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Regulation of Photosynthesis during Leaf Development in *Rbc*S Antisense DNA Mutants of Tobacco[1]

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We have previously characterized *Rbc*S antisense DNA mutants of tobacco that have drastic reductions in their content of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; S.R. Rodermel, M.S. Abbott, L. Bogorad [1988] Cell 55: 673–681). In this report we examine the impact of Rubisco loss on photosynthesis during tobacco (*Nicotiana tabacum*) leaf development. Photosynthetic capacities are depressed in the antisense leaves, but the patterns of change in photosynthetic rates during the development of these leaves are similar to those in wild-type plants: after attaining a maximum in young leaves, photosynthetic capacities undergo a prolonged senescence decline in older leaves. The alterations in photosynthetic capacities in both the wild type and mutant are closely correlated with changes in Rubisco activity and content. During wild-type leaf development, Rubisco accumulation is regulated by coordinate changes in *Rbc*S and *rbc*L transcript accumulation, whereas in the antisense leaves, Rubisco content is a function of *Rbc*S, but not *rbc*L, transcript abundance. This indicates that large subunit protein production is controlled posttranscriptionally in the mutants. The antisense leaves accumulate near-normal levels of chlorophyll and representative photosynthetic proteins throughout development, suggesting that photosynthetic gene expression is not feedback regulated by Rubisco abundance. Considered together, the data in this paper indicate that leaf developmental programs are generally insensitive to sharp reductions in Rubisco content and emphasize the metabolic plasticity of plant cells in achieving optimal photosynthetic rates.

Rubisco is the key regulatory enzyme of photosynthetic carbon assimilation, and in eukaryotes it is composed of eight SS proteins, coded for by a small multigene (*Rbc*S) family in the nucleus, and eight LS proteins, coded for by a single gene (*rbc*L) on the multicopy chloroplast genome (reviewed by Miziorko and Lorimer, 1983). The SS proteins are translated as precursors on 80S cytoplasmic ribosomes and then transported posttranslationally into the plastid, where they combine with mature LS proteins in a reaction mediated by chaperonin 60 to form the holoenzyme (reviewed by Keegstra et al., 1989; Ellis and van der Vies, 1991). We have previously generated and characterized transgenic tobacco (*Nicotiana tabacum*) plants that express *Rbc*S antisense RNAs as a tool to gain insight into regulatory interactions between the nucleus-cytoplasm and chloroplast (Rodermel et al., 1988). The antisense plants have reduced *Rbc*S sense mRNA amounts and corresponding, coordinate reductions in SS and LS protein accumulation. However, *rbc*L mRNA levels are unperturbed in the antisense plants, indicating that LS protein accumulation is regulated posttranscriptionally in the mutants. Antisense repression appears to be mediated by active degradation of *Rbc*S sense mRNAs by a mechanism that may involve re-utilization of the antisense transcript (Jiang et al., 1994a).

The antisense mutants display a wide range of Rubisco contents (from 5–100% of WT levels) that approximately correlate with antisense gene dosage—the higher the gene dosage, the lower the Rubisco level (Rodermel et al., 1988). Because of this variability, the antisense mutants have proven to be an attractive system for quantitative flux-control analyses (Kacser and Porteous, 1987) to assess the control that Rubisco exerts on photosynthesis and plant growth under a variety of short-term and long-term growth conditions (Quick et al., 1991a, 1991b, 1992; Stitt et al., 1991; Lauerer et al., 1993; Masle et al., 1993). Although these studies have focused on the biochemistry and physiology of mature leaf and whole plant responses to decreases in Rubisco content, little is understood about the impact of Rubisco loss on the regulation of photosynthesis during leaf development.

Dicot leaf ontogeny is characterized by a phase of increasing photosynthesis capacity, coincident with leaf expansion, a period of maximal photosynthetic capacity, and finally, a senescence phase of declining photosynthesis capacity (reviewed by Gepstein, 1988). The elements that control photosynthetic rates during leaf development have been broadly defined in a number of species and include Rubisco content and activity, as well as other components of the photosynthetic apparatus (reviewed by Brady, 1988; Gepstein, 1988; Jiang et al., 1993). However, little attention has been paid to the molecular mechanisms that regulate the accumulation of these components during leaf development. The primary purpose of the present investigation was to define the biochemical and molecular elements that control photosynthetic rates in developing tobacco leaves and to determine whether decreases in Rubisco amount affect these regulatory processes.

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Abbreviations: CAB, light-harvesting Chl *a*/*b*-binding proteins of PSII; *CER*, CO_2-exchange rate; *Ci*, intercellular CO_2 concentration; LS, large subunit of Rubisco; SS, small subunit of Rubisco; WT, wild type.

The changes in photosynthesis capacity that characterize leaf ontogeny appear to be part of a developmental program that specifies alterations in the expression of nuclear and chloroplast genes for photosynthetic proteins (reviewed by Gepstein, 1988; Huffaker, 1990). One issue that is poorly understood is whether these alterations are due, in part, to feedback regulation by the concentrations of various photosynthetic proteins. Therefore, another purpose of the present study was to take advantage of the specific genetic alterations in Rubisco content in the antisense plants to gain insight into those elements of the tobacco leaf developmental program that depend on Rubisco expression for their execution (linear pathways) and those that do not (parallel pathways), thereby providing entrance into the complicated regulatory circuitry that governs this process.

MATERIALS AND METHODS

Plant Material and Growth

T_1 progeny tobacco (*Nicotiana tabacum*) from the selfing of the original T_0 Rubisco antisense DNA transformant 5, which contains four copies of the *Rbc*S antisense sequence (Rodermel et al., 1988), were used in the present experiments. Nontransformed SR1 tobacco plants (WT) served as controls. The plants were maintained in the greenhouse with one plant per pot (6 quarts) on a soil/vermiculite mixture and watered with 5 mM NH_4NO_3 (Quick et al., 1991b). Supplemental illumination was provided by 400-W high-pressure sodium lamps (16-h photoperiod). First fully expanded leaves of young antisense plants were screened by SDS-PAGE (described below), and mutants with the desired amount of Rubisco were maintained for further experimentation.

Photosynthesis Measurements

Measurements of *CER*, stomatal conductance, and *Ci* were made on WT and antisense leaves under saturating PPFD (>1200 μmol m^{-2} s^{-1}) using an LI-6200 portable photosynthesis system (Li-Cor Inc., Lincoln, NE) (described by Jiang et al., 1993). Leaf discs (four per leaf) were isolated from the same portion of the leaf on which the gas-exchange measurements had been taken and immediately frozen in liquid nitrogen. These manipulations were performed at midday on all the leaves from a given plant; there was minimal interference by shading of lower canopy by upper canopy leaves. For Chl, total protein, and Rubisco activity determinations, leaf discs were homogenized by grinding in a chilled mortar and pestle in an ice-cold buffer containing 50 mM Bicine (pH 8.2), 20 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM Na_2-EDTA, 0.1 mM PMSF, and 5 μM leupeptin. An aliquot was removed and extracted with 80% acetone for at least 2 h at 4°C in the dark, and the extract was centrifuged at 5000*g* for 5 min. The supernatant was removed for the determination of Chl *a* and Chl *b* (Arnon, 1949), and the pellet was solubilized for total protein analyses in 63 mM Tris-HCl (pH 6.8), 2% SDS, 10% β-mercaptoethanol, and 10% glycerol. The remainder of the leaf disc homogenate was centrifuged at 5000*g* for 1 min at 4°C, and the supernatant was collected for determination of Rubisco activity and soluble protein content by the Bradford method (Bio-Rad). Initial and total Rubisco activities were estimated by D-ribulose-1,5-bisphosphate-dependent incorporation of $^{14}CO_2$ into acid-stable products (described by Jiang et al., 1993).

