free from developmental or light regulation, because it is derived from a foreign source. In this expression cassette, *Rbc*S cDNA should be transcribed as a dicistron in the chloroplasts, because the upstream gene, *aad*A, is devoid of a 3′ UTR and the gene 10 5′ UTR is promoterless. In pLDAD*psb*A*Rbc*S, expression of the *Rbc*S gene is regulated by the *psb*A promoter/5′ UTR (Fig. 1*C*). Because of the presence of a separate promoter, this expression cassette should produce an *Rbc*S monocistron in addition to an *aad*A-*Rbc*S dicistron.

Confirmation of Transgene Integration and Determination of Homoplasmy. Both chloroplast transformation vectors were introduced into the chloroplasts of nuclear *Rbc*S antisense leaves via particle bombardment. The presence of an origin of replication in the chloroplast transformation vector allows the vector to replicate independently and provide more copies for integration, thus expediting the process of achieving homoplasmy even in the first round of selection (16, 17). After 4–5 weeks, resistant shoots were obtained on RMOP-spectinomycin (500 mg/liter) medium. Putative chloroplast transgenic clones were screened initially for site-specific transgene integration by using primer pair 3P and 3M. Primer 3P anneals to the DNA sequence upstream of the flanking region used in the chloroplast transformation vector, and primer 3M anneals within the *aad*A coding sequence (Fig. 1*B*). True chloroplast transgenic shoots should produce an amplicon of 1.65 kb that should be absent in nuclear transgenic plants or mutants. PCR data from two clones, each of which has incorporated the transgene cassette, harboring pLDADg10*Rbc*S and pLDAD*psb*A*Rbc*S are shown (Fig. 1*D*). The presence of a 1.65-kb PCR product in the chloroplast transgenic plants confirms site-specific integration of the transgene cassette. To verify the presence of the complete transgene cassette, primers 5P and 2M were used. Primer 5P anneals within the *aad*A coding region, and 2M anneals to the right flanking region of the chloroplast transformation vector (Fig. 1 *B* and *C*). Shoots that have incorporated the pLDADg10*Rbc*S cassette produced a 2.15-kb amplicon, whereas shoots successfully transformed with the pLDAD*psb*A*Rbc*S vector produced a 2.3-kb amplicon (Fig. 1*E*). These PCR-positive shoots were divided into small pieces and placed on RMOP-spectinomycin medium for a second round of selection to achieve homoplasmy.

Homoplasmy was investigated by Southern blot analysis. Restriction digestion of total plant DNA with *Sma*I should produce a 4.01-kb fragment in the wild-type plastome when probed with a *Bam*HI/*Bgl*II radiolabeled fragment P1 (Fig. 1*A*). Plants successfully transformed with the pLDADg10*Rbc*S and pLDAD*psb*A*Rbc*S should produce a fragment of 6.0 and 6.15 kb, respectively (Fig. 1 *B* and *C*). Southern blot analysis confirmed the presence of a transformed plastid genome in the selected lines. The wild-type sample generated the predicted fragment of 4.01 kb, whereas samples from the Nt-pLDADg10*Rbc*S and Nt-pLDAD*psb*A*Rbc*S generated the predicted fragment of 6.0 and 6.15 kb, respectively (Fig. 1*F*). Absence of a wild-type fragment of 4.01 kb in the chloroplast transgenic plants confirmed the homoplasmy in these plants; i.e., all the chloroplast genomes were uniformly transformed with *Rbc*S cDNA. To confirm the presence of *Rbc*S cDNA, total plant DNA from Nt-pLDADg10*Rbc*S plants was digested with *Xba*I, and that from Nt-pLDAD*psb*A*Rbc*S was digested with *Spe*I. The presence

of 0.60-kb (Nt-pLDADg10*Rbc*S) and 0.73-kb (Nt-pLDAD*psb*A*Rbc*S) fragments in the Southern blots probed with a radiolabeled *Rbc*S cDNA fragment confirmed the presence of *Rbc*S cDNA (Fig. 1*G*).

