The Catalytic Properties of Hybrid Rubisco Comprising Tobacco Small and Sunflower Large Subunits Mirror the Kinetically Equivalent Source Rubiscos and Can Support Tobacco Growth\textsuperscript{1[W][OA]}

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Plastomic replacement of the tobacco (\textit{Nicotiana tabacum}) Rubisco large subunit gene (\textit{rbcL}) with that from sunflower (\textit{Helianthus annuus}; \textit{rbcL}) produced tobacco\textsuperscript{Rst} transformants that produced a hybrid Rubisco consisting of sunflower large and tobacco small subunits (L\textsuperscript{S}). The tobacco\textsuperscript{Rat} plants required CO\textsubscript{2} (0.5\% v/v) supplementation to grow autotrophically from seed despite the substrate saturated carboxylation rate, \(K_c\), for CO\textsubscript{2} and CO\textsubscript{2}/O\textsubscript{2} selectivity of the L\textsuperscript{S} enzyme mirroring the kinetically equivalent tobacco and sunflower Rubiscos. Consequently, at the onset of exponential growth when the source strength and leaf L\textsuperscript{S} content were sufficient, tobacco\textsuperscript{Rat} plants grew to maturity without CO\textsubscript{2} supplementation. When grown under a high pCO\textsubscript{2}, the tobacco\textsuperscript{Rst} seedlings grew slower than tobacco and exhibited unique growth phenotypes: Juvenile plants formed clusters of 10 to 20 structurally simple oblongate leaves, developed multiple apical meristems, and the mature leaves displayed marginal curling and dimpling. Depending on developmental stage, the L\textsuperscript{S} content in tobacco\textsuperscript{Rst} leaves was 4- to 7-fold less than tobacco, and gas exchange coupled with chlorophyll fluorescence showed that at 2 mbar \(p\text{CO}_2\), CO\textsubscript{2} assimilation in mature tobacco\textsuperscript{Rst} leaves remained limited by Rubisco activity and its rate (approximately 11 \(\mu\text{mol m}^{-2} \text{s}^{-1}\)) was half that of tobacco controls. \(^{35}\text{S}-\)methionine labeling showed the stability of assembled L\textsuperscript{S} was similar to tobacco Rubisco and measurements of light transient CO\textsubscript{2} assimilation rates showed L\textsuperscript{S} was adequately regulated by tobacco Rubisco activase. We conclude limitations to tobacco\textsuperscript{Rst} growth primarily stem from reduced tobacco\textsuperscript{Rst} Rubisco's catalytic bifunctionality that sees it not only catalyze the productive carboxylation of ribulose-P\textsubscript{2} into two molecules of 3-phosphoglycerate (3-PGA), but also the oxygenation of ribulose-P\textsubscript{2} producing one 3-PGA and one 2-PG molecule. The 2-PG is considered a waste product since its recycling via photorespiration is energetically demanding and also reduces net carbon assimilation due to CO\textsubscript{2} release during Gly decarboxylation in the mitochondria (Wingler et al., 2000; Kebeish et al., 2007). The catalytic properties of Rubisco therefore play a critical, frequently rate-limiting, role in photosynthetic carbon assimilation and have a pervasive influence on the efficiency with which plants use their resources of light, water, and nitrogen. Accordingly, improving the catalytic properties of Rubisco and transplanting such improvements into crop plants has been a longstanding goal toward improving growth efficiencies (Andrews and Whitney, 2003; Raines, 2006; Parry et al., 2007). These efforts are spurred on by the natural variation in Rubisco's kinetic properties, particularly the kinetically better versions from nongreen algae that have the capacity to profoundly improve crop growth (Zhu et al., 2004). The natural variability in the catalytic properties of Rubisco also indicates engineering improvements into higher-plant versions are significant biotechnological effort and expense is being devoted to engineering improvements to CO\textsubscript{2} assimilation in C\textsubscript{3} plants using various strategies (for review, see Raines, 2006; Parry et al., 2007), with recent success coming by transplanting the glycolate catabolic pathway from \textit{Escherichia coli} into \textit{Arabidopsis} (\textit{Arabi-dopsis thaliana}) chloroplasts that localized recycling of 2-phosphoglycolate (2-PG) to glycerate in the stroma, thereby increasing the CO\textsubscript{2} levels around the photosynthetic CO\textsubscript{2}-fixing enzyme, Rubisco (Kebeish et al., 2007). The production of 2-PG is a consequence of...
unlikely to be immutable and theoretically provides a means to improve the efficiency of photosynthesis through genetic manipulation of a single protein rather than by introducing an entire foreign pathway.

Genetic manipulation of Rubisco in crop plants is hindered by several factors that are particularly complicated by the disparate location of the genes coding for the approximately 54 kD large subunit (L) and the approximately 14 kD small subunits (S) in or out of the plastids. Assembly of the cytosolically synthesized S with the stromally synthesized L that contain the catalytic active sites into hexadecameric enzyme (LsSt) within the stroma occurs via a complex, highly organized chaperone-assisted mechanism that further restrains genetic manipulation of Rubisco in higher plants (Whitney and Andrews, 2001a; Zhang et al., 2002). It has been shown the preferred position of rbcL in is the chloroplast genome (plastome; Kanevski and Maliga, 1994), while an appropriate means for the engineering changes to the native (or inserting a foreign) RbcS in higher plants has remained elusive. Directly transplanting in catalytically more efficient Rubiscos from nongreen algae by transplastomic insertion of their genes for L and S into the tobacco (Nicotiana tabacum) plastome was frustrated by folding incompatibilities that precluded productive assembly of the foreign enzyme in tobacco chloroplasts (Whitney et al., 2001). Directed manipulation of RbcS in higher plants is complicated by the multiple copies that essentially precludes them from targeted mutagenic or replacement strategies. The difficulty with engineering changes to S via transplastomic manipulation is further compounded by the apparent preferential assembly of cytosolically synthesized S with the L subunits (Whitney and Andrews, 2001a; Zhang et al., 2002). Only by reducing the level of cytosolic S by >90% by antisensing RbcS and inserting a copy of rbcL in a highly transcribed region of the tobacco chloroplast genome could sufficient quantities of plastid synthesized S be incorporated into assembly tobacco Rubisco hexadecamers (Dhingra et al., 2004).

The generation of fully autotrophic and reproductive transplastomic tobacco-rubrum lines where the native LsSt tobacco Rubisco has been replaced by the structurally simple L2 form from the bacterium Rhodospirillum rubrum demonstrated the feasibility of replacing Rubisco in higher plants (Whitney and Andrews, 2001b). These lines were produced via homologous replacement of the tobacco rbcL gene with the R. rubrum Rubisco gene, rbcM. Importantly, the CO2 response of photosynthesis in the tobacco-rubrum transformants was consistent with the content and kinetic properties of R. rubrum Rubisco (Whitney and Andrews, 2003). Earlier attempts to replace tobacco rbcL with the comparable gene from a cyanobacterium (Synechococcus PCC6301) or sunflower (Helianthus annuus) produced transformants that were nonautotrophic since they produced either no Rubisco (those containing the PCC6301 rbcL) or assembled a hybrid Rubisco hexadecamer (LsSt) comprising sunflower L and tobacco S (Kanevski et al., 1999). In the leaves of the latter transformant (line Nt-pIK83-1; Kanevski et al., 1999) grown in tissue culture the amount of hybrid enzyme was reduced by approximately 70%. The impeded autotrophic growth of line Nt-pIK83-1 in air necessitated grafting of the tissue onto wild-type tobacco to procure T1 seed (Kanevski et al., 1999).

In this study we show that line Nt-pIK83-1 can be grown photoautotrophically at elevated CO2 levels and demonstrate that the kinetic properties of the hybrid LsSt mirror those of the source Rubiscos and that the hybrid enzyme is regulated appropriately by tobacco activase in vivo. We show the inability of juvenile Nt-pIK83-1 plants to grow in air and their delayed development at high pCO2 is likely due to inadequate amounts of hybrid LsSt enzyme in the young vegetative tissue to provide sufficient source strength for development.