Detection of Proteins

Total and soluble proteins were electrophoresed through 12.5% discontinuous SDS-polyacrylamide gels as described by Fling and Gregerson (1986); this procedure facilitates the separation of low mol wt proteins. After electrophoresis, the gels were either stained with Coomassie blue or blotted onto nitrocellulose filters for western immunoblot analysis (described by Jiang et al., 1994b). The antibodies in these experiments were specific for the tobacco LS and SS of Rubisco (Rodermel et al., 1988), spinach Rubisco activase (a gift of Dr. Ray Zielinski, University of Illinois), spinach Cyt *f* (the plastid *pet*A gene product), and the *N. tabacum* CAB proteins (both a gift of Dr. Jean Haley, Colby College). Antibody specificity was monitored using ^{125}I-conjugated protein A at a concentration of 1 μCi mL^{-1}. Relative protein amounts on the western blots were quantified using a Molecular Dynamics PhosphorImager 400E (Sunnyvale, CA).

Isolation and Detection of RNA and DNA

Total cell RNA and DNA were isolated from frozen leaf discs (Jiang et al., 1993) and used for either RNA gel blot, RNA slot blot, or DNA hybridization analysis (Jiang et al., 1993, 1994a). Following hybridization, the filters were either autoradiographed or subjected to PhosphorImage analysis to quantify relative transcript or plastid DNA amounts. The probes in these experiments included pSEM1, specific for the *N. tabacum Rbc*S gene family (Pinck et al., 1984), and pTB5, containing a portion of the *N. tabacum* plastid *rbc*L gene (Shinozaki and Sugiura, 1982).

RESULTS

Plant Growth

To monitor the growth and development of WT and Rubisco antisense DNA plants, we measured plant height every several days after planting until maximal heights were attained. Representative growth curves are shown in Figure 1 for WT and mutant plants with either 40% (mutant 1) or 20% (mutant 2) of WT Rubisco protein amounts. We observed that the WT and antisense plants have similar growth profiles but that the mutants grow much more slowly, with flowering delayed up to 3 weeks. The leaves on the antisense plants are also much longer-lived (data not shown). Despite the differences in growth rates, the mutant and WT plants eventually attain the same height and form the same number of leaves. In other experiments we found that mutants with extremely depressed levels of Rubisco (less than 10% of the WT amount) not only have much reduced rates of growth but also do not attain the stature of WT plants (data not shown). Moreover, these plants

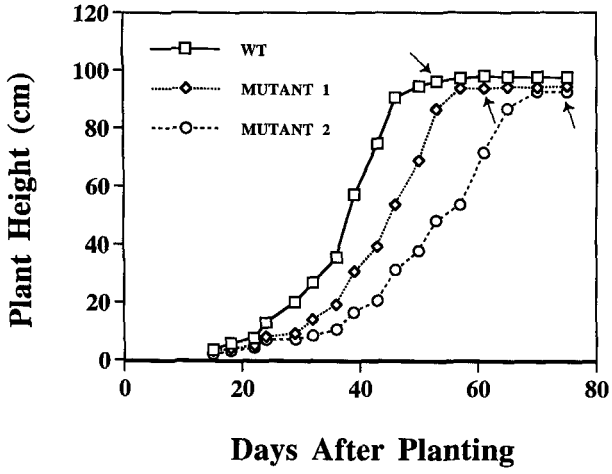

Figure 1. Growth of the WT and antisense plants. Plant height (cm) was monitored at 3- to 4-d intervals for nearly 12 weeks postplanting. The samples included a WT and antisense mutant plants with either 40% (mutant 1) or 20% (mutant 2) of WT Rubisco amounts. Arrows signify the beginning of flowering.

form reduced numbers of leaves prior to flowering. Thus, it seems that severe reductions in Rubisco amount can significantly disturb normal patterns of growth and development.

Leaf development is commonly studied by examining the development of a single leaf that occupies a given nodal position on the plant. Because invasive procedures that destroy the leaf are frequently needed, these kinds of experiments require a large number of replicate isogenic plants (Brady, 1988; Hensel et al., 1993; Jiang et al., 1993). However, isogenic lines of the antisense plants are not available, making this approach impractical with these plants. As an alternative, we chose to examine the developmental gradient of fully expanded leaves at progressive nodes on morphologically similar WT and antisense plants (i.e. on plants that had attained the same height and formed the same number of total leaves, just prior to flowering). Direct comparisons could then be made between developmentally similar leaves (at the same nodal position) of these plants. This system has previously been used to study leaf development in tobacco (Harris and Arnott, 1973; Aharoni et al., 1979; Dhillon and Miksche, 1981; Ursin and Shewmaker, 1993) and other species (Kura-Hotta et al., 1987; Hashimoto et al., 1989; Marshall and Vos, 1991).

Photosynthetic Activities

To assess the impact of decreased Rubisco on photosynthesis during leaf development, we measured *CER*s on all of the leaves from three WT, three mutant 1, and three mutant 2 plants (Fig. 2A). In all three sets of plants, photosynthetic capacities decline in older, fully expanded leaves after attaining a maximum in younger, fully expanded leaves; this pattern is similar to that normally observed during the development of a single leaf (reviewed by Gepstein, 1988; Matile, 1992). However, photosynthetic rates are depressed in the antisense plants, and maximal *CER*s are delayed and attained in developmentally older leaves of these plants (leaf 14 in the WT versus leaf 13 in the mutants). Since antisense leaves are longer-lived than WT leaves (data not shown), this indicates that maximal *CER*s are also delayed temporally by up to several weeks in leaves from the antisense plants. Figure 2A further reveals that photosynthetic rates reach the same low levels in senescent leaves from the WT and antisense plants but that the patterns of decline differ: *CER*s appear to decline more rapidly in the WT. Considered together, the data in Figure 2A suggest that reductions in Rubisco affect

