

Plastome-encoded bacterial ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) supports photosynthesis and growth in tobacco

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Edited by George H. Lorimer, University of Maryland, College Park, MD, and approved October 18, 2001 (received for review August 8, 2001)

The efficiency with which crop plants use their resources of light, water, and fertilizer nitrogen could be enhanced by replacing their CO₂-fixing enzyme, D-ribulose-1,5-bisphosphate carboxylase-oxygenase (RubisCO), with more efficient forms, such as those found in some algae, for example. This important challenge has been frustrated by failure of all previous attempts to substitute a fully functional, foreign RubisCO (efficient or inefficient) into higher plants. This failure could be caused by incompatibility between the plastid-encoded large subunits and the nucleus-encoded small subunits or by inability of the foreign RubisCO subunits to fold or assemble efficiently in the plastid. Mismatch between the regulatory requirements of the foreign RubisCO and conditions in the chloroplast also might render the substituted enzyme inactive but, previously, it has not been possible to test this. To answer the general question of whether a foreign RubisCO can support photosynthesis in a plant, we used plastid transformation to replace RubisCO in tobacco with the simple homodimeric form of the enzyme from the α -proteobacterium, *Rhodospirillum rubrum*, which has no small subunits and no special assembly requirements. The transplastomic plants so obtained are fully autotrophic and reproductive but require CO₂ supplementation, consistent with the kinetic properties of the bacterial RubisCO. This establishes that the activity of a RubisCO from a very different phylogeny can be integrated into chloroplast photosynthetic metabolism without prohibitive problems.

The productivity of plants is governed by the efficiency with which they use their resources of light, water, and fertilizer N, and this depends to a considerable degree on the effectiveness of their CO₂-fixing enzyme, D-ribulose-1,5-bisphosphate carboxylase-oxygenase (RubisCO) (1). RubisCO is a very slow catalyst, turning over only a few times each second even when saturated by its substrates, and its performance is compromised further by its innate tendency to confuse its substrate, CO₂, with O₂, which is much more abundant (2–6). There is natural variation in RubisCO's kinetic properties (7), and the higher-plant enzyme is by no means the most efficient. RubisCOs from some non-green algae, in particular, have higher specificities for CO₂, compared with O₂, than the higher-plant enzyme (8, 9). In at least one case, this occurs in combination with other kinetic parameters that would allow faster rates of photosynthetic CO₂ fixation at current and likely future atmospheric CO₂ partial pressures, without additional inputs of energy or other resources (10). These observations provide impetus for attempts to substitute RubisCO in higher plants with homologs from other sources.

There are two major types of RubisCO, deeply divided phylogenetically. Form I, found in plants, algae (except certain dinoflagellates), and some bacteria, is a hexadecameric protein composed of eight 50- to 55-kDa large subunits that bear the catalytic sites and eight 12- to 18-kDa small subunits. Form II is restricted to some bacteria and dinoflagellates and has only large subunits in differing degrees of oligomerization with no small subunits (6). Within form I RubisCOs, there is a further, deep divergence between the “green” subclass found in bacteria, cyanobacteria, green algae, and higher plants and the “red”

subclass found in bacteria and non-green algae (4, 11). In higher plants and green algae, the large subunit of the “green,” form I RubisCO is encoded by the plastomic *rbcL* gene, whereas the small subunits are nucleus-encoded. On the other hand, the *rbcL* and *rbcS* genes of the “red,” form I RubisCO are found together in an operon in the chromosomes (and resident plasmids) of prokaryotes and the plastomes of non-green algae (4, 11, 12).

Attempts to introduce foreign RubisCOs into higher-plant plastids by plastid transformation must contend with the nuclear location of the *RbcS* gene family. Replacement of the plastomic *rbcL* gene of tobacco with homologs from sunflower or the cyanobacterium, *Synechococcus* PCC 6301, did not result in hybrid hexadecameric RubisCOs capable of supporting photosynthetic growth. In the former case, the hybrid enzyme produced was crippled by mismatch between the large and small subunits. In the latter case, foreign, large subunits were not produced in detectable quantities, either because the cyanobacterial large subunits were not synthesized or folded efficiently or because mismatch between them and the cytoplasmically derived tobacco small subunits impaired assembly of the hexadecamer, leading to degradation of the unassembled subunits (13). A tobacco *RbcS* gene relocated from the nucleus to the plastome was capable of directing synthesis of small subunits in the plastid, and these assembled into RubisCO. However, despite strong transcription of the relocated gene, the plastid-synthesized small subunits were not abundant, suggesting that translation of the transcript or access of the product to the RubisCO assembly pathway were not efficient (14). Insertion of the *rbcLS* operons of two non-green algae into the inverted-repeat regions of the tobacco plastome, without disturbing the tobacco *rbcL* gene in the large, single-copy region, led to abundant production of both large and small subunits of the foreign enzymes. However, the subunits were insoluble and no functional foreign RubisCO was assembled, presumably because their folding or assembly requirements were not satisfied in the plant plastid (10).

