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Developmental and Age-Related Processes That Influence the Longevity and Senescence of Photosynthetic Tissues in Arabidopsis

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Factors that influence the longevity and senescence of photosynthetic tissues of Arabidopsis were investigated. To determine the influence of reproductive development on the timing of somatic tissue senescence, the longevity of rosette leaves of the Landsberg *erecta* strain and of isogenic mutant lines in which flowering is delayed (*co-2*) or sterile flowers are produced (*ms1-1*) were compared. No difference in the timing of senescence of individual leaves was observed between these lines, indicating that somatic tissue longevity is not governed by reproductive development in this species. To examine the role of differential gene expression in the process of leaf senescence, cDNA clones representing genes that are differentially expressed in senescing tissues were isolated. Sequence analysis of one such clone indicated homology to previously cloned cysteine proteinases, which is consistent with a role for the product of this gene in nitrogen salvage. RNA gel blot analysis revealed that increased expression of senescence-associated genes is preceded by declines in photosynthesis and in the expression of photosynthesis-associated genes. A model is presented in which it is postulated that leaf senescence is triggered by age-related declines in photosynthetic processes.

INTRODUCTION

The coupling of whole-plant senescence to reproduction is a life history trait that is common to many annual and some perennial plant species and is referred to as the monocarpic habit (Hildebrand, 1881). In nature, monocarpy is most characteristic of plant species classified as ruderals (Stebbins, 1950; Grimes, 1979). This ecological classification refers to plants, such as Arabidopsis, that are adapted to growth in disturbed environments. According to theory, ruderals have evolved life history traits that are compatible with the high mortality risks from the environment: a propensity for early reproductive development and high fecundity are two particularly noteworthy traits. The early diversion of resources from vegetative to reproductive development in ruderals is thought to have contributed to the evolution of the monocarpic habit characteristic of these species (Molisch, 1928).

The partitioning of resources between vegetative and reproductive development in monocarpic plants involves a complex interplay of generative and degenerative processes that are thought to be under developmental control. The physiological basis for these processes has been extensively studied in a few annual crop species, such as soybean, cotton, and maize (Eaton, 1955; Wittenbach, 1982; Crafts-Brandner et al., 1984a; Crafts-Brandner and Egli, 1987; Ford and Shibles, 1988). In general, monocarpic plant senescence involves a degeneration of existing vegetative tissues and a concomitant cessation of vegetative meristem activity that prevents the development of new photosynthetic tissues (Woolhouse, 1983; Noodén, 1988b). The two processes appear to be coordinately regulated in some cases but independently controlled in other species (see Noodén, 1988a, for a discussion).

The timing of both leaf senescence and apical arrest is thought to involve interacting signals between the affected tissues and the developing fruit. These interactions between vegetative and reproductive structures are generally referred to as correlative controls. A number of hypotheses concerning the nature of these correlative control signals have been presented (reviewed in Woolhouse, 1983; Kelly and Davies, 1988; Noodén, 1988b), but the biochemical nature of the signaling mechanisms has not been unequivocally established for any higher plant.

Although the regulatory mechanisms that govern the timing of leaf senescence remain elusive, the actual processes associated with senescence of photosynthetic tissue have been extensively characterized in a number of plant species. Common features observed across a range of species have led to the concept of the senescence syndrome: an orderly sequence of events involving the turnover of macromolecules and lipids and the transport of mobilized nutrients out of the senescing tissue. The most widely used biomarker for the senescence syndrome is the rapid loss of chlorophyll associated with the degeneration of chloroplast internal structure

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(Thomson and Plat-Aloia, 1987; see also Woolhouse, 1982). Experimental evidence indicates that the senescence syndrome is under genetic control by the nucleus (Yoshida, 1961; Ness and Woolhouse, 1980; Thomas et al., 1992). Recent studies indicate that differential expression of specific genes is associated with the senescence syndrome (Davies and Grierson, 1989; Graham et al., 1992).

The monocarpic habit is exemplified by Arabidopsis, which may undergo its entire life cycle in 8 to 10 weeks. Sexual reproduction in Arabidopsis involves the generation of thousands of offspring. Associated with this massive reproductive effort, the leaves, stems, and fruits of the adult plant undergo progressive senescence that ultimately results in the death of the plant. We are interested in determining the mechanisms involved in the coordination of these processes. In this report, we make use of single-gene mutations that affect reproductive development to evaluate the role of reproduction in the patterns of growth, development, and senescence of the Arabidopsis rosette leaf. In addition, we examine the differential expression of genes in the leaf associated with the senescence syndrome.

RESULTS

Experimental Set Up

Environmental conditions such as light quality and quantity, nutrient and water availability, temperature, and humidity have a strong influence on the course of development in Arabidopsis. For this reason, we established culture conditions that minimized environmental fluctuations. Although these conditions of constant light, temperature, humidity, and soil moisture are not necessarily optimum for growth and development, plants were healthy and uniform in appearance, and the Landsberg *erecta* (Ler) strain produced more than 10,000 seeds within the 50-day life cycle. Under these same conditions, delayed flowering varieties grew vigorously for several months. In some experiments, plants were grown in a 16-hr-light/8-hr-dark photoperiod. Plants of the Ler strain grown under these conditions were indistinguishable from plants grown under constant light.

Life History Traits in Arabidopsis

Figures 1A through 1D depict the life history of an Arabidopsis plant. Under the above constant environmental conditions, the Ler strain of Arabidopsis developed initially as a rosette of seven to eight leaves (Figure 1A). At ~12 days postgermination, the transition from vegetative to inflorescence meristem occurred at the apex, resulting in the production of the highly branched inflorescence (Figures 1B and 1C) (Vaughan, 1955; Bowman et al., 1989; Schultz and Haughn, 1991; Shannon and Meeks-Wagner, 1991). The most active period of flowering and

