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Molecular Genetic Analysis of Leaf Senescence in *Arabidopsis*

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ABSTRACT Senescence is a sequence of biochemical and physiological events that lead to death of a cell, organ, or whole organism. Senescence is now clearly regarded as a genetically determined and evolutionarilly acquired developmental process comprising the final stage of development. However, in spite of the biological and practical importance, genetic mechanism of senescence has been very limited. Through forward and reverse genetic approaches, we are trying to reveal the molecular and genetic mechanism of senescence in plants, employing leaf organs of *Arabidopsis* as a model system. Using forward genetic approach, we have initially isolated several delayed senescence mutants either from T-DNA insertional lines or chemical-mutagenized lines. In the case of *ore* 4 and *ore* 9 mutants, the mutated genes were identified. The recent progress on characterization of mutants and identification of the mutated genes will be reported. We are also screening mutations from other various sources of mutant pools, such as activation tagging lines and promoter trap lines. Two dominant senescence-delayed mutants were isolated from the activation tagging pool. Cloning of the genes responsible for this phenotype is in progress.

For reverse genetic approach, the genes that induced during leaf senescence were first isolated by differential screening method. We are currently using PCR-based suppression subtractive hybridization, designed to enrich a cDNA library for rare differentially expressed transcripts. Using this method, we have identified over 35 new sequences that are upregulated at leaf senescence stage. We are investigating the function of these novel genes by systemically generating antisense lines.

Introduction

Senescence is a sequence of biochemical and physiological events that comprise the final stage of development, the stage from the mature, fully developed state of a whole plant or plant organs to their death. Senescence in plants is now regarded as an evolutionarily acquired and genetically programmed developmental strategy rather than a simple and passive degenerative process. Identification of genes that alter senescence through molecular genetic approaches will be critical not just for understanding the mechanism of senescence but also for practical purposes such as improvement of plant productivity, pre- or post-harvest storage, and stress tolerance.

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Senescence in higher plants is a type of programmed cell death (PCD). Cell death is a widely observed phenomenon in higher plants. When cell death occurs by a genetic program as a part of developmental processes, it is regarded as PCD. PCD in higher plants occurs throughout development of most organs and can be triggered by environmental factors such as pathogen infection and physical injury. Among various plant PCDs, senescence is such an obvious and critical developmental process. However, despite that biochemical and cellular events during senescence have been extensively studied, the exact definition of senescence is not clear yet. Cell death during senescence is mostly observed in a broad area of plant bodies, for example in organs such as leaves, petals, fruits, or in whole plant bodies. In contrast, other PCDs involve rather localized cell death. Cell death during senescence also occurs more slowly than in other PCDs that involve more acute cell death. The slower cell death during senescence is in part associated

with efficient recycling of nutrients that are degraded during senescence.

Senescence and aging are different concepts, although these terms often used interchangeably in animals. The term senescence is applicable to a process that leads to death of a cell, an organ, or a whole plant and occurs at the final stage of their development. In contrast, aging is addition of timing to a cell, organ, or a whole plant and occurs throughout development; the timing could be either physical or developmental. In this sense, aging would be a major determinant of senescence but not senescence itself. It is also worth to distinguish replicative or mitotic senescence from post-mitotic senescence. Replicative senescence of a cell is referred to loss of capacity for further cell division upon aging. Post-mitotic senescence is a degenerative process that occurs after cellular maturation or differentiation and leads to death of a cell. Here, we mention about the post-mitotic senescence, since senescence at a tissue, organ, or whole body is accompanied by the post-mitotic senescence.

Senescence is not a random disintegration process but a highly regulated process that involves orderly and sequential change of cellular physiology, biochemistry, and gene expression. In fact, it appears that, during senescence, activities or quantities of most cellular components including expression of a majority of genes are changed. Thus, it is well expected there are many genes that alter senescence. However, in spite of the biological and practical importance of senescence, the molecular and genetic analyses on the mechanism have been actively investigated only recently [2, 6, 8]. Accordingly, there are a surprisingly low number of genes reported to alter senescence so far. In addition, many genes claimed to alter senescence were minimally analyzed with respects to senescence symptoms or markers, while use of a single senescence symptom such as yellowing or loss of chlorophyll may well be misleading.

We are trying to reveal the molecular and genetic mechanism of senescence in plants, employing leaf organs of *Arabidopsis* as a model system.

Isolation of delayed senescence mutants, *ore1*, *ore3*, and *ore9* from *Arabidopsis*

Even before much was known about the detailed cellular physiology of senescence, senescence was assumed to be an internally programmed process, since it appeared to be highly specific and orderly in terms of when, where, and how it occurs [10].

Based on the notion that senescence is a genetically controlled developmental process, molecular and genetic approaches have been employed to understand the mechanism of senescence in our laboratory.

