

## Update on Development

# Making Sense of Senescence<sup>1</sup>

## Molecular Genetic Regulation and Manipulation of Leaf Senescence

Susheng Gan<sup>2</sup> and Richard M. Amasino\*

Department of Biochemistry, University of Wisconsin, 420 Henry Mall, Madison, Wisconsin 53706–1569

Leaf senescence is the final stage of leaf development. In forests of deciduous trees, the autumn colors that develop during leaf senescence are of great aesthetic value. This process is also of great practical value because during leaf senescence, nutrients are recycled to other parts of the plant. For example, nitrogen from leaves of deciduous trees is used for the synthesis of storage proteins in stems that will support growth during the following spring (Clausen and Apel, 1991). However, in an agricultural setting, leaf senescence may limit yield in certain crops. Senescence also contributes to the postharvest loss of vegetable crops. Therefore, studying leaf senescence will not only contribute to our knowledge about this fundamental developmental process, but may also lead to ways of manipulating senescence for agricultural applications.

There have been many physiological, biochemical, and molecular studies of leaf senescence. These studies show that during senescence leaf cells undergo highly coordinated changes in cell structure, metabolism, and gene expression. The earliest and most significant change in cell structure is the breakdown of the chloroplast, the organelle that contains up to 70% of the leaf protein. Metabolically, carbon assimilation (photosynthesis) is replaced by catabolism of chlorophyll and macromolecules such as proteins, membrane lipids, and RNA so that some of the released nutrients can be recycled. At the molecular level, these changes are accompanied by, or perhaps driven by, changes in gene expression. In this *Update*, we summarize physiological and biochemical studies that have contributed to the present understanding of leaf senescence, then we discuss current molecular investigations into the regulatory mechanism(s) underlying leaf senescence, and, fi-

nally, we review some molecular approaches toward the manipulation of leaf senescence.

### GENETIC REGULATION OF LEAF SENESCENCE: LEAF SENESCENCE AS A FORM OF PCD

PCD is a broad term that refers to a process by which cells promote their own death through the activation of self-destruction systems. It is now generally accepted that many plant developmental processes and stress responses are achieved through the operation of PCD; these include senescence, xylogenesis, embryogenesis, sex determination in monoecious plants such as maize, abscission zone formation, and the hypersensitive reaction to pathogen infection (Greenberg, 1996; Jones and Dangl, 1996). The evidence that leaf senescence is a genetically defined program of cell death comes mainly from the following observations.

Functionally, leaf senescence is not simply a degenerative process, but is also a recycling process in which nutrients are translocated from the senescing cells to young leaves, developing seeds, or storage tissues (Fig. 1A). In many species, the pattern of leaf senescence illustrates this point: tissues around the vascular system, which are required for nutrient export, are the last to senesce (see Fig. 1B).

Structurally, there is a distinct pattern of senescence at the cellular level. Several studies have shown that leaf cells undergo defined subcellular changes during senescence (e.g. Thomson and Platt-Aloia, 1987). For example, the loss of chloroplast integrity occurs first, whereas the breakdown of the nucleus is a relatively late event. Because a large portion of the nitrogen in a leaf cell is in the chloroplast (Makino and Osmond, 1991), it is expected that during leaf senescence chloroplasts are broken down, while other cellular constituents such as the nucleus remain intact to accomplish the recycling process.

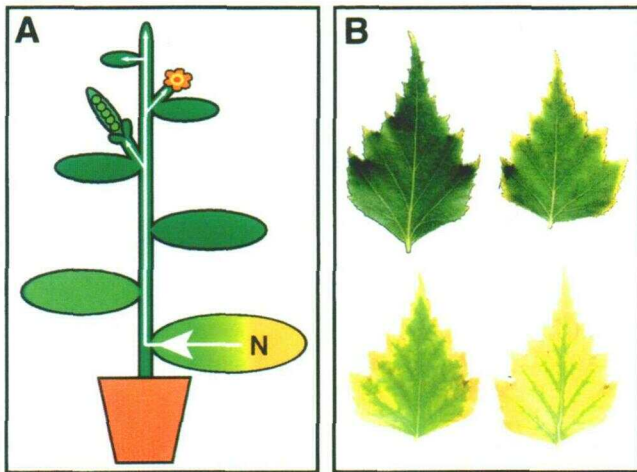
Chloroplast senescence is indeed under direct nuclear control, as demonstrated in enucleation studies. When *Elo-dea* leaves were exposed to hypertonic conditions, the protoplast in some cells separated into two nearly equal halves, both of which contained chloroplasts but only one of which contained a nucleus. Chloroplasts in the nucleated