RESULTS

TobaccoRst Variants Grown in Soil at High CO2 Have Abnormal Phenotypic Features

Successful replacement of the plastome rbcL copy in tobacco with sunflower rbcL (rbcLs) by Kanevski et al. (1999) produced tobacco transformants that produced a hybrid Rubisco comprising eight sunflower large subunits (Ls) and eight tobacco small subunits (St). We identify the transformed line Nt-pIK83-1 (Kanevski et al., 1999) as tobaccoRst (tobacco with a hybrid Rubisco comprising sunflower L and tobacco S) and its hybrid hexadecameric Rubisco as LsSt. Limitations in the catalytic properties and content of LsSt were proposed to perturb autotrophic growth in air of tobaccoRst plants grown in soil. Analogous to other tobacco transgenic lines expressing less Rubisco (Rodermel et al., 1984; Hudson et al., 1992) or catalytically perturbed foreign or mutated Rubiscos (Whitney et al., 1999; Whitney and Andrews, 2001b), the T1 generation of the tobaccoRst line Nt-pIK83-1 (Kanevski et al., 1999) could be grown in soil in air enriched with CO2. The phenotype of the T1 progeny was different to wild-type tobacco (see figure 2 in Andrews and Whitney, 2003) and to ensure the phenotype was not a consequence of unwanted nuclear mutations the tobaccoRst flowers were repeatedly backcrossed with wild-type pollen. No change in the phenotype of multiple plants screened in the T2 or T3 progeny has been found and sequencing of the rbcLs and flanking plastome sequence (approximately 0.2 kb) on either side showed no unwanted mutations.

A growth analysis of four tobaccoRst plants from the T2 progeny found, like the nontransformed tobacco controls, wild-type-like cotyledons emerged approximately 8 d after sowing the seed into soil. The morphology of the ensuing tobaccoRst leaves differed to wild type and even 35 d post cotyledon emergence (when the tobacco controls were approximately 70 cm...
in height and flowering; Fig. 1A) the tobacco\textsuperscript{Rst} plants were without a clearly defined apical meristem and primarily comprised clusters of 10 to 20 narrow, structurally simple, ob lanceolate leaves that lacked a defined midrib and lateral venation and displayed marginal folding (Fig. 1, B and C). Only after the production of wild-type-like leaves (30–40 d post cotyledon emergence) did a shoot apical meristem, and frequently more than one, develop, enabling the plants to mature to the exponential fast growth phase. In this experiment only one of the tobacco\textsuperscript{Rst} plants developed a single primary shoot, with the other three plants developing either two or four primary shoots. The rate of exponential growth of the tobacco\textsuperscript{Rst} plants, calculated from changes in height of the first primary shoot that developed, showed the rate of shoot elongation was approximately 2-fold slower than the wild-type controls (Fig. 1D). Curiously, although the production of more than one vegetative shoot delayed the onset of the fast growth phase in tobacco\textsuperscript{Rst} the production of multiple shoots had only a modest influence on the exponential growth rate of the initial emerging axial shoot. Despite the slower growth of the tobacco\textsuperscript{Rst} plants they all grew to approximately the same height (approximately 80–95 cm) as the wild-type plants, produced lateral branches at the onset of floral inflorescence (approximately 70–80 d post cotyledon emergence), and produced normal looking fertile flowers that yielded viable seed (Fig. 1, B and C).

The mature leaves of tobacco\textsuperscript{Rst} were phenotypically different to wild type in that they exhibited curling and dimpling around the leaf margin (Fig. 1, E and F). The severity of this abnormal leaf phenotype was less noticeable in younger tobacco\textsuperscript{Rst} leaves and was absent in the leaves of tissue cultured tobacco\textsuperscript{Rst} plants grown on Suc-containing media (Fig. 1G). The width of the mature leaves produced by the tobacco\textsuperscript{Rst} plants, in particular those with multiple shoots, were smaller than the corresponding leaves from wild-type controls of comparable physiological development (Fig. 1, E and F). For example, the fifth leaf from the apical meristem from the 60 cm high plants used for biochemical analyses (Table I) had average widths of 14.3 \pm 0.6 cm in the tobacco\textsuperscript{Rst} plants (n = 5), which was approximately 20% less than the analogous leaf from the wild-type controls (17.7 \pm 1.2 cm, n = 6).

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Growth and phenotype of tobacco\textsuperscript{Rst} plants compared with wild-type tobacco grown in air containing 0.5% (v/v) CO\textsubscript{2} (see "Material and Methods" for further details). A, Phenotype of wild-type \textsuperscript{Rst} (B and C) plants (tob\textsuperscript{Rst}) and tob\textsuperscript{Rst}, both germinated from the same seed stock of a T\textsubscript{2} plant derived from previous generations only backcrossed with wild-type pollen) at the juvenile (top sections) and mature growth stage. The number of days since cotyledon emergence is shown. The white arrows indicate the normal ob lanceolate leaves (A and B) and irregular ob lanceolate leaves (C) analyzed in Figure 7. D, Change in height of the primary axial shoot for wild-type (●, n = 6) and tobacco\textsuperscript{Rst} plants with one (tob\textsuperscript{Rst}, ○, n = 1), two (□, n = 2), or four (tob\textsuperscript{Rst}, △, n = 1) primary axial shoots. The exponential growth rates (black lines) were modeled to the height data according to the equation

\[
y = A_1 \exp^{t / t} + Y_0
\]

where A\textsubscript{1} is the amplitude, t\textsubscript{1} the time constant (calculated values are shown), and Y\textsubscript{0} is the offset. E, Comparison of a mature wild-type and tob\textsuperscript{Rst} leaf (F) with the marginal dimpling phenotype in the tob\textsuperscript{Rst} lines not evident when grown in Suc containing tissue culture medium (G; Svb and Maliga, 1993).

| Table 1. Leaf metabolite and biochemical measurements |
|-----------------|-----------------|-----------------|
| Leaf Measurement | Wild-Type Tobacco | Tobacco\textsuperscript{Rst} |
| Fresh weight (g m\textsuperscript{-2}) | 233.7 ± 16.9 | 234.8 ± 26.8 |
| Dry weight (g m\textsuperscript{-2}) | 69.8 ± 5.9 | 47.1 ± 5.7 |
| Chlorophyll a and b content (mg m\textsuperscript{-2}) | 2.2 ± 0.1 | 2.85 ± 0.7 |
| Chlorophyll a/b ratio | 2.5 ± 0.1 | 2.7 ± 0.2 |
| $F_o/F_m$ | 0.80 ± 0.01 | 0.77 ± 0.02 |
| Protein (g m\textsuperscript{-2}) | 7.2 ± 1.0 | 4.5 ± 1.7 |
| Rubisco (g m\textsuperscript{-2}) | 1.33 ± 0.3 | 0.31 ± 0.2 |
| Ribulose-P\textsubscript{5} (mg m\textsuperscript{-2}) | 31.0 ± 8.2 | 64.0 ± 22.1 |
| P-glycerate and triose P (mg m\textsuperscript{-2}) | 111.1 ± 28.5 | 81.7 ± 5.8 |
| Ribulose-P\textsubscript{5} to P-glycerate ratio | 0.4 | 3.6 |
| Starch (g m\textsuperscript{-2}) | 23.7 ± 2.7 | 14.9 ± 1.2 |
To unequivocally demonstrate the phenotypic anomalies of the Nt-pK83-1 tobacco\textsuperscript{Rst} line are a direct consequence of replacing rbcL\textsubscript{L}, with rbcL\textsubscript{S} additional tobacco plastomastic lines t\textsuperscript{Rst}LA7 and t\textsuperscript{Rst}LA13 that replace the tobacco L with the sunflower homolog were independently generated (Supplemental Fig. S1, A and B). The phenotype of the T\textsubscript{1} progeny of both plastomastic lines mimics that displayed in the T\textsubscript{1} tobacco\textsuperscript{Rst} progeny (Supplemental Fig. S1, C and D).