Transcript Analysis of *RbcS* in Chloroplast Transgenic Plants. The chloroplast-expressed *Rbc*S cDNA is composed of the transit peptide sequence along with the coding sequence for the SSU of Rubisco. The nuclear *Rbc*S transcript is ≈900 nt long, and it encodes the SSU precursor (12). In Nt-pLDADg10*Rbc*S plants, *Rbc*S cDNA is expressed under the regulation of a promoterless gene 10 5′ UTR. The *Rbc*S cDNA thus should be transcribed as a dicistron of ≈1,630 nt. Total cellular RNA isolated from a nuclear *Rbc*S antisense plant, an Nt-pLDADg10*Rbc*S plant grown under normal photoperiod (0D) or exposed to continuous light for 1, 3, or 5 days, and a wild-type tobacco plant were probed with radiolabeled *Rbc*S cDNA. From the autoradiogram, it is evident that the *aad*A/*Rbc*S dicistron (1,630 nt) is highly abundant, whereas the nuclear transcript (900 nt) is barely detectable (Fig. 2*A*, lane 2). The blot had to be exposed for a longer duration to perform densitometry analysis (Fig. 2*A*, lanes 1 and 3). Densitometry analysis revealed that at 0D, the plastid-derived *Rbc*S transcript was 143-fold more than the transcript levels in the nuclear *Rbc*S antisense plant (Fig. 2*B*), which is in sharp contrast to an only 20-fold increase in plastid-derived *Rbc*S transcript level observed in an earlier study in which the transgene was integrated into the single-copy region of the tobacco chloroplast genome (15). The *Rbc*S transcript levels in the wild type were only 6.8-fold that of the antisense, similar to that reported previously (12, 15). As expected, there was no increase in *Rbc*S transcription during continuous exposure to light in the chloroplast-engineered plant. The Northern blot had to be overexposed to observe the nuclear transcript in the nuclear *Rbc*S antisense and wild-type plants.

In Nt-pLDAD*psb*A*Rbc*S line, *Rbc*S is expected to be transcribed as a monocistron as well as an *aad*A-*Rbc*S dicistron. The monocistron and the dicistron should be 890 and 1,760 nt long, respectively. (Fig. 2*C*). In this case also, the nuclear transcripts were undetectable after normal exposure of the blot (Fig. 2*C*, lane 2). Therefore, the blot was exposed for a longer duration to perform densitometry analysis. Based on densitometry analysis, the chloroplast-derived *Rbc*S transcripts were 165-fold more than the transcript levels in the nuclear *Rbc*S antisense plants (Fig. 2*D*). In an earlier study, *Rbc*S was engineered into a transcriptionally silent region of the chloroplast genome, but the transcript abundance was only 5-fold more than the nuclear-derived wild-type *Rbc*S transcript levels (6); no *Rbc*S antisense plants were used in this study, and a direct comparison could not be made. However, we report here a 25-fold increase in *Rbc*S transcript abundance over the wild-type nuclear transcript level. As expected, there was no discernible effect of continuous light exposure on transcription of *Rbc*S cDNA integrated into plastid genomes (Fig. 2*C*) in these plants as well. Engineering of another transgene coding for the human serum albumin at the same integration site, under the regulation of *psb*A promoter, resulted in similar transcript abundance under light and dark, again confirming that light does not influence transcription (19). The native *psb*A transcript composed of the 5′ and 3′ UTRs has been shown to be stable for ≈40 h (23). Therefore, transcript stability should result in similar *Rbc*S transcript levels even under continuous light. For controls, transcript analysis also was performed on a wild-type and a nuclear *Rbc*S antisense plant maintained under normal photoperiod (0D) or exposed to continuous light for 1 and 3 days (Fig. 2*E*). Continued light exposure initially had only a slight enhancing effect on the steady-state transcript levels in the nuclear antisense plants, and the levels decreased on the third day (Fig. 2*E*). As expected, there was a 2-fold increase in *Rbc*S transcript levels in the wild type after continued light exposure, because the *Rbc*S transcription is known to be light-regulated (24).