To circumvent these difficulties and determine whether a foreign RubisCO could, in principle, support higher-plant photosynthesis, we chose to replace tobacco RubisCO with its simplest known homolog. The form II RubisCO from *Rhodospirillum rubrum* is a dimer of large subunits without small subunits, and it is synthesized abundantly in properly assembled and fully functional form in both *Escherichia coli* (15) and a cyanobacterium (16). *R. rubrum* is a photosynthetic anaerobe, and the kinetic properties of its RubisCO are quite unsuited to higher plants. Compared with the C-3 plant enzyme, its K_m for CO₂ is more than 5-fold higher and, most importantly, its CO₂/O₂

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: RubisCO, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; CPBP, 2'-carboxypentitol-1,5-bisphosphate; 5' UTR, 5' untranslated region.

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specificity is 7-fold poorer (7, 17, 18). Nevertheless, its substrate-saturated activity is high (17), and we reasoned that its unfavorable attributes might be complemented by CO₂ enrichment, thus allowing the central question about the feasibility of integrating the activity of a very different RubisCO into chloroplast metabolism to be answered.

Methods

Construction of Plastid-Transforming Plasmid. The transforming plasmid used in this study, pRubLev14, contained the promoter, 5' untranslated region (5' UTR) and 42 5' coding nucleotides of the tobacco plastid *rbcL* gene fused to a bicistronic operon composed of the coding region of the *rbcM* gene of *R. rubrum* linked by a synthetic sequence incorporating a ribosome-binding site to the *aadA* selectable-marker gene and a 3' terminator element derived from the *rps* 16 gene of the tobacco plastid. This operon was flanked by sequences derived from the tobacco plastome adjacent to *rbcL* (19) that targeted the operon to replace *rbcL* and its terminator sequence in the large, single-copy region of the tobacco plastome (Fig. 1).

The pRubLev14 plasmid was derived from the *rbcL*-targeted transforming plasmid used previously, pLEV1 (20). The 1,752-bp *Sac*II-*Bcl*II fragment containing parts of the *rbcL* and *aadA* coding sequences was replaced with a 223-bp *Sac*II-*Bcl*II fragment from the 383-bp product amplified from pLEV1 by using primers ScXhaadA (5'-TTTCCGCGGCTCGAGTTGTAGGGAGGATTTATG-3', *Sac*II and *Xho*I sites underlined, *aadA* initiator codon in bold) and AADr (20). The resulting plasmid, pΔTobrbcL, contained a reconstituted *aadA* gene with restriction sites appropriate for inserting the *rbcM* gene upstream.

The *rbcM* gene was amplified from pRR1 (17) by using primers RUB2 (5'-GTCCACATATGGACCAGTCATC-3', *Nde*I site underlined, *rbcM* initiator codon in bold) and RUB3 (5'-GGTGGCAAGCTTTTACGCCGGAAGG-3', *Hind*III site underlined, complement of *rbcM* terminator codon in bold). The 1,407-bp *Nde*I-*Hind*III fragment was ligated into pET28a(+) to give pRrHis. The 1,482-bp *Nco*I-*Xho*I fragment from pRrHis was inserted into pΔTobrbcL to give plasmid pΔRub. Use of the *Nco*I site for this insertion truncated the *rbcL* 5' UTR and incorporated 53 bp of unwanted sequence derived from pET28a(+). Therefore, sequence spanning the promoter, 5' UTR, and the 5' 42 coding nucleotides of tobacco *rbcL* was amplified from pLEV1 with primers TrbcLClA (5'-CAGCATATCGATTTATGCCTAGCC-3', *ClA*I site underlined) and NdeHis15 (5'-CCATATGTTGAATCCAACACTTGCTTTA-3', *Nde*I site underlined), and the 463-bp *ClA*I-*Nde*I fragment was used to replace the 319-bp *ClA*I-*Nde*I fragment in pΔRub. In the resulting transforming plasmid, pRubLev14, 42 bp of the *rbcL* 5' coding sequence plus a further histidine codon derived from the *Nde*I site were fused in-frame to the 5' coding region of the *rbcM* gene.

Plastid Transformation. Biolistic delivery and homologous recombination of this sequence into the tobacco (*Nicotiana tabacum* L. cv Petit Havana [*N*,*N*]) plastome and subsequent selection and regeneration of transformants in tissue culture were carried out as described (21) in air containing 5% (vol/vol) CO₂.

Plant Growth. Transformed plants and nontransformed controls were germinated and grown in 5-liter pots of soil in an air-conditioned cabinet as described (20) with artificial illumination (200 μmol quanta m⁻²s⁻¹) in an atmosphere of 2.5% (vol/vol) CO₂ in air. For growth analyses, the height of the plants and their number of leaves were recorded every 2–4 days until floral buds appeared. To remove progressively any unwanted nuclear mutations that might have been induced during tissue culture,