Arabidopsis thaliana, as a representative monocarpic plant, displays a relatively reproducible senescence pattern [6, 8]. In addition, *Arabidopsis* has a short life span and amenability to genetic analysis, making this plant as a suitable model system to study senescence and to find regulatory genes by forward genetic approach. In addition to the advantages *Arabidopsis* has as a general genetic model, *Arabidopsis* leaf has a short life span and is to assay for senescence phenotype. Furthermore, senescence in *Arabidopsis* leaf occurs in a predictable manner, whether senescence is induced by age or by other factors. Using *Arabidopsis* as a model system, therefore, a systematic genetic screening has been taken to identify the genes that control senescence in our laboratory.

We are screening for genetic mutations that show delayed senescence phenotype mutations. We are not screening for early senescence mutations, since any mutations that affects homeostasis of a cell would give apparent early senescence phenotype. The phenotype we screened for was delayed yellowing of leaf, since chlorophyll loss is one of the major symptoms of leaf senescence. Initially, by screening 34,000 of individual M2 plants that were derived from EMS-mutagenized seeds, we were able to isolate 4 genetic mutations (*ore1*, *ore3*, *ore9*, and *ore11*) that showed delayed yellowing of leaf. However, yellowing is only one of the symptoms associated with senescence. After examining other senescence-associated parameters, we found that the *ore11* mutation was only delayed in chlorophyll loss but not in other senescence parameters, suggesting that they have only a lesion in chlorophyll loss but not in ifunctional senescenceî. The other three genetic mutations showed delayed senescence symptoms with various senescence markers.

The three mutations (Figure 1) were named *oresara1*, *3* and *9* (or *ore1*, *ore3*, and *ore9*, for short), respectively, oresara meaning "long-living in Korea" [12]. Delayed senescence symptoms further observed in these

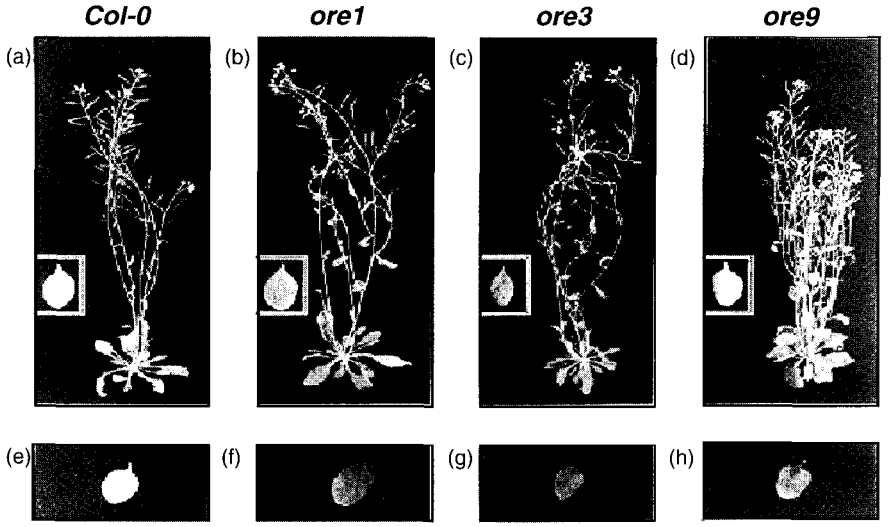

Figure 1. The senescence phenotype of oresara mutations. (a-d) Phenotypes of a whole plant and the fourth foliar leaf of wild type (a) and *oresara1* (b), *oresara3* (c), and *oresara9* (d) at 50 days after germination. The plants were grown with 16 hour light/8 hour dark cycle at 23°C in a growth chamber. Neither the age of the leaves, as measured from the time of leaf emergence, nor the time of flowering between wild type and the mutant plants was significantly different enough to account for the apparent phenotypic difference in leaf senescence (see Results). (e-f) Phenotype of the fourth foliar leaf of wild type (e), *oresara1* (f), *oresara3* (g), or *oresara9* (h) upon induction of senescence by incubation in darkness. The leaves at a same age (13 days after leaf emergence) were detached and incubated on 3 mM 2-N-morpholinoethanesulfonic acid buffer (MES, pH 5.8) for 4 days in darkness.