The large increase (20- to 25-fold) in steady-state *Rbc*S transcript levels in chloroplast transgenic plants as compared with the wild type can be attributed partly to the high copy number (≈20,000) of the chloroplast-engineered gene when integrated into the inverted repeat region. All the earlier efforts of manipulating the chloroplast genome for Rubisco engineering integrated the transgene into transcriptionally silent regions. The site of integration used here is the intergenic region between the *trn*I-*trn*A genes in the *rrn* operon present in the inverted repeat regions of the tobacco chloroplast genome. This site is transcriptionally active because of the read-through transcription from the upstream 16S promoter (P*rrn*), which is responsible for transcribing six native genes. This is responsible for the high accumulation of the foreign transcripts when transgenes are integrated at this site; chloroplast foreign transcripts were observed to be 169-fold higher than the best nuclear transgenic lines even in the absence of a promoter for this transgene (25). To rule out any loading artifact, the blots were probed with 16S rRNA-encoding DNA fragment. An equal amount of transcripts corresponding to 16S rRNA (1,490 nt) was detected in all the samples, confirming equal loading of total RNA (Fig. 2 *A* and *C*, lane 4).

Qualitative Analysis of SSU. Two different 5′ UTRs were used in this study to enhance the translation of *Rbc*S cDNA. The T7 gene 10 5′ UTR is independent of any cellular control in plants and should facilitate constitutive translation of the transgene. The second chloroplast transformation vector has the *psb*A promoter/5′ UTR regulating the expression of *Rbc*S cDNA. All plants were analyzed further for total protein and the level of SSUs and LSUs (Fig. 3 *A* and *C*). On an equal fresh-weight basis, both transformed lines Nt-pLDADg10*Rbc*S and Nt-pLDAD*psb*A*Rbc*S contained a higher level of total soluble protein when compared with nuclear antisense and the wild-type plants. This result is in contrast to observations made earlier in which *Rbc*S cDNA was expressed under the regulation of the Shine–Dalgarno (GGAGG) sequence in the chloroplast genome of nuclear *Rbc*S antisense plants. In that study, the total soluble protein on an equal leaf-area basis in the chloroplast transgenic plant was similar to the nuclear *Rbc*S antisense plants and one third of wild type (15). In the present study, an equal volume (10 μl) of protein sample derived from equal fresh weight of leaf tissue (100 mg) was subjected to SDS/PAGE. The total protein content in leaves of Nt-pLDADg10*Rbc*S under a normal photoperiod (0D) was 2.4- and 2.0-fold more when compared with the nuclear antisense and wild type, respectively (Fig. 3*A*). Furthermore, there was a 2.3-fold increase in the total protein content in these transgenic plants by the fifth day of continuous light exposure (Fig. 3*A*). The higher level of protein under a normal photoperiod (0D) was accompanied by an increased level of SSU, which was 102% when compared with the wild type by Western blot analysis (Fig. 3*B*). A corresponding increase in the level of LSU is clearly visible in the stained protein gel.

Similarly, the total soluble protein in leaves of Nt-pLDAD*psb*A*Rbc*S under a normal photoperiod (0D) was 1.6- and 1.3-fold more when compared with nuclear antisense and wild type, respectively (Fig. 3*C*). There was a 3.0-fold increase in the total soluble protein in these transformed plants on the fifth day of continuous light exposure. Western blot analysis revealed that SSU levels in Nt-pLDAD*psb*A*Rbc*S plant exposed to continuous light for 3 days were 107% of wild-type levels (Fig. 3*D*).

Quantitative Analysis of SSU. SDS gel electrophoresis and Western blots were carried out also by using an equal quantity of protein (i.e., 10 μg per sample). In the Nt-pLDADg10*Rbc*S plant, the percentage SSU level was 58% greater under normal photope-