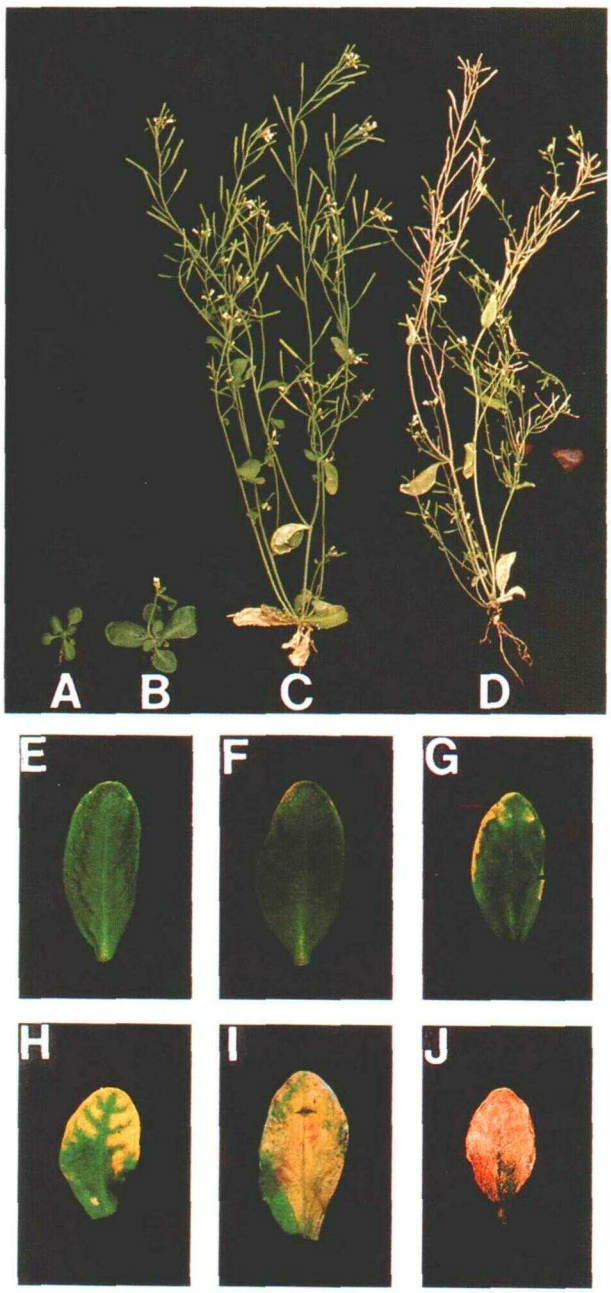

Figure 1. Stages in the Life Cycle of the Landsberg *erecta* Strain of Arabidopsis and Age-Related Changes in Rosette Leaf Number Five.

(A) 14 days after planting.
(B) 21 days after planting.
(C) 37 days after planting.
(D) 53 days after planting.
(E) 0 days after full leaf expansion.
(F) 3 days after full leaf expansion.
(G) 5 days after full leaf expansion.
(H) 7 days after full leaf expansion.
(I) 9 days after full leaf expansion.
(J) 11 days after full leaf expansion.

fruit development is accompanied by the sequential senescence of the original leaves of the rosette, as indicated by an initial stage of chlorophyll loss, followed by the complete disintegration of the leaf tissue (Figure 1C). Developmental arrest of the main inflorescence stem, characterized by a cessation of proliferative activity at the apex and a degeneration of the youngest flower buds, occurred at approximately day 40 (Vaughan, 1955; Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). During this later phase, the stem, cauline leaves, and seed pods became senescent and the seeds of the next generation reached maturity (Figure 1D).

Based on these general observations, we considered two distinct processes that govern the course of monocarpic senescence in Arabidopsis: (1) the senescence of the developed organ systems, such as leaves, stems, and siliques, which we define as somatic senescence and (2) the cessation of generative activity at the inflorescence meristems, which we refer to as apical arrest. In this study, we focused on the senescence of somatic tissues using primarily the fifth and sixth leaves of the rosette as our model.

The fifth and sixth leaves of the rosette represent the first consistently adult leaves of the Ler strain (as defined for the Wassilewskija strain by Medford et al., 1992). Under the culture conditions used, individual leaves progressed through full expansion to maturity followed by a period of progressive leaf yellowing and, finally, a degeneration and complete desiccation of the tissue. This developmental sequence is shown for leaf 5 in Figures 1E through 1J. To obtain data from large populations of plants in a noninvasive way, we performed our initial experiments to assess tissue longevity by simply recording the time from visible emergence (1 to 2 mm) of an individual leaf or stem internode to the time at which more than 50% visible degreening of that tissue had occurred. For leaves, the latter stage was invariably followed by a complete degeneration and death of the tissue within 2 days. In the case of stem tissue, senescence refers only to the degreening of the photosynthetic cortical tissues because stems may continue to function in nutrient transport beyond this stage. Preliminary ultrastructural analysis of the leaf and stem indicated that degreening of both mesophyll cells of the leaf and cortical cells of the stem is associated with the loss of thylakoid structure and increases in plastoglobuli characteristic of the senescence syndrome (data not shown) (Thomson and Platt-Aloia, 1987).

Relationship between Reproduction and Somatic Tissue Senescence

The sequential senescence of the leaves of the rosette is temporally correlated with inflorescence development (Figure 1). In correlative control models for monocarpic senescence, it is hypothesized that signals or processes associated with inflorescence and/or fruit development are responsible for the induction of the senescence syndrome in leaves (reviewed in Noodén, 1988b). To investigate the relationship between reproductive development and senescence in Arabidopsis, somatic tissue senescence was first compared between the wild-type Ler strain and *ms1-1*, a male-sterile mutant line in the Ler background (Koornneef et al., 1983). As shown in Figure 2, the absence of fruit development in the *ms1-1* line did not appreciably affect the timing of senescence of either leaf or inflorescence stem tissue. A delay in reproductive development also failed to have an effect on the longevity of rosette leaves. Leaves of a late-flowering mutant line, *co-2* (Rédei, 1962; Koornneef et al., 1991), senesce over the same period of time as leaves of wild-type plants, as shown in the mortality curves of Figure 3A. A second late-flowering line, *fca* (Martinez-Zapater and Somerville, 1990; Koornneef et al., 1991), was also examined with similar results (data not shown).

An alternative hypothesis for correlative control is the nutrient drain hypothesis in which it is postulated that changes in source–sink relationships between tissues may govern the timing of leaf senescence (Molisch, 1928). One could argue that sink demand resulting from inflorescence development in the male-sterile line or from the development of additional leaves in the delayed flowering lines was governing leaf senescence. To test this possibility, the longevity of rosette leaves was measured in the *tfl1-2* (terminal flower) mutant line (Shannon and

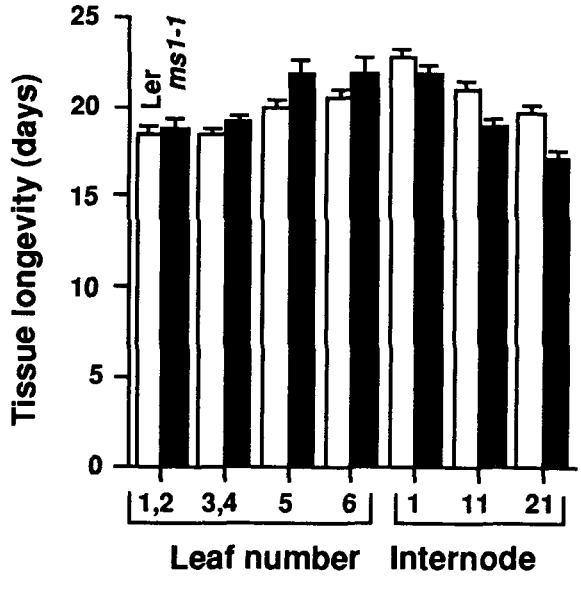

Figure 2. Longevity of Individual Leaves and Stem Segments of Wild-Type (Ler) and Male-Sterile (*ms1-1*) Lines.