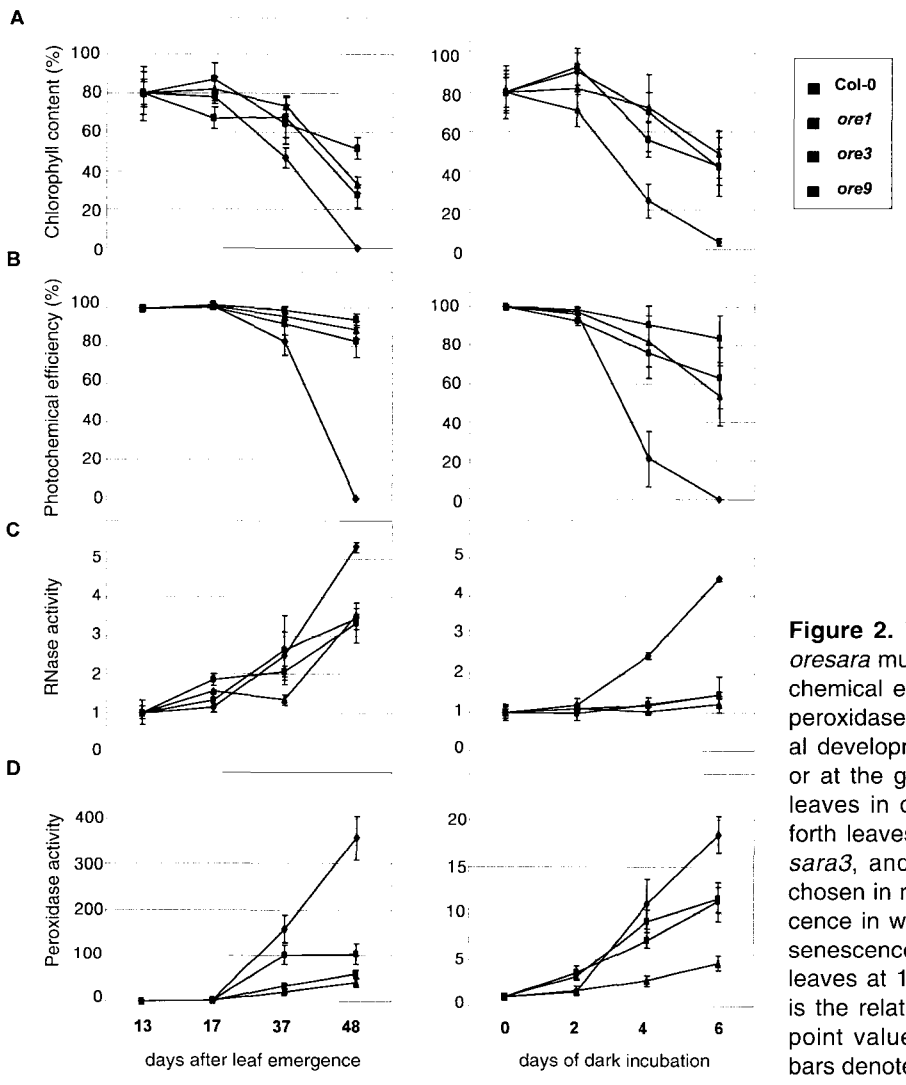

Figure 2. Physiological characterization of the *oresara* mutants. Chlorophyll content (A), photochemical efficiency (B), RNase activity (C), and peroxidase activity (D) were examined at several developmental ages of leaves *in planta* (left) or at the given times after incubating detached leaves in darkness (right), using the third and forth leaves of wild type (Col-0), *oresara1*, *oresara3*, and oresara9. The sampling time was chosen in relation to the advancement of senescence in wild type plants(12). The dark-induced senescence experiment was performed with the leaves at 13 days after leaf emergence. Shown is the relative value as percentage of the initial point value for each experiment. The vertical bars denote standard deviations.

mutants included delayed decrease of anabolic activities, including chlorophyll content, the photochemical efficiency of PS II (Fv/Fm), and the relative amount of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), as well as delayed increase in catabolic activities such as RNase and peroxidase activities during *in planta* senescence and dark-induced senescence (Figure 2). Genetic analysis showed that all of the mutations are inherited as monogenic recessive traits, and fall into three complementation groups (Table 1).

Existence of *ore1*, *ore3*, and *ore9* provides clear genetic evidence that functional leaf senescence in *Arabidopsis* is a genetically controlled event involving several monogenic genetic elements. Since these mutations affect a wide variety of senescence symptoms, these genes must be regulatory genes of senescence. All of the mutations were recessive, suggesting that the genes defined by these mutations are positive regulators of senescence. The result also suggested that senescence proceed through multiple pathways, since senescence is only delayed but not blocked in all of the mutants although there is a possibility that all of these mutations are weak alleles. Studies on expression of senescence-associated genes in the ore mutants are being progressed to further define their role in controlling senescence. In addition, double mutant analyses are being progressed to elucidate the interaction among these genes in controlling senescence.