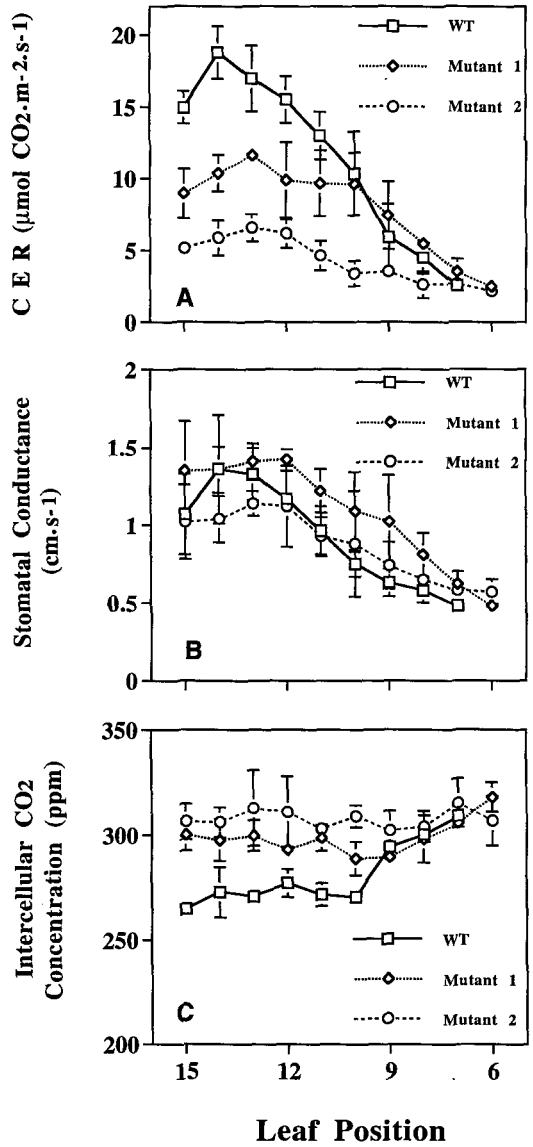

Figure 2. Gas-exchange analyses. Measurements of *CER* (A) and stomatal conductance (B) were performed on all leaves from three WT, three mutant 1, and three mutant 2 plants. C, The calculated *Ci*. Leaf 15 is the youngest fully expanded leaf at the top of plant just prior to flowering, and leaf 6 is the oldest leaf still remaining at the bottom of the plant. Each data point and bar represents the mean ± SD of three measurements from each of the three plants (a total of nine replicates per data point).

the magnitude, as well as the timing, of developmentally associated changes in photosynthesis capacity during leaf development.

Figure 2B shows that stomatal conductances are either similar to the WT (mutant 2 plants) or somewhat higher than the WT (in older leaves of mutant 1 plants). *Ci* is also generally higher in the antisense plants, especially in younger, fully expanded leaves (Fig. 2C). Because the alterations in stomatal conductance are not accompanied by corresponding changes in *Ci*, it is unlikely that stomatal aperture plays a central role in controlling photosynthetic activity during WT and antisense leaf development, at least under these conditions (reviewed by Farquhar and Sharkey, 1982). The data in Figure 2, B and C, confirm and extend earlier observations of enhanced stomatal conductances and *Ci* in first fully expanded leaves of the antisense versus WT plants under high-irradiance conditions (Quick et al., 1991b; Stitt et al., 1991; Lauerer et al., 1993).

Figure 3A shows that total Chl contents and the patterns of change in Chl accumulation are similar during WT and antisense leaf development. Because the mutants have normal levels of Chl, but much reduced photosynthetic rates, it is unlikely that light harvesting is limiting for photosynthesis in these plants. It is also unlikely that light harvesting is limiting for photosynthesis in the WT, especially in older, fully expanded leaves, because the rate of decline in *CER* in these leaves exceeds that of Chl (i.e. the *CER*/mg Chl declines). Figure 3B reveals that Chl *a/b* ratios do not change appreciably as a function of leaf age in the WT. However, overall Chl *a/b* ratios are lower in the mutants and decline as leaf development proceeds. This suggests that the composition of the photosynthetic apparatus is altered in the mutant versus WT plants and that the stoichiometries of various thylakoid components are changing during the course of antisense leaf development.

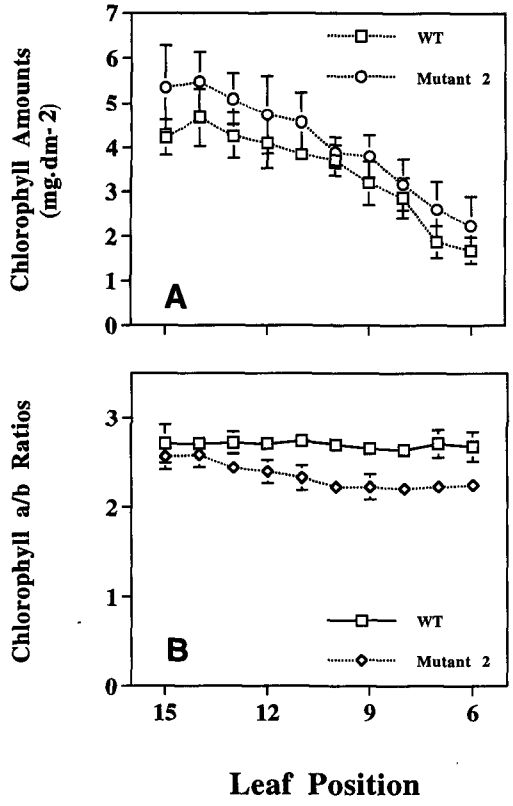

Figure 3. Pigment assays. Total Chl contents (A) and Chl *a/b* ratios (B) were measured on single leaf discs from three WT and three mutant 2 plants. Each data point and bar represents the mean ± SD of the three determinations.

Rubisco Activities and Content

To assess the contribution of Rubisco activity and activation state to the control of photosynthesis capacity during WT and antisense leaf development, we measured Rubisco initial and total activities. Initial activities provide an estimate of the amount of activated enzyme in the leaf sample at the time of harvest, whereas total activities provide a measure of the amount of Rubisco that is capable of being activated in the leaf sample. We found that alterations in both initial (Fig. 4A) and total activities (data not shown) closely parallel those of *CER*. This suggests that Rubisco activity is a primary determinant regulating photosynthetic rates during the development of both WT and antisense leaves.

To determine whether the changes in Rubisco activity are due to changes in enzyme content, we measured Rubisco amounts in leaves from the WT and mutant plants by PhosphorImage analysis of either western immunoblots (Fig. 4B) or stained SDS-polyacrylamide gels (Fig. 5). Both of these procedures gave similar results. These experiments showed, first, that LS and SS levels are coordinately decreased in amount in the antisense plants. This indicates that there is no overproduction of the LS in these plants. Second, these analyses revealed that the patterns of change in Rubisco content parallel those of Rubisco activity. This suggests that Rubisco activity is primarily a function of holoenzyme content throughout WT and antisense leaf development. Consistent with this hypothesis is the finding that the activation state of the enzyme (the ratio of initial to total activities) did not change appreciably during leaf development in either set of plants (Fig. 4C). It should be pointed out, however, that Rubisco activation states were slightly higher in the mutant plants. A similar observation was made on first fully expanded leaves of the antisense mutants (Quick et al., 1991b); we recently demonstrated that this may be due to enhanced Rubisco activase activities (Jiang et al., 1994b).