Plants were grown in 16-hr-light/8-hr-dark cycles. Mean tissue longevity (± SE) for rosette leaves 1 through 6 (numbered by order of emergence from the meristem) is shown. Data for the first two pairs of leaves are averaged because the time of emergence is often indistinguishable. Internode 1 is defined as the internode between the first and the second flower produced on the primary inflorescence meristem. Tissue longevity was measured as the time from visual emergence (1 to 2 mm) to more than or equal to 50% degreening. For leaf Ler, n = 137 plants; *ms1-1*, n = 45 plants. For internode Ler, n = 19; *ms1-1*, n = 11.

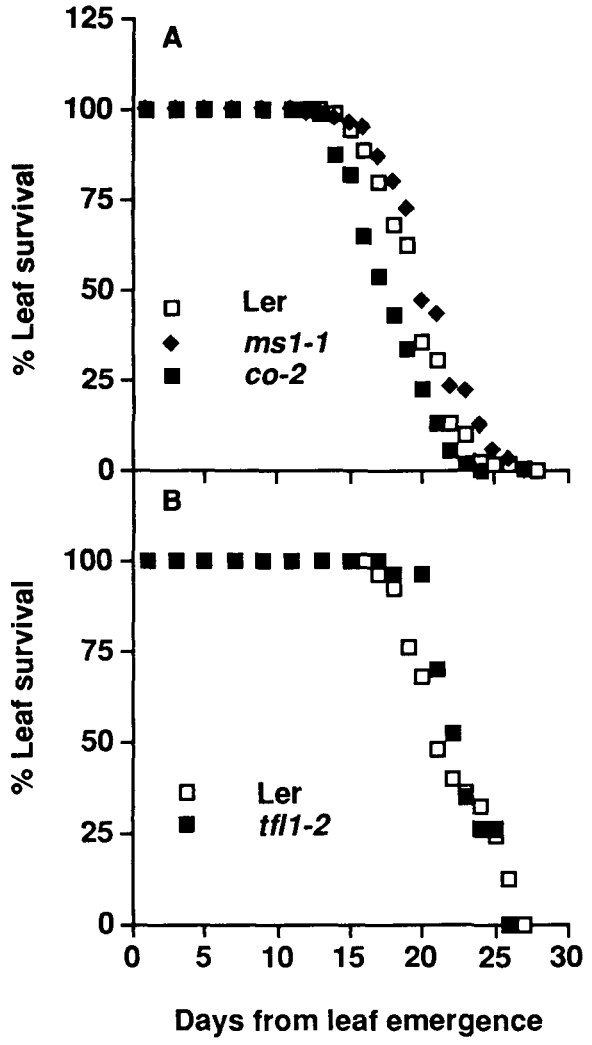

Figure 3. Survival Curves of Adult Leaves for Wild-Type (*Ler*), Late-Flowering (*co-2*), Male-Sterile (*ms1-1*), and Terminal Flower (*tfl1-2*) Lines.

Data from leaves 5 and 6 were combined for this analysis. Leaf longevity was measured as given in Figure 2.
(A) Ler, n = 322; *co-2*, n = 54; *ms1-1*, n = 74.
(B) Ler, n = 25; *tfl1-2*, n = 23.

Meeks-Wagner, 1991; Alvarez et al., 1992). The *tfl1-2* line produces a normal rosette and initiates flower development at the same time as the isogenic Ler wild type. However, the development of aberrant flower structures at the inflorescence meristem causes early termination of inflorescence development resulting in a 10-fold decrease in biomass accumulation relative to wild type, as shown in Table 1. Results shown in Figure 3B indicate that leaf longevity was not appreciably altered by the diminished sink demand in *tfl1-2*.

Rosette Leaf Senescence as an Age-Related Process

Results of the above experiments indicated that the timing of leaf and stem senescence is not coupled to reproductive development in Arabidopsis. Rather, somatic tissue longevity appears to be an intrinsic age-related property of the organ system.

To investigate the role of processes intrinsic to the leaf in the timing of leaf senescence, we examined a number of cellular parameters for age-related changes. Because the primary function of the mature leaf is photosynthesis, we focused on biomarkers associated with photosynthetic function. Results shown in Figure 4 indicate that CO_2 fixation rates, while initially variable, decline with leaf age almost from the time of full leaf expansion (Figure 4A). Extractable ribulose bisphosphate carboxylase (Rubisco) activity and soluble protein showed similar declines associated with age (Figures 4B and 4C). The slower decline in Rubisco activity relative to CO_2 fixation is due in part to the fact that fixation rates were measured on the whole leaf, and Rubisco activity was determined from leaf discs taken from the center of the leaf that senesces last (Figure 1). For leaf discs, the decline in Rubisco preceded the rapid loss in chlorophyll that is characteristic of the senescence syndrome (Figure 4D).

Isolation of cDNA Clones Representing Genes That Are Differentially Expressed during Senescence

Whereas biological markers for photosynthetic function were known, no such markers were known for the senescence phase of leaf development. As mentioned in the Introduction, several lines of evidence support the view that leaf senescence is a developmentally controlled process that requires expression of nuclear genes. To identify senescence-related biomarkers, we screened a cDNA library made from mRNA of senescing leaves using differential hybridization (Sambrook et al., 1989). Nine cDNA families that do not cross-hybridize were identified and further characterized. Data for two of these cDNA clones are shown here. These two senescence-associated (*SAG*) gene clones were designated *SAG2* and *SAG4*. As shown in Figure 5A, RNA gel blot analysis indicated that mRNA levels for both genes are elevated in senescing leaves and inflorescence stems. DNA gel blot analysis, shown

Table 1. Plant Dry Weight Produced by Wild Type (Ler) and Terminal Flower (*tfl1-2*)

Line	Flowers Produced per Primary Inflorescence	Dry Weight, Grams ± SE
Ler	34.5 ± 0.8	1.32 ± 0.16
tlf1-2	<4	0.17 ± 0.02

