Leaf Senescence

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Abstract

Leaf senescence constitutes the final stage of leaf development and is critical for plants' fitness as nutrient relocation from leaves to reproducing seeds is achieved through this process. Leaf senescence involves a coordinated action at the cellular, tissue, organ, and organism levels under the control of a highly regulated genetic program. Major breakthroughs in the molecular understanding of leaf senescence were achieved through characterization of various senescence mutants and senescence-associated genes, which revealed the nature of regulatory factors and a highly complex molecular regulatory network underlying leaf senescence. The genetically identified regulatory factors include transcription regulators, receptors and signaling components for hormones and stress responses, and regulators of metabolism. Key issues still need to be elucidated, including cellular-level analysis of senescence-associated cell death, the mechanism of coordination among cellular-, organ-, and organism-level senescence, the integration mechanism of various senescence-affecting signals, and the nature and control of leaf age.

Contents

INTRODUCTION

Senescence is the age-dependent deterioration process at the cellular, tissue, organ, or organismal level, leading to death or the end of the life span (48). Leaf senescence is an organ-level senescence but is often intimately associated with cellular or organismal death. Annual plants undergo leaf senescence along with the organismal-level senescence when they reach the end of their temporal niche, as we observe at the grain-filling and maturation stage of the crop fields of soybean, corn, or rice. For trees and other perennial plants, leaf senescence is illustrated by the splendid autumn scenery of color changes in leaves.

Leaf senescence is not a passive and unregulated degeneration process. During senescence, leaf cells undergo rather orderly 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 is replaced by catabolism of chlorophyll and macromolecules such as proteins, membrane lipids, and RNA. Increased catabolic activity is responsible for converting the cellular materials accumulated during the growth phase of leaf into exportable nutrients that are supplied to developing seeds or to other growing organs. Thus, although leaf senescence is a deleterious process for the sake of the leaf organ, it can be seen as an altruistic process: It critically contributes to the fitness of whole plants by ensuring optimal production of offspring and better survival of plants in their given temporal and spatial niches. Leaf senescence is thus an evolutionarily selected developmental process and comprises an important phase in the plant life cycle (7, 40, 46, 48). In agricultural aspects, however, leaf senescence may limit yield in crop plants by limiting the growth phase and may also cause postharvest spoilage such as leaf yellowing and nutrient loss in vegetable crops. Thus, studying leaf senescence will not only enhance our understanding of a fundamental biological process, but also may provide means to control leaf senescence to improve agricultural traits of crop plants.

Leaf senescence is basically governed by the developmental age. However, leaf senescence is also influenced by various internal and environmental signals that are integrated into the age information: Leaf senescence is

an integrated response of leaf cells to age information and other internal and environmental signals. This integrated senescence response provides plants with optimal fitness by incorporating the environmental and endogenous status of plants in a given ecological setting by fine-tuning the initiation timing, progression rate, and nature of leaf senescence. The environmental factors that influence leaf senescence include abiotic and biotic factors. The abiotic factors include drought, nutrient limitation, extreme temperature, and oxidative stress by UV-B irradiation and ozone, etc. The biotic factors include pathogen infection and shading by other plants. Leaf senescence can occur prematurely under these unfavorable environmental conditions (39).

In naturally senescing leaves, senescence occurs in a coordinated manner at the whole-leaf level, usually starting from the tips or the margins of a leaf toward the base of a leaf. However, when the uneven environmental stress is targeted locally on a leaf, the stressed leaf region undergoes earlier senescence than do the other parts. Thus, leaf cells show some degree of locality in a senescence program.

Leaf senescence can occur without an obvious correlation with senescence of other organs in some plants, such as many tree species, although it is often developmentally coordinated with senescence of other organs or whole plants, especially monocarpic plants. In some monocarpic plants, the reproductive development often governs senescence of leaves. This so-called correlative control is dramatically observed in pea and soybean, where removal of the reproductive organ can actually reverse the fate of senescing leaves to juvenile leaves. However, in some plants such as *Arabidopsis*, leaf senescence does not appear to be under correlative control, but the leaf senescence at the whole-plant level is somewhat correlated with the life span of the whole plant.

Arabidopsis thaliana is a favorite model for the molecular genetic study of leaf senescence (5, 7, 39). As a monocarpic plant, it has a short life cycle. Its leaves undergo readily distinguishable developmental stages and show a well-defined and reproducible senescence program (**Figure 1**), which makes genetic analysis of leaf senescence feasible. Extensive genomic resources available for *Arabidopsis* allow rapid identification and functional analysis of senescence regulatory genes.

In this review, we discuss recent progress toward molecular and genetic understanding of leaf senescence and longevity that has been achieved mostly from *Arabidopsis*. It is cautioned that *Arabidopsis* leaves have a senescence character different from that of some other monocarpic plants in that the leaf longevity in *Arabidopsis* is not controlled by the developing reproductive structures. Thus, the findings in *Arabidopsis* might not reveal some of the mechanisms involved in leaf senescence of other plants. Thus, wherever appropriate we also discuss the discoveries achieved from other plants.

Monocarpic plant: a plant that reproduces once and then dies at the end of its reproductive phase

Leaf longevity: reflects the period of a whole life span of a leaf from its emergence as a leaf primordium to death

LEAF SENESCENCE-ASSOCIATED CELL DEATH AS A PROGRAMMED CELL DEATH

Leaf senescence involves cell death that is controlled by age under the influence of other endogenous and environmental factors. Programmed cell death (PCD) is a self-destructing cellular process triggered by external or internal factors and mediated through an active genetic program. Cell death in leaf senescence is controlled by many active genetic programs (10). The cell death occurring in leaf senescence is thus a type of PCD. Leaf organs are composed of various cell types. Cell death in leaf senescence starts from mesophyll cells and then proceeds to other cell types. It also appears that cell death does not occur coherently but starts with local patches of early-dying cells and then propagates into the whole-leaf area.

