Photosynthesis, Rubisco Activity and Amount, and Their Regulation by Transcription in Senescing Soybean Leaves

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Senescence is a phase of leaf ontogeny marked by declining photosynthetic activity that, in soybean (Glycine max [L.] Merr.), is paralleled by a decline in chloroplast function. Soybean leaves have different patterns of decline in photosynthetic capacity and chloroplast function associated with nodal position and sink activity. The objective of this work was to determine whether leaves from nodes 3 and 6 of soybean, which show these different patterns, are similarly regulated with respect to ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and content and also to ascertain the degree of regulation of Rubisco content by transcription. Leaves from nodes 3 and 6 of field-grown soybean plants were sampled periodically from the time of their unfolding until near death. In situ CO₂-exchange rate (CER) increased to a maximal level in both leaves and then declined slowly. For node 3 leaves the decline was progressive, but for node 6 leaves the decline was arrested at about 75% of maximum CER for a period of about 20 d, coincident with the onset of rapid seed growth, before a short period of very rapid decline immediately preceding leaf death. Rubisco activities and Rubisco content were directly correlated with CER in the leaves exhibiting the two different patterns. Rubisco activation ratio was similar for the two leaves and did not change throughout development. The primary regulator of photosynthesis at the physiological level, thus, was the amount of Rubisco protein. Decreases in Rubisco holoenzyme during senescence of both leaves were accompanied by coordinate decreases in the levels of mRNAs for the small and large subunits of Rubisco, suggesting that the decrease in Rubisco enzyme amounts during soybean leaf senescence is due to slower transcription rates and that levels of these mRNAs are coordinately controlled during senescence as they are during chloroplast development. However, plastid DNA template availability and posttranscriptional controls may also influence Rubisco content during senescence of these leaves. We conclude that soybean leaf photosynthesis likely unfolds according to a single developmental program but that modifications can be superimposed upon this program to maximize photosynthetic rates.

Senescence is a phase of leaf development marked by a decline in cellular components, leaf-yellowing, and finally leaf death and abscission (reviewed by Brady, 1988). Overall, the process appears to play a fundamental role in the nutrient economy of the plant, allowing the plant to redistribute metabolites, particularly organic nitrogen, from one tissue (the senescing leaf) to other tissues, such as developing fruits and leaves (reviewed by Woolhouse, 1987). One of the most conspicuous features of senescence is a decline in photosynthetic capacity (reviewed by Shibles et al., 1987; Gepstein, 1988; Shibles et al., 1989). This decline is due to a process of chloroplast dismantling that may involve either a decrease in the number of chloroplasts (Wittenbach et al., 1982) or a decline in chloroplast function (Wardley et al., 1984). Ford and Shibles (1988) concluded that senescence was a two-stage process in soybean (Glycine max [L.] Merr.): first a decline in chloroplast function, followed by a brief terminal phase when chloroplasts are lost as well.

Relatively few investigators have rigorously examined whether decreased amounts of a given plastid component during senescence are due to decreased rates of synthesis of that component or, alternatively, to increased rates of degradation of that component. Consequently, the relative contributions of anabolic versus catabolic factors in regulating this process are not well understood (Woolhouse, 1987; Gepstein, 1988; Huffaker, 1990). Decreases in Rubisco activity are a hallmark of the senescence process (Brady, 1988; Gepstein, 1988), especially in soybean (Secor et al., 1984; Ford and Shibles, 1988; Crafts-Brandner, 1992). Although it is commonly assumed that these decreases reflect enhanced rates of protein degradation (Brady, 1988), several reports have shown a relationship with altered rates of Rubisco subunit synthesis at the transcriptional and translational levels (Brady, 1988; Bate et al., 1991). This suggests that accumulation of this protein during senescence may be regulated differently from species to species. The question arises whether the accumulation of this protein also is regulated differently among various leaves of a given species. Soybean is an ideal model system to address this question because our previous investigations have shown that soybean leaves have different patterns of decline in photosynthetic capacity during senescence that are directly correlated with changes in Rubisco activity (Secor et al., 1984). These differences arise primarily as a function of ontogeny (nodal position on the plant) and, perhaps, sink activity. Therefore, we hoped that analyses of such leaves might expose the spectrum of regulation used by soybean to control the amount of this enzyme during the senescence process.

In this paper, we first show that the regulation of photosynthetic activity is a contributing factor to North Central Regional Project NC-142. Support for the nucleic acid analyses was provided by the Iowa State University Biotechnology Council.

