The molecular genetic analysis of leaf senescence
Hong Gil Nam

The cloning of genes induced during leaf senescence and the study of their modes of regulation conducted in the past two years have revealed some of the molecular mechanisms underlying leaf senescence. The identification of genetic mutants that control leaf senescence in Arabidopsis thaliana opened up new possibilities for genetically analyzing leaf senescence in a model system. Encouraging experimental data with transgenic plants show that manipulation of leaf senescence may greatly contribute to the improvement of important agronomic traits such as crop yield and the storage life of the harvested tissues.

Introduction
Programmed cell death in plants is part of many developmental and environmental response processes. These processes include localized cell death, such as that that occurs in hypersensitive response (HR) reactions and in the differentiation of tracheary elements, as well as senescence of an organ or a whole plant. This review will focus on plant leaf senescence, and will also partially describe the similarities and differences between leaf senescence and other forms of programmed cell death.

Leaf cells undergo the sequential disorganization of cellular organelles and dramatic changes in cellular metabolism during leaf senescence [1–3,4••,5••,6,7]. Metabolic changes include loss of photosynthetic activities and hydrolysis of macromolecules that accumulate during the growth phase (Fig. 1). Degenerative activities are often concomitant with the massive remobilization of the hydrolyzed compounds to the growing parts of plants such as young leaves and developing seeds. Thus, leaf senescence, although deteriorative in nature, is a critical process for the fitness of plants and is regarded as an evolutionarily acquired genetic process.

Because of the biological importance and potential for improvement of crop characteristics such as plant productivity and post-harvest storage, there have been extensive physiological and biochemical studies conducted on plant leaf senescence during the past decade; however, molecular and genetic analyses of the mechanism of leaf senescence have only been actively investigated recently [8–10]. In this review, I describe the advances that have been made at the molecular and genetic level in the past two years. The progress prior to this period has been reviewed previously [2,4••].

Senescence-associated genes
In the past two years, continuing efforts have been made to identify genes associated with or induced during leaf senescence. Although the nature of many of these senescence-associated genes (SAGs) is still unknown, some genes are providing clues as to the molecular status of senescing leaf cells.

SAGs may be defined as genes whose expression increases during natural leaf senescence (i.e. developmental age-dependent senescence) relative to other developmental stages of the leaf (Fig. 1). Leaf senescence can be initiated or modulated by several internal and external factors as well as in an age-dependent manner. In contrast, many plant genes that respond to environmental factors may not be associated with leaf senescence and are regarded as genes specific to the environmental factors.

The spectrum of genes involved in protein turnover, such as cysteine protease-like genes ([11••,12•,13••]; CM Griffiths et al., personal communication), is being expanded continuously. The role of proteolytic degradation in leaf senescence is illustrated by the biochemical identification of a cysteine protease and a serine protease that catalyze the degradation of Rubisco (ribulose-1,5-biphosphate carboxylase/oxygenase), a major leaf protein undergoing degradation during leaf senescence [14], and by the immunological identification of alkaline endopeptidases that increase during leaf senescence [15]. The genes in the ubiquitin pathway such as those encoding polyubiquitin in potato [16•] and Arabidopsis (JH Park, SA Oh, HG Nam, unpublished data), are also induced during leaf senescence, consistent with the previous reports [17–19].

Other SAGs involved in hydrolytic activities include those that encode RNases such as the bean ribonuclease-like pathogenesis-related (PR) protein gene, Ypr10 [20••,21]. RNases have been suggested to function in phosphate remobilization during senescence [22,23]. A pumpkin gene for a key glyoxysomal protein, 3-ketoacyl-CoA thiolase [24••], which may be involved in the remobilization of
The molecular genetic analysis of leaf senescence