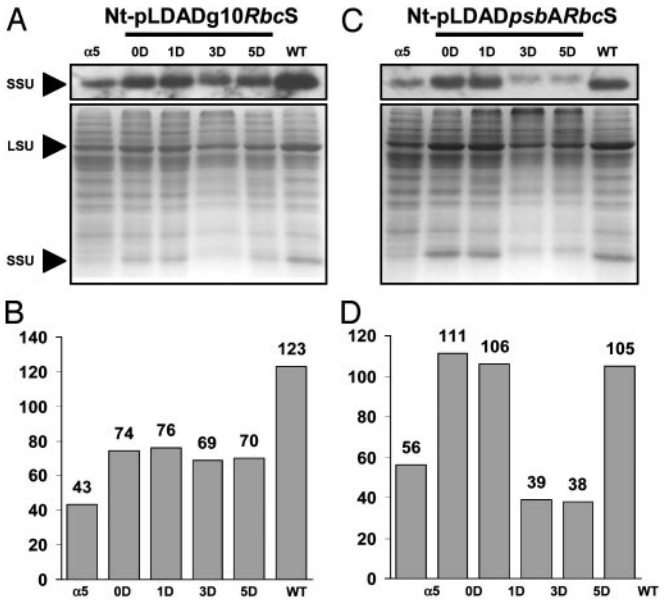

Fig. 4. Quantitative comparison of SSU protein level in 10 μg of total protein examined on a 14% SDS/PAGE gel. (*A* and *C*, *Upper*) Immunoblot probed with anti-Rubisco antibody. (*A* and *C*, *Lower*) GelCode blue-stained SDS/PAGE gel. Shown are the Nt-pLDADg10*Rbc*S line (*A*) or Nt-pLDAD*psb*A*Rbc*S (*C*) grown under normal photoperiod (0D) or exposed to continuous light for 1, 3, and 5 days. (*B* and *D*) Densitometry analysis of the immunoblots shown in *A* and *C*, respectively.

riod (0D) than the nuclear antisense plant, but it was still only 60% of the wild type (Fig. 4 *A* and *B*). On the other hand, in the Nt-pLDAD*psb*A*Rbc*S plant, SSU levels under a normal photoperiod (0D) were 106% of wild-type level (Fig. 4 *C* and *D*). In both transgenic lines, the levels decreased after 3 and 5 days of continuous light exposure (Fig. 4 *A* and *C*). The decrease was particularly prominent in the Nt-pLDAD*psb*A*Rbc*S plant (Fig. 4*C*). Similar observations were made in three different independent experiments. The dynamics of chloroplast-derived *Rbc*S expression in these plants are not understood completely at this point, although it is known that chloroplast proteases are activated under stress caused by prolonged light exposure (26).

From the protein analysis it is apparent that the use of 5′ UTRs for chloroplast-specific expression of *Rbc*S results in enhanced levels of the SSU. The increased levels of SSU are an index of the increased amount of Rubisco, which is also apparent in the GelCode blue-stained SDS/PAGE gels. The gene 10 5′ UTR enhanced the SSU level to 60% of the wild-type level. The Nt-pLDAD*psb*A*Rbc*S line accumulated 106% of SSU when compared with the wild type under normal photoperiod (0D). The *psb*A 5′ UTR has been shown to result in overexpression of several foreign proteins (19, 27), and it was found to be the best among the UTRs investigated in enhancing light-mediated translation (28). However, it is intriguing that in the present study, overexpression of SSU far beyond the wild-type levels was not observed. Continuous light had no enhancing effect on SSU levels after 1 day of exposure, and the levels actually dropped on the third and fifth days. Although the transcript levels remained very high, protein levels were not increased further. Lack of correlation between transcript levels and translation was evident also in an earlier study in which similar transcript levels of chloroplast-engineered human serum albumin resulted in a higher protein level during light but lower protein level under dark (19). Additionally, continued light exposure results in the activation of proteases in the chloroplast that may have contributed to the diminished amounts of SSU (26). It has been shown that the expression of *Rbc*S and *rbc*L is coordinated by adjustments of subunit stoichiometries in response to the abundance of unassembled subunits (13, 29). The question that arises then is: Do the *rbc*L transcripts (10,000 copies in the large single-copy region) and LSU levels become limiting for SSU assembly?