**Photosynthesis in Tobacco\textsuperscript{Rst} Leaves Corresponds to the Content and Catalytic Properties of the Hybrid Rubisco**

Photosynthetic gas-exchange CO\textsubscript{2} response measurements in young fully expanded tobacco\textsuperscript{Rst} leaves showed CO\textsubscript{2} assimilation rates were largely independent of light intensity below 2 mbar CO\textsubscript{2}, consistent with assimilation remaining Rubisco activity limited below this CO\textsubscript{2} concentration (Fig. 2A). This was confirmed by simultaneous chlorophyll fluorescence measurements that showed electron transport rate continued to increase above 1,600 μbar CO\textsubscript{2} while nonphotochemical quenching remained high (Supplemental Fig. S2). In wild-type controls assimilation was limited by light-dependent regeneration of ribulose-P\textsubscript{2} at approximately 300 to 600 μbar CO\textsubscript{2}, depending on the illumination intensity. The maximum assimilation rates for tobacco\textsuperscript{Rst}(approximately 11 μmol m\textsuperscript{-2} s\textsuperscript{-1}) were half of wild type (approximately 22 μmol m\textsuperscript{-2} s\textsuperscript{-1}) at growth illumination (350 μmol quanta m\textsuperscript{-2} s\textsuperscript{-1}) with CO\textsubscript{2} compensation points of 97 and 49 μbar CO\textsubscript{2}, respectively. Simultaneous measurements of stomatal conductance showed little difference between tobacco\textsuperscript{Rst} (0.21 ± 0.02 μmol m\textsuperscript{-2} s\textsuperscript{-1}) and wild-type leaves (0.26 ± 0.02 μmol m\textsuperscript{-2} s\textsuperscript{-1}) under growth light. Likewise, the measured F\textsubscript{v}/F\textsubscript{m}, chlorophyll content, and chlorophyll a/b ratio in the same tobacco\textsuperscript{Rst} leaves mirrored the wild-type controls, indicating no apparent perturbation in photochemical efficiency or elevated photoinhibition in tobacco\textsuperscript{Rst} under the growth conditions (Table I).

After taking into consideration the reduced Rubisco content, the initial slope of the assimilation curves for the tobacco\textsuperscript{Rst} leaves were inconsistent with the hybrid L\textsuperscript{S}\textsuperscript{L} having a 5-fold higher K\textsubscript{m} for CO\textsubscript{2} (K\textsubscript{o}) and 4-fold lower substrate-saturated carboxylase activity (V\textsubscript{c,max}) as reported previously (Kanevski et al., 1999). When limited by carboxylase activity, the slope of the CO\textsubscript{2} assimilation rate in response to changing pCO\textsubscript{2} is dependent on Rubisco content, V\textsubscript{c,max}, and K\textsuperscript{215O\textsubscript{2}} (K\textsubscript{m} for CO\textsubscript{2} at ambient pO\textsubscript{2} and equals K\textsubscript{o}[1+O/ K\textsubscript{o}] where K\textsubscript{o} is the K\textsubscript{m} for O\textsubscript{2} and O is the O\textsubscript{2} concentration; Farquhar et al., 1980; von Caemmerer et al., 1994). Both K\textsubscript{c} and V\textsubscript{c,max} were measured for the tobacco\textsuperscript{Rst}, tobacco, and sunflower Rubiscos in vitro using rapidly sampled soluble protein extracts from mature leaves (Fig. 2B). The measured values for wild-type tobacco Rubisco matched those measured previously and were comparable to that measured for the sunflower and tobacco\textsuperscript{Rst} Rubiscos, indicating all three enzymes show similar kinetics and, most importantly, that the catalytic turnover and substrate CO\textsubscript{2} affinity of the hybrid enzyme was not impaired. Comparable rates of catalytic turnover were measured using leaf protein extracts from tobacco\textsuperscript{Rst} and tobacco plants grown in tissue culture (Fig. 2B) even when extracted and assayed in the extraction buffer used by Kanevski et al. (1999; Supplemental Fig. S3).

Purified Rubisco from tobacco\textsuperscript{Rst}, tobacco, and sunflower was used to measure their CO\textsubscript{2}/O\textsubscript{2} selectivity, which again were highly similar (Fig. 2B). As evident in Figure 2A, the modeled assimilation rate of tobacco\textsuperscript{Rst} using the in vitro kinetic (V\textsubscript{c,max} = 3.3 s\textsuperscript{-1}, K\textsuperscript{215O\textsubscript{2}} = 20.8 μM) and Rubisco content measurements (determined by 	extsuperscript{14}C]carboxyarabinitol-P\textsubscript{2} binding, see below) closely matched that measured by whole leaf gas exchange.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** CO\textsubscript{2} assimilation rates in tobacco\textsuperscript{Rst} and wild-type tobacco plants and their Rubisco kinetics. A. Gas-exchange measurements of CO\textsubscript{2} assimilation rates in response to intercellular pCO\textsubscript{2} measured at an irradiance of 350 (white symbols) or 950 (black symbols) μmol quanta m\textsuperscript{-2} s\textsuperscript{-1} and leaf temperature of 25°C. Measurements were made on attached leaves from two wild-type plants (wt1, 13.5 cm leaf diameter; wt2, 15 cm leaf diameter containing 33.2 and 30.6 μmol Rubisco sites m\textsuperscript{-2}, respectively) and two tobacco\textsuperscript{Rst} plants (tob\textsuperscript{Rst}1 and tob\textsuperscript{Rst}2, both 12 cm leaf diameters with 5.8 and 4.9 μmol Rubisco sites m\textsuperscript{-2}, respectively). The Rubisco limited CO\textsubscript{2} assimilation rates for wt1 (○) and tob\textsuperscript{Rst}1 (●) were modeled according to Farquhar et al. (1980) using estimates of maximal ribulose-P\textsubscript{2} dependent carboxylase activity, B, V\textsubscript{c,max}, K\textsubscript{c} for CO\textsubscript{2} under ambient O\textsubscript{2} levels (K\textsuperscript{215O\textsubscript{2}}), and CO\textsubscript{2}/O\textsubscript{2} specificity (S\textsubscript{o}). B. Measurements were made using leaf protein extracts and purified Rubisco preparations. See “Materials and Methods” for further details. V\textsubscript{c,max} measurements in bold italics are those made using leaf tissue from tissue culture grown plants; see Supplemental Figure S3 for details. Superscript b indicates data from Whitney et al. (1999) and superscript c indicates data from Kanevski et al. (1999).
supporting the validity of the measured kinetic parameters.

Even in air containing 0.5% (v/v) CO₂ the growth of tobacco remained Rubisco activity limited. Ribulose-P₂ content in young fully expanded tobacco leaves was approximately 50% higher and the 3-PGA content approximately 3-fold lower compared to wild-type controls, resulting in a 9-fold increase in the ribulose-P₂ to 3-PGA ratio (Table I). Both the dry weight and starch content of tobacco leaves were similarly reduced one-third while the fresh weight of the tobacco leaves matched wild type, indicating the leaves of the mutants were highly hydrated.

Rubisco and Rubisco Activase Content in Mature Leaves

As Rubisco content varies considerably with leaf age (Rodermel, 1999; Miller et al., 2000) significant care was made to undertake all biochemical measurements on the expanding fifth leaf from plants of similar height during the fast growth phase. In both wild-type and tobacco the width of the fifth leaf was 10% to 15% less than the widest seventh leaf and positioned at similar heights in the canopy. The binding affinity of the hybrid LsSt enzyme for carboxyarabinitol-P₂ was tested using the [¹⁴C]/[¹²C]-carboxyarabinitol-P₂ exchange method (Schloss, 1988; Whitney and Andrews, 2001b). Over a 24 h period there was no displacement of [¹⁴C]carboxyarabinitol-P₂ bound to either purified LsSt or tobacco Rubisco when incubated with a 500-fold excess of [¹²C]carboxyarabinitol-P₂, validating its use for quantifying Rubisco content. Indeed, Coomassie staining, immunoblot analyses, and [¹⁴C]carboxyarabinitol-P₂ showed the Rubisco content in fully expanded tobacco leaves was approximately one-quarter that of the wild-type controls (Fig. A–C; Table I). To partially compensate for the reduced content, the carbamylation status (i.e. the number of activated sites that have activator CO₂ bound to Lys 201 and coordinated with Mg²⁺) of LsSt under the high-CO₂ growth conditions was twice that of wild type (Fig. 3D).

