Rubisco Oligomers Composed of Linked Small and Large Subunits Assemble in Tobacco Plastids and Have Higher Affinities for CO₂ and O₂

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Manipulation of Rubisco within higher plants is complicated by the different genomic locations of the large (L; rbcL) and small (S; rbcS) subunit genes. Although rbcL can be accurately modified by plastome transformation, directed genetic manipulation of the multiple nuclear-encoded RbcS genes is more challenging. Here we demonstrate the viability of linking the S and L subunits of tobacco (Nicotiana tabacum) Rubisco using a flexible 40-amino acid tether. By replacing the rbcL in tobacco plastids with an artificial gene coding for a S40L fusion peptide, we found that the fusions readily assemble into catalytic (S40L)₈ and (S40L)₁₆ oligomers that are devoid of unlinked S subunits. While there was little or no change in CO₂/O₂ specificity or carboxylation rate of the Rubisco oligomers, their Kₗₕ for CO₂ and O₂ were reduced 10% to 20% and 45%, respectively. In young maturing leaves of the plastome transformants (called ANₙSₐ40L), the S40L-Rubisco levels were approximately 20% that of wild-type controls despite turnover of the S40L-Rubisco oligomers being only slightly enhanced relative to wild-type. The reduced Rubisco content in ANₙSₐ40L leaves is partly attributed to problems with folding and assembly of the S40L peptides in tobacco plastids that relegate approximately 30% to 50% of the S40L pool to the insoluble protein fraction. Leaf CO₂-assimilation rates in ANₙSₐ40L at varying partial pressures and its tendency to confuse substrate affinities for CO₂ and O₂ were reduced 10% to 20% and 45%, respectively. In young maturing leaves of the plastome transformants (called ANₙSₐ40L), the S40L-Rubisco levels were approximately 20% that of wild-type controls despite turnover of the S40L-Rubisco oligomers being only slightly enhanced relative to wild-type. The reduced Rubisco content in ANₙSₐ40L leaves is partly attributed to problems with folding and assembly of the S40L peptides in tobacco plastids that relegate approximately 30% to 50% of the S40L pool to the insoluble protein fraction. Leaf CO₂-assimilation rates in ANₙSₐ40L at varying pCO₂ corresponded with the kinetics and reduced content of the Rubisco oligomers. This fusion strategy provides a novel platform to begin simultaneously engineering Rubisco L and S subunits in tobacco plastids.

Rubisco in higher plant leaves is notorious for its catalytic inefficiency (Zhu et al., 2004; Farrey et al., 2007; Zhu et al., 2007). Its slow turnover rate at limiting CO₂ partial pressures and its tendency to confuse substrate CO₂ with the more abundant O₂ encumbers photosynthesis in higher plants with both a requirement to invest large amounts of protein in Rubisco, and also a requirement for an energy-intensive photorespiratory metabolism to recycle the oxygenated waste product. To what extent a plant’s growth would alter if transplanted with a more efficient Rubisco has been modeled at both the individual and crop levels, with both highlighting the significant benefits of replacing the Rubisco in C₃ crops with more efficient variants from some C₄ plants and the more CO₂-specific forms naturally found in nongreen algae (Whitney et al., 2001; Andrews and Whitney, 2003; Zhu et al., 2004; Zhu et al., 2007). One key advantage shown by the models is the improvements come at no additional energy or resource cost. This challenge has prompted significant research into finding, or engineering, better Rubiscos and elucidating ways to effectively transplant these improvements into crop plants.

