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A mechanism for intergenomic integration: Abundance of ribulose bisphosphate carboxylase small-subunit protein influences the translation of the large-subunit mRNA

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ABSTRACT **Multimeric protein complexes in chloroplasts and mitochondria are generally composed of products of both nuclear and organelle genes of the cell. A central problem of eukaryotic cell biology is to identify and understand the molecular mechanisms for integrating the production and accumulation of the products of the two separate genomes. Ribulose bisphosphate carboxylase (Rubisco) is localized in the chloroplasts of photosynthetic eukaryotic cells and is composed of small subunits (SS) and large subunits (LS) coded for by nuclear *rbcS* and chloroplast *rbcL* genes, respectively. Transgenic tobacco plants containing antisense *rbcS* DNA have reduced levels of *rbcS* mRNA, normal levels of *rbcL* mRNA, and coordinately reduced LS and SS proteins. Our previous experiments indicated that the rate of translation of *rbcL* mRNA might be reduced in some antisense plants; direct evidence is presented here. After a short-term pulse there is less labeled LS protein in the transgenic plants than in wild-type plants, indicating that LS accumulation is controlled in the mutants at the translational and/or posttranslational levels. Consistent with a primary restriction at translation, fewer *rbcL* mRNAs are associated with polysomes of normal size and more are free or are associated with only a few ribosomes in the antisense plants. Effects of the *rbcS* antisense mutation on mRNA and protein accumulation, as well as on the distribution of mRNAs on polysomes, appear to be minimal for other chloroplast and nuclear photosynthetic genes. Our results suggest that SS protein abundance specifically contributes to the regulation of LS protein accumulation at the level of *rbcL* translation initiation.**

Multimeric protein complexes in chloroplasts of eukaryotic cells generally contain subunits that are encoded in the nuclear genome and other subunits that are encoded in the plastid genome (1). One such complex is ribulose bisphosphate carboxylase (Rubisco), which is the key regulatory enzyme of photosynthetic carbon assimilation (2). Rubisco is localized in the chloroplast stroma and is composed of eight small-subunit (SS) proteins, coded for by a small nuclear (3) multigene (*rbcS*) family, and eight large-subunit (LS) proteins, coded for by a single gene (*rbcL*) on the multicopy chloroplast genome (4, 5). The *rbcS* transcripts are translated into SS precursor polypeptides on 80S ribosomes in the cytosol, then transported posttranslationally into the chloroplast, where they are processed to their mature form (6). The LS are translated on 70S ribosomes in the chloroplast and then assembled with mature SS in a chaperonin-mediated reaction to yield the holoenzyme (2). Because Rubisco is composed of single types of nuclear and plastid DNA-encoded subunits, the biosynthesis of this enzyme is an attractive model system to study the mechanisms that coordinate and integrate metabolic processes in the nuclear-cytosolic and chloroplast compartments.

LS and SS production are controlled by a variety of stimuli, including internal (e.g., developmental and hormonal) and external (e.g., light) signals (7, 8). Coordination of subunit production can occur at the transcriptional, translational, and/or posttranslational levels, depending on the system (1, 9–13). However, the coordinating factors are poorly understood. To gain insight into these factors, we asked whether the abundance of SS proteins influences LS metabolism. Given the prokaryotic nature of the chloroplast (14), this type of regulatory circuit might not be unexpected because bacterial genes are often end product-inhibited at the transcriptional and/or translational levels (15).

To address this question, we generated transgenic tobacco plants that have reduced amounts of *rbcS* mRNAs and SS proteins because of the expression of *rbcS* antisense RNAs (16). LS protein is coordinately reduced in amount in mutant plants, but *rbcL* mRNA levels are normal. The present studies show that most of the *rbcL* mRNA in mutant plants is not associated with polysomes of normal size, and thus that the abundance of SS protein, directly or indirectly, affects the translation of *rbcL* mRNA.