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<sup>2</sup> Present address: Department of Agronomy and Tobacco and Health Research Institute, University of Kentucky, Lexington, KY 40546.

\* Corresponding author; e-mail amasino@biochem.wisc.edu; fax 1-608-262-3453.

Abbreviations: IPT, isopentenyl transferase; PCD, programmed cell death.



**Figure 1.** A, Schematic illustration of nutrition recycling during leaf senescence. Some of the released nutrients (N) such as nitrogen are transported to developing seeds and young organs at the shoot apex. B, Progression of leaf senescence in a species of birch. Senescence proceeds from leaf margins toward the center. Note that cells surrounding the vascular tissues senesce relatively late to facilitate nutrient mobilization from adjacent senescing cells.

half senesced at the expected time, whereas those in the enucleated half remained green and photosynthetic (Yoshida, 1961). Further evidence that nuclear gene expression is required for a leaf to senesce is that leaf senescence can be blocked by inhibitors of RNA and protein synthesis. The majority of the experiments using cycloheximide (an inhibitor of protein synthesis) and actinomycin D (an inhibitor of RNA synthesis) show that a variety of senescence-related changes in leaves of many plant species are blocked by these inhibitors (for review, see Noodén, 1988).

The concept of leaf senescence as a type of PCD has been recognized for many years (Noodén and Leopold, 1978). However, it is unclear whether leaf senescence shares any biochemical or genetic pathways with other types of PCD in plants or animals.

#### ENVIRONMENTAL AND AUTONOMOUS REGULATION OF LEAF SENESCENCE

Like many other genetically programmed developmental processes, leaf senescence, particularly its initiation, is subject to regulation by many environmental and autonomous (internal) factors. The environmental cues include stresses such as extremes of temperature, drought, ozone, nutrient deficiency, pathogen infection, wounding, and shading, whereas the autonomous factors include age, reproductive development, and phytohormone levels.

Among the environmental cues, limited water and nutrient availability (especially nitrogen) are major factors that adversely affect plant life in many ecosystems. Plants have evolved mechanisms by which leaf senescence can be induced by these stresses to reallocate nutrients to reproductive organs and to eliminate water consumption by older, less productive leaves. This regulation of leaf senes-

cence has an obvious adaptive value, allowing the plant to complete its life cycle even under stressful conditions.

Shading of lower leaves under a canopy is another factor that in certain species can induce senescence. Plants can respond to shade by increasing stem elongation to reach higher intensity radiation (known as the "shade-avoidance response"). However, growing to reach a sunnier location is not an option for a fully developed leaf; instead, leaf senescence may be initiated in the shaded leaves. Light that has passed through a canopy of leaves has a lower ratio of red to far-red light as a result of preferential absorbance of red photons by chlorophyll in upper leaves. It has been shown that reduced PAR and a decreased red/far-red ratio are the senescence-triggering signals in shaded leaves (Rousseaux et al., 1996), suggesting that a phytochrome signal pathway and photosynthate levels may be involved in triggering the leaf senescence program. Indeed, transgenic plants that overexpress phytochrome A exhibit delayed senescence in shaded leaves; the accumulation of phytochrome A to a higher level in transgenic plants may cause the leaf to sense that it is under more intense light or may interfere with its ability to sense the red/far-red ratio (Cherry et al., 1991). An extreme example of shading is darkness. The ability of darkness to induce leaf senescence has been used to synchronize the senescence program in many studies. During this artificially induced senescence, however, there are some differences in gene expression compared with natural leaf senescence, as discussed below.