An impediment to engineering higher plant Rubisco is the enzyme’s complex assembly mechanism that necessitates the coordinated expression and assembly of eight plastid-encoded large (L) and eight nuclear-encoded small (S) subunits into a form I hexadecameric (L₈S₈) enzyme (Roy and Andrews, 2000). The L subunit contains the catalytic site but the S subunits, whose precise role in the structure and function of Rubisco remains poorly understood, are essential for catalytic viability (Spreitzer, 2003). The canonical belief that there are catalytic trade-offs in hybrid Rubiscos formed from heterologous L and S subunits appears valid for those comprising cyanobacterial L subunits (Read and Tabita, 1992a; Wang et al., 2001) but may not be true for those comprising heterologous higher plant Rubisco subunits (Sharwood et al., 2008). However,
there is strong evidence that amino acid substitutions in the S subunits can augment changes made in the L subunit (Read and Tabita, 1992b; Spreitzer et al., 2005), indicating engineering improvements to Rubisco are likely to require complementary changes to both subunits. While genetic manipulation of the Rubisco L subunit gene (\textit{rbcL}), located in the plastome, by homologous recombination in the model plant tobacco (\textit{Nicotiana tabacum}) is routine (Whitney and Sharwood, 2008), there is no appropriate means for efficiently engineering the S subunit genes (\textit{RbcS}) with comparable precision. The multiple \textit{RbcS} copies in the nucleus preclude targeted mutagenic or replacement strategies and limitations on the translation and/or assembly of chloroplast-synthesized S subunits currently limit their genetic manipulation by plastome transformation in higher plants (Whitney and Andrews, 2001a; Zhang et al., 2002; Dhingra et al., 2004).

Recently we demonstrated a means by which both Rubisco subunits can be simultaneously engineered by linking them together as a single fusion peptide (Whitney and Sharwood, 2007). Using the form I Rubisco by linking them together as a single fusion peptide Rubisco subunits can be simultaneously engineered (Zhang et al., 2002; Dhingra et al., 2004). their genetic manipulation by plastome transformation to evaluate the applicability of the SL fusion strategy to engineer Rubisco in tobacco chloroplasts by examining the stability and expression of SL fusion peptides in tobacco plastids, evaluate their capacity to fold and assemble into Rubisco complexes, and measure their catalytic prowess.

**RESULTS**

The Transplastomic \textit{A}\textsuperscript{Ns}40L Lines Produce Two Fusion-Rubisco Complexes

The plastome of the selectable marker gene (\textit{aadA})-free tobacco marker line \textit{cmtrL} (Whitney and Sharwood, 2008) was biolistically transformed with \textit{pA}\textsuperscript{Ns}40L to direct the replacement of the dimeric \textit{Rhodospirillum rubrum} Rubisco-coding gene \textit{rbcM} with \textit{aadA} and a synthetic \textit{rbcS}40L gene coding a \textit{1S}40L fusion peptide comprising the tobacco \textit{S} and \textit{L} subunits tethered together by a 40-amino acid linker (Whitney and Sharwood, 2007; Fig. 1A). Previous use of this 40-amino acid linker with \textit{L} subunits synthesizing \textit{Synechococcus} PCC6301 (cyanobacterial) Rubisco subunits was found to be optimal for catalytic activity and maximized the amount of Rubisco assembled in \textit{E. coli} (Whitney and Sharwood, 2007). The native \textit{rbcL} promoter–5′-untranslated region (UTR) was not used to regulate \textit{rbcS}40L expression as the \textit{rbcL} translational control region may extend into the 5′-end of the \textit{rbcL} coding region (Kuroda and Maliga, 2001; Maliga, 2003), necessitating inclusion of additional residues on the S-subunit N terminus. Instead the \textit{rbcS}40L gene was equipped with the constitutive tobacco plastomic \textit{rrn} (16S rDNA) promoter and synthetic 63-bp T7 phage gene 10 (T7g10) 5′-UTR sequence (Fig. 1A), which has been shown to enhance foreign protein expression in tobacco plastids (Maliga, 2003).

Twenty-one spectinomycin-resistant (\textit{spec}) plantlets were obtained 30 to 40 d postbombardment with \textit{pA}\textsuperscript{Ns}40L. The soluble leaf proteins from only two
lines were analyzed by non-denaturing PAGE and both lines produced two distinct fusion-Rubisco oligomeric complexes larger than the native tobacco L_8S_8 enzyme (Fig. 1B). This is identical to that seen previously with the cyanobacterial fusion Rubiscos (Whitney and Sharwood, 2007), supporting the assertion that these complexes were octamers [(TS40L)_8] and adjoined octamers [i.e. (TS40L)_16 hexadecamers] of TS40L peptides. While no R. rubrum L_2 Rubisco was evident in the ANtS40L line, it was evident in ANtS40L and the control LEV1 transformant producing tobacco L_8S_8 (heteroplasmic line 2 from Whitney and Sharwood [2008]). Both ANtS40L lines were put through another round of regeneration on selective medium to ensure homoplasmicity.