Figure 4D shows that the antisense plants have less total protein than the WT plants on a leaf area basis. Much of the change in total protein in the WT plants is due to Rubisco, which can make up to 50% of the soluble leaf protein in tobacco (Quick et al., 1991b). However, much less of this change is due to Rubisco in the mutants, since Rubisco constitutes a much smaller proportion of the soluble protein pool in these plants.

Regulation of Rubisco Content

To assess the contribution of transcriptional factors to the regulation of Rubisco content during leaf development, we

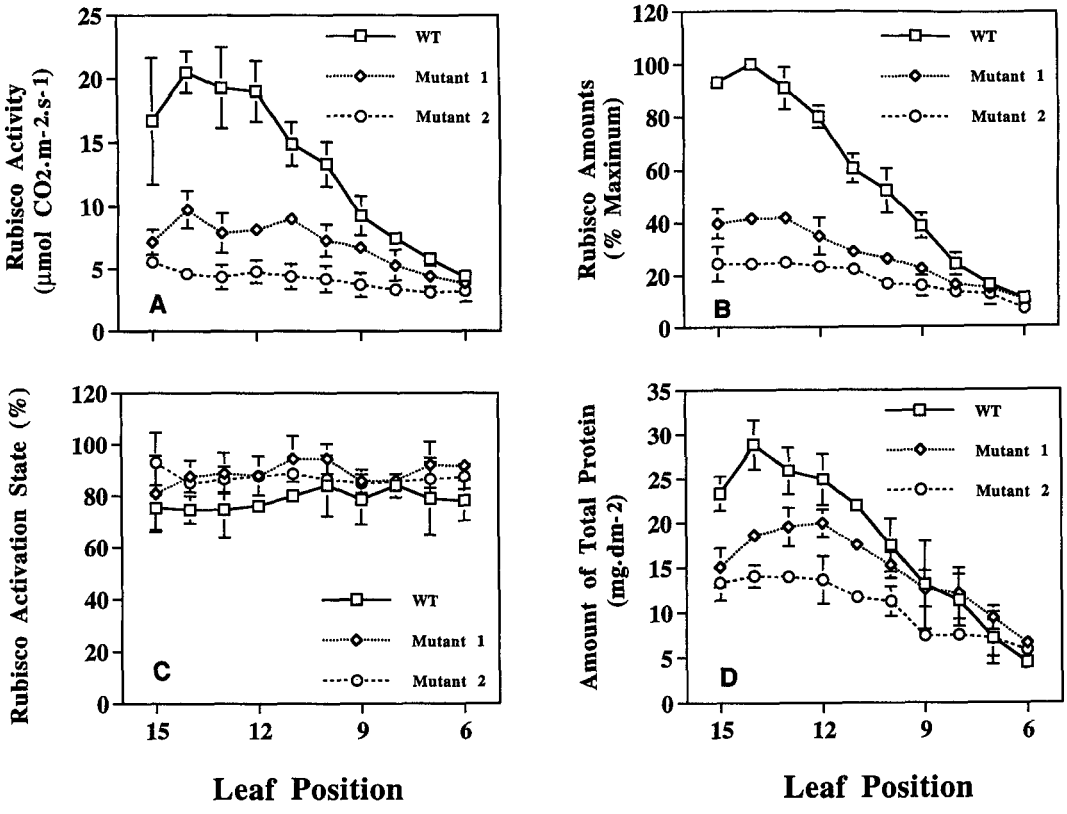

Figure 4. Rubisco activity, Rubisco activation state, Rubisco content, and total protein content. Rubisco initial (A) and total activities were determined on a leaf area basis, and the activation state of the enzyme was calculated as the ratio of initial to total activities (C). Soluble proteins were isolated from leaf discs, and proteins were electrophoresed through discontinuous 12.5% SDS-polyacrylamide gels; proteins were loaded on the gel on a leaf area basis. The gels were immunoblotted with tobacco SS and LS antibodies, and band intensities on the filters were quantified by PhophorImage analysis. The results for the LS and SS were similar; the LS results are shown in B and are expressed as a percentage of the maximum WT value. The total protein contents of leaf discs (D) were determined by Bradford analysis. Leaf discs were from the same plants described in the legend to Figure 2. Each data point and bar represents the mean ± SD of three replicates (leaves from three different plants, one disc per leaf).

examined profiles of *rbc*L and *Rbc*S mRNA abundance by slot blot analysis. Figure 6, A and B, show the results of PhosphorImage analysis of replicate slot blot filters of RNAs from WT and mutant 2 leaves. In the WT leaves there are coordinate changes in *rbc*L and *Rbc*S abundance during development; after the attainment of maxima in younger leaves, the levels of both mRNAs decrease approximately 70 to 80% in senescent leaves. These changes are closely correlated with alterations in Rubisco abundance (Figs. 4B and 5), indicating that Rubisco content during WT leaf development is regulated primarily at the level of *rbc*L and *Rbc*S transcript abundance. In the antisense plants, alterations in *rbc*L mRNA levels match those in the WT, whereas alterations in *Rbc*S abundance correlate with Rubisco amounts. This indicates that the content of the holoenzyme in the mutants is regulated primarily at the level of *Rbc*S transcript accumulation. We have verified the results in Figure 6, A and B, by RNA gel blot analysis (data not shown).

To estimate *rbc*L DNA template availability, Southern filters containing *Bam*HI-digested total cell DNA from WT and mutant 2 leaves were probed with a tobacco plastid *rbc*L DNA sequence. PhosphorImage analysis of replicate filters (Fig. 6C) reveals that the patterns of change in *rbc*L DNA levels are similar in the WT and mutant leaves and that *rbc*L DNA amounts decline approximately 50% during the course of leaf development in both types of plants. This suggests that restrictions in DNA template availability may contribute to decreased *rbc*L mRNA levels during the development of WT and antisense leaves.

Patterns of Photosynthetic Protein Expression

Quick et al. (1991b) and Hudson et al. (1992) have shown that the contents and/or activities of some enzymes are normal, and others depressed, in first fully expanded leaves of the antisense versus WT plants. It has also been suggested that decreases in Rubisco have little impact on overall protein accumulation, at least in plants with up to 80% reductions in Rubisco amount (Quick et al., 1991b; Hudson et al., 1992; Masle et al., 1993). To further explore the impact of Rubisco loss on photosynthetic protein accu-