In the absence of external stimuli that accelerate the senescence program, leaf age has a major influence on the initiation of senescence. Leaves of many annual species exhibit a progressive decline in the rate of photosynthesis after full leaf expansion (Batt and Woolhouse, 1975; Hensel et al., 1993; Jiang et al., 1993). The rate of this decline varies among species. *Arabidopsis thaliana* is an extreme example of a rapidly aging leaf: when grown in continuous light, there is a 50% decline in photosynthetic capacity between 4 and 6 d after full leaf expansion (Hensel et al., 1993).

In one general model of what triggers the senescence program, leaf senescence is initiated when the photosynthetic rate drops below a certain threshold. That threshold may be at or near the compensation point at which the leaf no longer contributes fixed carbon to the rest of the plant. The mechanism(s) that initiates the senescence program in response to declining rates of photosynthesis is unknown; models in which the levels of sugars or other photosynthetic metabolites are monitored have been proposed (Hensel et al., 1993; King et al., 1995). Consistent with this kind of model, when yeast invertase is expressed in the extracellular space of tomato, *Arabidopsis*, and tobacco leaves, carbohydrates are accumulated, photosynthesis is inhibited, and the leaves exhibit symptoms that resemble premature senescence (Dickinson et al., 1991; Ding et al., 1993).

In many annual species the number of leaves entering the senescence program increases during flowering and seed development, allowing the assimilated nutrients in leaves (the source) to be transported to and stored in developing seeds (the sink) for the next generation (Hayati

et al., 1995). This process is often referred to as "correlative control" of leaf senescence and has been well studied in soybean, in which surgical removal of flowers or physical restriction of pod growth delays leaf senescence (e.g. Noodén, 1988; Miceli et al., 1995). There are two major models to explain this phenomenon: in one, strong nitrogen demand from the reproductive tissues causes leaf senescence; in the other, reproductive tissues produce a senescence hormone that is transported to leaves to activate the senescence program (discussed in Hayati et al., 1995). Little is known, however, about the molecular mechanisms underlying correlative control of leaf senescence.

The five major classes of plant hormones, namely auxins, cytokinins, GAs, ABAs, and ethylene, and other plant growth regulators such as jasmonates, have been implicated in the regulation of leaf senescence. The first three classes of hormones typically inhibit senescence, whereas the remainder promote it (for recent reviews, see Smart, 1994; Gan and Amasino, 1996). The role of cytokinins and ethylene has been examined in studies with transgenic plants, as described below.

### DIFFERENTIAL GENE EXPRESSION DURING LEAF SENESCENCE

The leaf senescence program is accompanied by changes in gene expression. This was first demonstrated using *in vitro* translation followed by gel electrophoresis to detect changes that occur in translatable mRNA populations during leaf senescence (Watanabe and Imaseki, 1982). Analysis of the *in vitro* translated proteins revealed that an abundance of most leaf mRNAs significantly diminished during the progression of senescence, whereas some translatable mRNAs increased during senescence (Watanabe and Imaseki, 1982; Davies and Grierson, 1989; Becker and Apel, 1993; Buchanan-Wollaston, 1994; Smart et al., 1995).

Differential screening of cDNA libraries made from mRNAs of senescent leaf tissues also demonstrated that the