The presence of abundant SSU in the chloroplast transgenic lines indicates that posttranslational cleavage of its transit peptide and assembly into the holoenzyme was achieved successfully inside the chloroplast. In light of this observation, most of the arguments put forth in an earlier, unsuccessful study regarding inefficient posttranslational modification of chloroplast-synthesized SSU (11) have been overcome in our investigations. Expression of foreign proteins from the transcriptionally active *trn*I-*trn*A intergenic region has resulted in the highest level of foreign protein accumulation ever reported in plants, i.e., 46.1% (30). Also, proteins ranging from 11 to 83 kDa in size have been expressed successfully (31, 32) at very high levels. The *trn*I-*trn*A intergenic region has also allowed for single-step multigene engineering for insect resistance and phytoremediation (30, 33, 34). Successful engineering of 11 biopharmaceutical proteins, vaccine antigens, and 6 agronomic traits (for herbicide, insect, disease resistance, drought/salt tolerance, phytoremediation, etc) at this intergenic site make it a preferred site for chloroplast genetic engineering in general (18, 35).

Plant Growth and Photosynthetic CO_2 Assimilation Rate. The nuclear *Rbc*S antisense plants are easily distinguishable from wild-type

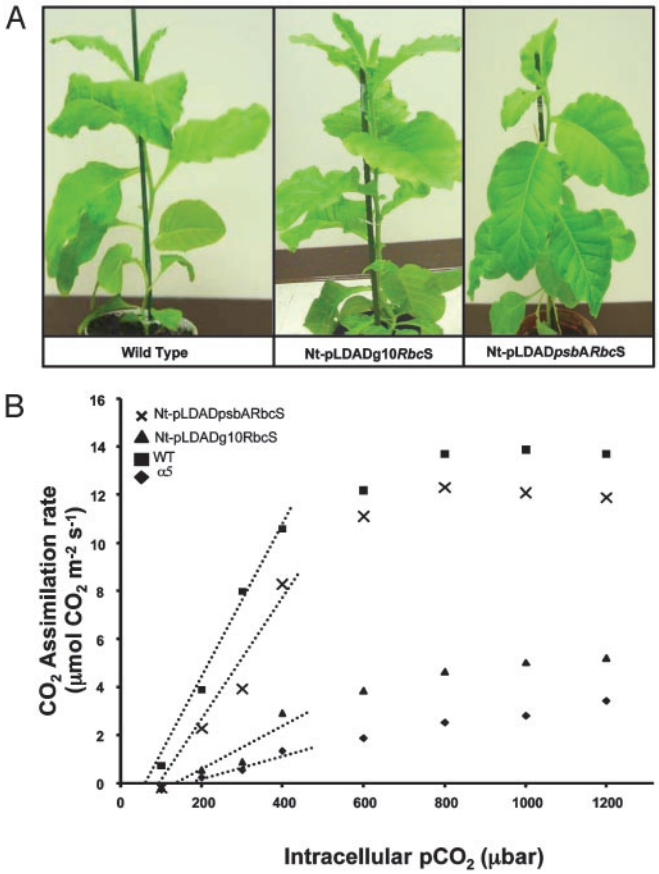

Fig. 5. (*A*) Comparative growth of wild-type, Nt-pLDADg10*Rbc*S, and Nt-pLDAD*psb*A*Rbc*S plants 29 days after transfer to soil in pots. (*B*) Photosynthetic CO_2 assimilation rates under 21% O_2. The calculated (dotted lines) initial slopes of CO_2 assimilation (μmol CO_2 m^{-2} s^{-1} μbar^{-1}) were: α5, 0.0051; Nt-pLDADg10*Rbc*S, 0.0096; Nt-pLDAD*psb*A*Rbc*S, 0.0271; and wild type, 0.0336. These data were determined from linear regressions of the data from 100–400 μbar CO_2.

plants, because they have retarded growth and delayed flowering (14). Expression of a promoterless gene 10 5′-UTR-regulated *Rbc*S cDNA in the chloroplasts of nuclear *Rbc*S antisense plant (Nt-pLDADg10*Rbc*S line) resulted in a phenotype that was similar to the wild-type plants under the growth conditions used. However, these plants started flowering ≈27–35 days earlier than the wild-type plants (data not shown) for reasons that remain to be determined. A similar observation was made for five different Nt-pLDADg10*Rbc*S transgenic lines. Comparison between the 29-day-old (after transfer to soil) Nt-pLDADg10*Rbc*S line and wild-type plant is shown in Fig. 5*A*. Expression of *psb*A 5′-UTR-regulated *Rbc*S cDNA in the Nt-pLDAD-*psb*A*Rbc*S plants also resulted in the restoration of normal growth and flowering time.