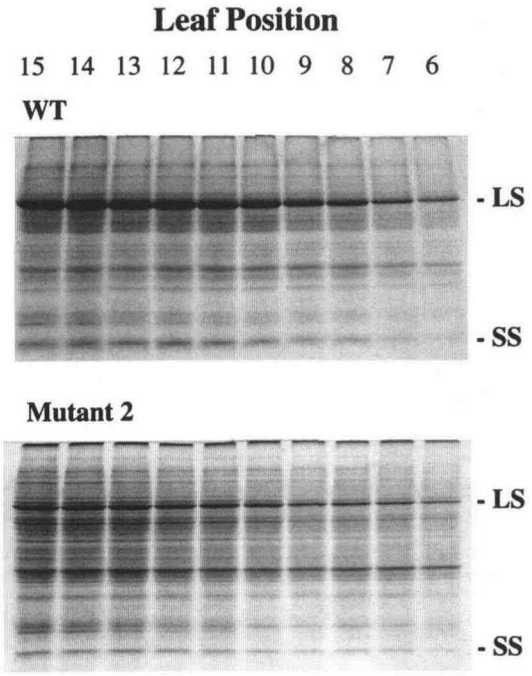

Figure 5. SDS-PAGE of soluble leaf proteins from the WT and antisense plants. The protein samples were electrophoresed through discontinuous 12.5% SDS-polyacrylamide gels on a leaf area basis. The gel was stained with Coomassie blue.

mulation, we examined the abundance of several representative photosynthetic proteins during leaf development in the WT and antisense plants by western immunoblot analysis. These included Rubisco activase, Cyt *f*, and CAB. The results of quantitative PhosphorImage analysis are illustrated in Figure 7. Figure 7A shows that alterations in activase content are similar throughout development of the WT and mutant leaves. These alterations are superimposable on those of Rubisco content in leaves of the WT plants. Similar conclusions can be drawn for Cyt *f* (Fig. 7B), with the exception that declines in the abundance of this protein during senescence may be somewhat accelerated in the mutants. Changes in CAB content, like those of activase and Cyt *f*, are similar during WT and antisense leaf development (Fig. 7C), although CAB levels do not appear to decline as sharply in older leaves of the mutants. However, in contrast to the other two proteins, CAB levels do not decrease as much as WT Rubisco levels in senescing leaves of either the WT or antisense plants (i.e. overall reductions of 40–50% for CAB versus 70–90% for Rubisco, activase, and Cyt *f*).

DISCUSSION

Quantitative flux-control analyses have been conducted on first fully expanded leaves of the Rubisco antisense DNA mutants to assess the control that Rubisco exerts on photosynthesis under a variety of short-term and long-term conditions (Quick et al., 1991a, 1991b, 1992; Stitt et al., 1991; Lauerer et al., 1993; Masle et al., 1993). These analyses revealed that under high-irradiance conditions, similar to those in the present experiments, Rubisco activity is primarily limiting for photosynthesis but that control is also shared with other factors. Stomatal conductance is one of these factors (Stitt et al., 1991), and CO_2 diffusion through the aqueous phase of the leaf may be another (Lauerer et al., 1993). Our results are consistent with these conclusions and suggest that the control exerted by Rubisco on photosynthesis is considerable throughout leaf development. Yet, because the relationships among the various photosynthetic parameters we measured were not constant (e.g.

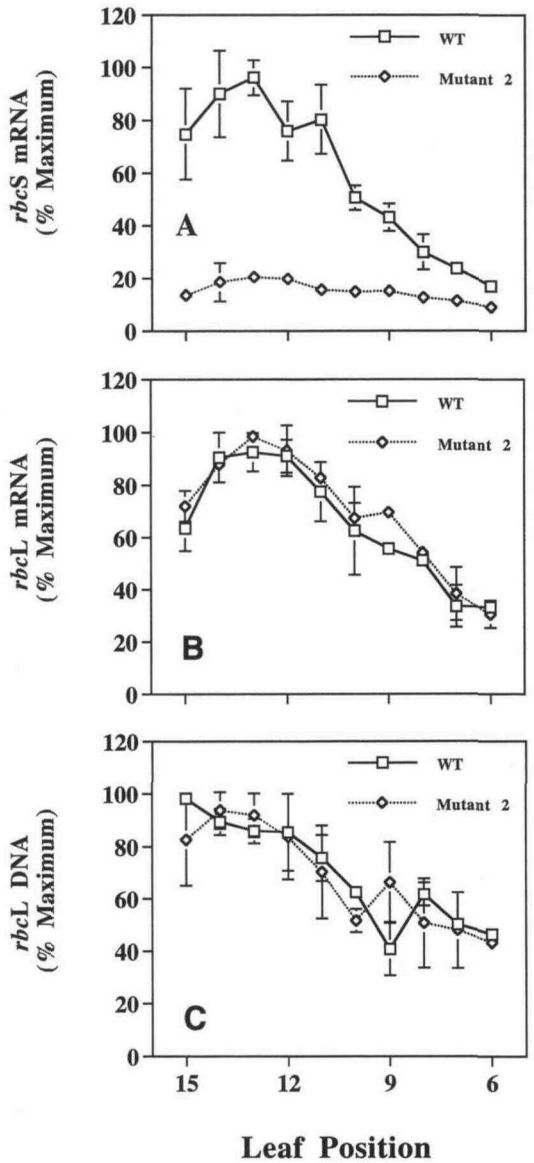

Figure 6. *Rbc*S and *rbc*L transcript abundance and *rbc*L DNA template availability. For the slot blot analyses in A and B, each data point and bar represents the mean ± SD from PhosphorImage analysis of two replicate filters (leaves from two different plants, one disc per leaf). To determine *rbc*L DNA template availability (C), Southern hybridizations were conducted on equal amounts of *Bam*HI-digested total cell DNA from WT and antisense mutant 2 leaves; the filters were probed with pTB5, specific for the tobacco plastid *rbc*L gene (Shinozaki and Sugiura, 1982). Results from PhosphorImage analysis of two replicate Southern filters are shown.