Growth Phenotype and CO₂ Requirement by Both ANtS40L Lines

In soil both T₀ ANtS40L lines were unable to grow in air without CO₂ supplementation. In air containing 0.5% (v/v) CO₂ their phenotype mimicked wild-type tobacco but grew more slowly and had paler green leaves during early exponential growth (Fig. 2A). With further maturity the leaves became darker green and produced normal-looking fertile flowers that were backcrossed with wild-type pollen (Supplemental Fig. S1).

Rubisco Content and mRNA Levels

DNA-blot analysis of the ANtS40L T₁ progeny confirmed the plants were homoplasmic (Fig. 2B) and sequencing of the inserted DNA and surrounding plastome sequence confirmed both lines were identical. Blots of total RNA from comparable young near fully expanded leaves showed the rrn promoter-T7g10 5′-UTR maintained high steady-state rbcS40L mRNA levels in the ANtS40L lines, exceeding rbcL mRNA levels in wild type by approximately 10% to 30% (Fig. 2C). In contrast, the corresponding level of RbcS transcripts were reduced 40% in the ANtS40L lines (Fig. 2C). The larger rbcS40L mRNA was not detected by the RbcS probe as it shares only 65% identity with the codon-modified S-subunit coding sequence in rbcS40L (Whitney and Sharwood, 2007).

Production of a 70-kD TS40L peptide in ANtS40L leaves was readily detected by SDS-PAGE and immunoblot analysis, with no wild-type 52-kD Rubisco L subunit and only a finite amount of cytosolic synthesized Rubisco S subunits (100-fold less than wild type) detected (Fig. 2D). In wild-type leaves no insoluble Rubisco L or S subunits were detected (data not shown), while in ANtS40L leaves approximately 50% of the TS40L peptide pool was insoluble, indicating problems with the folding and/or assembly of the 70-kD peptides into oligomeric complexes in tobacco chloroplasts (Fig. 2D).

Both the (TS40L)_8 and (TS40L)_16 Rubisco complexes tightly bind the Rubisco-specific inhibitor 2-carboxyarabinitol-1,5-bisphosphate (CABP). Consistent with
the levels of soluble $^{15}$S40L detected by SDS-PAGE, $[^{14}$C]CABP-binding analyses indicated the Rubisco content in the ANtS40L leaves was approximately one-fifth that of the wild-type controls (Fig. 2E). This amount was validated by the $[^{14}$C]CABP-binding/$[^{12}$C]CABP-exchange procedure (Schloss, 1988) where less than 3% of the Rubisco-bound $[^{14}$C]CABP in the ANtS40L and wild-type leaf protein extracts were displaced when incubated with a 180-fold molar excess of $[^{12}$C]CABP for 30 min (Fig. 2E). To compensate for the reduced Rubisco content the activation (carbamylation) status of Rubisco in the ANtS40L leaves (72% $\pm$ 8%) was almost double that in wild type (39% $\pm$ 5%) while the content of Rubisco activase—the protein that regulates Rubisco activity by facilitating the release of bound sugar phosphate inhibitors from the active site (Portis et al., 2008)—remained unchanged (Fig. 2D).

Measurements of Rubisco content in different aged leaves from an ANtS40L line during exponential growth (28 cm in height) showed the Rubisco levels were highest in the young fully expanded leaves (Fig. 2F), consistent with the relative variation in $L_8S_8$ Rubisco content in different aged tobacco leaves (Jiang and Rodermel, 1995; Rodermel, 1999). The amount of insoluble $^{15}$S40L in the different aged leaves constituted between 30% to 50% of the total $^{15}$S40L pool, indicating the insoluble protein can be degraded by proteolysis and its products likely reassimilated (Fig. 2F).

Stability of the ($^{15}$S40L)$_8$ and ($^{15}$S40L)$_{16}$ Rubisco Complexes

The turnover of both Rubisco complexes in ANtS40L and the $L_8S_8$ Rubisco in wild-type tobacco were compared by $[^{35}$S]-Met pulse-chase labeling using detached leaf discs. Following separation by nonnondenaturing PAGE, autoradiograph analysis showed the amount of radiolabel incorporated into the ($^{15}$S40L)$_8$ and ($^{15}$S40L)$_{16}$ complexes declined slightly more rapidly than $L_8S_8$ (Fig. 3A), while no change in the amount of Rubisco was evident by Coomassie staining (Supplemental Fig. S2A). Although the extent to which this higher turnover of the ($^{15}$S40L)$_8$ and ($^{15}$S40L)$_{16}$ complexes contributed to their reduced content in ANtS40L leaves was not quantified, analysis of the soluble (assembled) and insoluble $^{15}$S40L peptides showed their rates of turnover were similar (Fig. 3B). This is consistent with observations that the level of insoluble $^{15}$S40L does not accumulate with leaf age (Fig. 2F).