PCD plays crucial roles in various developmental and defense responses in plants. Typical examples of PCD in plants are observed in the formation of tracheary elements, germination-related degeneration of aleuron

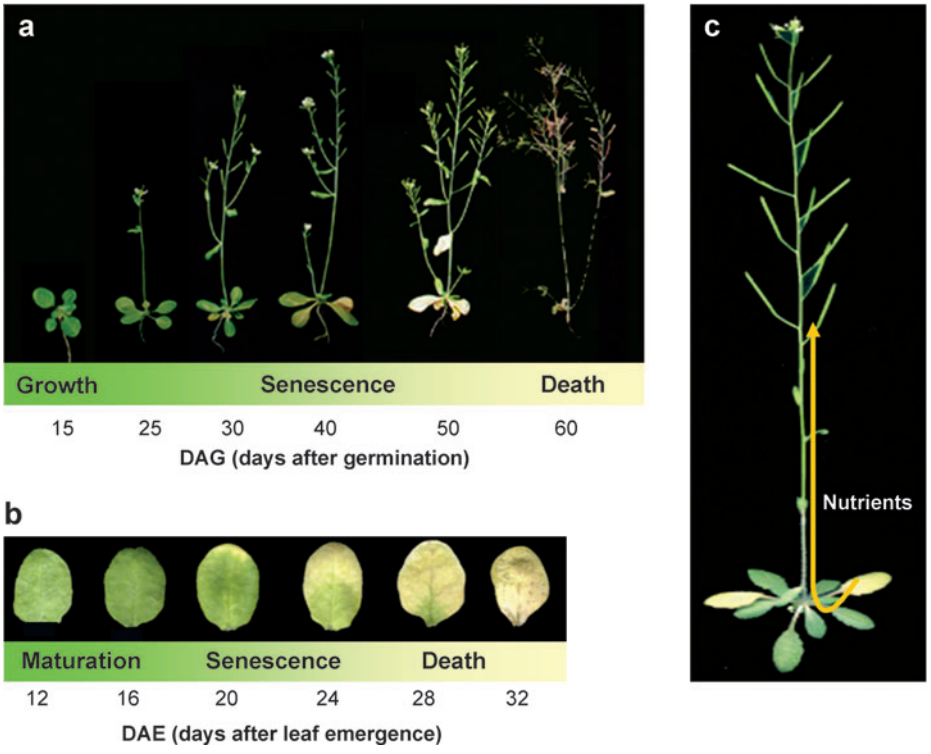

Figure 1

Characteristics of whole-plant senescence and leaf senescence in *Arabidopsis*. (*a*) Stages in the life cycle of whole plants. Plants are pictured at 15, 25, 30, 40, 50, and 60 days after germination. (*b*) An age-dependent senescence phenotype in the third rosette leaf. Leaves are pictured at 12, 16, 20, 24, 28, and 32 days after emergence. (*c*) As leaves senesce, nutrients such as nitrogen, phosphorus, and metals are relocated to other parts of the plants such as developing seeds and leaves.

layer cells, and pathogen-induced hypersensitive response (HR) (34, 70). PCD in leaf senescence has some features distinctive from other PCDs (71). First, leaf senescence involves an organ-level cell death that eventually encompasses the entire leaf, whereas other PCDs involve rather localized cell death or occur in limited tissues and cell types. Second, cell death rate during leaf senescence is slower than that in the other PCDs. Third, in terms of the biological function, PCD in leaf senescence is mostly for remobilization of nutrients from the leaf to other organs including developing seeds. The leaf organ is the major photosynthetic organ. Thus, optimal utilization of nutrients accumulated during the photosynthetic period is critical for plants' fitness and is critically affected by fine control of senescence process. In this regard, the slow degeneration of cells during leaf senescence is in part to ensure effective remobilization of nutrients that are generated by macromolecular hydrolysis during senescence. Many molecular events during leaf senescence can be readily understood from the viewpoint of this altruistic remobilization activity.

Structural and Biochemical Changes in Leaf Senescence-Associated Cell Death

Leaf cells at the senescence stage show some distinctive structural and biochemical

changes. A notable feature of cellular structural change during leaf senescence is the order of disintegration of intracellular organelles (48, 68). The earliest structural changes occur in the chloroplast, i.e., changes in the grana structure and content and formation of lipid droplet called plastoglobuli. In contrast, the nucleus and mitochondria that are essential for gene expression and energy production, respectively, remain intact until the last stages of senescence. This reflects that the leaf cells need to remain functional for progression of senescence until a late stage of senescence, possibly for effective mobilization of the cellular materials. In the last stage of leaf senescence, typical symptoms of PCD such as controlled vacuolar collapse, chromatin condensation, and DNA laddering are detected in naturally senescing leaves from a variety of plants including *Arabidopsis*, tobacco, and five trees (10, 62, 78). These observations imply that leaf senescence involves cellular events that ultimately lead to PCD. Eventually, visible disintegration of the plasma and vacuolar membranes appears. The loss of integrity of the plasma membrane then leads to disruption of cellular homeostasis, ending the life of a cell in senescing leaves.

The cellular biochemical changes in senescing leaves are first accompanied by reduced anabolism (4, 5, 76). The overall cellular content of polysomes and ribosomes decreases fairly early, reflecting a decrease in protein synthesis. This occurs concomitantly with reduced synthesis of rRNAs and tRNAs. Further cellular biochemical changes are most easily understood from the viewpoint of nutrient salvage, e.g., hydrolysis of macromolecules and subsequent remobilization, which requires operating a complex array of metabolic pathways. Chloroplast degeneration is accompanied by chlorophyll degradation and the progressive loss of proteins in the chloroplast, such as ribulose biphosphate carboxylase (Rubisco) and chlorophyll *a/b* binding protein (CAB). Hydrolysis of proteins to free amino acids depends on the actions of several endo- and exopeptidases (6, 28, 52). Senescence-associated cystein proteases, which are accumulated in the vacuole, also play a role in protein degradation. Lipid-degrading enzymes, such as phospholipase D, phosphatidic acid phosphatase, lytic acyl hydrolase, and lipoxygenase appear to be involved in hydrolysis and metabolism of the membrane lipid in senescing leaves (66, 67). Most of the fatty acids are either oxidized to provide energy for the senescence process or converted to α-ketoglutarate via the glyoxylate cycle. The α-ketoglutarate can be converted into phloem-mobile sugars through gluconeogenesis or used to mobilize amino acids released during leaf protein degradation (28, 66). A massive decrease in nucleic acids occurs during leaf senescence (65). Total RNA levels are rapidly reduced along with progression of senescence. The initial decrease in the RNA levels is distinctively observed for the chloroplast rRNAs and cytoplasmic rRNAs. The amount of various rRNA species is likely regulated coordinately, although this aspect has not been analyzed. The decrease of the amount of rRNAs is followed by that of the cytoplasmic mRNA and tRNA. The decrease in the RNA levels is accompanied by increased activity of several RNases.

Molecular Comparison of Leaf Senescence-Associated Cell Death with Other Programmed Cell Deaths

One obvious question regarding leaf senescence-associated cell death is how the cell death pathways during leaf senescence are distinct from those of other types of PCDs at the molecular level. Cell death in pathogen-induced HR is best-characterized among plant PCDs. Pathogenesis-related (PR) proteins are associated with PCD in HR. A few earlier works showed that many PR genes are induced during leaf senescence in several plant species (55, 56). A comparative study of leaf senescence and HR showed that *HIN1*, an HR cell death marker, is also expressed at late stages of leaf senescence (63). Furthermore, defense-related genes

Photochemical efficiency: deduced from the characteristics of chlorophyll fluorescence of PSII. The ratio of maximum variable fluorescence (Fv) to maximum yield of fluorescence (Fm), which corresponds to the potential quantum yield of the photochemical reactions of PSII, is used as the measure of the photochemical efficiency of PSII

including the *Arabidopsis ELI3* gene showed a senescence-associated induction as well (56). The *LSC54* gene encoding a metallothionine is also highly induced during both senescence and pathogen-related cell death (9). These observations indicate that, at the molecular level, some common steps or crosstalks exist between senescence-associated and pathogen-induced cell death.