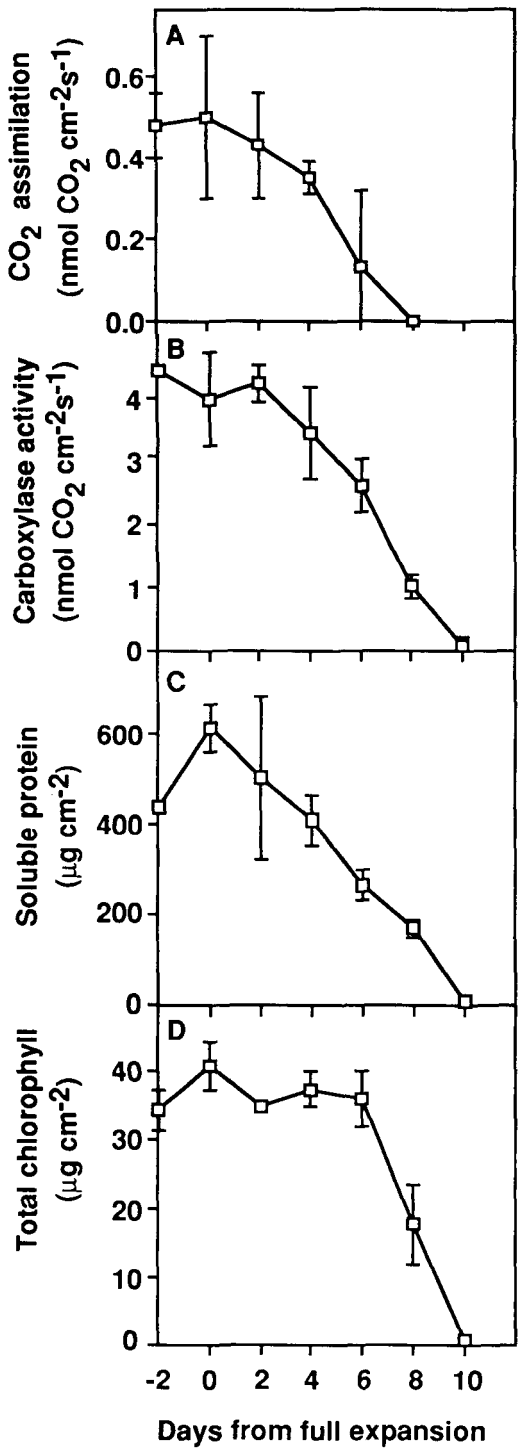

Figure 4. Age-Related Changes in Photosynthetic Parameters of Adult Rosette Leaves.

(A) CO_2 assimilation rates.
(B) Extractable Rubisco activity.
(C) Soluble protein.
(D) Chlorophyll content.

in Figure 5B, indicated that both *SAG2* and *SAG4* are single-copy genes within the Arabidopsis genome. Partial sequence analysis (Figure 5C) indicated that *SAG2* contains an open reading frame that shares a high degree of derived amino acid sequence similarity with a family of cysteine proteinases identified in plants and animals (Fuchs and Gassen, 1989; Watanabe et al., 1991). The identification of the product of *SAG2* as a proteinase is consistent with a role for this protein in the mobilization of nitrogen within the leaf during the senescence phase (Thayer et al., 1987).

Changing Patterns of Gene Expression Associated with Senescence

The process of leaf senescence involves a shift in leaf function from that of a photosynthetic organ to that of a storage organ. To examine the role of differential gene expression during the progression from a photosynthetic function to remobilization, the expression patterns of marker genes representing each stage in leaf development were used for RNA gel blot analysis. Cloned probes representing chlorophyll *a/b* binding protein (*CAB*) and ribulose bisphosphate carboxylase small subunit (*rbcS*) were used as markers for the photosynthetic stage of leaf development. An elongation factor (EF-1α) gene (Curie et al., 1991) probe was used to represent cytoplasmic housekeeping functions (protein synthesis). The two *SAG* cDNA clones described in the previous section served as markers for the senescence phase of leaf development.

To determine changes in steady state mRNA levels for the marker genes, total RNA was extracted from leaves (leaves 7 and 8, Columbia strain) of increasing age. Representative autoradiographs are shown and quantitative analysis of mRNA levels for all marker genes are depicted in Figure 6. Results indicated that transcript levels of nuclear-encoded, photosynthesis-associated (*PAG*) genes were highest in expanding leaves, dropped precipitously as the leaf reached full expansion, and then declined slowly with age relative to total RNA. On the other hand, transcript levels for EF-1α remained fairly constant relative to total RNA after full leaf expansion. Messages for the two *SAG* genes were expressed at lowest levels in the young fully expanded leaf and increased 10- to 20-fold in the senescing leaves. It should be noted that RNA levels depicted in Figure 6 are based on equal total RNA loads. However, the amount of extractable rRNA per leaf declined as the leaf senesced, as shown in Figure 7. Two factors may contribute to the observed decline in rRNA: (1) decreases in rRNA in senescing cells and (2) decreases in the number of live cells per leaf as necrotic areas developed. It was not possible to differentiate between these possibilities from the experiments described. Correcting for the changes in rRNA, it was calculated that *SAG2* and *SAG4* increased fourfold and threefold, respectively, on a per leaf basis during senescence. By the same analysis, *PAG* gene expression was 16-fold lower in senescing leaves than in fully expanded leaves on a per leaf basis.

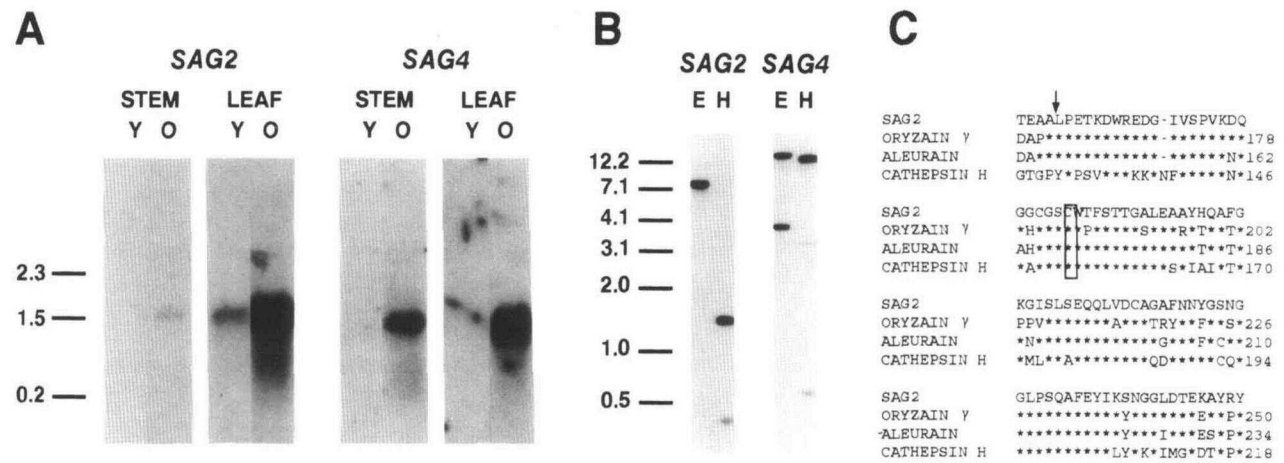

Figure 5. *SAG* Gene Analysis.