MATERIALS AND METHODS

Plant Material and Growth. T1 progeny from the self-fertilization of primary (T0) Rubisco antisense DNA transformants 3 and 5 (16) were grown from seed. Transformant 3 contained one copy of the antisense DNA sequence and had only about one-third of the normal amount of *rbcS* mRNA and about 50% of the normal amount of Rubisco protein, whereas transformant 5, which contained at least four copies of the antisense sequence, had only approximately 12% of normal *rbcS* mRNA and about 38% of normal Rubisco protein (16). Untransformed SR1 tobacco (*Nicotiana tabacum*) served as controls. Cuttings from the transformed and wild-type plants were maintained under tissue culture conditions on Gamborg's B-5 medium supplemented with 1% sucrose. The progeny from two different primary transformants were used in the present experiments to rule out the possibility that any observed effects were due to position effects of the inserted sequences in the tobacco genome.

Detection of RNA and Protein. RNA isolation and RNA gel blot procedures have been described (16). The probes in the present analyses included: pSEM1, specific for the *N. tabacum rbcS* gene family (17); pTB5, containing a portion of the *N. tabacum rbcL* gene (18); pJQ4, containing an *N. tabacum Rca* cDNA for Rubisco activase (19); *cab*40, containing a 0.8-kb *N. tabacum* cDNA specific for the light-harvesting chlorophyll *a*/*b*-binding (CAB) proteins of photosystem II (D. Jin and L.B., unpublished); pZmc427, containing the maize chloroplast *psbA* gene for the D1 reaction center polypeptide of

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Abbreviations: Rubisco, ribulose bisphosphate carboxylase; SS, small subunits; LS, large subunits.

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photosystem II (20); T18, a 2.3-kb *N. tabacum Bam*HI chloroplast DNA fragment containing the fused *atpB* and *atpE* genes, for the β and ε subunits of the chloroplast ATP synthase, cloned into pTZ19R (gift of Jonathan Wendel, Iowa State University); T22, a 3.0-kb *N. tabacum Bam*HI/*Sac* I chloroplast DNA fragment containing the *petA* gene for cytochrome *f*, cloned into pTZ19R (gift of Jonathan Wendel); and T14, a 2.5-kb *N. tabacum Bam*HI chloroplast DNA fragment containing the duplicated *psaA* and *psaB* genes for the Ia and Ib P700 apoprotein subunits of photosystem I, cloned into pTZ19R (gift of Jonathan Wendel).

Protein isolation, SDS/PAGE, and Western immunoblotting procedures have been described (16). Antibody specificity was monitored using ^{125}I-conjugated protein A at a concentration of 1 μCi/ml. Relative protein amounts on the Western blots were quantified using a Molecular Dynamics PhosphorImager 400E. Antibodies included spinach Rubisco activase, spinach cytochrome *f*, and the *N. tabacum* CAB proteins, all described in Jiang and Rodermel (21), and spinach antibodies to the chloroplast *atpB* and *psaA* gene products (gift of Alice Barkan, University of Oregon).

To examine *in vivo* LS synthesis, [^{35}S]methionine (150 μCi in 1% Tween 20) was spotted onto the top three expanding leaves of tissue culture-growth plants. After 10 min, the leaves were rapidly detached and frozen in liquid nitrogen. Soluble proteins were isolated from the leaves, trichloroacetic acid-precipitable cpm were determined, and equal cpm of the soluble protein extracts were electrophoresed through an SDS/7.5–12.5% polyacrylamide gel (16). The gels were dried and either autoradiographed or subjected to PhosphorImager analysis.

Polysome Analysis. The top three expanding leaves of plants grown under sterile tissue culture conditions were harvested and ground in liquid nitrogen. Leaf extracts containing total ribosomal material (subunits, monosomes, polysomes) were prepared and centrifuged on 12.5–55% analytical sucrose gradients according to the method of Klaff and Gruissem (22). After centrifugation, fractions were removed from the gradients, brought to 0.5% SDS and 20 mM EDTA, and extracted with phenol-chloroform. Nucleic acids were recovered from each fraction by ethanol precipitation, then separated on 1.2% Mops-formaldehyde gels (16). Hybridizations were conducted as described above. As controls for polysome dissociation, leaf lysates were brought to 20 mM EDTA before loading on sucrose gradients that contained 1 mM EDTA instead of 10 mM $MgCl_2$ (22).