Abbreviations: CER, CO₂-exchange rate; DAP, days after planting; rbcL, large subunit of Rubisco; rbcS, small subunit of Rubisco; RuBP, ribulose-1,5-bisphosphate.
synthesis during the senescence of two soybean leaves that display markedly different photosynthetic patterns is mediated primarily by Rubisco activity and content. Then, we show that decreases in the accumulation of the holoenzyme in both leaves are accompanied by coordinate decreases in the levels of the rbcL and rbcS mRNAs. This suggests that control over Rubisco enzyme amounts during soybean leaf senescence is exerted primarily at the transcriptional level. However, we further show that part of the decrease in rbcL mRNA amounts during the senescence process in both types of leaves may be due to decreases in plastid DNA template availability and that posttranslational events associated with sink development may also play a role in controlling Rubisco content during this process. We conclude that soybean leaf senescence likely unfolds according to a single developmental program but that modifications can be superimposed upon this program to maximize photosynthetic rates. We further suggest that part of this program involves transcriptional coordinating signals between the nucleus and the chloroplast.

**MATERIALS AND METHODS**

**Plant Material and Growth**

Soybean (Glycine max [L.] Merr. cv Hodgson 78) seeds were sown in rows 1.05 m wide in a silt loam soil at the Hinds Research Site near Ames, IA. After emergence, the plants were thinned at the unifoliolate stage to a uniform density of 10 plants per m of row. A combination of Bravo 500 and Benomyl was sprayed twice to prevent bacterial and fungal infection, and the field was irrigated as needed to maintain adequate water availability. A randomized complete-block design with four replications was used for these experiments. To reduce sample variation, 2-m row sections were marked, and eight plants of uniform age and development were tagged in each section before measurements and sampling began. A different 2-m section was randomly chosen for each sampling date. All measurements and other samplings were performed on node 3 and node 6 leaves of the tagged plants.

**Photosynthetic Measurements**

We measured CER and stomatal conductance on attached leaves using an LI-6200 Portable Photosynthesis System (Li-Cor Inc., Lincoln, NE). Measurements were made under saturating PPFD (>1200 μmol m⁻² s⁻¹ at the leaf surface) and between 1000 and 1400 h Central Daylight Time. Leaves from five of the eight tagged plants in each 2-m row section were measured, and four replicates were performed at each sampling date. All measurements and other samplings were performed on node 3 and node 6 leaves of the tagged plants.

Rubisco activities were estimated by RuBP-dependent incorporation of 14CO₂ into acid-stable products. In brief, "initial" activities were measured at 25°C by injecting 10 μL of 20 mM RuBP and 10 μL of the soluble leaf extract into an assay mixture (480 μL) containing 100 mM Bicine (pH 8.2), 20 mM MgCl₂, 5 mM DTT, and 200 mM NaH14CO₃ (8.14 Bq/μmol). The reaction was terminated after 60 s by the addition of 100 μL of 2 N HCl and dried at 90°C for 3 h; then, acid-stable 14C was estimated by liquid scintillation counting. "Total" Rubisco activities were determined in a similar manner, with the exception that 10 μL of the soluble leaf extract and 480 μL of the assay mixture were incubated together at 25°C for 5 min before 10 μL of 20 mM RuBP were added.

Rubisco amounts were determined by single radial immunodiffusion (Vaerman, 1981) with rabbit polyclonal antibodies against purified soybean Rubisco. In brief, soluble leaf extracts were applied to agarose gels (1%) that contained 50 mM Tris-HCl (pH 7.0), 0.9% (w/v) NaCl, 15 mM NaN₃, and Rubisco antibody (1:200–1:500 dilution). The gels were incubated at 25°C for 48 h and then dried on plastic films. After drying, the gels were stained in a solution containing 0.2% (w/v) Coomassie brilliant blue R-200, 10% acetic acid, and 25% isopropanol, destained in 10% acetic acid and 25% isopropanol, and then dried again. The resulting diameters were measured and compared with standards from purified soybean Rubisco (0.04–2 μg) that had been spotted in duplicate on each gel.