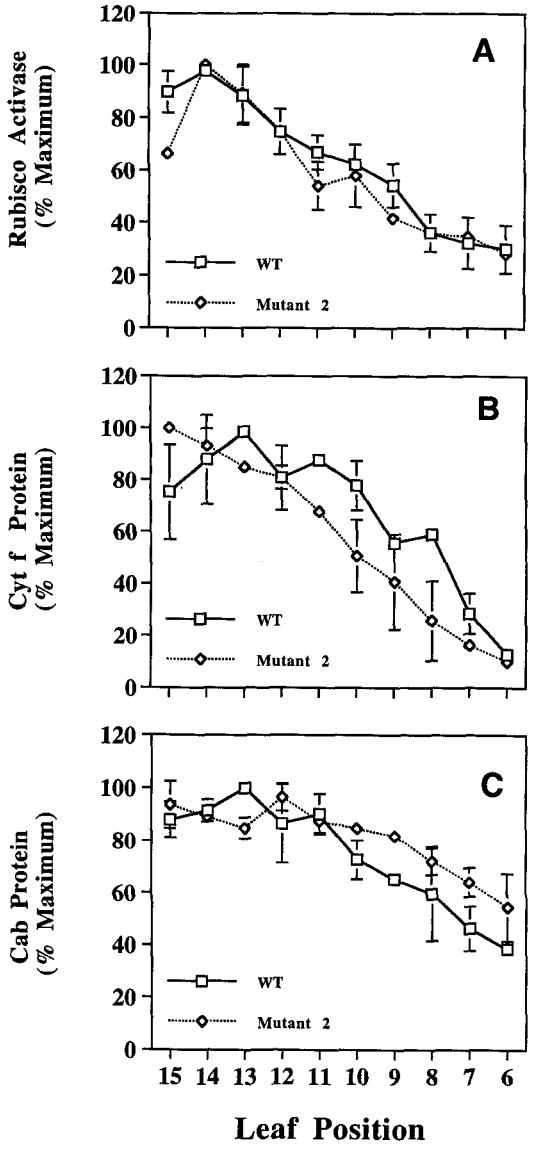

Figure 7. Rubisco activase, Cyt *f*, and CAB protein levels. Total proteins were isolated from leaf discs and electrophoresed though discontinuous 12.5% SDS-polyacrylamide gels; the proteins were loaded on the gel on a leaf area basis. Western immunoblot filters were prepared and incubated with antibodies specific for the spinach activase (A), spinach Cyt *f* (B), or tobacco CAB (C) proteins. Each data point is expressed as a percentage of the maximum WT value, and each point and bar represents the mean ± SD from PhosphorImage analysis of two replicate filters (leaves from two different plants, one disc per leaf).

there were increases in *Ci* in older leaves of the WT but not mutant plants), our data suggest that the relative contribution of these components to the control of photosynthetic rates may be shifting during development and may be somewhat different in leaves of the WT versus antisense plants.

The present experiments revealed that maximal rates of photosynthesis are delayed in the antisense plants and attained in older, fully expanded leaves. This delay represents a temporal delay as well as a developmental delay, since the antisense leaves are much longer-lived than WT leaves. It is likely that the delay in the period of maximal photosynthesis capacity is due to a shift in Rubisco accumulation maxima mediated at the level of *Rbc*S and *rbc*L transcription. Quick et al. (1991a) have shown that the antisense plants have reduced sugar contents, and thus one possibility is that maximal *CER*s are delayed by the low carbohydrate status of the mutants, i.e. photosynthesis may be inhibited earlier in the WT because it has higher carbohydrate levels. Consistent with this possibility is the well-established finding that source leaves adjust their photosynthetic rates in response to the demand for photoassimilates in the rest of the plant, inhibiting photosynthesis when the demand is low and increasing photosynthesis when the demand is high (sink regulation of photosynthesis) (reviewed by Turgeon, 1989; von Schaewen et al., 1990; Stitt et al., 1990; Stitt, 1991; Sonnewald and Willmitzer, 1992). It has also been observed that the rate of leaf senescence is affected by the sink-source status of the plant (Christensen et al., 1981; Bin Lazan et al., 1983; Wittenbach, 1983; Shibles et al., 1987; Guitman et al., 1991) and that photosynthetic gene expression can be modulated by carbohydrates at the transcriptional level (Sheen, 1990; Krapp et al., 1993). We are currently investigating the impact of carbohydrate levels on photosynthetic rates and Rubisco gene expression during leaf development in the antisense and WT plants.

One of the central findings in this study was that the alterations in Rubisco content during WT leaf development are primarily a function of coordinate changes in *Rbc*S and *rbc*L transcript abundance. We have also found this to be true in soybean (Jiang et al., 1993), and similar conclusions have been drawn in wheat and *Phaseolus* (Brady, 1988; Bate et al., 1991). This is not a general phenomenon, however, since in some species posttranscriptional controls regulate Rubisco accumulation during at least part of the leaf developmental process (reviewed by Brady, 1988; Mullet, 1988; Huffaker, 1990). The signals that integrate *Rbc*S transcription in the nuclear-cytoplasmic compartment with *rbc*L transcription in the plastid are poorly understood but involve both endogenous (e.g. hormones) and exogenous, environmental factors (e.g. light) (reviewed by Mullet, 1988; Taylor, 1989; Susek and Chory, 1992). As illustrated in the present studies, the factors that regulate *rbc*L transcription during leaf development do not appear to include feedback signals generated in response to the levels of *Rbc*S transcription or translation products, as predicted by the cytoplasmic control principle (Ellis, 1977). The present data further show that only stoichiometric amounts of the LS and SS proteins accumulate in the antisense plants: excess LS is not produced. Since normal *rbc*L mRNA levels are found in the antisense plants, this indicates that LS protein accumulation is regulated posttranscriptionally throughout leaf development in the mutants. The current experiments, which were conducted on greenhouse-grown T_1 plants, thus confirm and extend earlier studies of primary (T_0) *Rbc*S antisense DNA transformants growing on tissue-culture medium supplemented with Suc (Rodermel et al., 1988).

Although we cannot distinguish whether the alterations in *rbc*L and *Rbc*S transcript abundance are due to alterations in transcription initiation or transcript stability, at least part of the change in *rbc*L mRNA amounts in older, senescing leaves may be due to decreased plastid DNA template availability. This assumes that each of the multiple plastid chromosomes in the cell is capable of being transcribed during the senescence process. We do not know whether this is a valid assumption. However, our findings raise the question whether the decreases in plastid DNA during senescence occur on a per-plastid basis, as suggested in rice (Sodmergen et al., 1991), or whether they are due to the destruction of entire plastids. Although reduced chloroplast numbers are observed in some species throughout the senescence process (Lamppa et al., 1980; Camp et al., 1982; Wittenbach et al., 1982; Kura-Hotta et al., 1990), in other species plastid function is uniformly impaired during early senescence and whole plastids are lost only late in the process (Mae et al., 1984; Wardley et al., 1984; Ford and Shibles, 1988). This topic has not yet been investigated in tobacco.

In this study we took advantage of a specific genetic alteration in Rubisco content in the antisense plants to ascertain how this perturbation affects the expression of genes for photosynthetic proteins during leaf development. It was hoped that these studies might lend insight into those elements of the leaf developmental program that depend on Rubisco protein expression for their implementation (a linear pathway) and those that do not (parallel pathways). We found that the relative stoichiometries of some photosynthetic proteins are constant during WT leaf development, e.g. Rubisco and activase, which argues that the expression of genes for these proteins is coordinately regulated. On the other hand, the relative stoichiometries of other photosynthetic proteins appear to be altered during development, e.g. decreases in CAB in senescing leaves are not as severe as those of Rubisco. We do not know how the accumulation of activase, Cyt *f*, and CAB are regulated during tobacco leaf development, but the levels of these proteins are approximately similar in the WT and antisense plants and are generally insensitive to the drastic reductions in Rubisco content. This uncoupling suggests that the expression of genes for these proteins is not feedback regulated by Rubisco; integrative and coordinating mechanisms must reside elsewhere. This is consistent with earlier findings on first fully expanded leaves of the mutant plants (Quick et al., 1991b; Hudson et al., 1992; Masle et al., 1993). Thus, the present findings are generally in accord with studies of stay-green senescence mutants of fescue grass (Hilditch et al., 1989; Thomas, 1992), *Phaseolus* (Ronning et al., 1991), and soybean (Guiamét et al., 1991) and emphasize the notion that leaf development is a coordinated series of events that are not obligatorily connected (parallel pathways).