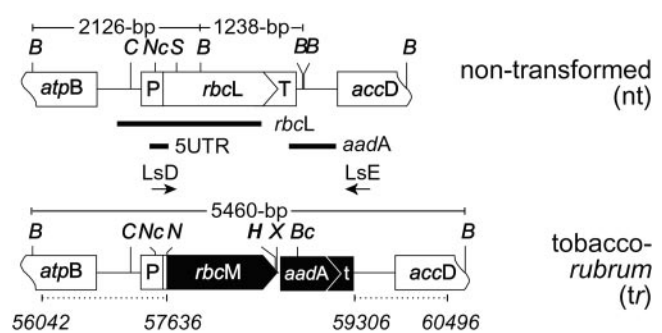


Fig. 1. Homologous replacement of the *rbcL* gene in the tobacco plastome with an operon containing the *R. rubrum* *rbcM* gene linked to the *aadA* selectable-marker gene. The organization of the nontransformed tobacco plastome in the region of *rbcL* (nontransformed, Upper) and the analogous region of the plastome after transformation with plasmid, pRubLev14 (tobacco-rubrum, Lower). The dashed lines and numbering (19) indicate the plastome-derived flanking sequences incorporated into pRubLev14 to direct the replacement of tobacco *rbcL* with a 2,404-bp *rbcM*-*aadA* operon (solid shapes). The annealing positions of the *rbcL*, 5'UTR, and *aadA* probes and primers LsD and LsE are indicated, together with the *Bam*HI (*B*) sites and the sizes of the *Bam*HI fragments that hybridize to the *rbcL* probe. *Bc*, *Bcl*I; *C*, *ClA*I; *H*, *Hind*III; *N*, *Nde*I; *Nc*, *Nco*I; *S*, *Sac*II; *X*, *Xho*I; *P*, *rbcL* promoter plus 5' UTR; *T*, *rbcL* terminator sequence; *t*, *rps*16 terminator sequence.

flowers of the transformants were pollinated artificially with nontransformed pollen at each generation.

Expression of pRubLev14 in *E. coli*. The plasmid pRubLev14 was transformed into *E. coli* XL1 Blue (Novagen) and grown in Luria-Bertani medium containing 200 μg·ml⁻¹ ampicillin and 250 μg·ml⁻¹ spectinomycin. Constitutive expression of *R. rubrum* RubisCO was so strong that the cells grew very slowly, tending to select for disablement of *rbcM* expression by transposon insertion (see Results). Such disablement was minimized by reducing the transformation and growth temperatures to 22°C. *E. coli*-synthesized *R. rubrum* RubisCO, used for validating the procedure for measuring the carbamylation status of the enzyme in leaves of the tobacco transformants (see below), was partially purified as follows. The cells were disrupted in 50 mM Hepes-NaOH buffer, pH 7.8, containing 5 mM DTT, 0.5 mM EDTA, and 0.5 mM PMSF, by passage through an ice-cold French press at 140 MPa. Insoluble material was removed by centrifugation (35,000 × *g*, 15 min, 4°C), and MgSO₄ was added to the supernatant solution to 20 mM before fractionation with polyethylene glycol (*M*_r 3,350; Sigma) to collect the protein precipitating between 10% and 20% (wt/vol) polyethylene glycol. This fraction was dissolved in 50 mM Hepes-NaOH buffer, pH 7.8, containing 0.5 mM EDTA, and 15% (vol/vol) glycerol and stored at -80°C.

DNA and RNA Blots. Total leaf DNA and RNA was extracted, electrophoresed, and blotted as described (14, 20). Blots were probed with alkaline-phosphatase-conjugated DNA probes (AlkPhos Direct labeling kit; APBiotech, Sydney) and visualized by using the AttoPhos reagent (Promega) with a Vistra Fluorimager (10). Densitometry was carried out with computer-generated images by using IMAGEQUANT software. For DNA blots, the 1,856-bp *rbcL* DNA probe (Fig. 1) was amplified from pLEV1 by using primers Usr (5'-CTCAAGTGGATGAATCAGAATC-3') and Lsb (5'-GGCACCTGGCGCATTACCC-3'). For RNA blots, the 228-bp 5'UTR and 814-bp *aadA* DNA probes (Fig. 1) were amplified from pRubLev14 by using the primer pairs NdeHis15/*rbcL*5'UTR (5'-TATTTGGCAAATCAAATACCATG-3') and *aadA*5 (5'-GAGGGATCCATGGCAGAAGC-3')/*aadA*3 (5'-CTCTATCTAGACATTATTTGCCG-3'), respectively.

PCR Analysis. Chloroplasts were purified from leaves (22) from plants grown aseptically on RMOP medium containing sucrose (21). DNA was extracted from chloroplasts the same way as for leaves (20) and used as template for PCR amplification with primers LsD (20) and LsE (5'-GAGGTGTGATACTTGGCT-TGATTC-3'). The single, 2,590-bp products amplified from transformants *tr1* and *tr3* were cloned into pGEM-T Easy (Promega) and sequenced (BigDye terminator cycle; Applied Biosystems) to confirm that no unwanted nucleotide changes had been introduced by the manipulations.

Immunoblots. Leaf protein was extracted from transformed and nontransformed leaves, separated on Bis-Tris-buffered 4–12% NuPAGE SDS gels (NOVEX, San Diego), blotted onto nitrocellulose, and probed with antibodies, and the immunoreactive polypeptides were visualized by using the AttoPhos reagent (Promega) as described (10). Antisera against spinach RubisCO, *R. rubrum* RubisCO, and spinach RubisCO activase (with an N-terminal glutathione *S*-transferase fusion) were raised in rabbits and used at 1:5,000 dilution.