The ($^{15}$S40L)$_8$ and ($^{15}$S40L)$_{16}$ Complexes Show Increased Affinity for $CO_2$ and $O_2$

Size exclusion chromatography of soluble leaf protein showed a single ribulose-1,5-bisphosphate (RuBP) carboxylase activity peak for wild-type $L_8S_8$ Rubisco and two activity peaks for ANtS40L corresponding to the ($^{15}$S40L)$_8$ and ($^{15}$S40L)$_{16}$ complexes (Fig. 4A). Unlike that shown by nonnondenaturing PAGE, the peak ($^{15}$S40L)$_8$ activity eluted slightly later than wild-type tobacco

![Figure 3. Rubisco turnover in ANtS40L and wild-type tobacco detached leaf discs. A, The discs (1 cm$^2$) were infiltrated with $[^{35}$S]Met and chased with 10 mM unlabeled Met for the times shown and the soluble Rubisco measured by densitometry as described (Whitney and Sharwood, 2008). B, Relative loss of $[^{35}$S]-incoportated into soluble (△) and insoluble (●) $^{15}$S40L in ANtS40L and wild-type samples. Shown are the average measurements ($\pm$SD) of three separate leaf samples for each time point. See Supplemental Figure S2 for more detail.

Rubisco, indicative of a lower molecular mass. The Rubisco content in the peak activity fractions was determined by $[^{14}$C]CABP binding and showed the carboxylase turnover rates ($k_i$) of both $^{15}$S40L fusion Rubiscos closely matched the $L_8S_8$ enzyme (Fig. 4A; Table I). Analysis of $CO_2/O_2$ specificity ($S_{CO_2}$) in the same fractions from replicate chromatography samples showed they were comparable for the ($^{15}$S40L)$_8$, ($^{15}$S40L)$_{16}$, and $L_8S_8$ enzymes, while the Michaelis constants ($K_m$) for $CO_2$ ($K_{CO_2}$) were reduced 10% to 14% for the $^{15}$S40L complexes (Table I). Comparable values for $K_i$ were measured using rapidly sampled leaf-soluble protein extracts, confirming the higher affinity of the $^{15}$S40L complexes for $O_2$. Likewise, the $^{15}$S40L Rubiscos had an increased affinity for $O_2$ with 45% lower values of $K_i$ for $O_2$ ($K_{O_2}$; Table I).

Unlinked S Subunits Do Not Assemble in the $^{15}$S40L-Rubisco Complexes

PAGE and immunoblot analyses with an antibody to tobacco Rubisco confirmed the RuBP-carboxylase activity peaks correlated with the elution of wild-type $L_8S_8$ (Fig. 4B) and the ANtS40L ($^{15}$S40L)$_8$ and ($^{15}$S40L)$_{16}$ complexes (Fig. 4C). No S subunits were detected by SDS-PAGE and immunoblot analysis of the fractions containing the $^{15}$S40L-Rubisco complexes (Fig. 4C), indicating the small amount of endogenous cytosolic synthesized S subunits detected in the soluble leaf protein extract (Fig. 2D) are not incorporated into Rubisco.
CO₂-Assimilation Rates in ANₜS₄₀L Leaves Match That Predicted

The low photosynthetic CO₂-assimilation rates by young fully expanded ANₜS₄₀L leaves (Fig. 2A) were consistent with those predicted by the model for photosynthetic gas exchange (Farquhar et al., 1980; Fig. 5). At an intercellular pCO₂ (Cᵢ) of approximately 400 μbar, assimilation in the wild-type leaf at the growth illumination (350 μmol quanta m⁻² s⁻¹) became limited by light-dependent regeneration of RuBP while photosynthesis by ANₜS₄₀L was still carboxylase limited at 1,600 μbar pCO₂ and had a higher CO₂ compensation point (approximately 200 μbar) relative to wild type (approximately 55 μbar). The maximum CO₂-assimilation rates by the young ANₜS₄₀L leaves (approximately 3.2 μmol m⁻² s⁻¹) were 9-fold lower than wild type (approximately 27 μmol m⁻² s⁻¹). In contrast, little difference was observed in the maximum photochemical efficiency of PSII in the dark-adapted state (variable fluorescence [Fᵥ]/maximum fluorescence [Fᵥ]m = 0.75) and stomatal conductance (approximately 0.16 μmol m⁻² s⁻¹) by ANₜS₄₀L under growth light relative to the wild type (0.80 and 0.21 μmol m⁻² s⁻¹, respectively), indicating no apparent problems with photochemical efficiency or limitations to leaf gas exchange in the transformants.