Young, fully expanded leaves were subjected to gas-exchange measurements for determining photosynthetic CO_2 assimilation. Photosynthesis of the Nt-pLDAD*psb*A*Rbc*S plants was nearly restored to the wild-type level (Fig. 5*B*), indicating increased Rubisco activity in the chloroplast transgenic line derived from nuclear *Rbc*S antisense plant. The initial slope of the CO_2 response curve, which is determined by the amount and activity of Rubisco, in the Nt-pLDAD*psb*A*Rbc*S line, is comparable with the wild type. Photosynthesis of the Nt-pLDADg10*Rbc*S line was not restored completely, but it was ≈2-fold greater than that of the nuclear *Rbc*S antisense plant as determined by comparison of the initial slope of CO_2 assimilation.

Conclusions

Successful complementation of the SSU deficiency in a nuclear *Rbc*S antisense line by chloroplast-derived SSU opens avenues for additional research in this field, which has been seriously limited by our inability to engineer *Rbc*S genes via the chloroplast genome. Genetic engineering of Rubisco has been complicated by the separate location of its genes, mechanisms that integrate gene expression in the chloroplast and nucleus, and the low catalytic ability of hybrid Rubisco proteins obtained previously with chloroplast or nuclear transformation. Existing eukaryotic Rubiscos with kinetic properties that can result in greater photosynthesis if introduced into C3 crop plants are being identified by biochemical analysis and improved modeling of photosynthesis, which takes into account the crop canopy structure (36). However, demonstrating that a foreign Rubisco will undergo possibly essential posttranslational modifications and whether it can be properly assembled and expressed at a high level in a foreign host has not been feasible yet. The ability to express the SSU gene successfully from the chloroplast genome, as we have demonstrated here, will facilitate the expression and analysis of foreign Rubisco genes, because only chloroplast transformation will be required and both genes can be engineered simultaneously in a single compartment. Then, the ability of a selected foreign Rubisco to increase photosynthesis and crop productivity can be evaluated further *in planta*. Use of a nuclear *rbc*S antisense line to hyperexpress mutant or foreign *Rbc*S genes in the chloroplast genome also can serve as a model system to study the dynamics of Rubisco assembly. For example, continued study of the transformed plants created here should provide additional insight into the mechanisms controlling Rubisco expression, because nuclear control via the regulation of SSU expression has been short-circuited. Specifically, consequences of the physical relocation of the *Rbc*S gene into its preevolutionary state on the processing of SSU and effect of light and developmental cues on the expression of *Rbc*S gene can be elucidated. It will be vital to determine whether nuclear control of Rubisco expression is necessary for a proper response to external signals in the environment and thus maximal growth. Consequently, the approach and methods developed here should greatly facilitate future Rubisco engineering to achieve the long-cherished goal of increasing plant productivity.