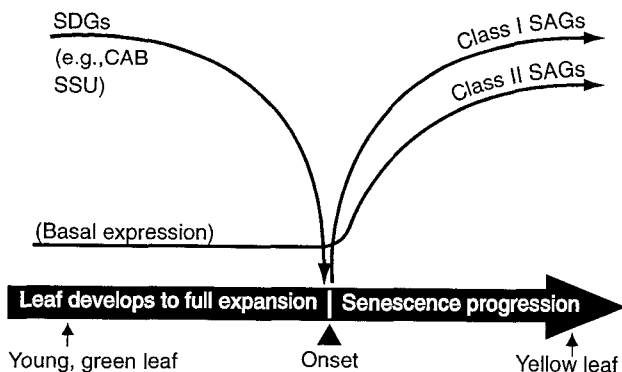
expression of the vast majority of genes is down-regulated, whereas the expression of other genes is up-regulated during senescence (Fig. 2). For example, the abundance of transcripts encoding proteins involved in photosynthesis decreases sharply during senescence (Bate et al., 1991; Hensel et al., 1993; Jiang et al., 1993; Humbeck et al., 1996); however, mRNA levels of certain genes increase with the progression of leaf senescence. To date, about 30 SAGs (senescence-associated genes; defined as genes with expression up-regulated during leaf senescence) have been identified from a variety of plant species, such as *Arabidopsis* (Hensel et al., 1993; Taylor et al., 1993; Lohman et al., 1994; Oh et al., 1996), asparagus (King et al., 1995), barley (Becker and Apel, 1993), *Brassica napus* (Buchanan-Wollaston, 1994), maize (Smart et al., 1995), radish (Azumi and Watanabe, 1991), and tomato (Davies and Grierson, 1989; Drake et al., 1996).

The function of many SAGs has been predicted by sequence comparison, whereas the enzymatic function of other SAGs has been demonstrated. It is not surprising that among them are genes encoding degradative enzymes such as RNases (Taylor et al., 1993), proteinases (Hensel et al., 1993; Lohman et al., 1994; Drake et al., 1996), and lipases (Ryu and Wang, 1995), and genes with products involved in nutrient translocation processes such as GS (Watanabe et al., 1994). GS incorporates ammonium into Gln for nitrogen recycling from senescing cells. In addition, genes homologous to short-chain alcohol dehydrogenases (*SAG13*; Gan, 1995), metallothioneins (Buchanan-Wollaston, 1994; Lohman et al., 1994), and pathogenesis-related proteins (Hanfrey et al., 1996) have been shown to be SAGs. *Tasselseed2* (*Ts2*) is also a member of the short-chain alcohol dehydrogenase family. *Ts2* is required for PCD during sex determination in maize (DeLong et al., 1993), and the existence of two members of this class of genes in different PCD programs is intriguing.

Although leaf senescence is characterized by both activation and inactivation of distinct sets of genes, gene inactivation per se is not sufficient for causing senescence; rather, gene expression within leaf cells is required for senescence to proceed. This is because, as discussed above, the senescence process can be blocked by inhibitors of RNA and protein synthesis and by enucleation. Current molecular studies on leaf senescence are mainly focused on genes up-regulated during senescence (i.e. SAGs).

### PLASTICITY OF LEAF SENESCENCE

Studying the regulation of SAG expression should help to decipher the molecular mechanisms underlying leaf senescence. Current investigations of the kinetics of SAG expression patterns under natural and artificial senescence induction conditions suggest that the regulation of SAG expression is rather complex, and that there may be multiple pathways that form a regulatory network to control leaf senescence. Therefore, blocking a particular pathway may not have a significant effect on the progression of senescence. We refer to this feature as the plasticity of leaf senescence.



**Figure 2.** Differential gene expression during leaf senescence. Senescence down-regulated genes (*SDGs*) include genes involved in photosynthesis such as the chlorophyll *a/b*-binding protein gene (*CAB*) and the Rubisco small subunit gene (*SSU*). *SAGs* are those genes with expression up-regulated during leaf senescence. Class I *SAGs* are expressed only during senescence (senescence-specific). Class II *SAGs* have a basal level of expression during early leaf development, but this level increases during senescence.