However, despite the difference in Rubisco content there was little difference in the leaf activase content (Fig. 3B), indicating an approximately 4-fold increase in the activase:Rubisco stoichiometry in tobacco leaves. Notably, the approximately 1 g m⁻² reduced Rubisco content within tobacco leaves did not account for the approximately 3 g m⁻² reduction in leaf protein that was approximately 40% lower than the wild-type controls (Table I).

Regulation of LsSt by Tobacco Rubisco Activase

Light transient CO₂ assimilation measurements, identical to those described by Hammond et al. (1998), were performed using the flow-through LI-6400 gas-exchange system to ascertain whether tobacco Rubisco activase could correctly regulate LsSt in tobacco leaves. Changes in assimilation rates were measured following the sudden transition from low to high light (110 and 1,200 μmol quanta m⁻² s⁻¹, respectively) at pCO₂ of 250 μbar where assimilation remained Rubisco activity limited under the high light. Both tobacco wild-type and wild-type controls showed the typical biphasic increase in CO₂ assimilation rate (Woodrow and Mott, 1989) where assimilation rapidly increased initially due to an upsurge in energy production by the photosynthetic light reactions that concomitantly increases ribulose-P₂ synthesis, stimulating assimilation rates to saturating levels, resulting in the stabilized second phase of assimilation (Fig. 4). The gradual rise in assimilation rate to a final steady state (A*) is dependent on the ability of Rubisco activase to activate Rubisco active sites that, under the low light, had bound sugar phosphates (primarily ribulose-P₂), rendering them unable to participate in catalysis (Hammond et al., 1998). By plotting the natural log of the difference between the corrected assimilation rate (A*) from A*, the apparent rate constants for Rubisco activation (k₄) were calculated from the regression of the linear part of the second phase. The k₄ for tobacco (0.16 ± 0.01 min⁻¹, n = 3) was less than wild type (0.21 ± 0.02).
min \( n = 3 \)), indicating a faster rate of activation of L\(^{S}\) by tobacco Rubisco activase (Fig. 4).

**Stability of Assembled L\(^{S}\)**

Pulse-chase labeling with \(^{35}\)S-Met was used to compare the turnover of the L\(^{S}\) and native tobacco Rubisco in detached leaf discs. Following separation of the \(^{35}\)S-labeled leaf soluble proteins by nondenaturing PAGE, Rubisco hexadecamers were identified as the prominent protein by Coomassie staining and autoradiography in both wild type and tobacco\(^{Rst}\) (Fig. 5A). Band densitometry analysis of the autoradiographs showed that the stability of the L\(^{S}\) hexadecamer was comparable to wild-type Rubisco, indicating the reduced Rubisco content in tobacco\(^{Rst}\) leaves is unlikely to be due to increased turnover of the hybrid enzyme (Fig. 5B).

**Relative Translational Efficiency of the Sunflower \(rbcL\) Transcripts**

The relative contents of \(rbcL\) and Rubisco in physiologically comparable expanding tobacco\(^{Rst}\) and tobacco leaves (the same leaves analyzed by gas exchange) were compared to examine to what extent \(rbcL\) transcription, its translational processing, or folding assembly of sunflower L with tobacco S impeded production of L\(^{S}\). The integration of the antibiotic resistance gene cassette (\(Pmn-aadA-Trps16\)) within the \(rbcL\) 3'-untranslated sequence within the plastome of tobacco\(^{Rst}\) (Fig. 6A) resulted in the production of a monocistronic \(rbcL\) transcript and a similarly abundant bicistronic one containing \(rbcL\) and \(aad\) (Fig. 6B). On a leaf area basis, the total RNA content in the tobacco\(^{Rst}\) expanding fifth leaf from plants during the fast growth phase were approximately 10% to 15% lower than the comparable leaves in wild-type controls. As the RNA blots contained similar amounts of total leaf RNA per lane, the equivalent leaf area sampled to obtain this amount of RNA was larger for the tobacco\(^{Rst}\) samples (Fig. 6B). When standardized on a leaf area basis, the steady-state pool of \(rbcL\) mRNA in wild-type leaves was 4-fold higher than the content of both \(rbcL\) mRNAs (Fig. 6C). This deficit indicated problems with transcription of the \(rbcL\) genes, problems with the stability of their mRNAs, and/or a difference in the developmental profiling of the \(rbcL\) transcripts during the ontogeny of tobacco\(^{Rst}\) leaves. Measurement of Rubisco content in these leaves by \(^{14}\)C-carboxyarabinitol-P\(_2\) binding showed the L\(^{S}\) content was reduced approximately 9-fold compared to wild type, corresponding to a 30% reduction in the relative translational efficiency.

![Figure 4.](image-url) Measurement of the activation constant for tobacco and tobacco\(^{Rst}\) Rubisco. Representative whole leaf gas-exchange measurements of the CO\(_2\) assimilation response for physiologically analogous leaves (12 cm leaf width, 35 cm plant height) from a wild-type tobacco (A) and a tobacco\(^{Rst}\) (B) before and after leaf irradiance was suddenly increased from 110 to 1,200 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\) (time zero). The chamber CO\(_2\) concentration and leaf temperature were set at 250 \(\mu\)bar and 25\(^{\circ}\)C, respectively. Bottom panels show the data replotted as the natural logarithm of the final assimilation rate \((\Delta A)\) minus the adjusted assimilation rate \((\Delta A)\) as described (Woodrow and Mott, 1989). See “Materials and Methods” for further details. The rate constant for Rubisco activation \((k)\) was calculated from the gradients of the linear second phase fitted to the wild-type (white dashed line) and tobacco\(^{Rst}\) (black dashed line) measurements.

![Figure 5.](image-url) Rubisco turnover in tobacco and tobacco\(^{Rst}\) leaves. Multiple leaf discs from the same leaf were infiltrated with 35S-Met and chased with 10 mM unlabeled Met for the times shown and the soluble protein from 18 mm\(^2\) of leaf separated by nondenaturing PAGE. A, Hexadecameric Rubisco from wild-type tobacco (L\(^{S}\) black arrow) and tobacco\(^{Rst}\) (L\(^{S}\), white arrow) leaves identified in the gels by Coomassie staining and autoradiography. B, Densitometry measurements of the \(^{35}\)S-labeled Rubiscos during the chase periods (±se of three separate leaf samples for each time point).
indicating photoautotrophic growth should be supported in air. When grown from seed in air the leaf tissue that developed following normal cotyledon development was necrotic, consistent with Kanevski et al. (1999). However, when grown at elevated pCO₂ and then transferred to growth in air once a defined apical meristem had developed and shoot elongation was imminent, growth to fertile mature plants was possible, albeit 4-fold slower than a tobacco<sup>Rst</sup> plant maintained in air with 0.5% (v/v) CO₂ (Supplemental Fig. S4). The leaves that developed in air were phenotypically distinct, being paler green, thinner, and consequently, extremely fragile.