RESULTS

LS Accumulation Is Controlled Posttranscriptionally in the Antisense Plants. Fig. 1 shows that LS and SS protein amounts are coordinately reduced in abundance in T1 progeny plants from primary transformants 3 (plant "E") and 5 (plants "B," "C," and "D"); untransformed SR1 plants served as controls (plants "A1" and "A2"). These reductions range from ≈12% (transformant "C") to ≈32% (transformant "E") of average wild-type protein levels (densitometric data not shown). Protein amounts were normalized to leaf area in Fig. 1, but similar results were obtained when gel loadings were normalized to chlorophyll content or protein amount. Fig. 2 shows that the reductions in Rubisco in the transformed plants are related to the accumulation of *rbcS*, but not *rbcL*, mRNAs. PhosphorImager analyses of replicate blots revealed that *rbcS* transcript levels are reduced up to ≈85% in some plants (e.g., plant "B"). Considered together, Figs. 1 and 2 demonstrate that there is appreciable posttranscriptional control of LS protein accumulation in the T1 Rubisco antisense mutants. These data confirm and extend our earlier findings on the primary transformants (T0 generation) (16).

LS Protein Synthesis. As a first approach to ascertaining whether LS abundance is regulated at the translational level,

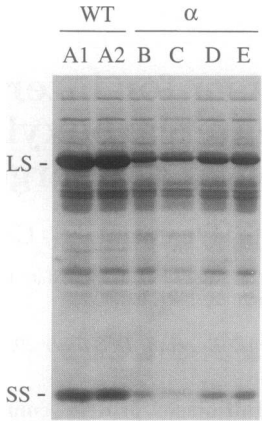

FIG. 1. LS and SS protein abundance. Leaf discs were isolated from the top three expanding leaves of antisense (α) and wild-type (WT) plants and solubilized in equal volumes of extraction buffer. After centrifugation, equal amounts of the soluble extracts (100 μl) were electrophoresed through an SDS/7.5–12.5% polyacrylamide gel; the gel was stained with Coomassie blue. Bands containing the LS and SS proteins were identified by Western immunoblot analysis (16).

we conducted *in vivo* pulse-labeling experiments with [^{35}S]methionine. The top three expanding leaves from plants growing on tissue culture medium were supplied with label for 10 min, then soluble proteins were isolated and aliquots containing equal cpm were electrophoresed through discontinuous SDS/polyacrylamide gels. The results of a representative experiment are illustrated in Fig. 3, and show that much less label was incorporated into the LS during the 10-min pulse in the antisense than in wild-type plants. There was also much less label incorporated into the SS in the mutants, as anticipated. In the presence of about equal amounts of *rbcL* mRNA, but lowered levels of the SS, LS synthesis could be unaffected but turnover could be accelerated, as we initially suggested (16), and/or translation of *rbcL* mRNA could be reduced.

Polysomal Distribution of *rbcL* mRNAs. To investigate whether *rbcL* mRNA translation is impeded in chloroplasts of antisense plants with low SS, we examined the distribution of *rbcL* mRNA on 12.5–55% analytical sucrose gradients. Polysomes have higher sedimentation constants than monosomes or free RNAs, and thus sedimentation analysis of a given mRNA species in these gradients reveals the proportion of an mRNA species in the cell that is in polysomes—and thus most likely being translated—and what proportion is not (e.g., 22–25). Fig. 4 shows that *rbcL* mRNAs from an antisense plant sediment higher in the gradient than *rbcL* mRNAs from a wild-type plant. We obtained similar results with other antisense and wild-type plants (data not shown). In the EDTA control, *rbcL* mRNAs were shifted to the top of the gradient, as expected. Collectively, these data indicate that *rbcL* mRNAs associate with fewer polysomes in the antisense plants, suggesting that *rbcL* mRNA translation initiation is defective in these plants.

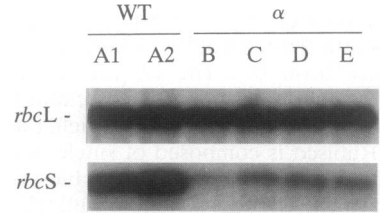

FIG. 2. *rbcL* and *rbcS* mRNA accumulation. RNA gel blot experiments were performed with 5 μg of total cell RNA from the top three expanding leaves of control and mutant plants. The filters were probed with pSEM1 (17) to detect *rbcS* mRNAs or pTB5 (18) to detect *rbcL* mRNAs. The plants are the same as those described in the legend to Fig. 1.