In contrast, a few molecular markers that are specific for each of the senescence-associated and HR-associated PCDs were also identified. For example, *HSR203J* is upregulated during HR but not during leaf senescence (54). Similarly, the *Arabidopsis SAG12* gene expression is associated with leaf senescence but is not detected in the HR PCD in tobacco. Thus, these two genes may be a specific part of signaling steps for HR PCD and senescence-associated cell death, respectively, indicating that there are specific branches of molecular pathways leading to these two types of PCD.

A comparison of changes in global gene expression patterns during natural leaf senescence with those during starvation-induced death of suspension culture cells has shown similarities as well as considerable differences between these two PCDs (8). Of the 827 senescence-enhanced genes, 326 showed at least threefold upregulation in the starvation-induced PCD of suspension culture. In contrast, the rest of the senescence-upregulated genes were not significantly upregulated in starvation-induced PCD of suspension culture. The result implies that distinctive pathways for the two PCD processes are present.

MOLECULAR AND GENETIC APPROACHES FOR ANALYZING LEAF SENESCENCE

As with any other biological phenomena, it was critical to develop an accurate and proper assay for leaf senescence. Two main points must be seriously considered in analyzing leaf senescence. First, leaf senescence should be measured on a single leaf base along with its age information. Measuring senescence parameters with a mixture of several leaves at a given age of a plant is not a valid analysis for leaf senescence because the individual leaves of a plant have different ages. Second, the senescence symptom should be measured with various senescence parameters and ideally with markers that cover various aspects of senescence physiology. Senescence results from a sum of various physiological changes and it is often possible that a single parameter may not reflect senescence but only the change of a specific physiology related to the measuring parameter.

Assay of Leaf Senescence

To quantitatively measure the leaf senescence symptom, a range of physiological and molecular parameters can be utilized. Well-established senescence markers include chlorophyll content, photochemical efficiency, senescence-associated enzyme activities, change of protein levels, membrane ion leakage, and gene expression, etc. Leaf yellowing is a convenient visible indicator of leaf senescence and reflects mainly chloroplast senescence of mesophyll cells, which is the first step in senescence-associated PCD. The survivorship curve assay based on visual examination of leaf yellowing (the time when the half of a leaf turns yellow) provides a reliable measure, although the assay is somewhat subjective. Measuring chlorophyll loss and photochemical efficiency is another convenient assay for chloroplast senescence (49, 74). The activation of catabolic or hydrolytic activities, such as RNase or peroxidase activity occurs during leaf senescence (1, 65). Thus, measuring these enzyme activities is also a reliable and quantitative way to assay leaf senescence. Senescence involves disruption of plasma membrane integrity as the final step of cell death, which can be conveniently quantified by monitoring membrane ion leakage (74). This provides one of most reliable assays for senescence-associated cell

death, although it measures the later step of senescence.

Leaf senescence is accompanied by decreased expression of genes related to photosynthesis (e.g., *CAB2*) and protein synthesis (e.g., *RPS*, *RBC*) and by increased expression of senescence-associated genes (*SAG*s). The expression pattern of these genes during leaf development can be monitored by RNA gel blot analysis or reverse transcription-polymerase chain reaction (RT-PCR) (74). Microarray analysis would provide a quantifiable and global picture of the senescence process at the gene expression level with a clue of which downstream pathways of leaf senescence are affected by a specific mutation or by a specific environmental condition (8, 11). Microarray data may be further utilized for systems-level analysis of leaf senescence.

Genetic Analysis of Leaf Senescence

Two main approaches were utilized for understanding regulation of leaf senescence: the genetic and molecular approaches. The genetic approach involves isolation and characterization of mutants that show altered senescence phenotypes. *Arabidopsis* is a suitable model plant in this regard (25). Considering the complex nature of leaf senescence, it is expected that regulation of senescence involves many regulatory elements composed of positive and negative elements to finely tune the initiation and progression of senescence. The positive elements must exist for senescence to proceed. The negative elements are also important to prevent senescence from occurring prematurely. Many of these regulatory elements may contribute subtly in the senescence phenotype due to redundant functions in senescence. In addition, senescence is inevitably affected by the previous developmental stages including leaf formation and growth. Thus, a screening scheme suitably focused on leaf senescence symptoms was developed and successfully employed for isolating senescence mutants. So far, most of the genetic screening was focused on identifying delayed senescence mutants from T-DNA or a chemical mutant pool, which allowed identification of various important positive elements of senescence (50, 74, 75, 79). Early-senescence mutants screened from T-DNA or chemical mutant pools would enable identification of negative factors involved in the leaf senescence process (80). However, this approach should be taken with the caution that mutations with apparent early-senescence symptoms may not be directly associated with control of senescence because mutations in many homeostatic or housekeeping genes could also give apparent early-senescence symptoms.

Molecular Approaches to Understanding Leaf Senescence

The alternative approach was to identify and characterize genes that show enhanced or reduced expression during leaf senescence. Recent technological advances have allowed investigation of the *SAG*s at the genome-wide scale (3, 8, 17, 19, 41, 72). For example, a DNA microarray with 13,490 aspen expressed sequence tags (ESTs) was used to analyze the transcriptom of aspen leaves during autumn senescence (3). In *Arabidopsis*, Affymetrix GeneChip arrays representing 24,000 genes were utilized for analyzing changes in global expression pattern during leaf senescence (8, 72). This analysis has identified more than 800 *SAG*s, illustrating the dramatic alteration in cellular physiology that underlies the developmental transition to the senescence stage. Unlike the genome-wide microarray analysis, microarray analysis of 402 potential transcription factors was carried out at different developmental stages and under various biotic and abiotic stresses, providing a clue to the transcriptional regulatory network during leaf senescence (11). Similar approaches for other crop plants should allow comparison of molecular pictures of leaf senescence in different species. The collection of T-DNA insertion lines available in *Arabidopsis* was effectively utilized for functional analysis of individual *SAG*s.

In particular, functional characterization of potential regulators such as signal transduction-related proteins and transcription factors was the primary target for this analysis. Analysis of these mutant lines has provided and will continue to provide important information for understanding regulatory pathways of leaf senescence.