Rubisco Content in Juvenile Leaves

The ability of more mature but not juvenile vegetative tobacco<sup>Rst</sup> tissue to grow in air led us to investigate whether a paucity of hybrid enzyme or Rubisco activase in the juvenile tobacco<sup>Rst</sup> oblaneolate leaves led to their demise in air. The levels of both enzymes in the total and soluble protein of juvenile leaves were examined (Fig. 7). Comparative immunoblot and [¹⁴C]carboxyarabinitol-P₂ binding analyses to wild-type controls showed no difference in leaf Rubisco activase levels while the level of hybrid Rubisco produced by tobacco<sup>Rst</sup> were approximately 7-fold less than the wild-type controls and appeared entirely

![Image of Rubisco content in juvenile leaves](https://example.com/image.png)

**Figure 7.** Comparison of Rubisco activase content and Rubisco production in juvenile wild-type tobacco and tobacco<sup>Rst</sup> leaves grown in air containing 0.5% (v/v) CO₂. Total and soluble protein from 0.3 cm² of normal ovate wild-type leaves (n = 6) and the strappy oblaneolate<sup>Rst</sup> leaves (n = 4; refer to white arrows in Fig. 1, A–C) were separated by SDS-PAGE and stained with Coomassie Blue (A) or replicate samples (B) blotted and probed with antibodies raised against spinach Rubisco (Rubisco L antibody) and tobacco Rubisco activase (activase antibody). For comparison, the content of Rubisco active sites measured in the soluble leaf protein by [¹⁴C]CABP binding are shown (C). The abundance of <i>rbcL</i> (black) and <i>rbcL<sub>-aadA</sub></i> transcripts calculated from the sum of the abundances of the <i>rbcL<sub>l</sub></i> and <i>rbcL<sub>-aadA</sub></i> mRNAs.
soluble (Fig. 7, A and B). On a leaf area basis, the RNA content in the juvenile tobacco\textsuperscript{Rst} leaves was reduced approximately 30\% relative to wild type and the abundance of \textit{rbcL}\textsuperscript{Rst} and \textit{rbcL}\textsuperscript{aadA} transcripts (present in a ratio of approximately 9:1) reduced 5-fold relative to the levels of \textit{rbcL} in wild type (Fig. 7C). This resulted in a 30\% lower RTE for L\textsuperscript{S}S compared with wild-type Rubisco in the juvenile plants indicating that, analogous to the mature leaves, the paucity of hybrid enzyme appears influenced by limitations in both the steady-state levels of the \textit{rbcL}\textsuperscript{S} transcripts, their translational processing, folding, and/or subunit assembly (Fig. 7C). Additional qualitative SDS-PAGE analyses showed even lower sunflower L content in juvenile tobacco\textsuperscript{Rst} leaves sampled earlier in development, suggesting juvenile tobacco\textsuperscript{Rst} vegetative tissue produces insufficient levels of the L\textsuperscript{S}S to support adequate levels of CO\textsubscript{2} assimilation for growth in air.

**DISCUSSION**

**Tobacco\textsuperscript{Rst} Can Grow Autotrophically**

We have shown using tobacco\textsuperscript{Rst} and independently generated \textit{t\textsuperscript{Rst}}LA transplastomic lines that tobacco only expressing a hybrid L\textsuperscript{S}S enzyme comprising sunflower L and tobacco S subunits can be grown autotrophically past cotyledon emergence to fertile maturity in air when supplemented with CO\textsubscript{2} (Fig. 1; Supplemental Fig. S1). Both photosynthetic gas-exchange measurements on mature leaves and kinetic measurements made on extracted L\textsuperscript{S}S showed that the catalytic properties of the hybrid enzyme are comparable to the native sunflower and tobacco Rubiscos (Fig. 2B). However, even when supplied with 0.5\% CO\textsubscript{2} (v/v) the tobacco\textsuperscript{Rst} line and the \textit{t\textsuperscript{Rst}}LA lines display atypical developmental phenotypes and slower growth.

**The Sustained Kinetics of the Hybrid L\textsuperscript{S}S Enzyme Is Unique**

Previous mutagenic studies using cyanobacterial and \textit{Chlamydomonas} Rubiscos have clearly shown the S subunits are required for catalytic competency, have a pervasive influence on the kinetic properties of Rubisco, and that the L subunits are catalytically impaired when assembled with heterologous or structurally altered S subunits (Spreitzer, 2003; Karkehabadi et al., 2005). This is not the case for L\textsuperscript{S}S, invalidating the canonical belief that the assembly of heterologous Rubisco L and S subunits will unfavorably influence catalytic efficiency. Indeed, although only sharing 76\% sequence identity we can only assume from the sustained kinetics of L\textsuperscript{S}S that there is sufficient similarity in the quaternary structure of the assembled tobacco S subunits so as to retain the normal active site geometry of the assembled sunflower L subunits. This raises the interesting possibility that there may be greater structural flexibility between heterologous Rubisco S and L peptides from higher plants that enables their assembly with little or no kinetic perturbation to the hybrid enzyme. Addressing this possibility will necessitate comparable transplastomic studies focused on replacing the tobacco \textit{rbcL} with other plant \textit{rbcL} variants.

Our in vitro measurements of \(V_{c,max}\) were made using rapidly isolated protein extracts from leaves from both tissue-culture and autotrophically grown L\textsuperscript{S}S plants using different extraction buffers containing different protease inhibitor additives without appreciable decline in activity after 30 min incubation at 25\°C (Supplemental Fig. S3). This use of rapidly isolated leaf protein extract is now commonplace within this laboratory and gives accurate, and reproducible, estimates of \(V_{c,max}\) (Whitney et al., 2001; Ghannoum et al., 2005) and also \(K_{c}\) whose values match those obtained previously using freshly purified Rubisco (Fig. 2B). Possibly the purified L\textsuperscript{S}S used by Kanevski et al. (1999) to measure its kinetics had been compromised by partial proteolysis of terminal residues during the extended purification process. Proteolysis of N- and/or C-terminal residues may explain their lower \(V_{c,max}\) (Fig. 2B) and high \(K_{c}(60\mu M)\) measurements for L\textsuperscript{S}S as these kinetic parameters (and to a much lesser extent \(S_{c/o}\)) have also been strongly compromised in \textit{R. rubrum}, cyanobacteria, and spinach (\textit{Spinacia oleracea}) Rubiscos upon removal of residues from their L subunit termini by partial proteolysis or mutagenesis (Gutteridge et al., 1986a; Kettleborough et al., 1987; Ranty et al., 1990).

**Rubisco Activity Limits Tobacco\textsuperscript{Rst} Growth**

Our results indicate the efficiency of the photosystems was uncompromised in tobacco\textsuperscript{Rst} at their growth illumination as their chlorophyll content, \(a/b\) ratio, and \(F_{v}/F_{m}\) ratio mirrored that observed for wild type. It is more likely the primary basis for the developmental differences resulted from limitations in L\textsuperscript{S}S production. Development was particularly perturbed during juvenile growth when L\textsuperscript{S}S levels were reduced more than 7-fold, resulting in the production of unique oblanceolate leaf clusters not seen previously in antisense \textit{RbcS} tobacco lines where Rubisco production was reduced 5-fold (Tsai et al., 1997), suggesting greater limitations to source strength in tobacco\textsuperscript{Rst}. With matura-

le and the leaf L\textsuperscript{S}S content improved to levels approximately 20\% that of Rubisco in wild-type tobacco, however, the photosynthetic capacity of tobacco\textsuperscript{Rst} remained Rubisco activity limited despite CO\textsubscript{2} supplementation (Fig. 3). This limitation was substantiated by an approximately 2-fold lower content of nonstructural carbohydrate, an approximately 9-fold higher ribulose-P\textsubscript{2} to P-glycerate ratio, and high nonphotochemical quenching up to an intracellular pCO\textsubscript{2} (C\textsubscript{c}) of 2 mbar (Supplemental Fig. S2), and the ineffectual response of CO\textsubscript{2} assimilation rate to illumination above the growth intensity of 350 \textmu mol quanta m\textsuperscript{-2} s\textsuperscript{-1} for tobacco\textsuperscript{Rst} (Fig. 2A). Consistent with the differences in their exponential growth rates (Fig. 1D) the predicted Rubisco limited CO\textsubscript{2} assimilation rate at the growth pCO\textsubscript{2} (approximately 5 mbar) in fully expanded
tobacco\textsuperscript{Rst} leaves (containing 5 \(\mu\)mol Rubisco active sites m\(^{-2}\)) modeled according to Farquhar et al. (1980) is 14 \(\mu\)mol CO\(_2\) m\(^{-2}\) s\(^{-1}\), which is approximately two-thirds that of the light limited assimilation rate in wild-type tobacco (23 \(\mu\)mol CO\(_2\) m\(^{-2}\) s\(^{-1}\); Fig. 2A). Also consistent with leaf gas-exchange measurements was the capacity for tobacco\textsuperscript{Rst} to grow without CO\(_2\) enrichment, albeit only once source strength and leaf L\textsuperscript{S}\textsuperscript{'} content were adequate. This necessitated the development of normal ovate leaves before growth through to reproductive maturity could be supported in air, albeit approximately 4-fold slower than if maintained at high \(pCO_2\) (Supplemental Fig. S4).