**Isolation of Nucleic Acids and Hybridization Analyses**

For each time, five leaf discs, each from different plants, were pooled and ground to a fine powder in liquid N₂. Total cell RNA and DNA were isolated by procedures described by Ausubel et al. (1992), and DNA amounts were quantitated by the diphenylamine assay (Munro and Fleck, 1966). For Southern hybridizations, samples of total cell DNA were digested with BglII, electrophoresed through a 1% Tris-acetate-EDTA agarose gel, and transferred to nylon filters (GeneScreen Plus, New England Nuclear) (Ausubel et al., 1992). For northern hybridizations, equal amounts (10 μg) of total cell RNA were electrophoresed through a 1.2% Mops-formaldehyde gel and transferred to Zeta-probe blotting membranes (Bio-Rad) (Ausubel et al., 1992). Both Southern and northern filters were hybridized and washed by methods described by Church and Gilbert (1984) using randomly primed DNA probes (Pharmacia) at a concentration of about 5 × 10⁵ dpm/mL. Following hybridization, the filters were either autoradiographed or exposed to Phosphorimage analysis (Molecular Dynamics) to quantitate the amount of radioactivity in labeled bands.

The DNA probes used in the present study included pTB5 (Shinozaki and Sugiuira, 1982) and pSRS0.8 (gift of R.B. Meagher, University of Georgia). The pTB5 probe contains a portion of the tobacco (Nicotiana tabacum L.) chloroplast gene for rbcL, and the pSRS0.8 probe contains a fragment of soybean nuclear DNA that includes a portion of the "SRS1" member of the gene family for rbcS. These latter sequences
include the third exon of the gene, which is highly conserved among the members of the family (Berry-Lowe et al., 1982).

**RESULTS**

**Photosynthetic Activities**

Node 3 leaves unfolded 30 DAP, reached full size 40 DAP, and then began to senesce, whereas node 6 leaves unfolded 42 DAP, were fully expanded by 55 DAP, and then began to senesce (Figs. 1A and 2). Flowers did not develop at node 3, but flowering began 50 DAP at node 6; subsequently, beginning 62 DAP there was a rapid, linear increase in pod dry weight (seed growth) at this node (Fig. 1B). Thus, node 3 leaves developed in the absence of reproduction, whereas the development of node 6 leaves overlapped pod and seed growth.

Figure 2 shows the patterns of change in photosynthetic capacities, estimated by CER, of node 3 and node 6 leaves. As observed previously (reviewed by Shibles et al., 1989), the development of leaves from both nodes is characterized by a phase of increasing photosynthetic rates (coincident with leaf expansion), a brief phase of maximal photosynthetic rates (just subsequent to full expansion), and finally a prolonged senescence phase of declining photosynthetic capacities. However, node 6 leaves display an arrest in photosynthetic capacity decline (from 65–85 DAP) that is not present during the senescence of node 3 leaves. This period of arrest is coincident with the onset of rapid seed growth (compare with Fig. 1B).

Initial (Fig. 3A) and total (data not shown) Rubisco activities and Rubisco content (Fig. 3B) showed patterns of change during senescence similar to that of CER. The activation state of the enzyme did not change appreciably in either of these leaves during senescence (Fig. 3C), and consequently, alterations in Rubisco activity during this process were primarily a reflection of alterations in enzyme content. Considered together, the data in Figures 2 and 3 indicate that differences in photosynthetic capacity during the senescence of node 3 and node 6 leaves are directly correlated with alterations in Rubisco activity and content (Fig. 4).

**Regulation of Rubisco Content**

To assess the contribution of transcriptional factors to the regulation of Rubisco content, northern hybridizations were performed to examine the profiles of rbcL and rbcS transcript abundance. Sample autoradiographs from these hybridiza-
Photosynthetic capacities of leaves from node 3 and node 6. Photosynthetic activities were estimated from CER as described in "Materials and Methods." Results are means ± SD from measurements of leaves from 20 different plants.

DISCUSSION

Senescence-correlated losses in photosynthetic capacity are usually associated with alterations in Rubisco activity (reviewed by Gepstein, 1988). These changes in activity may arise as a consequence of alterations in either the amount and/or activation state of the enzyme. Previous studies of soybean have demonstrated that the patterns of decline in photosynthetic capacity during senescence vary markedly from leaf to leaf, depending upon whether pod and seed growth overlap leaf development at a particular node (reviewed by Shibles et al., 1989). The results of the present studies confirm these findings and extend earlier observations by showing that Rubisco content and activity are directly correlated with these declines, regardless of the nodal position of the leaf on the plant. These correlations can be interpreted to mean that the decline in Rubisco protein is the major cause of the loss of Rubisco activity during soybean leaf senescence. Consistent with this interpretation, the present experiments with lower (node 3) and midcanopy (node 6) leaves showed that the activation state of Rubisco does not change appreciably during the course of senescence in either leaf.