Measurement of mRNA, Protein, and Content and Carbamylation Status of RubisCO Active Sites. Samples for these measurements were taken from the fifth leaf below the apical meristem of 37-day-old, nontransformed and 55-day-old, T₁ generation *tr1* transformants grown in 2.5% (vol/vol) CO₂ as described above. At these times, the plants were of similar physiological age (similar height and number of leaves; Fig. 4). The fifth leaf was the youngest mature leaf, and its size (16–17 cm wide) and distance from the apical meristem (27–35 cm) were similar in transformed and nontransformed plants.

The *rbcL* and *rbcM* mRNA contents were measured from RNA blots of 10 μg of total leaf RNA probed with the 5UTR probe (see above).

The content of RubisCO active sites and their carbamylation status were measured by binding of 2'-carboxypentitol-1,5-bisphosphate ([¹⁴C]CPBP), using a previously described procedure (20, 23, 24) modified to accommodate the CPBP-binding characteristics of both tobacco and *R. rubrum* RubisCOs. Leaf discs were punched from plants in the high-CO₂ growth cabinet near the middle of the photoperiod and frozen immediately in liquid N₂. The frozen discs were extracted rapidly in ice-cold glass homogenizers in CO₂-free 50 mM Hepes-NaOH buffer, pH 7.8, containing 1 mM EDTA, 10 mM DTT, 1% (wt/vol) polyvinylpyrrolidone, and 1 mM PMSF. The extract was centrifuged for 60 s at 13,000 × *g* at 4°C. Within 90 s of extraction, [¹⁴C]CPBP [10⁵ cpm·nmol⁻¹, prepared as described (25)] was added to a final concentration of 20 μM to one sample of the extract supernatant (sample A), and the mixture was stored on ice for 30–60 min before addition of [¹²C]CPBP to 1 mM. NaHCO₃ and MgCl₂ were added to a second sample of the supernatant (sample B) to final concentrations of 20 mM, followed by [¹⁴C]CPBP to 20 μM after 10 min of incubation at room temperature. Protein-bound ¹⁴C in both samples was separated from unbound ¹⁴C by gel filtration through 0.7 × 27-cm columns of Sephadex G-50 fine, equilibrated with 20 mM Hepes-NaOH buffer, pH 8, containing 75 mM NaCl, and measured by scintillation counting. Sample A was gel-filtered 5 min after addition of [¹²C]CPBP. The total concentration of RubisCO active sites was estimated from the bound ¹⁴C in sample B and the fraction carbamylated from the ratio between sample A and sample B.

Total protein in the extract supernatants was measured with a dye-binding assay (Pierce Coomassie Plus kit).

N-terminal Protein Sequencing. Equal amounts (5 pmol as judged by CPBP binding) of tobacco RubisCO large subunits from nontransformed plants, *R. rubrum* RubisCO from tobacco-*rubrum* plants,

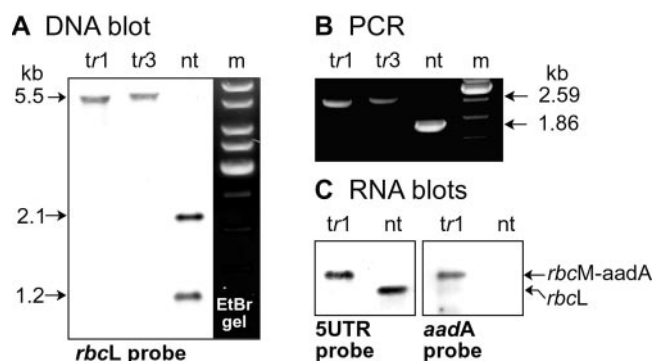


Fig. 2. Genetic and RNA-transcript analyses of nontransformed and tobacco-*rubrum* (T₀ generation) plants. (A) DNA blot of *Bam*HI-digested DNA from two tobacco-*rubrum* transformants (*tr1* and *tr3*) and a nontransformed control plant (*nt*) probed with the *rbcL* DNA probe. *m*, 1-kb DNA ladder (MBI Fermentas, ethidium bromide [EtBr] stain). (B) Agarose-gel electrophoresis (EtBr stain) of products of PCR amplification of chloroplast DNA by using primers LsD and LsE. (C) Blots of total leaf RNA from *tr1* (10 μg) and *nt* (5 μg) plants probed with the 5UTR and *aadA* probes.

and *R. rubrum* RubisCO from *E. coli* expressing pRubLev14 were separated by SDS/PAGE (see above) and subjected to N-terminal amino acid sequencing as described (14).

Leaf Gas Exchange. Photosynthetic gas exchange by leaves of a 31-day-old, nontransformed plant and 46-day-old tobacco-*rubrum* transformants was measured after transfer to the laboratory by using a Li-Cor flowthrough photosynthesis system (LI-6400; Li-Cor, Lincoln, NE) at 600 μmol quanta m⁻²s⁻¹ illumination and a leaf temperature of 25°C (20).

Results

The Plastid-Transforming Plasmid, pRubLev14, Is Very Active in *E. coli*.