Nam 201

Figure 1

© 1997 Current Opinion in Biotechnology

A simple model for leaf growth and senescence. The regulation of senescence is viewed as a matter of balance between two antagonistic self-maintenance and senescence gene activities. The self-maintenance gene activity increases during leaf growth and declines at the senescence stage. This activity includes genes involved in many anabolic activities such as photosynthesis. Other basic cellular maintenance activities include genes associated with RNA and protein synthesis and repair activities. Gene activity associated with senescence may start to accumulate at earlier stages but the resulting symptoms are not apparent until the loss of self-maintenance gene activity at a later stage. In the intermediate stage, both activities coexist and the plant may retain self-maintenance or proceed towards senescence, depending on the conditions. Because of this, the initiation point for senescence cannot be clearly defined. The process is developmentally programmed but once a certain threshold level of senescence gene activity has been reached, the leaf cell proceeds towards death. Developmental age-dependent and environmental factors influence the timing and progression rate of senescence.

Many stress-related genes are also induced during senescence. These include metallothionein-like genes [10,30•, 31•,32••] that may be involved in the chelation of metal ions released during cellular degradation. The genes involved in the oxidative stress response are also induced, including the genes for Fe^{2+}-ascorbate oxidase [29••], anionic peroxidase [12•], glutathione S-transferase ([13••,33]; SA Oh, JH Park, personal communication) and a blue copper-binding protein ([34]; JH Jun, SA Oh, JH Park, personal communication). Several pathogenesis-related protein genes are induced in senescent leaves [20•,35••,36••]. The radish din1 gene [37] and its Arabidopsis counterpart, sent [38••], are induced by dark treatment. These stress-related genes may participate in protecting the cellular integrity required for progression and completion of senescence.

Thus, the SAGs identified so far include genes executing the senescence program such as genes involved in disintegration or remobilization of macromolecules, genes involved in protecting cell viability for completion of the senescence process; however, it is expected that SAGs may also include genes involved in the initiation or triggering of leaf senescence, and genes controlling the progression rate of senescence (some of the genes involved in disintegration or remobilization of macromolecules, including RNase, protease or ubiquitin pathway genes, may also function in this latter category).

Studying genes downregulated during leaf senescence may also provide some information on the molecular mechanism of leaf senescence (Fig. 1). Senescence may involve the downregulation of not only many metabolic enzymes but also critical components that repress the senescence program. The downregulated genes identified recently include the genes for ATP sulfurylase [13••], a photosystem II 10 kDa polypeptide [36••], and a few stromal enzymes [5••].

**Regulation of senescence-associated genes**

Analysis of the modes of regulation of SAGs will provide important clues to the molecular mechanism of initiation and progression of leaf senescence.
Many leaf SAGs are not uniquely induced during senescence. For example, many genes are shown to be induced in both seed germination and leaf senescence ([11**,24**,28]; CM Griffiths et al., personal communication), and seed maturation may have gene activity in common with senescence [35**]. The *Arabidopsis* *meri*-5 gene (JH Park, SA Oh, HG Nam, unpublished data) and a potato ubiquitin–ribosomal protein gene [17] expressed in leaf meristem are induced during leaf senescence. Some SAGs show a biphasic induction pattern during leaf development, suggesting that they have other roles in leaf development as well as in senescence [8,13**,**36**]. Some leaf SAGs are also expressed during senescence of other organs or cell types and do not function uniquely in leaf senescence.

Many SAGs are induced by adverse internal and external environmental factors, including the presence of heavy metals [30*], salicylic acids [20*,35**], dark [20*,37,38**], senescence-affecting hormones (ethylene [38**], abscisic acid [38**] and methyl jasmonate [16*]), wounding [16*], heat shock [17,30*] and nutrient starvation [22,23,32**]. Thus, many SAGs also function in the plant’s response to these environmental factors. Indirectly, this may mean that the senescing cells are highly stressed and require the expression of these stress-responsive genes to cope with the stressful conditions. It is likely that many other stress-related genes are also induced during leaf senescence.