These findings lead us to hypothesize that Rubisco is the primary determinant of photosynthetic capacity during leaf senescence in field-grown soybean plants. Consistent with
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Figure 4. Relation of CER with initial Rubisco activity (A) and Rubisco content (B) for leaves at node 3 and node 6.

Figure 5. Changes in rbcS and rbcL transcript abundance during the senescence of node 3 and node 6 leaves. The northern hybridizations were conducted as described in “Materials and Methods.” As expected, the rbcS probe detected transcripts of approximately 900 bp (Berry-Lowe et al., 1982), whereas the rbcL probe detected a single 1.6-kb mRNA species (Shinozaki and Sugiura, 1982). Lanes 1 to 6, Node 6 leaves corresponding to 47, 51, 53, 63, 81, and 94 DAP. Lanes 7 to 11, Node 3 leaves, corresponding to 33, 39, 45, 51, and 68 DAP.

Figure 6. Changes in rbcS and rbcL transcript abundance and rbcL template availability during the senescence of node 3 and node 6 leaves. For the northern analyses (A and B), results represent the means ± so from Phosphorlmage analysis of hybridizations to two separate northern filters containing samples of RNA from five pooled leaf discs, each disc from a different plant. For the Southern analyses (C), results represent the means ± so from Phosphorlmage analysis of two hybridizations to a Southern filter containing samples of DNA from five pooled leaf discs, each disc from a different plant. A, B, and C show the relative amounts of rbcL mRNA, rbcS mRNA, and rbcL template (expressed as percentage of maximum), respectively, during the senescence of leaves from node 3 and node 6.

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this hypothesis and with our previous findings (Secor et al., 1984; Ford and Shibles, 1988), we found that changes in Chl content (data not shown) correspond only approximately to alterations in photosynthetic capacity during the senescence of lower and midcanopy soybean leaves. This indicates that light harvesting is not limiting for photosynthesis under these conditions. Whereas stomatal conductance was directly correlated with photosynthetic activity during the senescence of these leaves, we believe it unlikely that stomatal aperture played a central role in controlling the senescence-associated declines in photosynthetic activity in these leaves because internal CO₂ concentrations did not decrease but, rather,
remained constant during the course of senescence in both leaves (reviewed by Farquhar and Sharkey, 1982). We did not examine other components of the photosynthetic apparatus in these experiments, e.g. electron transport capacities. However, a preponderance of evidence indicates that Rubisco is limiting for photosynthesis in soybean (reviewed by Shibles et al., 1987, 1989). It should be pointed out, however, that the kinds of experiments described in this paper provide only correlative types of data, and a rigorous demonstration of this conclusion requires the ability to perturb the system in a specific manner.

Given the likely importance of Rubisco content in determining photosynthetic capacity, it was of interest to elucidate those mechanisms that regulate Rubisco content during soybean leaf senescence. At the outset, we hoped that an examination of this question in leaves with markedly different patterns of decline in photosynthetic capacity (viz. mid-canopy versus lower canopy leaves) might reveal whether the accumulation of the holoenzyme is regulated differently during the senescence process in leaves at different nodal positions on the plant. We observed that overall reductions in the abundance of Rubisco are accompanied by coordinate reductions in the levels of rbcL and rbcS mRNAs during the senescence of leaves from both nodes, indicating that, regardless of leaf nodal position, transcriptional controls seem primarily responsible for the level of the holoenzyme during the senescence process in soybean. This coordination suggests that both leaves have the same underlying developmental program that, for example, sets the rates of transcription of the rbcS and rbcL genes. We cannot rule out the possibility, however, that superimposed upon this "coarse" control mechanism is a "fine-tune" control mechanism that operates posttranscriptionally to adjust Rubisco amounts. For example, it is possible that reductions in rbcS and rbcL mRNA amounts are not directly matched by corresponding reductions in Rubisco protein amounts during the phase of arrest in photosynthetic capacity decline during the senescence of mid-canopy leaves. If so, this might indicate that the basic senescence program common to both leaves can be temporally modified at the translational and/or posttranslational levels, perhaps by factors associated with sink development. The nature of such factors is unknown, but their existence has been inferred in numerous other studies showing that sinks can have marked effects on photosynthesis during the senescence of source leaves (Brady, 1988; Diethelm and Shibles, 1989).