As expected, the tobacco *rbcL* promoter was constitutively active in *E. coli*. In combination with the 5' UTR and the 5' 14 codons of the coding region of tobacco *rbcL*, it directed so much synthesis of recombinant *R. rubrum* RubisCO that the cells grew very slowly unless the chimeric *rbcL/M* gene became disabled. Plasmid isolated from colonies grown at 37°C frequently contained *E. coli* transposon sequences inserted in the 5' UTR of *rbcL*. Transposon-free plasmid could be obtained by reducing the transformation and growth temperatures to 22°C. Under these conditions, *R. rubrum* RubisCO (measured by CPBP binding, see *Methods*) constituted 40–50% of the soluble protein in cell extracts. Microsequencing (see *Methods*) revealed Ser-Pro-Gln-Thr-Glu-Thr as the N-terminal hexapeptide, consistent with the nucleotide sequence of the 5' extension fused to the *rbcM* gene and with cleavage of the N-terminal Met residue according to the general rules for N-terminal modifications in *E. coli* (26).

Plastid Transformation. The tobacco plastome was transformed by using pRubLev14, and two independent, homoplasmic transformants (*tr1* and *tr3*) were obtained after several rounds of regeneration on spectinomycin-containing medium. No trace of the uninterrupted wild-type plastomic sequence could be detected by either DNA-blot (Fig. 2A) or PCR analysis (Fig. 2B). RNA blots of *tr1* total RNA probed with the 5UTR probe (Fig. 1) detected a larger mRNA transcript than that present in the nontransformed control, consistent with the expected bicistronic size (Fig. 2C). No trace of a smaller transcript that might have indicated a monocistronic product of the *rbcM* transgene or continued persistence of the nontransformed plastome was apparent. As expected, the larger transcript was also detected by

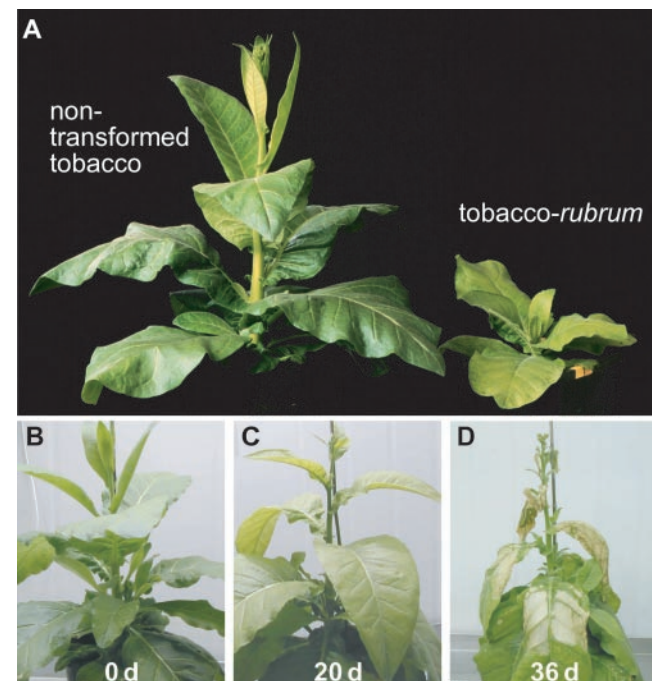


Fig. 3. Growth of nontransformed and transformed plants in an atmosphere of 2.5% (vol/vol) CO₂ in air and deterioration of transformed plants after transfer to unenriched air (0.036% [vol/vol] CO₂). (A) A nontransformed plant and a T₁-generation tobacco-*rubrum* transformant (tr1) 37 days after emergence of the cotyledons. (B–D) Demise of a 55-day-old tr1 transformant after growth in high CO₂ for 55 days before transfer to air without CO₂ enrichment for the further periods shown.

a probe that hybridized with the *aadA* sequence. The DNA inserted into the plastomes of the transformants was fully sequenced (see *Methods*). Transformants tr1 and tr3 yielded identical sequences, confirming that these independent transformants have identical plastomes.

Tobacco-*rubrum* Requires CO₂ Enrichment. The tobacco-*rubrum* transformants were unable to grow beyond the cotyledon stage without CO₂ enrichment. However, when the atmosphere was supplemented with 2.5% (vol/vol) CO₂, they grew, flowered, and set viable seed. The plants appeared normal (Fig. 3A) but grew more slowly than the nontransformed controls under the same conditions, although they eventually attained the same height (Fig. 4A) and the same number of leaves (Fig. 4B). When tobacco-*rubrum* plants that had been grown at high CO₂ to the point of flowering (Fig. 3B) were transferred to unenriched air,

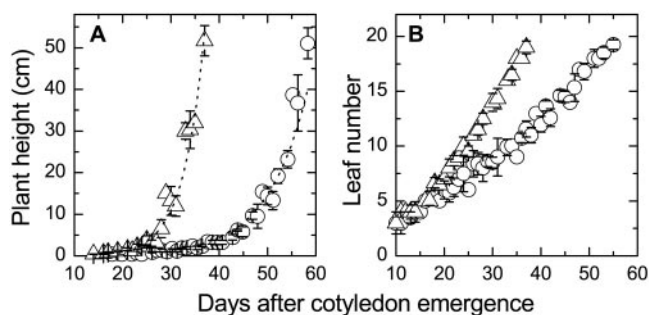


Fig. 4. Plant height (A) and leaf number (B) (mean \pm SE) during growth of nontransformed (Δ , $n = 6$) and tobacco-*rubrum* (tr1) plants (\circ , $n = 14$) plants in 2.5% (vol/vol) CO₂ in air.