(A) Age-related expression in leaves and inflorescence stem. RNA gel blot analysis of young, fully expanded green tissue (Y) and old (>50%) degreening tissue (O).

(B) DNA gel blot analysis of genomic DNA digested with EcoRI (E) or HindIII (H) and probed with either *SAG2* or *SAG4* cDNA clones. Molecular size markers are given at the left in kilobases.

(C) DNA sequence analysis of the *SAG2* cDNA open reading frame. Comparison of the predicted SAG2 protein sequence with three related cysteine proteinases: oryzain γ from rice (Watanabe et al., 1991); aleurain from barley (Rogers et al., 1985); and cathepsin H from humans (Fuchs and Gassen, 1989). Gaps are indicated by dashes and sequence identities by asterisks. The arrow marks the cleavage site that is processed to produce the N terminus of the mature protein.

DISCUSSION

The sequential senescence of Arabidopsis rosette leaves is associated with the time of maximum inflorescence development. We were surprised to discover that although these two processes are normally juxtaposed in time, they are not coupled in a causal way. Under the growth conditions used, the rosette leaves appear to have an intrinsic maximum life span that is independent of the reproductive status of the plant. This is clearly not the case with other monocarpic plants. In the legumes, for instance, monocarpic senescence of leaves is dependent on fruit development and affects both younger and older leaves (Noodén, 1988b). In some pea varieties, senescence may even progress from youngest to oldest leaves (Proebsting et al., 1976). The age-related nature of senescence in Arabidopsis is not restricted to rosette leaves. The photosynthetic tissues of the inflorescence stem also show age-related patterns of senescence. Based on our observations, all photosynthetic tissues of the plant exhibit equally short life spans. Therefore, the age of specific tissues appears to be the best predictor of when senescence will occur. Whether vascular tissues are also subject to age-related declines in function is a question that can only be answered with a detailed anatomical analysis.

The lack of evidence for extrinsic, correlative controls on leaf senescence in Arabidopsis led to a more detailed examination of intrinsic processes within the leaf that might contribute to the observed age-related senescence. Analysis of photosynthetic processes revealed that carbon fixation rates declined progressively from the time of full leaf expansion (Figure 4). This age-related, progressive loss of photosynthetic function from the time of leaf maturity may be a general feature of annual plants because this phenomenon has been observed in cucumber (Callow, 1974), *Perilla* (Batt and Woolhouse, 1975), barley (Friedrich and Huffaker, 1980), wheat (Peoples et al., 1980), maize (Crafts-Brandner et al., 1984a), and soybean (Wittenbach, 1982; Ford and Shibles, 1988). Wittenbach referred to the photosynthetic decline as "functional senescence" to distinguish this process from the subsequent rapid loss of chlorophyll and macromolecular turnover associated with the senescence syndrome (Wittenbach, 1983). The physiological basis for functional senescence is not known, although a number of explanations have been suggested (Callow, 1974; Woolhouse, 1982; Noodén, 1988b). Perhaps, the simplest explanation for the observed functional decline in photosynthesis may be that maintenance and repair of the photosynthetic apparatus does not keep pace with the damaging effects of the free-radical byproducts of photosynthesis in the leaf (Bowler et al., 1992). In this sense, functional senescence of leaves may conform to free-radical theories of aging applied to animal systems (Harman, 1981; Bolli et al., 1989).

Whatever the mechanism, the consequence of age-related decline in photosynthesis is that the leaf will ultimately reach the compensation point at which carbon assimilation and respiration are equal. At this point, the leaf will no longer contribute

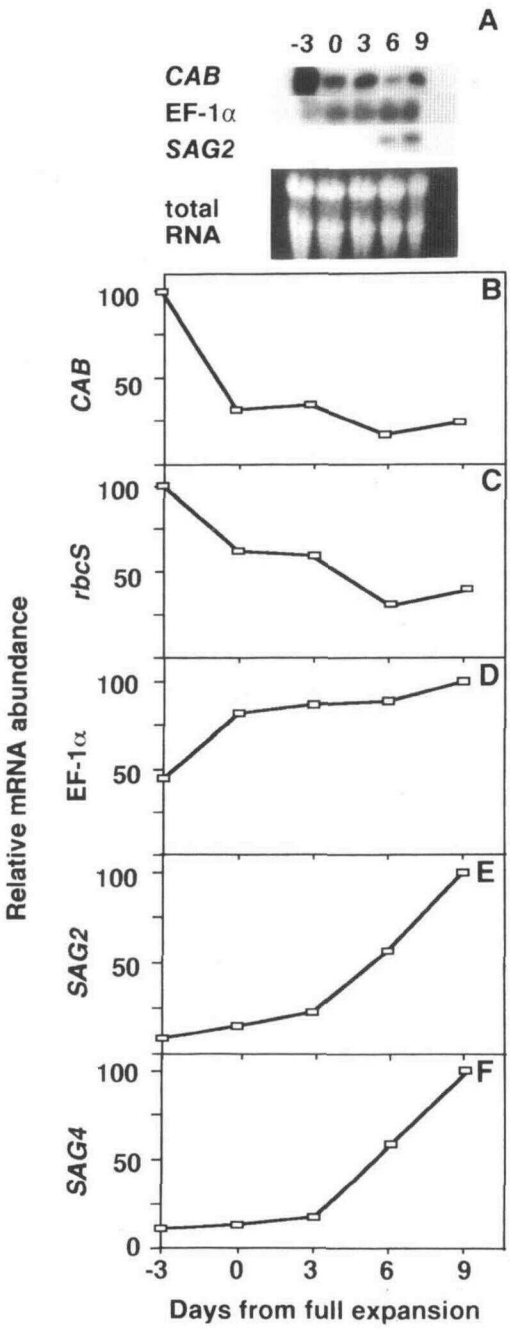

Figure 6. Age-Related Changes in Transcript Levels for Selected Genes.