MOLECULAR GENETIC REGULATION OF LEAF SENESCENCE

Leaf senescence is an integral part of plant development and constitutes the final stage of development. The timing of leaf senescence is thus controlled by developmental age. However, the senescence process including senescence rate and molecular nature is intimately influenced by various environmental and internal factors. The environmental cues that affect leaf senescence include stresses such as high or low temperature, drought, ozone, nutrient deficiency, pathogen infection, and shading, etc. The internal factors include various phytohormones and reproductive development as well as developmental age (**Figure 2**). It is obvious that multiple pathways responding to various internal and external factors should exist and are interconnected to form a complex network of regulatory pathways for senescence (24). It is also obvious that, although the apparent symptoms of leaf senescence appear similar during senescence,

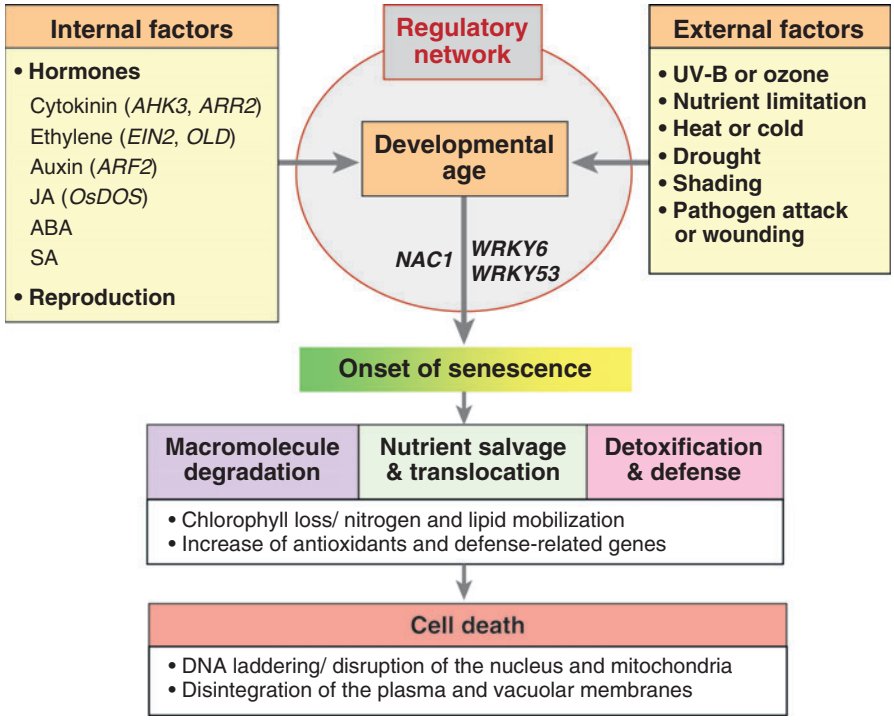

Figure 2

A model for regulatory pathways in leaf senescence. Leaf senescence is considered a complex process in which the effects of various internal and external signals are integrated into the developmental age-dependent senescence pathways. Multiple pathways that respond to various factors are possibly interconnected to form regulatory networks. These regulatory pathways activate distinct sets of senescence-associated genes, which are responsible for executing the degeneration process and ultimately lead to cell death.

the molecular nature of the senescence state influenced by these factors will be distinctive (53).

Leaf senescence should be a finely regulated process, considering its potential role in plants' fitness and the various factors involved in senescence control. Below we discuss the progress regarding molecular and genetic understanding of leaf senescence.

Onset of Leaf Senescence

A few of the central and unanswered questions regarding leaf senescence are how the leaf senescence is initiated, what the nature of the threshold that triggers leaf senescence is, how the developmental age is recognized to initiate the senescence program, and what the nature of the developmental age is. There are some indications that lead to answers to these questions. In plants, sugar status modulates and coordinates internal regulators and environmental cues that govern growth and development. Several lines of evidence suggest that a high concentration of sugars lowers photosynthetic activity and induces leaf senescence (12, 31, 44, 55). Senescence would be triggered when the level of sugars is above an acceptable window. In that sense, sugar metabolic rate would affect leaf longevity and might be the mechanism that regulates the developmental aging process, as shown in a range of organisms from yeast to mammals (15, 36).

An interesting finding was obtained from studies of the *oresara 4-1* (*ore4-1*) mutation, which causes a delay in leaf senescence during age-dependent senescence, but not in hormone- or dark-induced senescence (75). The *ore4-1* mutant has a partial lesion in chloroplast functions, including photosynthesis, which results from reduced expression of the plastid ribosomal protein small subunit 17 (*PRPS17*) gene. It was suggested that the delayed leaf senescence phenotype observed in the *ore4-1* mutant is likely due to a reduced metabolic rate because the chloroplasts, the major energy source for plant growth via photosynthesis, are only partially functional in the mutant. Reduced metabolic rate could lead to less oxidative stress, which might be a crucial factor in senescence.

Leaf senescence should be intimately related to the previous developmental stages of leaf, such as leaf initiation, growth, and maturation. Thus, it is possible that genes controlling these processes, including meristematic activity, could influence age-dependent senescence. In this respect, we observed that the leaves of the *blade on petiole 1-1* (*bop1-1*) mutant that showed enhanced meristematic activity in leaves exhibited a prolonged life span (21). Exact mechanisms by which this gene regulates leaf senescence need to be investigated.

Aging: an addition of timing to a cell, organ, or a whole plant that occurs throughout development. In this sense, aging would be a major determinant of senescence but not senescence itself

Environmental Factors and Leaf Senescence

Senescence is an integrated response of plants to endogenous developmental and external environmental signals. Thus, some of the genes involved in the response to environmental changes are expected to regulate leaf senescence. A comparison of gene expression patterns between stress responses and leaf senescence indicated that considerable crosstalk exists between these processes. For example, among the 43 transcription factor genes that are induced during senescence, 28 genes are also induced by various stresses. Our current understanding of the relationship between environmental responses and leaf senescence mostly comes from the study of senescence response to the phytohormones such as abscisic acid (ABA), jasmonic acid (JA), ethylene, and salicylic acid (SA) that are extensively involved in response to various abiotic and biotic stresses. These stresses affect synthesis and/or signaling pathways of the hormones to eventually trigger expression of stress-responsive genes, which in turn appears to affect leaf senescence. The involvement of these hormonal pathways is discussed below. However, we emphasize the need to directly examine the relationship between the stress

responses and leaf senescence by, for example, utilizing various stress response mutants existing in *Arabidopsis*.

AHK3: *Arabidopsis* Histidine Kinase 3

***Arabidopsis* response regulators (ARRs):** classified into two distinct subtypes, type A and type B, by the receiver domain sequences and by C-terminal characteristics

Apoplastic phloem unloading pathway: Sucrose is released via a sucrose transporter from the sieve elements of the phloem in the apoplast, where it is irreversibly hydrolyzed by an extracellular invertase

Involvement of Phytohormone Pathways in Leaf Senescence

Hormone signaling pathways often mediate or influence development and environmental responses in plants. For leaf senescence, an especially intimate interplay of many of these hormonal pathways is involved along with age-controlled senescence. This would be a way for plants to ensure proper control of leaf senescence response to endogenous and/or environmental signals. The hormonal pathways appear to play at all the stages of leaf senescence, including the initiation phase of senescence, progression, and the terminal phases. Each plant hormone affects various developmental and/or environmental events in a complex manner. This causes difficulties in assaying the roles of the hormonal pathways in leaf senescence. Nonetheless, the roles of these hormones in regulating leaf senescence are becoming evident through characterization of genetic mutants and the global gene expression analysis, providing important molecular information about how the hormonal signaling pathways lead to changes in pattern of gene expression during leaf senescence.