Although activase, Cyt *f*, and CAB protein expression are generally similar in the mutant and WT plants, there do appear to be subtle differences. For instance, Cyt *f* may be lost somewhat more rapidly in older leaves of the antisense plants. This may have important consequences for the regulation of photosynthesis rates in these plants; as observed in other systems, the Cyt b_6/f complex is often rate limiting for photosynthesis during the senescence process (reviewed by Brady, 1988). In addition to the alterations in Cyt *f*, CAB levels may be decreasing somewhat less rapidly in senescing leaves of the antisense plants. Because Chl *b* is exclusively associated with the light-harvesting complexes (I and II), and predominantly with light-harvesting complex II (Kaplan and Arntzen, 1982), an enhanced relative accumulation of this protein would be consistent with the observation that Chl *a/b* ratios are lower in the mutants and progressively decline during antisense leaf development. However, the reason for such an apparent reallocation of nitrogen to light-harvesting complex II is not clear, since photosynthesis is not light limited in the antisense plants. Alternatively, the altered Chl *a/b* ratios in the mutants could be due to differences in the levels of other thylakoid membrane proteins (e.g. reduced reaction center complexes).

Regardless of how the changes in photosynthetic protein abundance are achieved, the present results suggest that the antisense plants do not adapt to genetic alterations in Rubisco content by significantly altering nuclear and chloroplast gene expression. Other data in this report are consistent with this notion (e.g. the finding that chloroplast DNA levels decrease in concert throughout leaf development in the WT and antisense plants) and suggest that leaf developmental programs are generally unaffected by the drastic changes in Rubisco content. This is especially interesting given the fact that leaf development is delayed temporally in the mutants: the mutant leaves undergo the same sequence of developmental events, but the overall process is prolonged. It has been suggested that photosynthesis rates are dependent on gene expression as a coarse control to set enzyme amounts and, consequently, maximal potential enzyme activities; rates are fine-tune controlled by adjustments in substrate and effector levels (Servaites et al., 1991; Geiger and Servaites, 1994). Considered in this light, the current results emphasize the metabolic flexibility of plant cells and their remarkable ability, in the absence of marked changes in gene expression, to adapt to a wide range of concentrations of photosynthetic components to achieve optimal photosynthesis rates.

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LITERATURE CITED

Aharoni N, Lieberman M, Sisler HD (1979) Patterns of ethylene production in senescing leaves. Plant Physiol **64:** 796–800

Arnon DI (1949) Copper enzymes in chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol **24:** 1–15

Bate NJ, Rothstein SJ, Thompson JE (1991) Expression of nuclear and chloroplast photosynthesis-specific genes during leaf senescence. J Exp Bot **42:** 801–811

Bin Lazan H, Barlow EWR, Brady CJ (1983) The significance of vascular connection in regulating senescence of the detached flag leaf of wheat. J Exp Bot **143:** 726–736

Brady CJ (1988) Nucleic acid and protein synthesis. *In* LD Noodén, AC Leopold, eds, Senescence and Aging in Plants. Academic Press, San Diego, CA, pp 147–179

Camp PJ, Huber SC, Burke JJ, Moreland DE (1982) Biochemical changes that occur during senescence of wheat leaves. I. Basis for the reduction of photosynthesis. Plant Physiol **70:** 1641–1648

Christensen LE, Below FE, Hageman RH (1981) The effects of ear removal on senescence and metabolism of maize. Plant Physiol **68:** 1180–1185

Dhillon S, Miksche JP (1981) DNA changes during sequential leaf senescence of tobacco (*Nicotiana tabacum*). Physiol Plant **51:** 291–298

Ellis RJ (1977) Protein synthesis by isolated chloroplasts. Biochim Biophys Acta **463:** 185–215

Ellis RJ, van der Vies SM (1991) Molecular chaperones. Annu Rev Biochem **60:** 321–347

Farquhar GD, Sharkey TD (1982) Stomatal conductance and photosynthesis. Annu Rev Plant Physiol **33:** 317–345

Fling SP, Gregerson DS (1986) Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. Anal Biochem **155:** 83–88

Ford DM, Shibles R (1988) Photosynthesis and other traits in relation to chloroplast number during soybean leaf senescence. Plant Physiol **86:** 108–111

Geiger DR, Servaites JC (1994) Dynamics of self-regulation of photosynthetic carbon metabolism. Plant Physiol Biochem **32:** 173–183

Gepstein S (1988) Photosynthesis. *In* LD Noodén, AC Leopold, eds, Senescence and Aging in Plants. Academic Press, San Diego, CA, pp 85–109

Guiamét JJ, Schwartz E, Pichersky E, Noodén LD (1991) Characterization of cytoplasmic and nuclear mutations affecting chlorophyll and chlorophyll-binding proteins during senescence in soybean. Plant Physiol **96:** 227–231

Guitman MR, Arnozis PA, Barneix AJ (1991) Effect of source-sink relations and nitrogen nutrition on senescence and N remobilization in the flag leaf of wheat. Physiol Plant **82:** 278–284

Harris JB, Arnott HJ (1973) Effects of senescence on chloroplasts of the tobacco leaf. Tissue Cell **5:** 527–544

Hashimoto H, Kura-Hotta M, Katoh S (1989) Changes in protein content and in the structure and number of chloroplasts during senescence in rice seedlings. Plant Cell Physiol **30:** 707–715

Hensel LL, Grbic V, Baumgarten DA, Bleecker AB (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis*. Plant Cell **5:** 553–564

Hilditch P, Thomas H, Thomas BJ, Rogers LJ (1989) Leaf senescence in a non-yellowing mutant of *Festuca pratensis*: proteins of photosystem II. Planta **177:** 265–272

Hudson GS, Evans JR, von Caemmerer S, Arvidsson YBC, Andrews TJ (1992) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. Plant Physiol **98:** 294–302

Huffaker RC (1990) Tansley Review No. 25. Proteolytic activity during senescence of plants. New Phytol **116:** 199–231

Jiang CZ, Kliebenstein D, Ke N, Rodermel S (1994a) Destabilization of *rbc*S sense transcripts by antisense RNA. Plant Mol Biol **25:** 569–576

Jiang CZ, Quick WP, Alred R, Kliebenstein D, Rodermel SR (1994b) Antisense RNA inhibition of Rubisco activase expression. Plant J **5:** 787–798

Jiang CZ, Rodermel SR, Shibles RM (1993) Photosynthesis, Rubisco activity and amount, and their regulation by transcription in senescing soybean leaves. Plant Physiol **101:** 105–112

Kacser H, Porteous JW (1987) Control of metabolism: what do we have to measure? Trends Biochem Sci **12:** 5–14

Kaplan S, Arntzen CJ (1982) Photosynthetic membrane structure and function. *In* Energy Conversion by Plants and Bacteria. Academic Press, New York, pp 65–140

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. Annu Rev Plant Physiol Plant Mol Biol **40:** 471–501