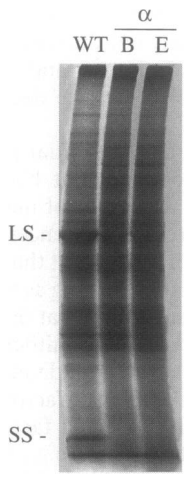

FIG. 3. *In vivo* LS synthesis. [^{35}S]Methionine was spotted onto the top three leaves of two antisense ("B" and "E") and a wild-type ("A2") plant. After a 10-min pulse, equal cpm of soluble protein were electrophoresed through an SDS/7.5–12.5% polyacrylamide gel, and the gel was autoradiographed. The LS and SS bands were identified by Western immunoblot analysis, as described in the legend to Fig. 1.

The Translation Initiation Defect Is Specific for *rbcL* mRNAs. Because the antisense plants have lower rates of photosynthesis (21, 26), it is possible that reductions in Rubisco have pleiotropic effects that include a generalized repression of chloroplast translation. To a first approximation, analysis by SDS/PAGE shows that, other than Rubisco, the amounts of soluble (see Fig. 1) and total protein (data not shown) do not appear to be grossly affected in the mutant plants. This was confirmed by Western immunoblot analysis of representative chloroplast proteins. For example, Fig. 5 shows that the amounts of the *petA, psaA*, and *atpB* gene products (coded for in the chloroplast genome) and the *cab* and *Rca* gene products (coded for in the nuclear genome) are unperturbed in the transgenic plants.

Consistent with the protein accumulation data, Fig. 6 shows that the abundance of transcripts of representative nuclear and chloroplast genes, including *cab, atpBE, psaA/B, petA*, and *psbA*, is relatively unaffected in the antisense plants. There are also no major differences between wild-type and mutant plants in the distributions of these mRNAs on "polysome gradients" (Fig. 7). It might be noted that in both the wild-type and mutant plants a higher proportion of *cab, psaA/B* and *atpBE*

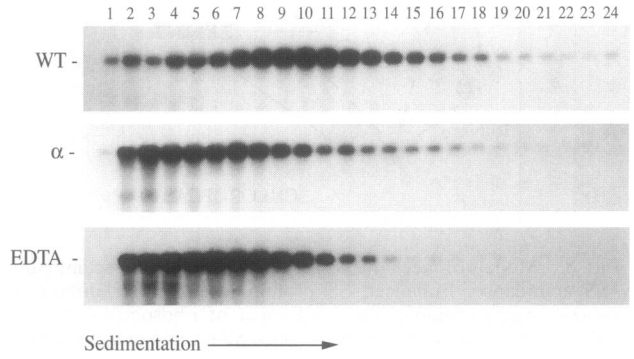

FIG. 4. Distribution of *rbcL* mRNA on sucrose gradients. Leaf homogenates from the top three expanding leaves of antisense ("B") and wild-type ("A1") plants were size-fractionated on 12.5–55% sucrose gradients. Fractions of equal volume were collected from the gradient, and RNA was isolated from each fraction. *rbcL* mRNA levels were assayed by RNA gel blot hybridization. Sedimentation is from the top of the gradient to the bottom. In the EDTA control, wild-type homogenates were treated with 20 mM EDTA before loading on a gradient containing 1 mM EDTA in the absence of $MgCl_2$.

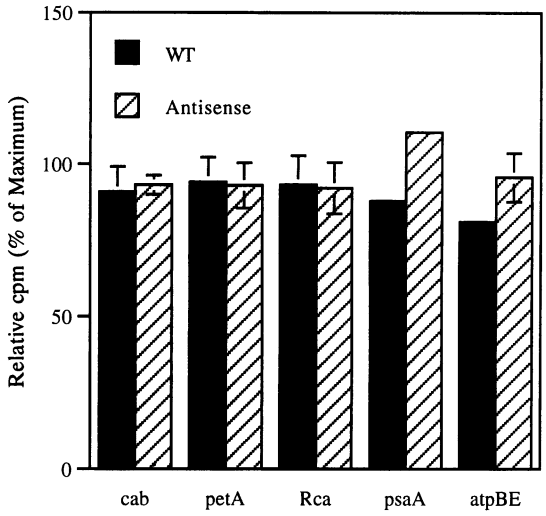

FIG. 5. Photosynthetic protein accumulation. Total proteins were isolated from leaf discs of wild-type ("A2") and antisense ("C") plants and electrophoresed through discontinuous SDS/7.5–12.5% polyacrylamide gels; the proteins were loaded on a leaf area basis. Western immunoblot filters were prepared and incubated with antibodies specific for the products of the genes shown. Each data point is expressed as a percentage of the maximum wild-type value, and represents the mean ± SD from PhosphorImager analysis of 2 to 3 replicate filters.