In a few cases, the activation of SAGs was observed during leaf senescence induced by environmental factors. Expression of tomato SENU mRNAs increases during leaf senescence caused by aging, heat shock or drought [36**]. The *Arabidopsis* *sen1* gene is activated during leaf senescence induced by age, dark, abscisic acid (ABA) or ethylene [38**]. It will be interesting in these cases to ask whether the genes are specifically responsive to a certain environmental factor that accelerates leaf senescence or whether they are induced by a presumed senescence signal that is generated by this factor. The identification and examination of cis-acting elements that are responsive to age or environmental factors should provide an answer to the question. It is likely that either one or both mechanisms are operating to regulate senescence and stress-responsive genes.

As described above, leaf senescence is not conducted by a unique set of senescence-specific genes but primarily utilizes genes that are also involved in other cellular processes. Plant cells may have recruited these genes during the evolution of the leaf senescence program and may have modulated their expression to be senescence-associated in the leaf; however, it is certainly conceivable that leaf cells utilize genes specific to leaf senescence. It will be important to identify such genes to reveal the mechanism of leaf senescence. Such genes may include those that control the basic senescence program, that is, the age-dependent developmental program.

One question regarding the mechanism of leaf senescence is whether the molecular states (the kinds and expression patterns of genes or proteins) of senescence are the same regardless of the senescence factors that induce the condition. Some SAGs [36**,38**] are induced upon senescence caused by all the factors examined; however, the modes of regulation of other SAGs suggest that leaf senescence caused by different senescence factors (such as aging, dark, ethylene, ABA or methyl jasmonate [MeJA]) involves the differential induction of SAGs (JH Park, SA Oh, HG Nam, unpublished data). The results of these studies indicate that the molecular states of leaf senescence caused by different senescence factors are different (Fig. 2). This may imply that the process of leaf senescence involves fine-tuning the expression of SAGs to incorporate complex environmental signals into the senescence program. Thus, leaf senescence may be envisioned as a complex process in which various environmental influences are superimposed on the age-dependent development program (Fig. 3). This mechanism should enhance the fitness of plants in ecological settings with ever-changing environments.

![Figure 2](image-url)

The involvement of different sets of genes during leaf senescence affected by various senescence factors. Leaf senescence is affected by several factors and involves the induction of different sets of genes. Thus, although the apparent symptoms of senescence may look the same, the detailed molecular states of senescent leaves are different depending on the senescence factors. For example, the *sen5* gene may be induced in ABA-, age- and dark-induced senescence but not in ethylene- or MeJA-induced senescence. In contrast, the *sen4* gene is involved in senescence affected by all the factors. This, in turn, suggests that senescence induced by different senescence factors may proceed through a common pathway and pathway(s) specific for one or more of the senescence factors.

Metabolite repression is involved in regulating SAGs. Sugar starvation induces glyoxysomal genes [24**,25,39] and the rice metallothionein-like gene [30*]. The promoter of the *Arabidopsis* *sen1* gene is activated upon sugar starvation and is repressed by exogenous sugar com-
Genetic pathways of leaf senescence. In this model, which is partly hypothetical and partly reflects recent findings, leaf senescence is viewed as a complex process in which the effects of various environmental factors are superimposed on the developmental age-dependent senescence process. The Arabidopsis genes any1, any2 and any3 defined by genetic mutations lie in a common pathway of leaf senescence (thick black line). The etr1 gene [62] is in the developmental age-, dark- and ethylene-induced pathway but not in the ABA pathway; the effects of ABA and MejA are not known. The etr1 gene is in the developmental age- and ethylene-induced pathway; the effects of ABA, darkness and MejA are unknown. Leaf senescence ultimately occurs in all the mutants, suggesting there may be an additional pathway to senescence (thin black line); there may be differences in the contribution of each pathway to senescence. Furthermore, leaf senescence may not go through common pathways; there may be pathways specific to one or more of the senescence factors (dotted lines) and the specific pathways may utilize only a subset of SAGs. In addition, the environmental factors may interact before they merge into the age-dependent pathway.