Equal loads of total RNA were loaded in each lane and blots were hybridized with ^{32}P-labeled probes.

(A) Representative autoradiographs of **(B)**, **(C)**, **(D)**, **(E)**, and **(F)**.

(B) *CAB*.

(C) *rbcS*.

(D) EF-1α.

(E) *SAG2*.

(F) *SAG4*.

as a photosynthetic organ to the assimilatory needs of the rest of the plant. However, the functionally senescent leaf contains a significant pool of nutrients in the form of lipids and macromolecules. The chloroplasts of a mesophyll cell are of particular importance in this regard because it has been estimated that these organelles may contain over 50% of the protein and 75% of the lipid of the cell (Forde and Steer, 1976; Dean and Leech, 1982). The mobilization of these nutrients is thought to occur via salvage pathways that increase in activity at the terminal stages of leaf development (Woolhouse, 1984; Noodén, 1988a).

As outlined below, we postulate that in Arabidopsis the activation of salvage pathways associated with senescence is directly coupled to photosynthetic decline. This regulatory feature may be the critical one that distinguishes Arabidopsis from other monocarpic plants such as soybean, in which the senescence syndrome is correlatively controlled by reproductive development. Interestingly, depodding of soybean plants will delay the final stages of senescence in the leaf but does not prevent photosynthetic decline (Wittenbach, 1982; Crafts-Brandner et al., 1984b). In fact, the Rubisco enzyme may be degraded in leaves of depodded plants, but the mobilized N is apparently refixed in the paraveinal mesophyll as storage protein (Franceschi et al., 1983; Wittenbach, 1983). Thus, in soybean, depodding appears to result in uncoupling of photosynthetic decline from activation of the senescence syndrome. In this case, full activation of salvage pathways presumably

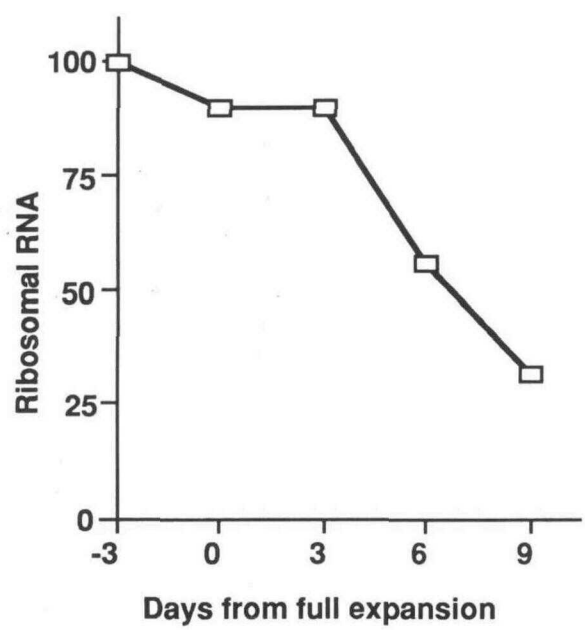

Figure 7. Age-Related Changes in Extractable Ribosomal RNA per Leaf.

RNA quantities were evaluated by slot-blot analysis of RNA obtained on a per leaf basis. Values were corrected for RNA losses during purification.

requires some form of signal from the developing fruit. In contrast, we have not yet found a similar situation in Arabidopsis leaves. In addition to the analysis of the Arabidopsis mutants affecting reproduction reported in this paper, we have examined existing hormone-deficient and insensitive mutants as well as newly isolated mutants with delayed leaf senescence. In no case have we found a condition in which the senescence syndrome has been uncoupled from photosynthetic decline (L.L. Hensel, V. Grbić, D.A. Baumgarten, and A.B. Bleecker, unpublished results).

To consider the possible mechanisms by which the activation of salvage pathways may be coupled to photosynthetic decline, we turn to the paradigm of differential gene expression. Based on our analysis of *PAG* and *SAG* gene expression, we consider these biomarkers as representatives of two competing developmental programs within the leaf. *PAG* genes code for components of the photosynthetic apparatus and are consequently expressed at highest levels during leaf expansion when chloroplast biogenesis is occurring (Figure 6; see also Mullet, 1988). The lower *PAG* transcript levels observed after full leaf expansion presumably represent messages utilized in the maintenance and repair of existing chloroplasts because chloroplast fission and biogenesis are thought to cease at full leaf expansion (Pyke and Leech, 1992). The *SAG* genes appear to code for salvage-related functions that are antagonistic to photosynthetic maintenance. The observed age-related expression patterns of *SAG* genes are consistent with this concept: *SAG* transcripts are expressed at low levels in young leaf tissues and increase in expression as photosynthesis declines (Figure 6).

Potential regulatory processes that drive the expression of *PAG* and *SAG* gene transcripts are presented diagrammatically in Figure 8. These possible regulatory pathways can form the basis for mechanistic models that simulate the regulation of these two sets of genes. As indicated in the diagram, the regulation of the genes under consideration is complex. As one working model from which to generate and test specific hypotheses, we suggest a two-step process in which (1) age-related declines in photosynthesis trigger the activation of *SAG* gene expression and (2) the products of the *SAG* genes act to mobilize nutrients and, as a consequence, reduce the viability of the tissues to the point of a loss of homeostasis and death. According to this model, *PAG* transcript levels, which influence the rate of damage repair in the mature leaf, may be determinants in the rate of photosynthetic decline and, thus, contribute indirectly to the timing of leaf senescence. An additional key feature of this model is the speculation that *SAG* gene expression is repressed in younger leaves by metabolites of photosynthesis, and it is the derepression of these genes resulting from photosynthetic decline that initiates the senescence syndrome. According to this model, the rate of decline in photosynthesis after full leaf expansion will determine the timing of *SAG* gene expression and, therefore, the longevity of the leaf. The chemical identity of the metabolite(s) responsible is not known, but it is unlikely to be sucrose because this transportable form of carbon may occur at high levels in senescent

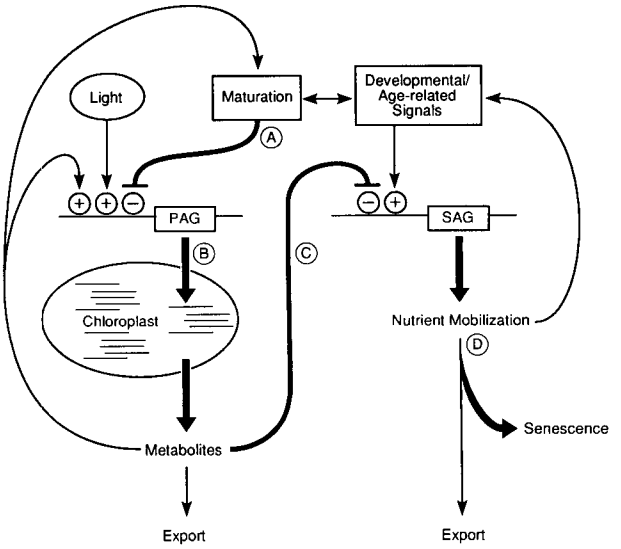

Figure 8. Schematic of Regulatory Pathways That May Influence Leaf Longevity and Senescence.