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1. Ogren, W. L. (2003) *Photosynth. Res.* **76,** 53–63.
2. Spreitzer, R. J. & Salvucci, M. E. (2002) *Annu. Rev. Plant Biol.* **53,** 449–475.
3. Houtz, R. L. & Portis, A. R. (2003) *Arch. Biochem. Biophys.* **414,** 150–158.
4. Kanevski, I. & Maliga, P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1969–1973.
5. Madgwick, P. J., Colliver, S. P., Banks, F. M., Habash, D. Z., Dulieu, H., Parry, M. A. J. & Paul, M. J. (2002) *Ann. Appl. Biol.* **140,** 13–19.
6. Whitney, S. M., Von Caemmerer, S., Hudson, G. S. & Andrews, T. J. (1999) *Plant Physiol.* **121,** 579–588.
7. Kanevski, I., Maliga, P., Rhoades, D. F. & Gutteridge, S. (1999) *Plant Physiol.* **119,** 133–141.
8. Getzoff, T. P., Zhu, G. H., Bohnert, H. J. & Jensen, R. G. (1998) *Plant Physiol.* **116,** 695–702.
9. Whitney, S. M., Baldett, P., Hudson, G. S. & Andrews, T. J. (2001) *Plant J.* **26,** 535–547.
10. Whitney, S. M. & Andrews, T. J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 14738–14743.
11. Whitney, S. M. & Andrews, T. J. (2001) *Plant Cell* **13,** 193–205.
12. Rodermel, S. R., Abbott, M. S. & Bogorad, L. (1988) *Cell* **55,** 673–681.
13. Jiang, C. Z. & Rodermel, S. R. (1995) *Plant Physiol.* **107,** 215–224.
14. Tsai, C. H., Miller, A., Spalding, M. & Rodermel, S. (1997) *Plant Physiol.* **115,** 907–914.
15. Zhang, X. H., Ewy, R. G., Widholm, J. M. & Portis, A. R. (2002) *Plant Cell Physiol.* **43,** 1302–1313.
16. Guda, C., Lee, S. B. & Daniell, H. (2000) *Plant Cell Rep.* **19,** 257–262.
17. Daniell, H., Datta, R., Varma, S., Gray, S. & Lee, S. B. (1998) *Nat. Biotechnol.* **16,** 345–348.
18. Daniell, H., Ruiz, O. N. & Dhingra, A. (2004) *Methods Mol. Biol.* **286,** 111–137.
19. Fernández-San Millán, A., Mingo-Castel, A., Miller, M. & Daniell, H. (2003) *Plant Biotechnol. J.* **1,** 71–79.
20. Eckardt, N. A., Snyder, G. W., Portis, A. R. & Ogren, W. L. (1997) *Plant Physiol.* **113,** 575–586.
21. Stern, D. B., Higgs, D. C. & Yang, J. J. (1997) *Trends Plant Sci.* **2,** 308–315.
22. Staub, J. M., Garcia, B., Graves, J., Hajdukiewicz, P. T. J., Hunter, P., Nehra, N., Paradkar, V., Schlittler, M., Carroll, J. A., Spatola, L., *et al.* (2000) *Nat. Biotechnol.* **18,** 333–338.
23. Kim, J., Christopher, D. A. & Mullet, J. E. (1993) *Plant Mol. Biol.* **22,** 447–463.
24. Tobin, E. M. & Silverthorne, J. (1985) *Annu. Rev. Plant Physiol.* **36,** 569–593.
25. Lee, S. B., Kwon, H. B., Kwon, S. J., Park, S. C., Jeong, M. J., Han, S. E., Byun, M. O. & Daniell, H. (2003) *Mol. Breed.* **11,** 1–13.
26. Zheng, B., Halperin, T., Hruskova-Heidingsfeldova, O., Adam, Z. & Clarke, A. K. (2002) *Physiol. Plant.* **114,** 92–101.
27. Staub, J. M. & Maliga, P. (1994) *Plant J.* **6,** 547–553.
28. Eibl, C., Zou, Z. R., Beck, A., Kim, M., Mullet, J. & Koop, H. U. (1999) *Plant J.* **19,** 333–345.
29. Rodermel, S. (1999) *Photosynth. Res.* **59,** 105–123.
30. DeCosa, B., Moar, W., Lee, S. B., Miller, M. & Daniell, H. (2001) *Nat. Biotechnol.* **19,** 71–74.
31. Daniell, H., Lee, S. B., Panchal, T. & Wiebe, P. O. (2001) *J. Mol. Biol.* **311,** 1001–1009.
32. Daniell, H., Watson, J., Koya, V. & Leppla, S. H. (2004) *Vaccine*, in press.
33. Ruiz, O., Hussein, H., Terry, N. & Daniell, H. (2003) *Plant Physiol.* **132,** 1344–1352.
34. Daniell, H. & Dhingra, A. (2002) *Curr. Opin. Biotechnol.* **13,** 136–141.
35. Devine, A. L. & Daniell, H. (2004) in *Plastids*, Annual Plant Reviews, ed. Moller, S. (Blackwell, Oxford), Vol. 13, Chap. 10, in press.
36. Zhu, X.-G., Portis, A. R., Jr., & Long, S. P. (2004) *Plant Cell Environ.* **27,** 155–165.