mRNAs are associated with polysomes than *psbA* and *petA* transcripts. Although the reason for the reduced association of *psbA* and *petA* mRNAs with polysomes is not clear, a similar phenomenon has been documented for *psbA* mRNAs in spinach, maize, and barley (22, 25, 27). We have obtained results similar to those in Figs. 6 and 7 using other antisense and wild-type plants. Considered together, the data in Figs. 5, 6, and 7 indicate that there is not a global reduction in chloroplast mRNA translation initiation in the antisense plants—i.e., *rbcL* mRNAs are specifically altered in their association with polysomes in the mutant plants.

DISCUSSION

Our experiments have revealed that antisense plants with reduced SS protein incorporate less label into the LS during a

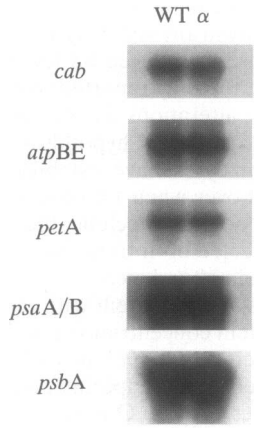

FIG. 6. Accumulation of mRNAs from photosynthetic genes. RNA gel blot experiments were conducted using 5 μg of total cell RNA from the top three expanding leaves of wild-type ("A1") and antisense ("C") plants. The filters were probed with sequences that detect all members of the *cab* gene family, and chloroplast mRNAs from *psbA, petA*, the duplicated *psaA* and *psaB* genes, and the fused *atpBE* gene (probes described in *Materials and Methods*).

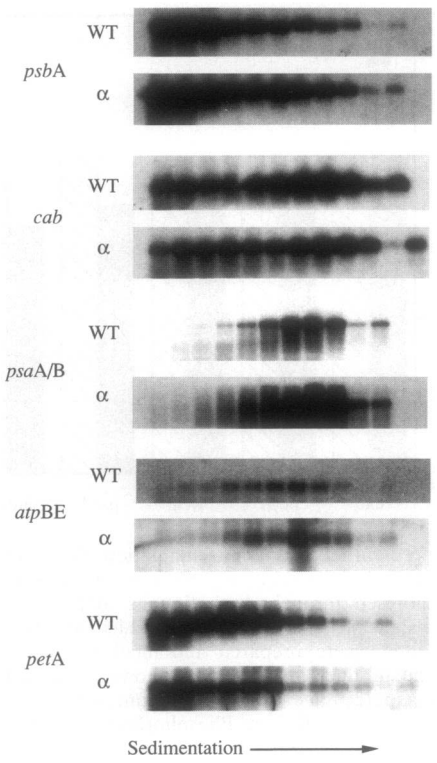

FIG. 7. Distribution of cytoplasmic and chloroplast mRNAs on sucrose density gradients. The distributions of *psbA, cab, psaA/B, atpBE*, and *petA* were determined as described in the legend to Fig. 4 using the same probes as in Fig. 6.

short-term pulse. In addition, *rbcL* mRNAs in these plants are not associated with polysomes of the normal size distribution. Inasmuch as *rbcL* mRNAs are present in normal amounts in the antisense plants (16), these observations indicate that LS protein levels are controlled primarily at the level of *rbcL* mRNA translation.

Our previous longer-term pulse-labeling experiments suggested that *rbcL* mRNA translation might be restricted in Rubisco antisense plants with severely reduced SS levels (16). Although the LS is a stable protein, with a half-life on the order of days (28), the relatively long duration of the pulse in the previous experiments (60 min) permitted the interpretation that the lower level of radiolabeled LS in mutants with very low SS might result from enhanced LS turnover. It has not been demonstrated directly in any system that excess LS proteins are degraded in response to reduced SS levels, because the *rbcS* antisense plants are the only reported mutants, to our knowledge, with specific alterations in SS protein amounts that would allow one to test this hypothesis unambiguously. In contrast, mutant analyses have established that excess SS proteins are turned over when LS production is limiting (12, 29–32). Our data do not preclude the possibility that LS accumulation is affected both translationally and posttranslationally by SS protein abundance.