The emphasis of this schematic is on the transcriptional control of *PAG* and *SAG* genes. The darker lines outline a hypothetical model in which the following sequence of events is proposed: (A) maturation signals suppress *PAG* gene expression at full leaf expansion; (B) photosynthetic function declines as a result of inadequate maintenance; (C) metabolites of photosynthesis, which act to repress *SAG* gene expression in the young leaf, decline in concentration, resulting in derepression of *SAG* genes; (D) *SAG* gene products act to mobilize nutrients and, as a consequence, promote the senescence of the leaf tissues.

tissues (Crafts-Brandner et al., 1984a). The source of carbon for sucrose synthesis in this case is unlikely to be photosynthesis, but is rather through the β-oxidation of lipids because glyoxysomal pathways are known to increase during senescence (Gut and Matile, 1988; DeBellis et al., 1990). In this regard, the metabolite repression model has previously been suggested for the senescence-associated expression of malate synthase in cucumber cotyledons (Graham et al., 1992).

The limited longevity of Arabidopsis somatic tissues is consistent with the early reproductive development and high fecundity of this species. In terms of evolutionary theory, the disposable soma hypothesis (Kirkwood and Cremer, 1982; Kirkwood and Rose, 1991) predicts that species adapted to early reproduction will tend to invest less energy in somatic maintenance because such maintenance requires resources that are then unavailable for reproductive effort. In specific terms, the dynamics of the Arabidopsis leaf represent a trade-off between high photosynthetic output, which supports new growth, particularly of reproductive structures, and maintenance and repair, which contribute to longevity of somatic tissues once they develop. The measure of this evolutionary trade-off for photosynthetic tissues may be in the age-related rate of photosynthetic decline characteristic of a particular species. At the opposite end of the continuum from Arabidopsis would be

the needles of some conifers, which are purported to maintain photosynthetic competence for years but are characterized by relatively low photosynthetic output (Oren et al., 1986).

The processes we have detailed also conform to the genetic theory of life span evolution known as the antagonistic pleiotropy hypothesis first elaborated by Williams (1957) and more recently discussed by Rose (Kirkwood and Rose, 1991; Rose, 1991). According to this hypothesis, individual genes that have a positive effect on reproductive fecundity but a negative effect on postreproductive survival of the parent will be selected for in evolution. The accumulation of these genes over evolutionary time is thought to determine the maximum life span for a given species. Although this theory is generally applied to animals, specific genes and associated mechanisms for antagonistic pleiotropy have not been well characterized in animal systems (Finch, 1990). On the other hand, the salvage pathways associated with the senescence syndrome in plants provide a clear example of the operation of processes that may favor reproductive success through remobilization of nutrients but also lead to decreased fitness (i.e., senescence) of the tissues in which they are expressed.

The *SAG* genes we have identified by differential hybridization conform to the theoretical definition of antagonistic pleiotropy if the encoded proteins function in the mobilization of nutrients, favoring reproduction, and also decrease the viability of the tissues in which they operate. The *SAG2* gene, which encodes a cysteine proteinase, may provide a specific case. The derived amino acid sequence of this gene shares a high degree of amino acid identity with oryzain γ and aleurain, which are specifically expressed in germinating grain seeds and are thought to function in the mobilization of nitrogen from protein reserves (Rogers et al., 1985; Watanabe et al., 1991). Thus, a role for the *SAG2* gene in nutrient mobilization seems likely. It is also plausible that the *SAG2* gene, in concert with other *SAG* genes, contributes actively to the autolytic process of senescence that leads to the death of the somatic tissues in which these genes are expressed. Considering the evolutionary origin of senescence syndrome–associated salvage pathways, most of the *SAG* genes we have identified are expressed at detectable levels in the young photosynthetically active leaf (Figure 6; V. Grbić, unpublished results), which is consistent with a role for some of these genes in the maintenance-related turnover of macromolecules (e.g., damaged proteins). This concept is supported by the high degree of amino acid sequence identity between *SAG2* and cathepsin H from animals, which is thought to function in general protein turnover in animal cell lysosomes (Takio et al., 1983). Sequence analysis of additional *SAG* gene clones should provide information about how these differentially expressed genes function in cellular maintenance and senescence.

Assuming that *SAG* genes are responsible for the progression of senescence in somatic tissues of plants, the regulatory pathways that govern their expression patterns may actually determine the longevity of these tissues. We have presented an argument that in Arabidopsis, *SAG* genes are activated as a consequence of age-related declines in photosynthesis. Not all monocarpic plants conform to the predictions of this model. As discussed above, the senescence of leaves can be delayed by removal of fruit in a number of species (Noodén, 1988b) even though this treatment does not prevent age-related declines in photosynthesis. In these systems, the activation of *SAG* gene expression is apparently not coupled to photosynthetic decline but rather depends on other or additional developmental signals for activation. This possibility is accounted for in our model by the "developmental and age-related signals" box in Figure 8. The developing Arabidopsis plant apparently either does not require these additional regulatory systems or cannot afford them. In any case, the timing of leaf senescence in Arabidopsis is adapted to the reproductive requirements of the plant; the sequential senescence of the rosette leaves coincides with the period of maximum primary inflorescence development and seed fill (Figure 1).

What impact does the short life span of Arabidopsis somatic tissues have on the longevity of the whole plant? In theory, the iterative nature of plant development could allow for an indefinite life span. In practice, this is true for Arabidopsis only in cases where reproductive development is delayed. In this case, the primary meristem continues to generate new rosette leaves for many months (Napp-Zinn, 1985). The transition to flowering, however, marks the beginning of the end for the adult plant because all aerial meristems are converted over to reproductive development. While the tissues produced by the inflorescence and flower meristems are capable of photosynthesis, inflorescence meristems have a limited proliferative capacity; they cease meristematic activity after the production of a few dozen flowers (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). Thus, the combination of limited longevity of somatic tissues and the limited proliferative capacity of the inflorescence meristems ultimately limit the life span of the adult Arabidopsis plant.