What Is Limiting the Content of L\textsuperscript{S}\textsuperscript{'} in Tobacco\textsuperscript{Rst} Leaves?

While the comparable stability of the tobacco\textsuperscript{Rst} and wild-type tobacco Rubiscos indicated little or no perturbations to turnover of the assembled L\textsuperscript{S}\textsuperscript{'} hexadecamer (Fig. 4), reductions in the relative RNA content per leaf area and in the steady-state pool of the sunflower \(rbcL\textsuperscript{5}\) and \(rbcL\textsuperscript{5}-aad\textsuperscript{A}\) mRNAs contributed to the paucity of L\textsuperscript{S}\textsuperscript{'} produced. Compared with the \(rbcL\) pool in tobacco, the abundance of both the \(rbcL\textsuperscript{5}\) and dicistronic \(rbcL\textsuperscript{5}-aad\textsuperscript{A}\) transcripts in juvenile and mature tobacco\textsuperscript{Rst} leaves were approximately 5- and 4-fold lower, respectively. The stability of chloroplast transcripts are strongly influenced by appropriate endo- and exonucleolytic maturation of the 5'- and 3'-untranslated regions (UTRs; Bollenbach et al., 2004). The sequence of these regions, and that of the juxtaposed coding sequence, strongly influence appropriate mRNA folding and interaction with nucleus encoded chloroplast RNA-binding complexes that maintain the integrity of the transcript by correctly processing the 5' and 3' ends to prevent degradation by nucleases (Monde et al., 2000; Bollenbach et al., 2004; Zicker et al., 2007). As \(rbcL\textsuperscript{5}\) expression in tobacco\textsuperscript{Rst} is controlled by the same promoter and 5' UTR as tobacco \(rbcL\) it is likely changes to the 3' UTR and \(rbcL\textsuperscript{5}\) coding sequences perturbed proper maturation and stabilization of the transcripts making them more susceptible to degradation by plastid ribonucleases. The importance of the tobacco \(rbcL\) 3' UTR for maintaining steady-state levels of \(rbcL\) mRNA has been shown previously (Whitney and Andrews, 2003). In tobacco\textsuperscript{Rst} the \(aad\textsuperscript{A}\) cassette is inserted at the equivalent position 178-bp downstream of the \(rbcL\) stop codon after nucleotide 59251 in the tobacco plastome (GenBank accession no. Z00044). Intriguingly in subsequent RNA blots using fully denaturing formaldehyde gels we find the \(rbcL\textsuperscript{5}\) mRNA is smaller than the native \(rbcL\) mRNA, indicating the mature 3' UTR of \(rbcL\) extends further downstream (data not shown). In support of this, insertion of a promoterless \(aad\textsuperscript{A}\) gene a further 91-bp 3' to that in tobacco\textsuperscript{Rst} does not perturb \(rbcL\) abundance or Rubisco production and still enables transcription of an abundant, less abundant (approximately 20% of the \(rbcL\) mRNA) \(rbcL\textsuperscript{5}-aad\textsuperscript{A}\) transcript (Whitney et al., 1999; Whitney and Andrews, 2003). Clearly, the 3' UTR does have a pervasive influence on the steady-state levels of chloroplast mRNAs and to what extent lengthening the 3' UTR would improve \(rbcL\textsuperscript{5}\) transcript abundance, and reduce transcriptional read through of the \(rbcL\textsuperscript{5}-aad\textsuperscript{A}\) message, remains to be examined.

Problems with translation and/or assembly also contributed to the paucity of L\textsuperscript{S}\textsuperscript{'} produced in the tobacco\textsuperscript{Rst} leaves. Frequently in plastome transformation studies the equipping of foreign gene sequences with nonnative UTR sequences can perturb mRNA folding and slow translational processing of transgenes (Maliga, 2002). This necessitates the trialing of different UTR combinations to optimize expression (Maliga, 2003). The RTE of both \(rbcL\textsuperscript{5}\) mRNAs in both the juvenile and mature tobacco\textsuperscript{Rst} leaves was reduced 30% to 50%. One possible reason for this difference is the levels of \(rbcL\) and Rubisco holoenzyme during leaf ontogeny may differ between tobacco\textsuperscript{Rst} and wild type. That is, despite sampling expanding leaves from a comparable position in the canopy from wild type and tobacco\textsuperscript{Rst} of similar heights, their apparent developmental differences (Fig. 1) might extend to variations in the patterns of \(rbcL\) and Rubisco contents during leaf development that are otherwise generally correlated in tobacco (Miller et al., 2000) but not in all plants (see Suzuki et al., 2001 and refs. therein). Clearly this needs to be examined more comprehensively in the future by profiling the variation in plastome copy number, \(rbcL\), \(RbcS\), and Rubisco contents, and the rates of subunit translation (using rapid 35S-Met pulse methods; Rodermel et al., 1996) in both tobacco and tobacco\textsuperscript{Rst} leaves during their development.

The reduced RTE of \(rbcL\textsuperscript{5}\) mRNAs in tobacco\textsuperscript{Rst} might otherwise arise from problems related to translational processing of the transcripts due to the introduced \(NheI\) cloning site in codons 9 and 10. This introduces changes to nucleotides 27 and 30 in \(rbcL\textsuperscript{5}\) that otherwise shows absolute homology to tobacco \(rbcL\) for the first 56 nucleotides (GenBank accession no. AF097517). Unfortunately these nucleotide changes may potentially hamper translational processing of \(rbcL\textsuperscript{5}\) since the translational control region of \(rbcL\) includes both the 5' UTR and N-terminal coding sequence (approximately 42 nucleotides; Kuroda and Maliga, 2001). By examining the sedimentation behavior of the polyisomes associated with the \(rbcL\textsuperscript{5}\) mRNA it should be possible to identify whether translation initiation or translation elongation is impeded (Kim and Mullet, 1994). Alternatively, truncation of the mRNA 3' UTR may affect interactions between the 5' UTR and 3' UTR that are required for efficient recycling of mRNAs during translation (Kawaguchi and Bailey-Serres, 2002).

The lower RTE of the \(rbcL\textsuperscript{5}\) transcripts in mature tobacco\textsuperscript{Rst} leaves may also stem from limitations on the functional assembly of the L\textsuperscript{S}\textsuperscript{'} hexadecamer. This limitation may arise from incompatibilities between tobacco chloroplast chaperone complexes and the
The Hybrid Rubisco Is Adequately Regulated by Tobacco Rubisco Activase

The compatibility problems that can potentially limit the regulatory capacity of a Rubisco activase to Rubiscos from related species (Wang et al., 1992) did not affect the capacity of tobacco activase to adequately regulate L\textsubscript{S}S. This was initially evident from the approximately 2-fold higher carboxylation status of the hybrid Rubisco. As seen in transplastomic tobacco lines producing 50% less Rubisco (Whitney and Andrews, 2003) or a kinetically inferior mutant (Whitney et al., 1999), the high carboxylation status presumably compensates for the lower carboxylation potential. Light transient gas-exchange analyses also showed the activation constant for L\textsubscript{S}S was 25% faster than wild-type tobacco Rubisco, indicating the hybrid enzyme was adequately regulated by tobacco Rubisco activase (Fig. 4). Possibly this faster activation occurred as a consequence of the 4- to 7-fold (depending on leaf age) higher stoichiometry of activase to Rubisco in the tobacco\textsuperscript{Rst} leaves that resulted from a concomitant reduction in L\textsubscript{S}S levels but no discernable change in activase production. This higher stoichiometry might also conceal regulatory incompatibilities between L\textsubscript{S}S and tobacco Rubisco activase that will need to be resolved using appropriate in vitro enzyme assays (Wang et al., 1992), particularly if efforts are undertaken to increase production of L\textsubscript{S}S in tobacco chloroplasts.