Programmed cell death in plants includes processes similar to those in animal cell apoptosis, and some of the leaf SAGs are also induced during apoptosis-like processes. The genes induced during the senescence of cultured cells are induced in senescent leaf cells, and leaf SAGs are induced during the senescence of cultured cells [20••]; however, there are some discrepancies. For example, a gene encoding a 20 kDa cysteine protease and two RNase genes [41,42] in Zinnia elegans are induced in differentiating tracheary elements but not during leaf senescence. A typical apoptosis-like process in plants is found during the HR of plant cells to pathogen attack, which, as in animal apoptosis, involves DNA fragmentation [43,44]. The activation of HR-associated DNase activity, however, is not induced during leaf senescence [45] and no discrete fragmentation pattern of DNA has yet been reported in senescent leaves [44••]. This difference suggests that the HR may require a rather rapid and localized cellular death utilizing DNA fragmentation at an early stage of the response. In contrast, leaf senescence may require tight genetic control to maximize the mobilization of nutrients; this may require that the genetic material remains intact.

Genetic analysis of leaf senescence
Genetic analysis of leaf senescence has, for the most part, been limited to identifying ‘stay-green’ varieties from naturally occurring cultivars [44••,46]; such plants are the continuing subjects of research on leaf senescence [47•,48,49,50•]. Recent data indicate that two genetic loci of soybean, d1 and d2, together play an important regulatory role in leaf senescence. Soybean plants that contain both mutants experience a significant delay in the degradation of leaf soluble proteins [47•], the plasma membrane and chloroplasts [48]. The long studied ‘stay-green’ mutant of meadow fescue (Festuca pratensis Huds.), designated B993, is unable to carry out oxygenolytic cleavage of the porphyrin macrocycle, a key step in chlorophyll degradation [50•]. Recently, four genetic mutants with a delayed senescence phenotype have been identified in Arabidopsis (SA Oh, JH Park, HG Nam, unpublished data). They fall into three complementation groups. All of the mutations were monogenic and recessive, indicating that they are required for normal leaf senescence. Several parameters associated with leaf senescence, including a decrease in the amount of photosynthetic components (chlorophyll and Rubisco, and photosystem II activity) and an increase in catabolic activities (RNase and peroxidase), are delayed in the mutants, suggesting that the three genes defined by these mutations are key regulatory elements in leaf senescence. The delayed senescence phenotypes are observed during both developmental age-dependent and dark-induced senescence. Preliminary data derived from studies of these mutants suggest that leaf senescence caused by ABA, ethylene and MejA is also delayed. The genes may function at a common step of senescence caused by these factors. It should be noted, however, that senescence still occurs in these mutants, suggesting there might be a parallel senescence pathway that does not require these genes (Fig. 3).

The role of ethylene in leaf senescence
Ethylene plays a prominent role in senescence in some plant species. The role of ethylene in leaf senescence has been investigated in an ethylene-insensitive mutant of Arabidopsis [51••] and using transgenic tomato plants in which ethylene synthesis was blocked by the introduction of an antisense 1-amino cyclopropane-1-carboxylic
acid (ACC) oxidase gene [52••]. In these plants, leaf senescence is delayed; however, once senescence has been initiated, the expression level of SAGs does not differ greatly from that of wild-type plants. The results of these studies suggest that an age-dependent program that does not involve the ethylene-dependent pathway is necessary and sufficient to initiate leaf senescence. Although ethylene is not essential in initiating leaf senescence, it influences its timing. Ethylene concentration is increased by many adverse environmental and internal factors, and incorporating an ethylene signal into the basic, development-age-dependent pathway (Fig. 3) may be a way of adjusting the timing of leaf senescence to respond to environmental conditions. It is, therefore, not unexpected that two of the delayed senescence mutants of Arabidopsis isolated in our laboratory are alleles of ein2-1, an ethylene-insensitive mutation (SA Oh, JH Park, HG Nam, unpublished data). The Nr mutation of tomato causes delayed leaf senescence and has a defect in the etr1-like gene of tomato [53]. These results provide further genetic evidence for the importance of the ethylene pathway in leaf senescence.