Krapp A, Hofmann B, Schäfer C, Stitt M (1993) Regulation of the expression of *rbc*S and other photosynthetic genes by carbohydrates: a mechanism for the "sink regulation" of photosynthesis? Plant J **3:** 817–828

Kura-Hotta M, Hashimoto H, Satoh K, Katoh S (1990) Quantitative determination of changes in the number and size of chloroplasts in naturally senescing leaves of rice seedlings. Plant Cell Physiol **31:** 33–38

Kura-Hotta M, Satoh K, Katoh S (1987) Relationship between photosynthesis and chlorophyll content during leaf senescence of rice seedlings. Plant Cell Physiol **28:** 87–89

Lamppa GK, Elliot LV, Bendich AJ (1980) Changes in chloroplast number during pea development. Planta **148:** 437–443

Lauerer M, Saftic D, Quick WP, Labate C, Fichtner K, Schulze ED, Rodermel SR, Bogorad L, Stitt M (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S. VI. Effect on photosynthesis in plants grown at different irradiance. Planta **190:** 332–345

Mae T, Kai N, Makino A, Ohira K (1984) Relation between ribulose bisphosphate carboxylase content and chloroplast number in naturally senescing primary leaves of wheat. Plant Cell Physiol **25:** 333–336

Marshall B, Vos J (1991) The relation between the nitrogen concentration and photosynthetic capacity of potato (*Solanum tuberosum* L.) leaves. Ann Bot **68:** 33–39

Masle J, Hudson GS, Badger MR (1993) Effects of ambient CO_2 concentration on growth and nitrogen use in tobacco (*Nicotiana tabacum*) plants transformed with an antisense gene to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol **103:** 1075–1088

Matile P (1992) Chloroplast senescence. *In* NR Baker, H Thomas, eds, Crop Photosynthesis: Spatial and Temporal Determinations. Elsevier Science, Amsterdam, The Netherlands, pp 414–440

Miziorko HM, Lorimer GH (1983) Ribulose-1,5-bisphosphate carboxylase-oxygenase. Annu Rev Biochem **52:** 507–535

Mullet J (1988) Chloroplast development and gene expression. Annu Rev Plant Physiol Plant Mol Biol **39:** 475–502

Pinck M, Guilley E, Durr A, Hoff M, Pinck L, Fleck J (1984) Complete sequence of one of the mRNAs coding for the small subunit of ribulose bisphosphate carboxylase of *Nicotiana sylvestris*. Biochimie **66:** 539–545

Quick WP, Fichtner K, Schulze ED, Wendler R, Leegood RC, Mooney H, Rodermel SR, Bogorad L, Stitt M (1992) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S. IV. Impact on photosynthesis in conditions of altered nitrogen supply. Planta **188:** 522–531

Quick WP, Schurr U, Fichtner K, Schulze ED, Rodermel SR, Bogorad L, Stitt M (1991a) The impact of decreased Rubisco on photosynthesis, growth, allocation and storage in tobacco plants which have been transformed with antisense *rbc*S. Plant J **1:** 51–58

Quick WP, Schurr U, Scheibe R, Schulze ED, Rodermel SR, Bogorad L, Stitt M (1991b) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S. I. Impact on photosynthesis in ambient growth conditions. Planta **183:** 542–554

Rodermel SR, Abbott MS, Bogorad L (1988) Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. Cell **55:** 673–681

Ronning CM, Bounwkamp JC, Solomos T (1991) Observations on the senescence of a mutant non-yellowing genotype of *Phaseolus vulgaris*. J Exp Bot **42:** 235–241

Servaites JC, Shieh WJ, Geiger DR (1991) Regulation of photosynthetic carbon reduction cycle by ribulose bisphosphate and phosphoglyceric acid. Plant Physiol **97:** 1115–1121

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell **2:** 1027–1038

Shibles R, Secor J, Ford DM (1987) Carbon assimilation and metabolism. *In* JR Wilcox, ed, Soybeans: Improvement, Production, and Uses, Ed 2. American Society of Agronomy, Madison, WI, pp 535–588

Shinozaki K, Sugiura M (1982) The nucleotide sequence of the tobacco chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Gene **20:** 91–102

Sodmergen, Kawano S, Tano S, Kuroiwa T (1991) Degradation of chloroplast DNA in second leaves of rice (*Oryza sativa*) before leaf yellowing. Protoplasma **160:** 89–98

Sonnewald U, Willmitzer L (1992) Molecular approaches to sink-source interactions. Plant Physiol **99:** 1267–1270

Stitt M (1991) Rising CO_2 levels and their potential significance for carbon flow in photosynthetic cells. Plant Cell Environ **14:** 741–762

Stitt M, Quick WP, Schurr U, Schulze ED, Rodermel SR, Bogorad L (1991) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with "antisense" *rbc*S. II. Flux-control coefficients for photosynthesis in varying light, CO_2, and air humidity. Planta **183:** 555–566

Stitt M, von Schaewen A, Willmitzer L (1990) "Sink" regulation of photosynthetic metabolism in transgenic tobacco plants expressing yeast invertase in their cell wall involves a decrease of the Calvin-cycle enzymes and an increase of glycolytic enzymes. Planta **183:** 40–50

Susek RE, Chory J (1992) A tale of two genomes: role of a chloroplast signal in coordinating nuclear and plastid genome expression. Aust J Plant Physiol **19:** 387–399

Taylor WC (1989) Regulatory interactions between nuclear and plastid genomes. Annu Rev Plant Physiol Plant Mol Biol **40:** 211–233

Thomas H, Ougham HJ, Davies TGE (1992) Leaf senescence in a non-yellowing mutant of *Festuca pratensis*. Transcripts and translation products. J Plant Physiol **139:** 403–412

Turgeon R (1989) The sink-source transition in leaves. Annu Rev Plant Physiol Plant Mol Biol **40:** 119–138

Ursin VM, Shewmaker CK (1993) Demonstration of a senescence component in the regulation of the mannopine synthase promoter. Plant Physiol **102:** 1033–1036

von Schaewen A, Stitt M, Schmidt R, Sonnewald U, Willmitzer L (1990) Expression of a yeast-derived invertase in the cell wall of tobacco and *Arabidopsis* plants leads to accumulation of carbohydrate and inhibition of photosynthesis and strongly influences growth and phenotype of transgenic tobacco plants. EMBO J **9:** 3033–3044

Wardley TM, Bhalla PL, Dalling MJ (1984) Changes in the number and composition of chloroplasts during senescence of mesophyll cells of attached and detached primary leaves of wheat (*Triticum aestivum* L.). Plant Physiol **75:** 421–424

Wittenbach V (1983) Effect of pod removal on leaf photosynthesis and soluble protein composition of field grown soybeans. Plant Physiol **73:** 121–124

Wittenbach VA, Lin W, Heber RR (1982) Vacuolar localization of proteases and degradation of chloroplasts in mesophyll protoplasts from senescing primary wheat leaves. Plant Physiol **69:** 98–102