Reasons for the Abnormal Tobacco\textsuperscript{Rst} Leaf Phenotype Are Unclear

As the developmental phenotype of tobacco\textsuperscript{Rst} leaves has been maintained over three generations after backcrossing with wild-type pollen and is repeated in the independently generated tob\textsuperscript{Rst}LA7 and tob\textsuperscript{Rst}LA13 transplastomic lines (Supplemental Fig. S1), the phenotype is not caused by pleiotropic effects and results from the replacement of tobacco L with the sunflower L. More comprehensive developmental studies on tobacco\textsuperscript{Rst} and the tob\textsuperscript{Rst}LA lines are needed to uncover the underlying cause(s) of its aberrant phenotypic features. While sharing developmental similarities to anti-Rbc\textsubscript{S} and other Calvin cycle antisense mutants with reduced carbon assimilation capacity (Tsai et al., 1997; Raines and Paul, 2006), the unique production of leaf clusters and multiple shoots early in tobacco\textsuperscript{Rst} development and the marginal dimpling of mature leaves indicate their development process is more severely perturbed. Interestingly, juvenile tobacco\textsuperscript{rubrum} plants do not show this phenotype when grown in air containing 1% or 2.5% CO\textsubscript{2} (v/v), suggesting their rates of carbon assimilation are sufficiently nonlimiting at these high CO\textsubscript{2}. However, at 1% CO\textsubscript{2} some tobacco\textsuperscript{rubrum} leaves display slight marginal dimpling early in development that disappears as the leaves age and accumulate more Rubisco (data not shown). Whether the developmental anomalies of tobacco\textsuperscript{Rst} can be circumvented by growth under non-Rubisco limiting conditions (i.e. higher pCO\textsubscript{2}, lower light) have yet to be examined. Nevertheless, it is important to note that when grown in tissue culture on media containing Suc, tobacco\textsuperscript{Rst} only produces normal leaves consistent with limitations in carbon assimilation being the primary cause of the developmental differences during autotrophic growth.

Possibly the developmental differences of tobacco\textsuperscript{Rst} arise from greater limitations in carbohydrate production than in other anti-Calvin cycle mutants, particularly in the juvenile tissue where survival past cotyledon emergence necessitates CO\textsubscript{2} supplementation. Very low Rubisco levels would impose significant limitations on the acquisition of sufficient source strength, explaining the lengthy duration of the juvenile phase in tobacco\textsuperscript{Rst} (30–40 d) and phenotypic abnormalities that possibly arise from alterations in the developmental program of the shoot apical meristems in response to developmental delay. As shoot apical meristems regulate the development of lateral organs such as leaves and branches (Piazza et al., 2005), it is possible some (or all) of the multiple apical shoots produced by tobacco\textsuperscript{Rst} may really be lateral branches. Clearly a more thorough growth comparison of tobacco\textsuperscript{Rst} with anti-Rbc\textsubscript{S} tobacco (‘Petit Havana’) with comparable Rubisco reductions (particularly in juvenile leaves) is needed to identify differences in their development, carbon-nitrogen status, and metabolite profiles (Fritz et al., 2006) and improve our understanding of the regulatory signals derived from
Future Considerations for Rubisco Engineering in Tobacco Chloroplasts

While many unresolved problems associated with transplanting foreign Rubiscos into higher plant plastids remain to be addressed, our discovery that L5S is not catalytically impaired should motivate further plastomic replacement studies with foreign Rubisco L subunits, particularly those from other higher plants. As highlighted in this study, success will necessitate the introduced rbcL to be stably transcribed, efficiently translated, and the folding and assembly requirements of the L be sufficiently compatible with the tobacco chaperone complexes and the tobacco S and suitably regulated with the endogenous Rubisco activase. Only needing to engineer rbcL would be advantageous as it would negate the need for cotransplanting in the complementary RbcS and evade further obstacles with needing to silence the endogenous S or genetically manipulate it as this is complicated by the multiple RbcS copies in the nuclear genome (Andrews and Whitney, 2003). Candidate rbcL genes for testing would include those of the kinetically superior Rubiscos from C3 plants growing in hot arid conditions (Galmes et al., 2008), and the Rubiscos from the cabinet with a 14 h light (25 °C) photoperiod in air where CO2 assimilation rate (A) was zero. A corrected assimilation rate (A*) was calculated for each 10 s time point using the equation:

\[ A^* = \frac{A}{C_i - \Gamma} \]

where \( C_i \) represents a \( C_i \) value (170 \( \mu \)bar) chosen to normalize the varying \( C_i \) against.

Leaf Sampling, Metabolite, and Biochemical Measurements

Biochemical measurements were made on leaf samples taken 8 h into the 14 h photoperiod from either developing juvenile leaf tissue or from young expanding mature leaves at comparable positions in the canopy (the fifth leaf below the apical meristem) from plants during the fast growth phase that were either approximately 35 or 60 cm tall. For the mature leaves, samples were taken from the same leaf and frozen immediately in liquid nitrogen and stored at −80 °C for protein and carbon content analysis as described in Whitney and Sharwood (2007). The carbohydrate-phosphoryl-P, that binds stoichiometrically to CO2-Mg2+ activated Rubisco active sites almost irreversibly (Schloss, 1988) was purified from these preparations by ion exchange (Zhu and Jensen, 1991).

Materials and Methods

Materials

Substrate ribulose-P2 (both 1H labeled and unlabeled) was synthesized according to Kane et al. (1998). Unlabeled (13C) and carboxyl-13C-labeled (13C) carboxyphosphatidyl-P2 (an isomeric mixture of carboxyribulokinase-P2 and carboxyribulokinase-P2 from Limonium gibertii (Parry et al., 2007) whose L shows 92% identity to tobacco L. A wider examination might also include rbcL genes from C4 Rubiscos that characteristically have higher kcat values relative to C3 species, albeit at the expense of higher Kc values (Yeoh et al., 1981; Seemann et al., 1984).

Plant Growth

Tobacco (Nicotiana tabacum L. ‘Petit Havana’ [N.N.]) and transplastomic tobacco (line Nt-pRb-83-1; Kanervski et al., 1999; T2 seed obtained from Pal Maliga) were germinated and grown in a controlled environment cabinet with a 14 h light (25°C) and 10 h dark (20°C) photoperiod in air supplemented with 0.5% (v/v) CO2 during the light period where the artificial illumination was 350 μmol quanta m−2 s−1 and the humidity set at 65%. Each generation of tobacco was artificially pollinated with wild-type pollen and the T2 seed used for all analyses. The plants were grown in 5-L pots of soil and provided with Hoagland nutrient mix (Hoagland and Snyder, 1933) every 2 d. The height of the plants was measured every 2 to 3 d from the soil surface to the apical meristem of the primary aerial shoot until the emergence of the first floral apertures.