Our observations are consistent with inhibitor studies, suggesting that LS protein concentration is adjusted to that of the SS by translational control of *rbcL* mRNA expression (33, 34). However, a drawback to such experiments is that inhibitors can have multiple direct effects. Our studies revealed that the patterns of expression of photosynthetic genes (other than *rbcS* or *rbcL*) are not significantly altered in the antisense plants, indicating that reductions in SS in the antisense plants do not have a global impact on chloroplast translation. Inhibitor studies (22) and analyses of maize mutants with global defects in translation (25) have suggested that *rbcL* mRNAs are destabilized by lack of association with ribosomes. However, the lack of pleiotropy in the present system permits us to conclude that this is likely not the case (at least in the present system), because the antisense and wild-type plants have similar *rbcL* mRNA levels, despite decreased ribosome association in the mutants.

The factors that regulate the initiation of chloroplast translation are poorly understood, but biochemical and genetic studies have identified a number of nuclear factors that bind to the 5′-untranslated region sequences of several chloroplast mRNAs (9–11, 35, 36). It is thought that such factors facilitate ribosome binding, perhaps by acting as translational activators. Gillham *et al.* (9) have suggested that there are three classes of such factors for chloroplast and mitochondrial mRNAs, and that initiation of translation of individual organelle mRNAs may be governed by a complex of factors: general factors that bind to all mRNAs; class-specific factors that bind mRNAs from functionally related genes (e.g., photosynthetic genes); and gene-specific factors that bind a single mRNA species. To this must be added factors that could exert negative control on translation by blocking ribosome binding and polysome formation. The fact that SS proteins, either directly or indirectly, specifically affect recruitment of ribosomes to *rbcL* mRNA, either positively or negatively, suggests that control of *rbcL* mRNA translation initiation involves one or more gene-specific factors. As illustrated in Fig. 8, the SS could be acting, directly or indirectly, as a translational activator, or perhaps as a cofactor or coderepressor. With less SS, less cofactor (or coderepressor) would be present, resulting in less *rbcL* mRNA translation initiation. Alternatively, excess LS or its degradation products could serve to suppress *rbcL* mRNA translation initiation when SS is limiting. The latter mechanism would resemble end-product inhibition at translation as has been seen

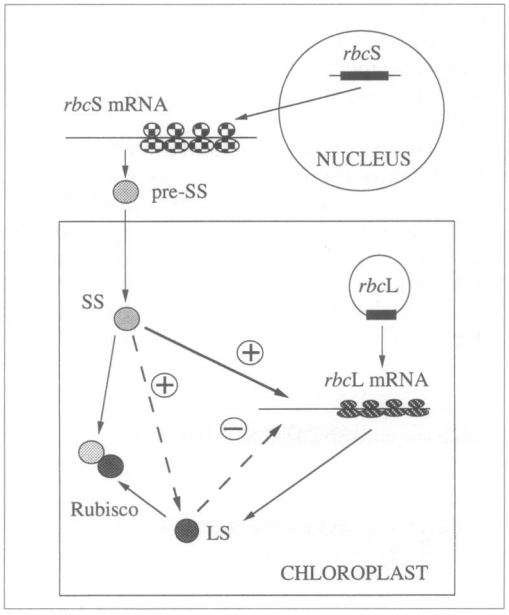

FIG. 8. Models of mechanisms for the control of LS accumulation by SS abundance. Enhanced SS protein levels, either directly or indirectly, might promote the recruitment of ribosomes onto *rbcL* mRNAs (bold line); alternatively, reduced SS levels can be viewed as, directly or indirectly, inhibiting ribosome recruitment. As an example of this positive control, SS proteins (or some product of a nuclear or plastid gene present when SS are abundant) could be cofactors required for attachment of chloroplast ribosomes to LS mRNA. As an example of negative control, excess LS or LS breakdown products that might accumulate when SS are scarce could block ribosomes from associating with *rbcL* mRNAs (dashed line extending from LS protein). Our data do not exclude the possibility that increased SS protein could also act posttranslationally as a signal to decrease LS turnover (dashed line extending from SS protein).

for some bacterial genes (e.g., r-protein genes) (15) and thus could be a relic of the prokaryotic endosymbiont origins of chloroplasts.

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