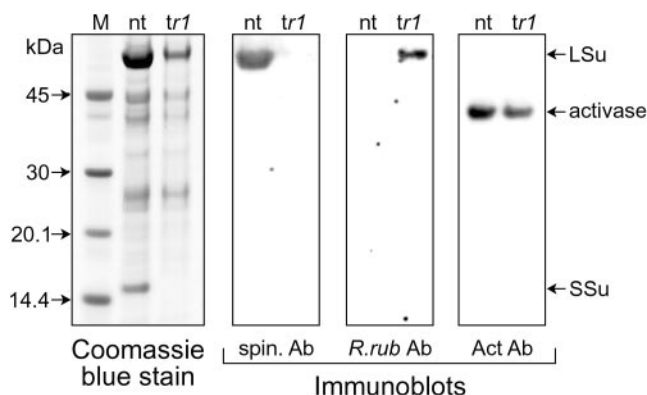


Fig. 5. Immunoblot analysis of RubisCO and RubisCO activase content of a nontransformed and a T₁-generation tobacco-*rubrum* plant grown in an atmosphere of 2.5% (vol/vol) CO₂ in air. Total protein was extracted from 1.1 mm² of young, mature leaves of a tobacco-*rubrum* plant (tr1, 2.3 μ g) and a nontransformed plant (nt, 4.2 μ g) and separated by SDS/PAGE. Part of the gel was stained with Coomassie blue, and replicate lanes were blotted and probed with antibodies raised against spinach RubisCO (spin Ab), *R. rubrum* RubisCO (*R. rub* Ab), and spinach RubisCO activase (Act Ab). M, molecular weight markers with molecular sizes indicated.

they ceased growing and developing immediately and the leaves became pale-green (Fig. 3C). After persisting in this condition for several weeks, severe chlorosis and leaf senescence eventually ensued (Fig. 3D).

Contents of RubisCO and RubisCO Activase. Leaf extracts of the tobacco-*rubrum* transformants contained no higher-plant RubisCO detectable by antibodies raised against the spinach enzyme, but a strong signal was observed with antibodies raised against the *R. rubrum* enzyme; leaf extracts of the nontransformed control displayed the reciprocal result (Fig. 5). Nearly all (>98%) of the *R. rubrum* RubisCO in the transformants was in the soluble fraction of the extracts (not shown). The transformants lacked RubisCO small subunits (Fig. 5), indicating that, as expected, the bacterial RubisCO did not bind the cytoplasmically sourced small subunits and protect them from degradation. RubisCO activase, the protein that regulates higher-plant RubisCO activity by facilitating release of inhibitory ligands (27–29), was present in similar amounts in both nontransformed and transformant leaves (Fig. 5). The higher-plant activase shows considerable species specificity for its cognate RubisCO (30) and is not likely to interact with the bacterial RubisCO. Therefore, we conclude that lack of a cognate RubisCO does not impair the synthesis of RubisCO activase.

Young, mature leaves of the transformants contained approximately one-third as much bacterial RubisCO as the nontransformed control's content of tobacco RubisCO (Fig. 6B). This reduction in RubisCO content was due to, in part, reduced abundance of the mRNA transcript compared with the nontransformed control (Fig. 6A) and, in part, reduced translation of the transcript.

Regulation of *R. rubrum* RubisCO in Tobacco. To investigate the carbamylation status of *R. rubrum* RubisCO in tobacco-*rubrum*, it was necessary to verify that the procedure for measuring carbamylation developed for higher-plant RubisCO (23, 24) could be applied to *R. rubrum* RubisCO. This was accomplished by showing that, whereas isolated *R. rubrum* RubisCO bound negligible [¹⁴C]CPBP when fully decarbamylated, the ligand bound to 90% of the sites present when nearly fully carbamylated (Fig. 6D). As observed previously (20), growth at high CO₂ concentrations reduced the degree of carbamylation of tobacco