Leaf Gas Exchange

Whole leaf photosynthetic gas exchange was measured using the portable, flow-through LI-6400 gas-exchange system (Li-COR). Tobacco and wild-type tobacco controls (approximately 30–35 cm tall) were brought from the high-CO2 growth cabinet to the laboratory for gas-exchange analysis and measurements made on the still expanding fifth leaf whose width was typically approximately 85% that of the largest seventh leaf. CO2 assimilation rates were measured using the 2 × 3 cm chamber fitted with a red/blue (10%) LED light source (Li-COR 6400-02B) with the leaf temperature set at 25°C. Transient light assays were performed similar to those described by Hammond et al. (1998). The plants were brought from the growth chamber early in the light period into the laboratory to ensure that the nocturnal inhibitor of tobacco Rubisco, 2-carboxyarabinitol-1-P (Gutteridge et al., 1986b) was absent from the Rubisco active sites. The plants were maintained under dim light (<60 μmol quanta m−2 s−1) before gently clamping into the LI-6400 system and equilibrating the leaf at 110 μmol quanta m−2 s−1 and approximately 200 μbar CO2 (to encourage stomata opening and ensure the conductance was >0.15 μmol m−2 s−1) for 45 min. The CO2 concentration was then raised to 250 μbar (where CO2 assimilation is still Rubisco activity limited) for 20 min before suddenly increasing the irradiance to 1,200 μmol quanta m−2 s−1. The flow rate of air into the chamber was set at 500 μmol s−1. At this flow rate, it takes 7 s for 63% of the air in the LiCOR chamber (83 cm3) to be refreshed. Assimilation measurements were continued until a final steady-state rate was reached (A). Photosynthetic CO2 assimilation rates were normalized to a constant C by assuming linear relationship between the CO2 compensation point (F) and each assimilation rate measurement (A); Brooks and Farquhar, 1985; Woodrow and Mott, 1989). Calculation of F was achieved by measuring assimilation rates, in replicate, at intercellular pCO2 (Ci) of approximately 50, 100, 150, and 250 μbar at an illumination of 350 μmol quanta m−2 s−1 (the growth irradiance). Using a linear fit, F was calculated for tobacco (97 ± 6.4 μbar) and wild type (49 ± 3.0 μbar) as the Ci where A was zero. A corrected assimilation rate (A*) was calculated for each 10 s time point using the equation:

\[ A^* = \frac{A}{C_i - \Gamma} \]

where \( C_i \) represents a \( C_i \) value (170 μbar) chosen to normalize the varying \( C_i \) against.
were calculated using comparable $^{14}$CO$_2$ fixation assays containing 15 mM NaH$^{14}$CO$_3$ and dividing the rate by the concentration of Rubisco active sites measured by $[^{14}$C]carboxyarabinitol-P$_2$ binding (see above).

Rubisco Purification and CO$_2$/O$_2$ Specificity

Rubisco was rapidly purified from approximately 50 g of freshly harvested leaves of tobacco, tobaccoRst, and sunflower (Helianthus annuus) plants grown at high CO$_2$. The leaves were homogenized in 40 mL ice cold extraction buffer, filtered through five layers of miracloth, centrifuged (35,000 g, 10 min, 4°C), and the soluble protein chromatographed on a 5 mL Q-sepharose column equilibrated with column buffer (50 mM Heppe-NAOH, pH 8.0, 1 mM EDTA). Proteins were eluted over a 0 to 400 mM NaCl gradient over 150 mL (5 mL min$^{-1}$ flow rate) and the two fractions (5 mL) containing peak Rubisco activities (measured by ribulose-P$_2$-dependent $^{14}$C fixation) were pooled and dialyzed for 16 h at 4°C against 1 L of storage buffer (25 mM Heppe-NAOH, pH 8.0, 1 mM EDTA, 20% [v/v] glycerol). This concentrated samples approximately 3-fold before they were frozen in liquid nitrogen and stored at −70°C. The purified Rubisco preparations were used to measure their CO$_2$/O$_2$ specificity ($S_{c/o}$) using the method of Kane et al. (1994).

SDS-PAGE and ImmunobLOTS

Leaf protein samples were separated by SDS-PAGE (Bio-Dirs-buffered 4%–12% NuPAGE gels, Invitrogen), blotted onto nitrocellulose, probed with appropriate antibodies, and the immunoreactive bands visualized using AstrolPhos (Promega) as described previously (Whitney and Andrews, 2001a).

$^{35}$S-Pulse Chase Labeling

Pulse chase labeling of leaf proteins was performed at 25°C using a modified protocol described by Whitney and Andrews (2001a). Thirty leaf discs (1 cm$^2$) were collected in the growth chamber from young fully expanded leaves and transferred to the laboratory in covered dishes onto prewetted Whatman paper. Within 5 min the discs were layered abaxial side down onto pulse solution (Murashige and Skoog nutrient solution [pH 7.2]; Murashige and Skoog, 1962) containing 10 mM NaHCO$_3$, 0.25% (v/v) silwet, and 2% ( v/v) [ $^{35}$S]Met (10.5 mCi/mL; ICN Biomedicals) and vacuum infiltrated at 200 mbar for 4 min before slowly releasing the vacuum. After 20 min incubation (600 μmol μm$^{-2}$ s$^{-1}$) the discs were quickly rinsed with three 100 mL washes of chase solution (pulse solution containing 10 mM Met) with intermittent blotting on Whatman paper to remove excess solution. Three leaf discs were snapshot frozen in liquid nitrogen (0 h chase samples) and the remainder layered onto 50 mL chase solution, vacuum infiltrated, and illuminated a further 0.5, 1, or 2 h before freezing in liquid nitrogen. The frozen discs were extracted in 0.5 mL extraction buffer on ice in Wheaton glass tubes then centrifuged (35,000 g, 10 min, 4°C) and the supernatant diluted 9:1 with native load buffer (60% [v/v] glycerol, 1% [w/v] bromphenol blue) and the proteins loaded onto Tris-Glycine buffered 4% to 20% NuPAGE gels and separated at 60 V for 18 h at 4°C in nondenaturing running buffer (19.2 mM Gly, 2.4 mM Tris, pH 8.3). The separated proteins were fixed in the gels and stained with Gel Code Blue Coomassie stain (Pierce) according to manufacturer instructions before vacuum drying the gels and exposing them to a storage phosphor screen for 21 d. The radioactive signals were detected using a Molecular Dynamics 400-B 2D PhosphorImager and the banding pattern quantified by densitometry analysis using ImageQuant software.

Leaf DNA, RNA, and PCR Analyses

Total leaf DNA from tobaccoRst transplastomic plants was used as template for PCR amplification using primers LoS (Whitney and Andrews, 2001b) and LoH (Fig. 6A). The 2.6 kb amplified product was cloned into pGEM-T Easy (Promega) and fully sequenced using BigDye terminator sequencing (Applied Biosystems) on an ABI 3730 sequencer (Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The genotype and phenotype of new tobaccoRst-Lox-ala/A transplastomic lines.

Supplemental Figure S2. Measurement of electron transport rate, non-photochemical quenching (NPQ), and CO$_2$ assimilation rate in response to change in intercellular pCO$_2$, using simultaneous leaf chlorophyll fluorescence and CO$_2$ assimilation measurements.

Supplemental Figure S3. Change in maximal ribulose-P$_2$-dependent carboxylase activity ($V_{\text{cmax}}$) in leaf protein extracts from tissue-cultured plants in response to CO$_2$-Mg$^{2+}$ activation during incubation at 25°C.

Supplemental Figure S4. Growth of tobaccoRst in air.

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LITERATURE CITED


Gutteridge S, Millard BN, Parry MAJ (1986a) Inactivation of ribulose-bisphosphate carboxylase by limited proteolysis—loss of the catalytic
activity without disruption of bisphosphate binding or carboxamylation. FEBS Lett 196: 263–268


Hammond ET, Andrews TJ, Mott KA, Woodrow IE (1998) Regulation of Rubisco activation in antisense plants of tobacco containing reduced levels of Rubisco activase. Plant J 14: 101–110

Haagland DR, Snyder WC (1933) Nutrition of strawberry plants under conditioned conditions: (a) effects of deficiencies of boron and certain other elements; (b) susceptibility to injury from sodium salts. Proc Am Soc Hortic Sci 30: 288–294


Whitney SM, Andrews TJ (2001a) The gene for the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit relocated to the plastid genome of tobacco directs the synthesis of small subunits that assemble into functional oligomers without impeding catalytic performance. EMBO J 19: 4738–4743