Cytokinins have many critical functions in plants, such as the control of cell proliferation, shoot formation, and shoot branching. Cytokinins have also been known for many decades to be senescence-delaying hormones (59), based on the findings that the endogenous cytokinin level drops during leaf senescence and exogenous application or endogenous enhancement of cytokinin content using the senescence-specific *SAG12* promoter delays senescence (16, 42, 51). Consistent with the physiological finding that the cytokinin level decreases during leaf senescence, genomic-scale molecular analysis revealed that genes involved in cytokinin synthesis, a cytokinin synthase and adenosine phosphate isopentenyl-transferase (*IPT*) genes, are downregulated and a gene for cytokinin degradation, cytokinin oxidase, is upregulated in senescing leaves (8).

How cytokinins affect leaf senescence is still unknown despite of the dramatic effect of cytokinin in delaying leaf senescence. A recent discovery showed that *Arabidopsis* Histidine Kinase 3 (AHK3), one of the three cytokinin receptors in *Arabidopsis*, plays a major role in controlling cytokinin-mediated leaf longevity (35). This conclusion was obtained through characterization of the gain-of-function *Arabidopsis* mutant, *ore12-1*, which shows delayed leaf senescence due to a missense mutation in the *AHK3* gene. A loss-of-function mutation of *AHK3*, but not of the other cytokinin receptors, conferred a reduced sensitivity to cytokinin in cytokinin-mediated delay of leaf senescence. This report also showed that the phosphorylation of the *Arabidopsis* response regulator 2 (ARR2) mediated by AHK3 is essential for controlling leaf longevity (**Figure 3**). The exact mechanism by which the phosphorylated ARR2 leads to induction or repression of genes regulating and/or executing leaf senescence needs further investigation.

An interesting link between the antisenescence effect of cytokinins and primary metabolism was suggested, based on the finding that cytokinin-mediated delay of senescence is correlated with the activity of extracellular invertase, the enzyme functionally linked in the apoplastic phloem unloading pathway (38). When the extracellular invertase activity was inhibited, cytokinin-mediated delay of leaf senescence was also inhibited. The result showed that extracellular invertase plays a role in mediating cytokinin action in delaying leaf senescence, suggesting that carbohydrate partitioning associated with invertase activity may be related to cytokinin-mediated delay of leaf senescence. This observation is particularly notable in that regulation of leaf senescence is related to changes in source-sink relationships of sugars and in that a tight link among cytokinin action, primary metabolism, and leaf senescence exists.

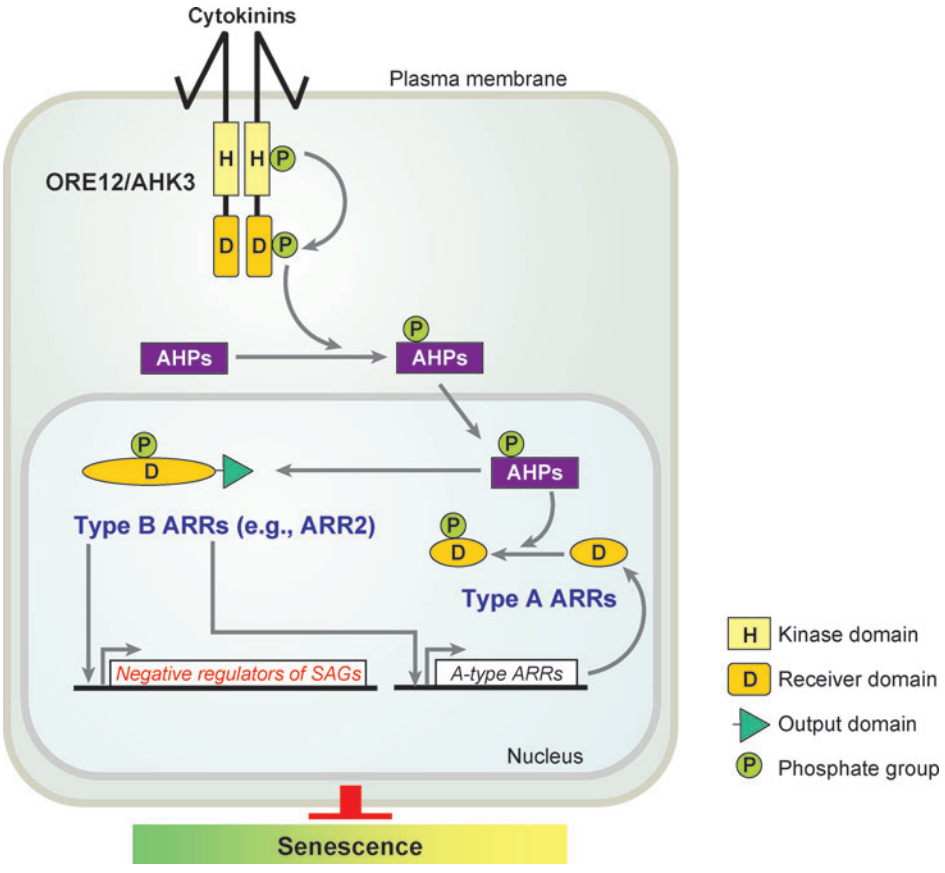

Figure 3

Hypothetical model for a function of ORE12/AHK3 in controlling cytokinin-mediated leaf longevity. Although the cytokinin signals may be perceived by the other cytokinin receptors in *Arabidopsis*, ARR2 phosphorylation is specifically mediated by ORE12/AHK3. Thus, the phosphorelay of ORE12/AHK3 to ARR2 is a signaling pathway specific to the control of cytokinin-mediated leaf longevity. The phosphorylated ARR2 then induces downstream cytokinin-responsive genes and, directly or indirectly, leads to induction of a set of target genes responsible for delaying the leaf senescence program, resulting in increased leaf longevity.