Met-1 of the TS₄₀L Peptides Is N-Methylated

N-terminal Edman microsequencing of the soluble TS₄₀L peptide yielded the sequence X-K-V-W-P as expected from other studies (Grimm et al., 1997). The first residue, X, chromatographed as a large broad peak just after the Glu phenylthiohydantoin derivative, consistent with it being N-methyl-Met as seen with the spinach (Spinacia oleracea) S-subunit N terminus (Ying et al., 1999). Analysis by mass spectrometry following Asp-N digestion identified a peptide at a mass-to-charge ratio (m/z) of 2,349, consistent with the N-terminal region of TS₄₀L with a mass shift of 14 mass-to-charge units (Supplemental Fig. S3A). Subsequent collision-induced dissociation and postsource decay fragmentation patterns localized the 14 m/z mass shift to the N terminus of the 2,349 parent ion (Supplemental Fig. S3B) consistent with N-methylation of Met-1.

DISCUSSION

Rubisco Comprising SL Fusions Can Assemble in Tobacco Chloroplasts with Minimal Catalytic Change

Here we demonstrate the feasibility of generating autotrophic tobacco lines producing Rubisco oligomers that only comprise approximately 70-kD TS₄₀L peptides that can assemble into both catalytically
Problems with the Synthesis, Folding, and Assembly of \(^{15}S40L\) Limit Rubisco Production in Chloroplasts

The Rubisco content in young near fully expanded leaves of the \(A^{Ns40L}\) lines was reduced at least 5-fold relative to wild-type controls, which limited photosynthesis and growth rates and necessitated CO\(_2\) supplementation during juvenile plant development. As observed previously in tobacco-producing foreign (Whitney and Andrews, 2001b) or hybrid (Sharwood et al., 2008) Rubiscos, the Rubisco activase content in \(A^{Ns40L}\) leaves remained unchanged. The higher carbamylation status of Rubisco in the \(A^{Ns40L}\) leaves suggests the activity of the S40L fusion complexes can be adequately regulated by Rubisco activase, although the efficiency of this regulation remains to be examined.

The highly transcribed \(rrn\) promoter produced steady-state \(rbcS40L\) mRNA levels slightly higher than \(rbcL\) levels in wild-type controls (Fig. 2C), indicating that \(S40L\) production was primarily limited posttranscriptionally. The paucity of assembled \(S40L\) Rubisco can partially be attributed to 30% to 50% of the leaf \(S40L\) pool accumulating as insoluble aggregates (Fig. 2, D and F). This indicates the \(S40L\) folding and assembly requirements are not fully met by the plastid molecular chaperone network and correlates with comparable impediments in the folding assembly of \(^{15}S40L\) peptides in \(E. coli\) (Whitney and Sharwood, 2007). Identifying the lesion point(s) impeding \(S40L\) folding and assembly in plastids is difficult given our rudimentary understanding of Rubisco assembly in plastids (Roy and Andrews, 2000). Possibility the capacity of the stromal Cpn21 chaperonins and their Cpn21 cofactors (known to be involved in Rubisco assembly) to properly fold the large 70-kD \(^{15}S40L\) peptides may be impeded, resulting in their rapid proteolysis and/or accumulation as misfolded insoluble aggregates (whose proteolytic turnover is comparable to that of properly assembled \(^{15}S40L\); Fig. 3). Notably, space limitations within chaperonin GroEL-GroES complexes in \(E. coli\) make it difficult for them to encapsulate and fold peptides greater than 60 kD in size (Kerner et al., 2005). It is also possible incompatibilities with other chaperones, such as the Rubisco L-subunit-specific BSDII (Bruttell et al., 1999; Wostrikoff and Stern, 2007), may also encumber prechaperonin processing of \(^{15}S40L\) also resulting in their misfolding and/or inadequate protection from stromal proteases. A strategy for identifying proteins involved in Rubisco assembly may be to introduce affinity tags within the \(^{15}S40L\) linker sequence (or just the L subunit itself [Rumeau et al., 2004]) and rapidly isolate unassembled Rubisco subunit-chaperone complexes.