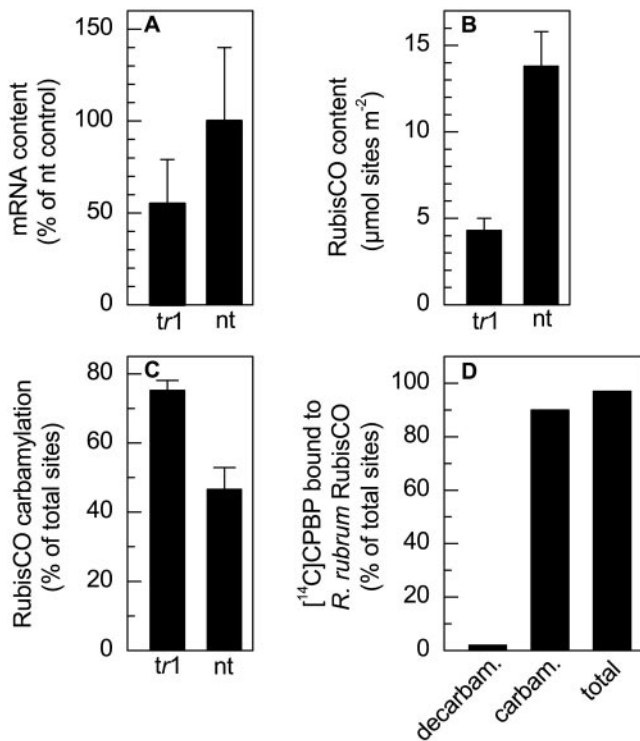


Fig. 6. Abundance of *rbcL* or *rbcM* transcripts and content and carbamylation status of RubisCO active sites in comparable young, mature leaves from nontransformed (nt, $n = 6$) and T₁-generation tobacco-*rubrum* (tr1, $n = 9$) plants of similar physiological age. (A) The amounts of *rbcL* or *rbcM* mRNA expressed as a percentage of *rbcL* mRNA content of the nontransformed plant. The content of RubisCO active sites (B) and their carbamylation status *in vivo* (C) (measured under the high-CO₂ growth conditions). (D) Validation of the [¹⁴C]CPBP-binding/[¹²C]CPBP-exchange procedure for measuring carbamylation with *R. rubrum* RubisCO. The partially purified RubisCO preparation derived from *E. coli* expressing pRubLev14 (see *Methods*) was decarbamylated by dialysis at 4°C for 16 h against CO₂-free 50 mM Hepes-NaOH buffer, pH 8, containing 2 mM EDTA with constant N₂ sparging. Two samples of this preparation were subjected to the [¹⁴C]CPBP-binding/[¹²C]CPBP-exchange procedure for measuring carbamylation in leaf extracts (sample A procedure, see *Methods*). One was used without additions (decarbam.); the other was activated before use by adding MgCl₂ and NaHCO₃ to final concentrations of 20 mM and incubating at room temperature for 10 min (carbam.). Total sites (total) were estimated in the usual manner with a third sample without exchange with [¹²C]CPBP (sample B procedure, see *Methods*).

RubisCO. However, *R. rubrum* RubisCO in tobacco-*rubrum* was nearly fully carbamylated under the same conditions (Fig. 6C).

Stability and N-terminal Modification of *R. rubrum* RubisCO in Tobacco Plastids. Once synthesized, the bacterial RubisCO was moderately stable in tobacco-*rubrum* leaves. As with tobacco RubisCO in nontransformed leaves (10, 14), little turnover could be detected in 3 hours after pulse-labeling with ³⁵S-labeled amino acids (not shown). As a positive control, rapid degradation of the D1 protein of photosystem II was readily detected in both genotypes.

N-terminal microsequencing of RubisCO isolated from nontransformed tobacco (see *Methods*) returned no sequence. This is expected because previous studies (31) showed that the N-terminal residue is *N*-acetyl-Pro, produced by removal of the N-terminal dipeptide predicted from the nucleotide sequence and acetylation of Pro-3. Thus, it is blocked to Edman degradation. RubisCO isolated from tobacco-*rubrum* returned Pro-Gln-Thr-Glu-Thr-Lys- as the N-terminal hexapeptide. This peptide, encoded by codons 3–8 of the 5' *rbcL* coding sequence

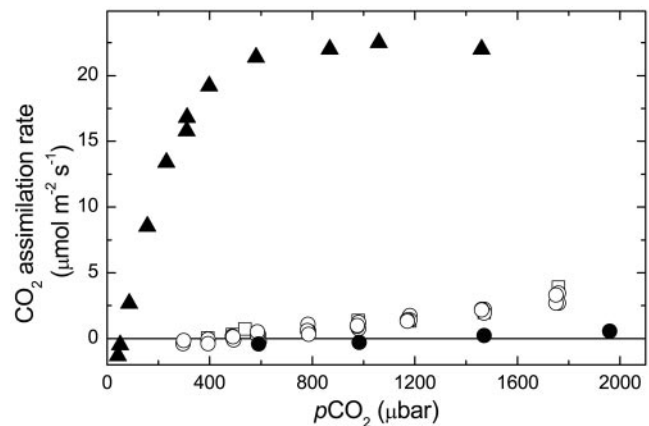


Fig. 7. Photosynthetic gas-exchange measurements at different O₂ partial pressures. The responses of CO₂-assimilation rate to intercellular CO₂ partial pressure (pCO₂) in leaves of nontransformed plants (▲) and tr1 (○, ●) and tr3 (□, ●) transformants. Assimilation was measured at O₂ partial pressures of 19.1 (○, □) and 200 (▲, ●) mbar.

fused to *rbcM*, establishes that removal of the N-terminal dipeptide occurs in the same way as for the tobacco large subunit, but acetylation of the Pro-3 residue so exposed is impaired in the fusion product. However, the areas of the peaks for the derivatives released at each cycle were only ≈20% as large as those observed for a similar quantity of the pRubLev14 product derived from *E. coli* (see above). This suggests that the acetylation step may be impaired only marginally by the succeeding context of the fusion protein. Some 80% of the N-terminally processed fusion protein may be blocked by acetylation in the normal manner.

Photosynthesis in Tobacco-*rubrum* Leaves Displays the Catalytic Properties of *R. rubrum* RubisCO.