Analyses of gene expression during natural leaf senescence have revealed that different SAGs exhibit different temporal expression patterns. In general, the SAGs can be placed into two classes. One class represents senescence-specific SAGs. mRNA of these SAGs can be detected only during senescence (class I, Fig. 2). The other class consists of those SAGs with transcripts detectable through early leaf development, i.e. with a basal level of expression; the abundance of these SAG mRNAs increases during senescence (class II, Fig. 2). To date, only a few SAGs appear to be highly senescence-specific class I genes. These include *SAG12* and *SAG13* from *Arabidopsis* (Gan, 1995) and *LSC54* from *B. napus* (Buchanan-Wollaston, 1994). It should be noted that both *SAG12* and *SAG13* are senescence-specific but not leaf-specific. In addition to senescing leaves, both genes are expressed in other senescing green tissues and floral organs such as stems, sepals, petals, and carpels. It is interesting that, in contrast to its senescence-specific expression in leaves, the *Brassica LSC54* gene encoding a metallothionein is also expressed in the nonsenescent inflorescence meristem (Buchanan-Wollaston, 1994).

The patterns of SAG expression change in response to different treatments or conditions. For example, one of three cDNA clones isolated from dark-induced senescing barley leaves is not expressed during natural senescence (Becker and Apel, 1993). *SAG12*, which is expressed during natural senescence, is not detectable in leaves during the initial stages of dark-, ethylene-, or ABA-induced senescence. In addition, the expression level of individual SAG genes may also change in response to different stimuli. This is exemplified by the *Arabidopsis SEN1* gene, which exhibits distinctive expression levels during natural and artificially induced (by darkness, ABA, or ethylene) leaf senescence (Oh et al., 1996). These results suggest that there may be multiple senescence regulatory pathways that activate distinct sets of genes; certain genes are likely to be shared by these pathways, whereas others may be unique to specific pathways (Fig. 3). The genes common to all pathways

may be involved in the execution of senescence, whereas the unique genes may be genetically upstream regulatory genes that regulate components of the senescence program. It is also likely that some of the genes induced by a particular treatment such as darkness do not play a role in the senescence program.

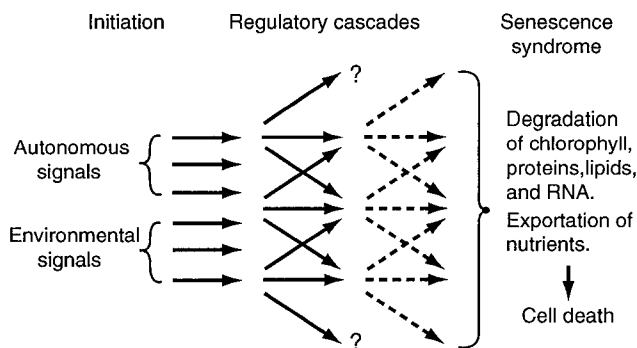
SAG promoter sequence analysis has suggested that the regulation of SAG expression is multifactorial. If a single regulatory transcriptional factor were involved in senescence-associated expression, SAGs should have a common *cis*-acting element in their promoter regions. However, comparison of the upstream sequences of *SAG12*, *SAG13*, *SAG15* (Gan, 1995), and *SEN1* (Oh et al., 1996) did not reveal any recognizable regions of sequence similarity. The regulatory elements conferring senescence expression remain to be identified.

Although the regulation of SAG expression is likely to be complex, the regulatory mechanisms of individual SAG expression are sometimes conserved among different plant species. Reporter gene studies show that the senescence-specific expression patterns of the GUS gene (*GUS*) directed by the *SAG12* or *SAG13* promoter are essentially identical in transgenic tobacco and *Arabidopsis* plants. The *din1* gene of the radish (Azumi and Watanabe, 1991) and the *SEN1* gene of *Arabidopsis* (Oh et al., 1996) are homologous genes; their expression during dark-induced senescence is conserved. However, the expression of two homologous metallothionein genes differs in *Brassica* versus *Arabidopsis*: *LSC54* is leaf-senescence-specific in *Brassica* (a class I pattern) (Buchanan-Wollaston, 1994), whereas *SAG17* in *Arabidopsis* exhibits a moderate basal expression as well as up-regulated expression during leaf senescence (Lohman et al., 1994).

### MOLECULAR GENETIC MANIPULATION OF LEAF SENESCENCE

Because the molecular mechanisms underlying leaf senescence are largely undefined, current molecular genetic strategies to manipulate leaf senescence are based on phytohormone physiology, either by enhancing cytokinin production or by blocking ethylene formation or perception. Although the ethylene strategy is used primarily for delaying fruit ripening, leaf senescence is also affected and will be discussed here.