Translational processing of the \(rbcS40L\) transcript may also account for the reduced Rubisco levels in tobacco (and possibly foreign) Rubisco to explore intersubunit residue interactions, their influence on catalytic activity, and whether leaf gas-exchange analysis (Fig. 5) continues to emulate the predicted changes in photosynthetic CO\(_2\)-assimilation capacity according to the models of Farquhar et al. (1980).
A\(^\text{N}_{\text{S40L}}\) leaves. In most plastome transformation studies the coupling of heterologous UTRs and transgene sequences perturbs mRNA folding, slows translational processing, and frequently necessitates the trialing of different UTR combinations to optimize expression (Maliga, 2003). Whether unfavorable folding of the \(rbcS40L\) mRNA impedes engagement and translation by ribosomes remains to be examined by analyzing the transcript association with polysomes (Barkan, 1998). Importantly, irrespective of the possible causes for limiting \(^\text{T}_{\text{S40L}}\) production, once assembled into \(^\text{T}_{\text{S40L}}\)\(_8\) or \(^\text{T}_{\text{S40L}}\)\(_16\) complexes the peptides are relatively stable (Fig. 3) and produced in quantities suitable for future mutagenic analyses.

Can a Plastid-Signaling Event Regulate \(RbcS\) mRNA Levels?

Biogenesis of \(L_3S_2\) Rubisco in higher plants is complicated by the disparate location of the \(RbcS\) and \(rbcL\) genes in different genomes and their translation in different cellular locations (the cytosol and stroma, respectively). Light, hormone, and particularly carbohydrate levels have been shown to play determinant roles in leaf developmental programming by a complex signal transduction pathway whose nucleus-plastid communication circuitry remains poorly defined, particularly with regard to Rubisco production (Rodermel, 1999). However, a recent advance showed expression of the \(L\) subunit in tobacco is tightly coordinated via a classical control by epistasis of synthesis paradigm wherein a peptide motif from unassembled \(L\) subunits appears to bind to its mRNA to autoregulate its translation (Wostrikoff and Stern, 2007). Whether translation of the \(rbcS40L\) mRNA is influenced by this process is uncertain; however, according to the sink (source strength) regulation of photosynthesis hypothesis (Koch, 1996) the 40% reduction in leaf \(RbcS\) mRNA levels in \(A\(^{\text{N}_{\text{S40L}}}\) is contradictory to the near absence of starch and Glc in its leaves (data not shown). Indeed even in comparable carbohydrate-limited tobacco leaves producing \(R.\ ru bru\)n\) Rubisco that does not require \(S\) subunits, the steady-state \(RbcS\) mRNA pool was elevated >2-fold (Whitney and Andrews, 2003) as expected in response to source-strength repression. Clearly further study is required to fully examine how changes in leaf ontogeny and source strength impact on the developmental programming in the \(A\(^{N_{\text{S40L}}}\) lines. In particular quantifying differences in \(RbcS\) transcription rate and/or stability is required to assess whether there exists a plastid-signaling event that can regulate \(RbcS\) mRNA levels to determine whether such a regulatory event correlates with the deletion of the \(rbcL\) promoter-5'-UTR sequence from the \(A\(^{\text{N}_{\text{S40L}}}\) plastome.

The Unique Posttranslational Methylation of a Plastid-Synthesized Protein

Our finding that Met-1 of \(^\text{T}_{\text{S40L}}\) and the native \(S\) subunits share the same posttranslational monomethyl-
products were sequenced using BigDye terminator sequencing on an ABI 37300 sequencer (Biomolecular Resource Facility, Australian National University).