Ethylene has long been known as a major hormone in hastening leaf senescence as well as fruit ripening and flower senescence (1). Although it was apparent that ethylene is an important positive regulator of leaf senescence, the molecular genetic mechanism of ethylene action in leaf senescence is only now being revealed. In many plant species, including *Arabidopsis*, the level of ethylene increases during leaf senescence. Accordingly, the ethylene biosynthetic genes encoding ACC synthase, ACC oxdiase, and nitrilase are upregulated in senescing leaves (72). Two of the *Arabidopsis* mutants, *ethylene-resistant 1* (*etr1*) and *ethylene-insensitive 2* (*ein2*), that are deficient in ethylene perception and signal transduction, respectively, exhibited significant delays in leaf senescence, revealing the importance of the endogenous ethylene signaling pathway as a positive regulator in leaf senescence. A recent study also showed that Enhanced Disease Resistance 1 (EDR1) might be a negative regulator for ethylene-mediated leaf senescence (64). However, transgenic

Abscission: the shedding of leaves, flowers, or fruits, usually at a weak area termed the abscission zone

Arabidopsis and tomato plants that constitutively overproduce ethylene do not exhibit earlier-onset leaf senescence, suggesting that ethylene alone is not sufficient to initiate leaf senescence. This is consistent with the postulation that age-dependent factors are required for ethylene-regulated leaf senescence. Furthermore, potential regulators involved in integrating ethylene signaling into age-dependent pathways have been reported. The *onset of leaf death 1* (*old1*) mutant of *Arabidopsis* displays a phenotype with earlier-onset senescence in an age-dependent manner (33). The early-senescence phenotype was further accelerated by exposure to ethylene, showing that the *old1* mutation resulted in alternation of both of the age- and ethylene signaling-dependent leaf senescence. However, in the *old1etr1* double mutant where ethylene perception was blocked by the mutation in the *ETR1* gene, age-dependent earlier-onset leaf senescence still occurred but was not further accelerated by ethylene treatment. These observations suggested that OLD1 negatively regulates integration of ethylene signaling into leaf senescence. Recent studies with several *old* mutants that exhibited an altered senescence response to ethylene treatment further supported the notion that the effect of ethylene on leaf senescence depends on age-related changes through these *OLD* genes (32).

ABA is a key plant hormone mediating plant responses to environmental stresses. It also functions in plant development such as seed germination and plant growth. Furthermore, it has been well known that exogenous application of ABA promotes leaf abscission and senescence (81). However, the role of ABA in leaf senescence has not been clearly defined aside from some circumstantial evidence. The ABA level increases in senescing leaves and exogenously applied ABA induces expression of several *SAG*s (73), which is consistent with the effect on leaf senescence. Environmental stresses such as drought, high salt condition, and low temperature positively affect leaf senescence, and under these stress conditions ABA content increases in leaves. Concurrently with the increased ABA level in senescing leaves (18), the genes encoding the key enzyme in ABA biosynthesis, 9-*cis*-epoxycarotenoid dioxygenase (*NECD*), and two aldehyde oxidase genes *AAO1* and *AAO3* show increased expression (8, 72). The ABA-inducible receptor-like kinase gene of *Arabidopsis*, *RPK1*, was found to be gradually upregulated during leaf senescence (J.C. Koo & H.G. Nam, unpublished data). Inducible expression of RPK1 hastened the onset of leaf senescence, supporting a role for ABA in leaf senescence. A recent report argued that ABA induces accumulation of H_2O_2 in senescing rice leaf, which in turn accelerates leaf senescence (30). Another possibility is that senescence accelerated by exogenous ABA treatment might cause increased H_2O_2 generation, since it is well known that there is an increase of reactive oxygen species during leaf senescence. ABA also induces expression of antioxidant genes and enhances the activities of antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APOD), and catalase (CAT) (29). These activities may play at least a partial role in protecting the cellular functions required for progression and completion of senescence. It appears that ABA controls activities of both the cellular protection activities and senescence activities. The balance between these two activities seems to be important in controlling progression of leaf senescence and may be adjusted by other senescence-affecting factors such as age. A crucial link between ABA and leaf senescence has yet to be discovered via genetic analysis.

Methyl jasmonate (MeJA) and its precursor JA promote senescence in detached oat (*Avena sativa*) leaves (69). Exogenously applied MeJA to detached *Arabidopsis* leaves leads to a rapid loss of chlorophyll content and photochemical efficiency of photosystem II (PSII) and increased expression of *SAG*s such as *SEN4*, *SEN5*, and *γVPE*. A more convincing support for the role of JA in leaf senescence comes from the observation that JA-dependent senescence is defective in the

JA-insensitive mutant *coronatine insensitive 1* (*coi1*), implying that the JA signaling pathway is required for JA to promote leaf senescence (23). Functional studies on a nuclear-localized CCCH-type zinc finger protein, OsDOS (*Oryza sativa* Delay of the Onset of Senescence), also supports involvement of MeJA in leaf senescence (37). The expression of the *OsDOS* gene was downregulated during leaf senescence. Notably, RNAi knockdown of *OsDOS* accelerated age-dependent leaf senescence, whereas its overexpression resulted in a marked delay of leaf senescence, showing that OsDOS acts as a negative regulator for leaf senescence. A genome-wide expression analysis revealed that many of the JA signaling-dependent genes in particular were upregulated in the RNAi transgenic lines but downregulated in the overexpressing transgenic lines. This implies that OsDOS acts as a negative regulator of leaf senescence in integrating the JA signaling pathway into age-dependent senescence.

SA is the hormone involved in pathogen response and pathogen-mediated cell death. A recent intriguing discovery in leaf senescence was the role of SA in age-dependent leaf senescence. The concentration of endogenous SA is four times higher in senescing leaves of *Arabidopsis*. The higher SA level in senescing leaves appears to be involved in upregulation of several *SAG*s during leaf senescence (45): expression of a number of *SAG*s such as *PR1a*, *chitinase*, and *SAG12* is considerably reduced or undetectable in *Arabidopsis* plants defective in the SA signaling or biosynthetic pathway (*npr1* and *pad4* mutants, and *NahG* transgenic plants). A surprising discovery was derived from transcriptome analysis: The change of transcriptome mediated by the SA pathway is highly similar to that mediated by age-dependent senescence. The fact that the SA pathway is specifically involved in age-dependent leaf senescence is further supported by the finding that age-dependent but not dark-induced leaf senescence is delayed in *NahG* overexpressing transgenic plants that produce dramatically reduced SA levels.

There is evidence indicating that SA might be involved in senescence-associated cell death. Leaves from *Arabidopsis pad4* mutants that are defective in the SA signaling pathway do not appear to undergo cell death as efficiently as the wild type (45). In this mutant, leaves often remain yellow during the senescence stage with a much-delayed cell death (57, 58). This result shows a clear involvement of the SA pathway in senescence-associated cell death. A hypersenescence mutant *hys1*, which showed an early-senescence phenotype, was found to be allelic to *cpr5*, which was isolated based on its constitutive expression of defense responses and spontaneous cell death. The enhanced levels of SA and defense-related gene expressions might cause precocious senescence, supporting a role of SA pathways in the senescence-associated cell death process.