Genetic engineering of leaf senescence

There has been considerable interest in breeding crop varieties with delayed senescence in the hope of increasing crop productivity and the storage life of leaves. For example, delayed senescence with concomitant preservation of the photosynthetic apparatus in the Gd1d2 mutant of soybean increases seed yield by 44% [54]. Despite the potential benefits of delayed leaf senescence in agriculture, the application of genetic engineering to manipulate leaf senescence has been limited, in contrast to the case of fruit ripening [55]. One recent result is particularly exciting and exceeded the expectations of the investigators [56••]. Cytokinin, a plant hormone, retards leaf senescence in many plants [57]. Although there have been several reports of the overproduction of cytokinin in plants [56••,58], when nonregulated promotors were used, the plants were abnormal. In contrast, when the level of cytokinin was autoregulated via a senescence-induced promotor, the transgenic plants exhibited a clear improvement of several traits important in agronomy, including a 50% increase in both seed yield and total biomass. Further tests are needed to determine if there are adverse effects of the introduced gene under various environmental conditions. It is important to note that cytokinin alone may not be sufficient to delay all of the symptoms associated with leaf senescence [38••]. Reducing the level of ethylene by the introduction of an antisense ACC oxidase gene [51••] under the control of a senescence-responsive promotor may be another way to delay senescence. It is notable that in the 'stay-green' mutation of Festuca, which retains greenness but not photosynthesis, dry matter production and tillering were lower in wild-type plants, implying that retention of photosynthetic activity is required for increased crop productivity (B Hauck et al., personal communication).

Conclusions

Knowledge related to molecular and genetic mechanisms of leaf senescence has increased rapidly in the past two years; however, we are still seeing only a glimpse of the molecular activities that occur during leaf senescence, and the in vivo functions of any of the SAGs have not been proven experimentally. It is necessary to identify additional SAGs and characterize their function(s). More importantly, we do not know the nature of any of the key genes that regulate the initiation or progression of leaf senescence, except in the examples of the Arabidopsis etr1 gene [59] and the tomato Nr gene [53,59]. Isolation of the regulatory genes, especially genes that control developmental age-dependent senescence, will be of fundamental importance in understanding the mechanism of leaf senescence. A report that telomeres are shortened during the aging of cultured cells is interesting in this respect [60]. The study of the regulation of SAGs is generating valuable information, but more efforts, including the study of the mechanisms of transcriptional and post-transcriptional regulation of SAGs, are certainly needed. In this respect, it is notable that the translational inhibitory activity of ribosome-inactivating proteins increases during leaf senescence [61] and there are reports of a lack of coordination between mRNA and protein levels for some SAGs during senescence [5••,24••,32••]. Expression of SAGs has been examined primarily at organ level thus far; however, the regulation of SAGs may not be the same in all cell types. The modes of regulation of SAGs at the cellular level need to be examined to further enhance our knowledge of leaf senescence [26].

It is very encouraging that leaf senescence can now be analyzed genetically in Arabidopsis, an experimentally amenable model plant and the relevant molecular clones should be obtainable with a reasonable amount of effort. The cloning of these genes along with the isolation of additional mutants and their characterization should provide critical clues to the mechanisms of leaf senescence.

The manipulation of leaf senescence is feasible through the use of senescence promotors and such studies contribute significantly to plant improvement. In the future, it should be possible to manipulate leaf senescence using regulatory senescence genes.

Acknowledgements

This article is dedicated to Larry Nooden, who pioneered work in this area. I am most grateful to Catherine Griffiths and Larry Nooden for helpful discussions and critical reading of this manuscript. The work in the author’s laboratory was supported by the Pohang University of Science and Technology (POSTECH) and the Plant Molecular Biology and Biotechnology Research Center (PMBBRC).
References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


6. Matile P, Hoertensteiner S, Thomas H, Kraeutler B: Coordination of protein and mRNA abundances of the clpC gene per unit total RNA is relatively constant during natural senescence of leaves and by salicylic acid or glutathione treatment. The gene is also highly induced in MeJA-treated leaves but not significantly in ABA-treated leaves.