**Transformation and Plant Growth**

pANtS40L was purified from *Escherichia coli* XL1-Blue cells (10-mL culture) using the Wizard mini-prep kit (Promega) and biologically transformed as described (Svab and Maliga, 1993) into five sterile leaves from the T2 progeny of *trrl1* (Whitney and Sharwood, 2008). Spectrinycin-resistant plants were selected on selective tissue culture medium (agar-solidified Murashige and Skoog salts containing 3% v/v Suc, hormones, and 0.5 mg mL<sup>-1</sup> spectrinycin [Svab and Maliga, 1993]) and two lines (13 and 14) passed through a second round of regeneration on selective media following confirmation they were plastome transformants by nondenaturing PAGE (Whitney and Sharwood, 2008). Both lines were grown to maturity in soil in a controlled environment cabinet as described (Sharwood et al., 2008), in air supplemented with 0.5% K<sub>2</sub>CO<sub>3</sub> and 350 μmol quanta m<sup>-2</sup> s<sup>-1</sup> illumination. The flowers were backcrossed with wild-type tobacco (cv Petit Havana [N,N]) pollen and the T2 progeny used for all subsequent analyses.

**DNA, RNA, SDS-PAGE, and <sup>35</sup>S-Met Pulse- Chase Analyses**

All analyses were made on samples from the fifth leaf (13 cm in width) of wild-type and A<sup>NS40L</sup> plants (15–18 cm in height). Total leaf DNA and RNA was extracted, agarose gel separated and blotted onto Hybond N<sup>-</sup> membranes, and hybridized with alkaline phosphate or [α-<sup>32</sup>P]dATP-labeled DNA probes as described (Whitney and Sharwood, 2008). The total (lysate) and soluble leaf protein was extracted, separated by SDS-PAGE, and immunoblotted with polyclonal antiserum to spinach (*Spinacia oleracea*) Rubisco activase (Whitney and Andrews, 2001b) and tobacco Rubisco (that strongly recognizes the S subunit [Sharwood et al., 2008]). Both lines were grown to maturity in soil in a controlled environment cabinet as described (Sharwood et al., 2008), in air supplemented with 0.5% K<sub>2</sub>CO<sub>3</sub> and 350 μmol quanta m<sup>-2</sup> s<sup>-1</sup> illumination. The flowers were backcrossed with wild-type tobacco (cv Petit Havana [N,N]) pollen and the T2 progeny used for all subsequent analyses.

**Rubisco Purification, Content, Carbamylation, and Kinetic Analysis**

Measurement of Rubisco content and carbamylation status by [<sup>15</sup>N]<sup>34</sup>CABP/[<sup>15</sup>N]<sup>34</sup>CAB exchange, the substrate saturated turnover rate (k<sub>s</sub>), and the apparent K<sub>m</sub> for CO<sub>2</sub> (K<sub>c</sub>) under varying v/v CO<sub>2</sub> and 350 μmol quanta m<sup>-2</sup> s<sup>-1</sup> illumination. The flowers were backcrossed with wild-type tobacco (cv Petit Havana [N,N]) pollen and the T2 progeny used for all subsequent analyses.

**Leaf Gas Exchange**

Whole-leaf gas-exchange measurements were made in the growth chamber using a LI-6400 gas-exchange system (LI-COR) as described (Sharwood et al., 2008).

**N-Terminal Sequence Analysis**

The A<sup>NS40L</sup> peptide bands in the anion-exchange-purified Rubisco sample were separated by SDS-PAGE and either blotted onto polyvinylidene difluoride membrane for Edman sequencing on an Applied Biosystems 494 Proceic Protein Sequencing Sequencing system at the Australian Proteome Analysis Facility, or excised directly from the gel for Asp-N-digestion and analyzed by mass spectrometry at the University of Kentucky, Center for Structural Biology Protein Core Facility (Supplemental Fig. S3).

**Supplemental Data**

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

**Supplemental Figure S1.** Comparative phenotype of a mature flowering T<sub>0</sub> A<sup>NS40L</sup> and wild-type tobacco plants at approximately 70 cm in height.

**Supplemental Figure S2.** Relative turnover of <sup>35</sup>S-labeled Rubisco in leaf discs from an A<sup>NS40L</sup> and a wild-type tobacco leaf.

**Supplemental Figure S3.** Mass spectrometry analysis of Asp-N peptides derived from <sup>35</sup>S40L.

**Supplemental Table S1.** Tobacco plastome sequences coding putative peptides with Met-Lys N termini.

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**LITERATURE CITED**


Linked Rubisco Subunits Correctly Assemble in Plastids