Physiological studies have shown that in many species cytokinins can inhibit leaf senescence and that the endogenous cytokinin level drops with the progression of leaf senescence (reviewed by Gan and Amasino, 1996). One obvious approach for manipulating senescence is to engineer plants that will overproduce cytokinins. In the cytokinin biosynthesis pathway, the first committed and controlling step is catalyzed by *IPT*. Therefore, expression of this enzyme will result in the production of cytokinins. The native plant *IPT* has not been identified, but a bacterial version is available. Many strategies (i.e. different promoters) have been used to manipulate the expression of *IPT* in transgenic plants, including heat-, wound-, and light-inducible promoters, and tissue- or development-specific promoters. Most of the transgenic plants containing such



**Figure 3.** Regulatory pathways and plasticity of leaf senescence. Multiple pathways that respond to various autonomous and environmental factors are possibly interconnected to form a regulatory network. In such a model, a single gene mutation that inactivates one component of the pathways may not have a significant effect on the progression of senescence. Question marks (?) represent genes that are induced by a particular signal but may not play a role in leaf senescence.

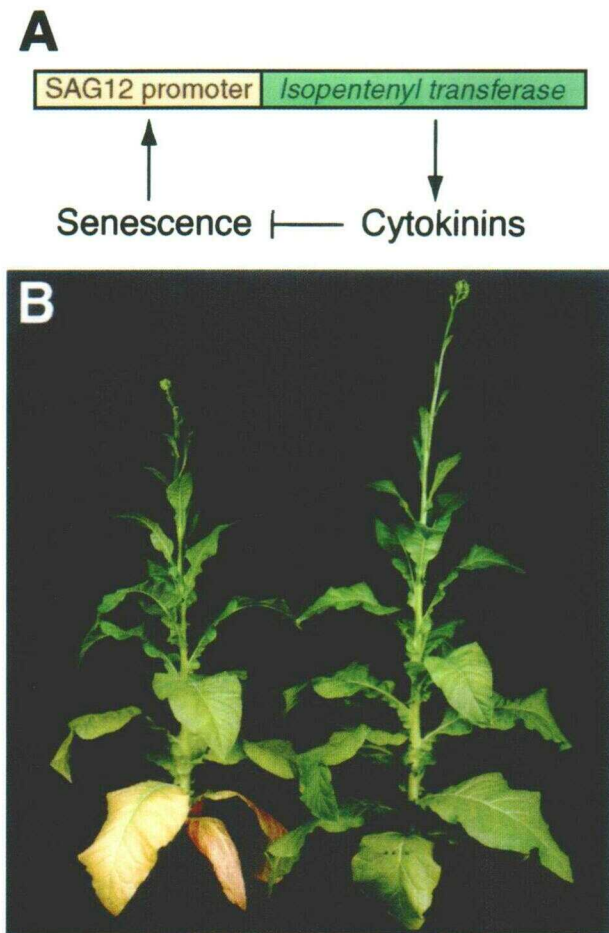
*IPT* constructs exhibit some delay in leaf senescence, as well as a variety of morphological and developmental abnormalities. This is because cytokinins may influence many developmental processes in addition to senescence, and overproduction of cytokinins before senescence will interfere with normal plant development. To avoid this problem, the highly senescence-specific *SAG12* promoter was used to direct *IPT* expression (Gan and Amasino, 1995). This promoter activates the expression of *IPT* in leaves only at the onset of senescence, resulting in an increase in the cytokinin level, which prevents the leaf from senescing. The inhibition of leaf senescence will in turn result in the attenuation of the senescence-specific promoter, therefore preventing cytokinin from accumulating to a level that would interfere with other aspects of plant development. This forms an autoregulatory loop (Fig. 4A). Leaf senescence

in transgenic tobacco plants containing this transgene was efficiently retarded with no other developmental abnormalities (Fig. 4B). This transgenic study confirms the regulatory role of cytokinins in leaf senescence in tobacco. Because exogenous cytokinin treatment can retard leaf senescence in a variety of monocotyledonous and dicotyledonous plant species, it is expected that this transgenic strategy of autoregulatory production of cytokinins may have the potential to delay senescence in a broad range of plants.