The role of auxin in leaf senescence has been elusive, particularly due to its involvement in various aspects of plant development. However, evidence suggests that auxin is also involved in the senescence process (61). The auxin level increases during leaf senescence. Consequently, IAA biosynthetic genes encoding tryptophan synthase (*TSA1*), IAAld oxidase (*AO1*), and nitrilases (*NIT1-3*) are upregulated during age-dependent leaf senescence (72). Exogenous application of auxin represses transcription of some *SAG*s (27, 47). Together, this implies that the auxin level increases during leaf senescence due to increased expression of auxin biosynthetic genes, which leads to delayed leaf senescence, leaving auxin as a negatively acting factor of leaf senescence. It has also been suggested that changes in auxin gradients rather than the endogenous auxin level itself could be important in modulating the senescence process (2). Expression of more than half of the genes related to auxin transport is reduced during senescence (72). This may cause aberrant distribution of auxin following leaf senescence.

Studies on the genetic mutation altered in auxin signaling support the involvement of auxin in controlling leaf senescence (14, 50).

***NahG*:** a gene-encoding bacterial salicylate hydroxylase that destroys SA by converting it to catechol

AUXIN RESPONSE FACTOR 2 (ARF2) is one of the transcription repressors in the auxin signaling pathway. Microarray analysis shows that expression of the *ARF2* gene is induced in senescing leaves. Disruption of *ARF2* by T-DNA insertion causes delay in leaf senescence. The phenotype canonically puts ARF2 as a positive regulator of leaf senescence. We also isolated another allele of *arf2* from an ethylmethane sulfonate (EMS)-mutagenized pool, which showed delayed leaf senescence along with an increased sensitivity to the exogenous auxin in hypocotyls growth inhibition. Together, these imply that the reduced ARF2 function in the mutant can cause reduced repression of auxin signaling with increased auxin sensitivity, leading to delayed senescence. However, it has yet to be seen whether the effect of auxin pathways is directly involved in leaf senescence or whether it indirectly influences leaf senescence because auxin and the *arf2* mutants also cause a pleiotropic effect in plant development.

Other Regulatory Genes of Senescence

Besides the regulatory genes mentioned above, several other regulatory genes of leaf senescence have been identified through genetic screening of senescence mutants and through functional identification of some *SAG*s.

Ubiquitin-dependent proteolysis is likely involved in regulation of leaf senescence. The *ORE9* gene encodes an F-box protein, a component of the SCF complex, which acts as an E3 ligase in ubiquitin-dependent proteolysis. Leaf senescence was delayed in the *ore9* mutant (74). It was also known that proteolysis by the N-end rule pathway has a function in senescence progression. The *delayed-leaf-senescence 1* (*dls1)* mutant, which is defective in arginyl tRNA:protein transferase (R-transferase), showed delayed development of leaf senescence symptoms (79). R-transferase is a component of the N-end rule proteolytic pathway.

A few regulatory genes identified in our laboratory by genetic screening include ORE7, ORE1, and SOR12. ORE7 is an AT-hook transcription factor that may be involved in controlling chromatin architecture. ORE1 is NO APICAL MERISTEM (NAM), ATAF1, and CUP-SHAPED COTYLEDONS2 (CUC2) (NAC) family transcription factor. SOR12 suppresses the delayed senescence phenotype of ORE12/AHK3. It also appears that miRNA is involved in controlling leaf senescence. Functional characterization of these genes and further genetic isolation of the senescence regulatory elements should be a critical asset in understanding leaf senescence.

A large number of *SAG*s have been identified in various plants through microarray analysis. Some of them have been found to encode potential regulatory factors that are components of signal perception and transductions, such as transcription factors and receptor-like kinases. Characterization of these potential regulatory genes led to discovery of a few important senescence regulatory genes and provided some insight into the regulatory mechanism of leaf senescence.

Genes for 96 transcription factors were identified in *Arabidopsis* to be upregulated at least threefold in senescing leaves. These belong to 20 different transcription factor families, the largest groups being NAC, WRKY, C2H2-type zinc finger, AP2/EREBP, and MYB proteins. Among the WRKY transcription factors, AtWRKY53 and WRKY6 have been further characterized in relation to leaf senescence. *WRKY53* is upregulated at a very early stage of leaf senescence but decreases again at later stages, implying that WRKY53 might play a regulatory role in the early events of leaf senescence (26). Putative target genes of WRKY53 include various *SAG*s, PR genes, stress-related genes, and transcription factors including other WRKY factors. A knockout line of the *WRKY53* gene showed delayed leaf senescence, whereas inducible overexpression caused precocious senescence, showing that it functions as a positive element in

leaf senescence (43). Identification of direct target genes of WRKY53 should further reveal the WRKY53-mediated senescence regulatory pathways. Another WRKY transcription factor gene, *WRKY6*, shows high-level upregulation during leaf senescence as well as during pathogen infection (60). WRKY6 regulates a set of genes through the W-box sequences in their promoter. Many WRKY6-regulated genes are associated with senescence and pathogen response, including the senescence-induced receptor-like kinase gene (*SIRK*). Although WRKY6 appears to have a functional role both in pathogen defense as well as senescence, the *SIRK* gene appeared to be expressed only during senescence but not during pathogen infection. The *wrky6* knockout mutation alters expression of *SAG*s but does not have any apparent effect on leaf senescence. The altered expression of *SAG*s in the knockout mutation may not be enough to be manifested into the apparent change of leaf senescence. It is also likely that functional redundancy exists among the WRKY transcription factors, considering the large number of members in the family.

NAC proteins are one of the largest families of plant-specific transcription factors with more than 100 members in *Arabidopsis.* NAC family genes play a role in embryo and shoot meristem development, lateral root formation, auxin signaling, and defense response. A total of 20 genes encoding the NAC transcription factor, representing almost one fifth of the NAC family members, showed enhanced expression during natural senescence and in dark-induced senescence (20). The T-DNA knockout mutant of *AtNAP*, a gene encoding an NAC family transcription factor, showed significantly delayed leaf senescence. Thus, AtNAP functions as a positive element in leaf senescence. *AtNAP* orthologs exist in kidney bean and rice and are also upregulated during leaf senescence. We also isolated a delayed leaf senescence mutant, which is due to a nonsense mutation in one of the NAC transcription factors (J.H. Kim & H.G. Nam, unpublished data). It is likely that several other senescence-upregulated NAC transcription factors play a regulatory role in leaf senescence. Transcriptional autoregulation and inter-regulation, as well as homodimerization and heterodimerization, among the NAC family members are important mechanisms in regulating NAC transcription factor-mediated developmental processes. Similar mechanisms are expected to be involved in the NAC transcription factor-mediated regulatory network of leaf senescence. The potential functions of most leaf senescence-associated transcription factors remain to be elucidated. Functional characterization of these genes including the signaling pathways they are involved in and the target genes they regulate will be invaluable in understanding the complex molecular pathways regulating leaf senescence.