METHODS

Strains

Seeds for Landsberg *erecta* (Ler) and the isogenic mutant lines *co-2*, *ms1-1*, and *fca* were obtained from A.R. Kranz (Kranz, 1978; Kirchheim and Kranz, 1981). Columbia seeds were obtained from Chris Somerville (Michigan State University, East Lansing). *tfl1-2* seeds were provided by D. Smyth (Monash University, Melbourne, Australia) (Alvarez et al., 1992).

Plant Material and Measurements

All plants were grown at a density of approximately one plant per 25 cm^2, at 22°C, and 65 to 85% relative humidity under fluorescent illumination supplemented with incandescent light (100 to 150 $\mu E\ m^{-2}\ sec^{-1}$) on a 2:1 mixture of Jiffy Mix (Jiffy Products of America, Batavia, IL) to perlite with a continuous wicking system of 10% Hoagland's solution (Hoagland and Arnon, 1938). All plants were grown under

continuous illumination unless otherwise stated. Seeds were surface sterilized with a 30% bleach and 0.5% Triton solution and stratified at 4°C for 24 to 48 hr prior to planting. Leaf and internode longevity were measured as the time from visual emergence (1 mm) to more than or equal to 50% degreening. Dry weight was determined from fully mature plants harvested 52 days after planting. Plants were dried for 3 days at 65°C prior to weighing.

Nucleic Acid Preparation

DNA for gel blot analysis was prepared from 5-week-old plants according to the method of Shure et al. (1983). Total RNA was prepared as described by Puissant and Houdebine (1990). Total RNA was isolated from a pool of 20 adult leaves (the seventh and eighth leaves to emerge from the primary inflorescence meristem). All nucleic acid preparations were isolated from tissues of the Columbia strain, except the RNA for Figure 5A, which was isolated from tissues of the Landsberg strain.

To correct for losses in total RNA during isolation, ^{35}S-labeled RNA was added as a tracer to leaf homogenates. The ^{35}S-labeled tracer was made from the 1.15-kb HindIII fragment of the 5′ end of the *TMK1* gene (Chang et al., 1992), which was cloned into the pGEM-7 plasmid (Promega). In vitro transcription from the SP6 promoter (Riboprobe kit; Promega) produced ^{35}S-labeled RNA of ~1 kb in length.

Screening the cDNA Library

The cDNA library made from RNA of senescing leaves was provided by M. Michael (CalGene Pacific, Australia). Clones from the amplified cDNA library were screened with ^{32}P-labeled cDNA from green (young) and yellow (old) leaves according to the method of Sambrook et al. (1989). The screening of 7000 recombinant cDNA clones resulted in identification of 40 differentially expressed clones belonging to nine families that do not cross-hybridize, designated senescence-associated (*SAG*)*1* through *SAG9*.

DNA and RNA Gel Blot Analysis

For DNA gel blots, 3 μg of DNA was digested with EcoRI and HindIII (Promega), fractionated on an 0.8% agarose gel (0.5 × Tris-borate/EDTA electrophoresis buffer), transferred to a nylon membrane (MSI-Magna NT; Micron Separations Inc., Westborough, MA), and hybridized at 68°C with random primer ^{32}P-labeled probes (Prime-A-Gene; Promega) according to standard protocols (Sambrook et al., 1989). For RNA gel blot analyses, 3 μg of total RNA was denatured in 70% formamide, 20% formaldehyde, 10% 10 × running buffer (0.25 3-(*N*-morpholino) propanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7.0), fractionated on an 0.8% agarose gel (1 × running buffer), transferred to a nylon membrane (MSI-Magna NT; Micron Separations Inc.) with CE (10 mM sodium citrate, pH 7.0, 1 mM EDTA), and hybridized as was done for DNA gel blot analysis. The chlorophyll *a/b* binding protein (*CAB*) probe, pAB 140, was obtained from E. Tobin (University of California, Los Angeles) (Leutwiler et al., 1986). The ribulose bisphosphate carboxylase small subunit (*rbcS*) probe, pATS-3, is a genomic clone that spans the promoter and the 5′ half of the coding sequence of the ATS3B member of the *rbcS* gene family (Krebbers et al., 1988). The elongation factor (EF)-1α probe was made by polymerase chain reaction amplification with oligonucleotides designed to be unique to EF-1α-A1 (Curie et al., 1991), and the amplified fragment was cloned into Bluescript KS+ (Stratagene). Hybridized filters were washed in 1 × SSPE (0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.2), 0.1% SDS, and then in 0.2 × SSPE, 0.1% SDS, each at 68°C for 45 min, followed by autoradiography overnight. Radioactivity was quantified with a Betagen Betascope 630.

For quantification of rRNA per leaf, 10-fold serial dilutions (10^3 to 10^6 of total RNA) were slot blotted on nylon membranes and probed with a pea rRNA gene, *pHA2* (Polans et al., 1986). rRNA levels were calculated from the linear range of signals on the slot blot and corrected for losses during extraction using recovery of the ^{35}S-labeled tracer RNA.

DNA Sequencing Analysis

The dideoxynucleotide chain termination method (Sanger et al., 1977) was used for sequencing *SAG2*. Sequencing was done using the Sequenase kit (U.S. Biochemicals) according to the manufacturer's protocol. The deduced amino acid sequence of the *SAG2* polypeptide was compared to the GenBank sequence data base using FASTA (Pearson and Lipman, 1988).

Biochemical Assays

For protein, chlorophyll, and carboxylase activity, each data point represents the average of three pools of three 0.5-cm^2 leaf discs taken from the middle of the leaf. The discs were frozen immediately in liquid nitrogen and stored at −80°C until measurements were made. Discs used for total chlorophyll measurements were ground in 2 mL of 80% acetone and quantified photometrically using the method of Arnon (1949). Initial carboxylase activity was determined by grinding the tissue in 2 mL of buffer (100 mM Bicine, pH 7.8, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 1.5% polyvinylpolypyrrolidone) and analyzed photometrically using the method of Sharkey et al. (1991). Soluble protein was quantified using 30 μL of the soluble extract from the carboxylase measurements by the method of Bradford (1976) using BSA as the standard.

Gas Exchange Measurements

CO_2 assimilation measurements were performed on the fifth leaf using the method of Loreto and Sharkey (1990). A 2.0-cm^2 area of leaf was clamped in a 1.59-cm^3 aluminum cuvette with glass windows, maintained at 24°C, and illuminated with 150 μE m^{-2} sec^{-1} white light. Data points represent the average of measurements from three leaves.

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