A question raised by these findings concerns the mechanisms that coordinate rbcS and rbcL transcript levels during the senescence process. Such mechanisms are not unique to senescence, because coordinate alterations in rbcS and rbcL mRNA levels also occur during various other developmental processes, most notably, the process of light-induced chloroplast development (reviewed by Tobin and Silverthorne, 1985). Because these mRNAs are transcribed in different genetic compartments, there may be some sort of communication between the nucleus and the plastid in the down-regulation of the transcription of these genes. The nature of such communication is not understood. However, mutant analyses have revealed that, if integrative signals between these two compartments do exist, they likely are not generated in response to the levels of transcription or translation products of nuclear or chloroplast genes for multimeric plastid components (Hildebrandt et al., 1984; Rodermel et al., 1988); rbcL transcription is not influenced by rbcS mRNA or small-subunit protein amounts, and rbcS transcription is not influenced by rbcL mRNA or large-subunit protein amounts.

Pulse-labeling experiments have shown that there are decreasing rates of accumulation of labeled protein during leaf senescence in a variety of species. It cannot be discerned
from these types of experiments, however, whether such decreases are due to enhanced rates of protein degradation or, alternatively, to decreased rates of synthesis (transcription and/or translation). Relatively few investigators have attempted to discriminate between these possibilities, but the results to date indicate that these mechanisms vary from system to system. Some studies, for example, have shown that posttranscriptional mechanisms predominate in regulating Rubisco accumulation during senescence (Brady, 1988; Vera et al., 1990), which is consistent with the notion that decreased amounts of plastid proteins during this process are primarily due to enhanced rates of protein degradation (Huf- faker, 1990). On the other hand, other studies (including the present one) have shown that transcriptional mechanisms may be paramount in regulating Rubisco amounts during senescence (Brady, 1988; Bate et al., 1991), suggesting that decreased biosynthesis of plastid components is an important determinant of protein levels during this process (Woolhouse, 1987). Because the relative rates of degradative and biosynthetic processes may well vary from system to system, it will be necessary to characterize each system in detail to obtain an estimate of the relative contributions of catabolic versus anabolic factors in regulating the senescence process.

It cannot be distinguished from the present data whether the coordinate decreases in rbcS and rbcL mRNAs during senescence are due to decreased levels of transcription initiation or, alternatively, to decreased levels of mRNA stability. However, it would appear that at least part of the decreases in rbcL mRNA may be due to decreased plastid DNA template availability. This assumes that each of the multiple plastid chromosomes in the cell (up to 104 copies [Bendich, 1987]) is equally capable of being transcribed during the senescence process. It is not known whether this is a valid assumption, but it is interesting that the overall decreases in plastid DNA (approximately 40%) and their profiles of change are similar during the course of senescence in both midcanopy and lower canopy leaves. This finding is consistent with the hypothesis that these leaves have the same fundamental senescence program.

The question arises: How are these decreases in plastid DNA brought about? Ford and Shibles (1988) have shown that chloroplast numbers remain relatively constant (on a per cell basis) during most of soybean senescence, declining only during the terminal phases of this process. Therefore, it is likely that, with the possible exception of the final time point, which occurred during this terminal phase, the decreases in plastid DNA observed in the present study are occurring on a per plastid basis. Fluorometric analyses have recently demonstrated that decreases in plastid DNA content (on a per plastid basis) also occur during the senescence of rice (Oryza sativa L.) leaves (Sodmergen et al., 1991). The cause for the senescence-associated decreases in plastid DNA content in soybean is not known, but in rice such decreases may be accomplished by the active destruction of plastid chromosomes by specific nucleases (Sodmergen et al., 1991). It is not known to what extent the abundance of proteins other than Rubisco is regulated at the transcriptional level during soybean senescence. However, it would appear that a general dampening of the synthesis of plastid DNA-encoded proteins brought about by gradual decreases in plastid DNA does not readily explain the observation that plastid proteins are not decreased uniformly in amount during this process (Brady, 1988). Nor does it explain the observation that some plastid mRNAs increase, rather than decrease, in abundance during senescence (Vera et al., 1990). Rather, as illustrated by the example of Rubisco in the present paper, the disassembly of the plastid likely involves the concerted activities of the nucleus and the chloroplast at multiple levels of control that vary from protein to protein and from species to species.

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