Another example of *SAG*s with which in vivo function was assayed is the autophagy genes. Autophagy is an intracellular process for vacuolar bulk degradation of cytoplasmic components and is required for nutrient cycling. Mutants carrying a T-DNA insertion within the *Arabidopsis* autophagy genes, *AtAPG7*, *AtAPG9*, and *AtAPG18a*, exhibited premature leaf senescence (13, 22, 77). In these mutants, nutrients may be less efficiently utilized during execution of senescence, or some of the components needed for progression of senescence may not be efficiently provided.

A few of the genes involved in lipid metabolism have a role in leaf senescence. Reduced expression of the *Arabidopsis* acyl hydrolase gene by antisense RNA interference in transgenic plants delayed the onset of leaf senescence, whereas chemically induced overexpression of the gene caused precocious senescence (20). In addition, transgenic plants with reduced expression of a senescence-induced lipase also showed delayed leaf senescence (67). It is likely that the delayed senescence in these transgenic lines with reduced lipase expression is due to prolonged maintenance of membrane integrity, indicating the importance of membrane integrity during senescence.

Autophagy: a regulated recycling process whereby cytosol and organelles are encapsulated in vesicles, which are then engulfed and digested by lytic vacuoles/lysosomes

CONCLUSIONS AND FUTURE CHALLENGES

With the aid of microarray, we now know that more than 800 genes are distinctively upregulated during senescence, which illustrates the dramatic alteration in cellular physiology that underlies leaf senescence. With the knowledge of the nature of these *SAG*s, we can now figure out the molecular landscape of leaf senescence. We need to see the dynamic changes of the transcriptome along more detailed windows of leaf senescence stages, which will enable identification of more *SAG*s. This will also allow us to understand the dynamic changes of the physiology undergoing senescence. The microarray data could be further examined using various bioinformatic tools for classifying the *SAG*s, for establishing a hierarchical relationship among the *SAG*s, and for systems-level analysis of molecular events underlying senescence. The senescence pathways affected by various signals are being revealed. Further microarray analyses of leaf senescence in various senescence mutants and under various senescence-affecting conditions should reveal a detailed molecular level of the senescence pathways. One important challenge will be to investigate how the *SAG*s are coordinately regulated during leaf senescence. DNA microarray and chromatin immunoprecipitation approaches could be helpful to answering this question.

Although isolating a few key regulatory genes of leaf senescence greatly aided the understanding of leaf senescence, there are many more regulatory elements of leaf senescence. Finding the senescence regulatory genes has been and will continue to be one of the main challenges. Some regulatory elements could be found fairy easily by functionally characterizing the potential regulatory *SAG*s. However, it should be noted that there are many other senescence regulatory genes that do not belong to *SAG*s. The in vivo function of the *SAG*s can now be easily assayed using the genomic tools available in *Arabidopsis*, including the large collection of T-DNA insertion lines or the Targeting-Induced Local Lesions in Genomes (TILLING) approach.

It is encouraging that senescence is now well assayed in the realm of genetics. The genetic screening of senescence mutants was fruitful in understanding the genetic regulatory mode and in isolating senescence regulatory genes. However, considering the complex nature of senescence, current genetic screening is far from saturated. It will be important to use various mutant pools to identify novel senescence regulatory elements. Chemically mutagenized pools in particular are valuable because they might provide novel alleles that cannot be obtained by T-DNA mutagenesis, as illustrated by the discovery of ORE12/AHK3. Global gene expression analysis of the senescence mutants could provide important clues for dissecting the regulatory pathways. Identifying suppressors to known mutants will be also useful to dissect genetic mechanisms governing senescence processes. More genetic mutants can certainly be isolated by designing a more elegant screening scheme, for example, for mutations defective in integrating environmental effects into a senescence program.

Leaf senescence occurs at the last step of leaf development. Accordingly, some genes that function in senescence could also be involved in other biological processes. The mutations in this type of gene may show difficulties in assaying their function in senescence because their effects on senescence can be masked by other early-mutant phenotypes. Through senescence-specific gene silencing or senescence-specific induction, using a senescence-specific promoter or chemically inducible promoters will partially circumvent the problem.

Although most of the molecular analyses on leaf senescence were based on mRNA expression, it should be noted that mRNA expression is only one aspect of functional gene regulation. Other regulatory mechanisms such as protein-level expression,

protein stability, or localization of regulatory proteins involved in senescence process should certainly be involved. An integrated informational analysis involving proteomic and metabolomic analyses during leaf senescence will eventually be needed to better understand leaf senescence.

One serious pitfall in the current assay for leaf senescence is that senescence symptoms are measured at the organ level. However, within a senescing leaf, individual cells are usually at different stages of developmental age or senescence. It is also unlikely that all the cells within an individual leaf undergo coherent cell death. To better understand the senescence process, it will be necessary to develop assays that can monitor senescence symptoms and senescence-associated cell death symptoms at the individual cellular level.

Leaf senescence is certainly an evolutionarily acquired process and thus the plants evolved in different ecological settings with different evolutionary tracks will show differences in the pattern and regulation of senescence. It would be interesting to do a comparative study utilizing the information obtained from *Arabidopsis*. This may even be pursued in various ecotypes of *Arabidopsis*.

Another important challenge in the area of leaf senescence is determining its biotechnological applications. Although manipulation of leaf senescence can greatly improve crop yield and other characteristics such as increased shelf life, the knowledge and materials obtained so far have been poorly utilized for this purpose. Considering the potential future food shortage and the use of plants as a source of bioenergy, improving crop productivity should be a top priority.

SUMMARY POINTS

1. Leaf senescence is a finely regulated and complex process that incorporates multiple developmental and environmental signals. Microarray analysis revealed the complex molecular network of the senescence pathways.
2. Leaf senescence involves age-dependent PCD. Senescence-associated cell death and other PCDs show common as well as distinctive signaling pathways.
3. The genetic scheme was established in screening leaf senescence mutants. Genetic analysis revealed that leaf senescence is controlled by various negative and positive genetic elements.
4. More than 800 genes were identified as *SAG*s, reflecting the dramatic alteration in cellular physiology that underlies the leaf senescence. Potential regulatory elements among the *SAG*s were characterized for their function in leaf senescence. The transcription factors WRKY53 and AtNAP act as positive regulators of leaf senescence.
5. Metabolic rate appears to be one mechanism involved in age-dependent leaf senescence.
6. A key molecule for cytokinin-mediated leaf longevity was identified as AHK3, one of the cytokinin receptors. ARR2 phosphorylation mediated by AHK3 is essential for controlling leaf longevity.
7. Signaling pathways of various phytohormones including ABA, SA, JA, and ethylene are intimately linked to leaf senescence. Interestingly, SA-mediated senescence pathway is highly similar to that of natural leaf senescence, as revealed by microarray analysis.

8. Ubiquitin-dependent proteolysis is likely involved in controlling leaf senescence. Other senescence-regulatory factors include acyl hydrolase, invertase, and autophagy, which suggest involvement of membrane integrity, apoplasitc sugar levels, and nutrient recycling, respectively.

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