10. Kato A, Hayashi M, Takeuchi Y, Nishimura M: A pumpkin gene for 3-ketoacyl-CoA thiolase that catalyzes the last step of β-oxidation of fatty acids was characterized. Expression of the mRNA and protein increases during cotyledon senescence and during germination. The expression pattern is coincident with the transition of microbodies from peroxisomes to glyoxysomes. The protein is exclusively found in microbodies degraded during leaf senescence. Post-transcriptional regulation of the thiolase gene expression during senescence is different from that of the malate synthase gene: the thiolase protein remained more persistently than malate synthase during senescence.


34. Van-Gysel A, Van-Montagu M, Inze DA: Negatively light-regulated was not detectable in senescent leaves. Two specific polypeptides was observed. Expression of the plastid gene psbA was examined. Changes in polypeptide pattern and gene expression were examined during leaf senescence. The mRNA for another cDNA clone increased up to mid-senescence and decreased thereafter. The mRNA for a cDNA clone encoding a metallothionein-like protein of Arabidopsis thaliana and also in senescent leaf. The mRNA for a cDNA clone encoding a metallothionein-like protein of Arabidopsis thaliana is expressed during leaf senescence. Furthermore, the SAG2 and SAG4 genes that are induced during natural leaf senescence are induced during the aging of the cultured cells. This result shows that many of the genes induced during the loss of cell viability in cultured cells and leaf senescence are common. Two of the cDNA clones encode proteins similar to Fe²⁺-ascorbate oxidase and β-glucosidase, respectively.


43. Guaitter J, Giannelli MC: Nuclear and cytoplasmic "stay-" green" mutations of soybean alter the loss of leaf soluble proteins during senescence. Physiol Plant 1996, 96:655–661. The effects of cytG and d12 mutations in soybean on leaf soluble protein contents were analyzed. The d12 mutant line retained 50% of soluble protein at the time of abscission whereas wild type retained ~10%. The cytG line did not show any difference. The decreased protein degradation in the d12 line was not due to lack of proteolytic activity.
The molecular genetic analysis of leaf senescence


The sid locus of Festuca pratensis, a mutant allele of which causes the 'stay-green' phenotype because of reduced chlorophyll loss during leaf senescence, encodes the gene for phaeophorbide a dioxygenase, an enzyme that is either involved in chlorophyll breakdown or regulates its synthesis or activity.


The ethylene-insensitive mutation of Arabidopsis etr1-1 was employed to reveal the role of ethylene in leaf senescence. The mutation causes a delay in leaf senescence symptoms such as the decrease of leaf longevity, the induction of a SAG, the repression of photosynthesis-associated genes, and chlorophyll loss. The level of the SAG mRNA was, however, not significantly increased in these mutant plants.


Transgenic tomato plants transformed with the antisense ACC oxidase gene contained a greatly reduced level of ethylene production in leaf and exhibited 10-14 days of delay in leaf senescence. The relative delay of leaf senescence symptoms such as the loss of chlorophyll and photosynthetic capacity and the induction of SAGs was observed in leaves of the transgenic plants when compared to those of wild-type plants of a similar age; however, once senescence was initiated, the senescence symptoms in the transgenic plants progressed similarly to those in wild-type plants.


Transgenic plants in which expression of the gene for isopentenyl transferase was controlled under the promoter of the Arabidopsis SAG12 gene were produced. The production of cytokinin was autoregulated, because activation of the promoter during senescence produced cytokinin, which, in turn, retards senescence and attenuates the senescence-induced expression of the promoter. The transgenic plants exhibited an extended, photosynthetically active life span with no apparent abnormalities. The plants produced 50% more seeds and dry mass.