In contrast to cytokinins, ethylene treatment often promotes senescence. This suggests another molecular strategy to interfere with leaf senescence: blocking ethylene production or perception in transgenic plants. Transgenic tomato plants expressing antisense genes that inhibit either of two ethylene biosynthetic enzymes, ACC synthase or ACC oxidase, showed significantly reduced production of ethylene and retarded senescence of fruits (Hamilton et al., 1990; Oeller et al., 1991) and leaves (John et al., 1995). Because leaf senescence is also delayed in *Arabidopsis* plants with the dominant *etr1* mutation, which renders plants insensitive to ethylene (Grbic and Bleeker, 1995), it is expected that transgenic plants overexpressing *etr1* and some other mutant genes involved in ethylene perception may result in a delayed senescence phenotype. The brassinosteroids may be another class of hormones that can be manipulated to control senescence. Recently, it has been reported that *Arabidopsis* mutants deficient in brassinosteroid biosynthesis exhibit delayed leaf senescence (reviewed by Clouse, 1996).

## CONCLUSIONS AND PERSPECTIVES

Leaf senescence is an integral part of plant development. Like many other developmental processes, it is a genetically controlled program regulated by a variety of environmental and autonomous factors. Distinct from other major developmental events in plants that involve mainly cell division, differentiation, and/or growth, leaf senescence is accompanied by an organ-wide operation of PCD. Recent progress has been made in the molecular biology of leaf senescence. Many *SAG*s have been identified from several plant species. Most of them encode enzymes that are thought to be involved in cell degeneration and nutrient mobilization, whereas the identity of some *SAG*s remains unknown. Although the regulatory mechanisms of *SAG* gene expression may be conserved among plant species, the regulation appears to be complex. Both the population of *SAG* genes and the expression kinetics of individual *SAG*s change during senescence under natural or artificial induction conditions. The lack of common sequence elements in the promoters of several *SAG*s also suggests that the regulation of *SAG* expression is multifactorial. It is postulated that there may be several genetic pathways that are interconnected to form a senescence regulatory network. This complexity may contribute to the lack of success in isolating mutants that do not undergo leaf senescence from *Arabidopsis* and other plants: eliminating the activity of one gene in the



**Figure 4.** Retardation of leaf senescence by autoregulated production of cytokinins. A, The senescence-specific *SAG12* promoter was fused to a cytokinin-synthesizing gene, *isopentenyl transferase*. The onset of senescence activates this promoter to direct the production of cytokinins. Cytokinins in turn inhibit senescence, thus attenuating the *SAG* promoter activity to prevent overproduction of cytokinins. B, A transgenic plant (right) containing this autoregulatory system is shown to exhibit delayed leaf senescence compared with an age-matched, wild-type tobacco (*Nicotiana tabacum* cv Wisconsin 38). (Fig. 4B was kindly provided by Dr. W. Jordi of AB-DLO, The Netherlands.)

regulatory network may have little effect on the overall progression of leaf senescence.

Using both molecular and genetic approaches to decipher the regulatory mechanisms underlying leaf senescence will be a very important tool in future studies of senescence. The identification of many SAGs has provided a foundation for further molecular analysis of gene regulation during senescence. The promoter elements that confer senescence-specific expression and the transcriptional factors that interact with these elements need to be defined. Also, it is now possible to screen for mutants in which the expression of one or a subset of SAGs is reduced or eliminated. This type of mutant screen will be necessary if, because of multiple pathways of senescence initiation, it is impossible to obtain single gene mutations that prevent senescence. Such mutations will most likely identify genes that play a role in the regulation of senescence. Once the regulatory mechanisms underlying leaf senescence are understood, it should be possible to devise more sophisticated ways to manipulate leaf senescence for agricultural application.

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