The photosynthetic gas-exchange properties of the leaves of tr1 and tr3 transformants (Fig. 7) were similar and consistent with the known kinetic properties of *R. rubrum* RubisCO. They explain the transformants' growth requirement for CO₂ supplementation. Approximately 1,200 μbar CO₂ was required for the transformants to reach CO₂ compensation in air and, even at 2,000 μbar (the highest CO₂ pressure accessible by the CO₂ analyzer), the assimilation rate was still only 3% of that of the nontransformed plant. Substantial stimulation of CO₂ assimilation at low O₂ concentration (Fig. 7) accords with the low CO₂/O₂ specificity of the *R. rubrum* RubisCO (7, 18). The low assimilation rates of the transformants were not the result of stomatal closure. Leaf conductances to water vapor transfer were consistently ≈30% higher in the transformants than in nontransformed plants throughout the measurement range (not shown).

Less precise CO₂-assimilation measurements were carried out at much higher CO₂ concentrations with leaf discs sealed in an illuminated (240 μmol quanta m⁻²s⁻¹) cuvette (25°C) attached to a membrane-inlet mass spectrometer (32). At 2% (vol/vol) CO₂, the tobacco-*rubrum* plants assimilated CO₂ at 50–70% of the rate of the nontransformed controls under the same conditions (not shown). This is consistent with the observed growth rates at high CO₂ (Fig. 4) and with preliminary measurements (according to ref. 33) showing only moderate increases (≈40%) in the pool sizes of D-ribulose-1,5-bisphosphate in the transformants compared with the nontransformed controls under the growth conditions (not shown). Apparently, the 13-residue extension fused to the N terminus of the *R. rubrum* enzyme in our transformation construct has no serious effect on activity, con-

sistent with previous observations with another, longer N-terminal extension (34).

Discussion

Successful growth of tobacco-*rubrum* establishes that no prohibitive problems impede replacement of the RubisCO of the higher-plant chloroplast with a homolog from a widely divergent phylogeny. The folding and assembly requirements of the *R. rubrum* enzyme, unlike those of form I RubisCOs from non-green algae (10), are satisfied in chloroplasts, as they are in *E. coli* (15) and cyanobacteria (16). As shown previously with the “cyano-*rubrum*” strain of the cyanobacterium, *Synechocystis* PCC6803 (16), CO₂ supplementation adequately complements the *R. rubrum* enzyme’s poor affinity and specificity for CO₂, permitting it to support oxygenic photosynthesis.

The tobacco-*rubrum* transformant’s RubisCO content was only one-third of that of the nontransformed control. This is compensated partly at high CO₂ concentrations because the bacterial enzyme’s CO₂-saturated specific activity is approximately double that of tobacco RubisCO. It is possible that the bicistronic structure of the mRNA produced by the transgene both reduces its abundance (by either slowing translation or enhancing degradation) and retards its translation. The possibility of improving both transcription and translation by using transforming constructs in which the RubisCO and selectable-marker genes form separate transcriptional units will be addressed in future studies.

There can be no doubt that the observed growth of the tobacco-*rubrum* transformants is supported entirely by the bacterial RubisCO’s activity. No trace of the gene, mRNA, or protein corresponding to the tobacco large subunit could be detected in the transformants by using blotting procedures capable of detecting <2% of the nontransformed control’s content of these species (Figs. 2 and 5). Even the many-fold more sensitive PCR procedure failed to amplify any wild-type *rbcL* sequence from the transformants (Fig. 2B). Residual traces of tobacco RubisCO

below these detection limits could neither support the substantial CO₂-assimilation rates observed at high CO₂ nor confer the distinctive CO₂ and O₂ responses that are the signature of the kinetic properties of the bacterial RubisCO. Further convincing proof of the transformants’ homoplasmy (and, hence, total lack of functional tobacco RubisCO) is supplied by the complete stability of the transformants through several generations of growth in the absence of antibiotic selection.

In view of the species specificity of RubisCO activase (30), it is unlikely that tobacco activase can regulate *R. rubrum* RubisCO. Therefore, the complex mechanisms that regulate the activity of the higher-plant enzyme, which depend, in part, on the activity of activase, apparently are dispensable, at least under the conditions in which we grew the transformants. Nearly full carbamylation of the bacterial RubisCO was observed under the high-CO₂ growth conditions (Fig. 6C). This is consistent with the equilibria of carbamylation and the associated binding of Mg²⁺ reported for the *R. rubrum* enzyme (35). Carbamylation of purified *R. rubrum* RubisCO is promoted strongly by D-ribulose-1,5-bisphosphate, rather than being inhibited by it, as is the case for the higher-plant enzyme (36). This may mean that, in the tobacco chloroplast in the light, *R. rubrum* RubisCO is fully activated at all times. This could be confirmed in future studies by observing the way the activation of the introduced bacterial RubisCO responds to changing light and CO₂ concentrations. It will also be interesting to study how the photosynthetic carbon cycle as a whole reacts to the presence of an unregulated or differently regulated primary CO₂-fixing catalyst.

This demonstration that a foreign RubisCO’s activity can integrate with chloroplast metabolism and support photosynthesis and growth encourages attempts to replace higher-plant RubisCO with more kinetically efficient homologs (10). This will require better understanding of the translation, folding, and assembly processes of both the existing higher-plant RubisCO (6) and its intended replacements.

We thank R. Sharwood for assistance.

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