Senescence is affected by several environmental and endogenous factors. We have examined the effect of several senescence-affecting factors on senescence of these mutations. In addition to the delayed senescence phenotype of the mutants in age and dark-induced senescence, we are obtaining supporting data that leaf senescence symptoms caused by several phytohormones such as ethylene, abscisic acid (ABA), and methyl jasmonate (MeJA) are also delayed in these mutant plants (Woo, Park, and Nam, unpublished data). The *ORE1*, *ORE3*, and *ORE9* genes may be required for proper progression of leaf senescence induced by the phytohormones, as well as age and darkness. In fact, *ore3*, also designated as *ein2-34*, is an allele of *ein2* (*ethylene-insensitive2*) *mutations*. However, the other mutants do not have a defect in general hormone responses but in hormone-induced senescence.

Age-specific senescence-mutants

Senescence is an age-dependent process and thus there should be a cellular mechanism(s) that measures age of a cell, tissue, organ, or whole body. The evidence for the presence of genes that alter senescence by controlling developmental aging is accumulating. Senescence occurs in an age-dependent manner and

Table 1. Genetic analysis of the oresara mutants.

Cross	Progeny	Total	Phenotype[a]		x^2
			+	-	
oresara1 × Col-O	F_1	30	30	0	
	F_2	432	319	113	0.309 (p>0.5)
oresara2 × Col-O	F_1	89	89	0	
	F_2	230	177	53	0.470 (p>0.1)
oresara3 × Col-O	F_1	22	22	0	
	F_2	130	98	32	0.010 (p>0.9)
oresara9 × Col-O	F_1	25	25	0	
	F_2	196	142	54	0.680 (p>0.1)
oresara2 × *oresara3*	F_1	25	0	25	
oresara1 × *oresara3*	F_1	32	32	0	
oresara1 × *oresara9*	F_1	29	29	0	
oresara3 × *oresara9*	F_1	25	25	0	

The phenotypes of the progenies were scored with the two senescence parameters, chlorophyll content and photochemical efficiency of PS II, during age-dependent and dark-induced senescence in each plant. In all the progenies examined, the two phenotypes cosegregated. The x^2 value is for an expected ratio of 3:1 (wild type:delayed senescence).

[a]+, wild type; -, delayed senescence.

the acceleration or initiation of senescence by plant hormone ethylene was shown to be age-dependent. We identified two independent genetic mutations in *Arabidopsis* that delay leaf senescence during natural senescence but not in hormone or dark-induced senescence (unpublished data). These genes may be candidates for the genes that affect senescence by altering aging process.

Other senescence mutants screened in *Arabidopsis*

We are also screening mutations from other various sources of mutant pools, such as T-DNA insertion lines, activation tagging lines, and promoter trap lines.

Screening of delayed senescence mutants from activation tagging lines will be especially useful in identify-

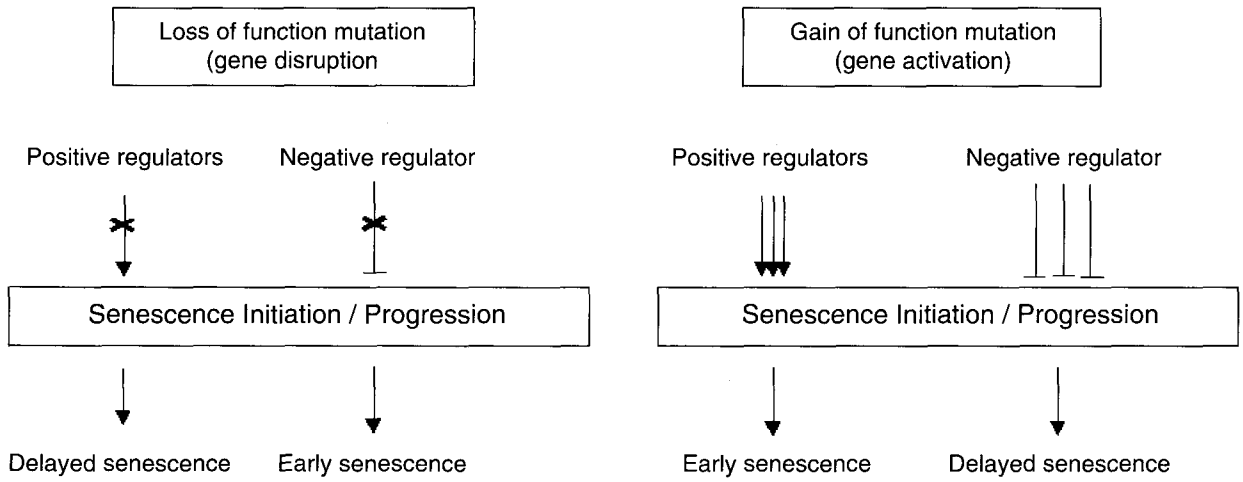

Figure 3. Screening of negative regulators from activation tagging.

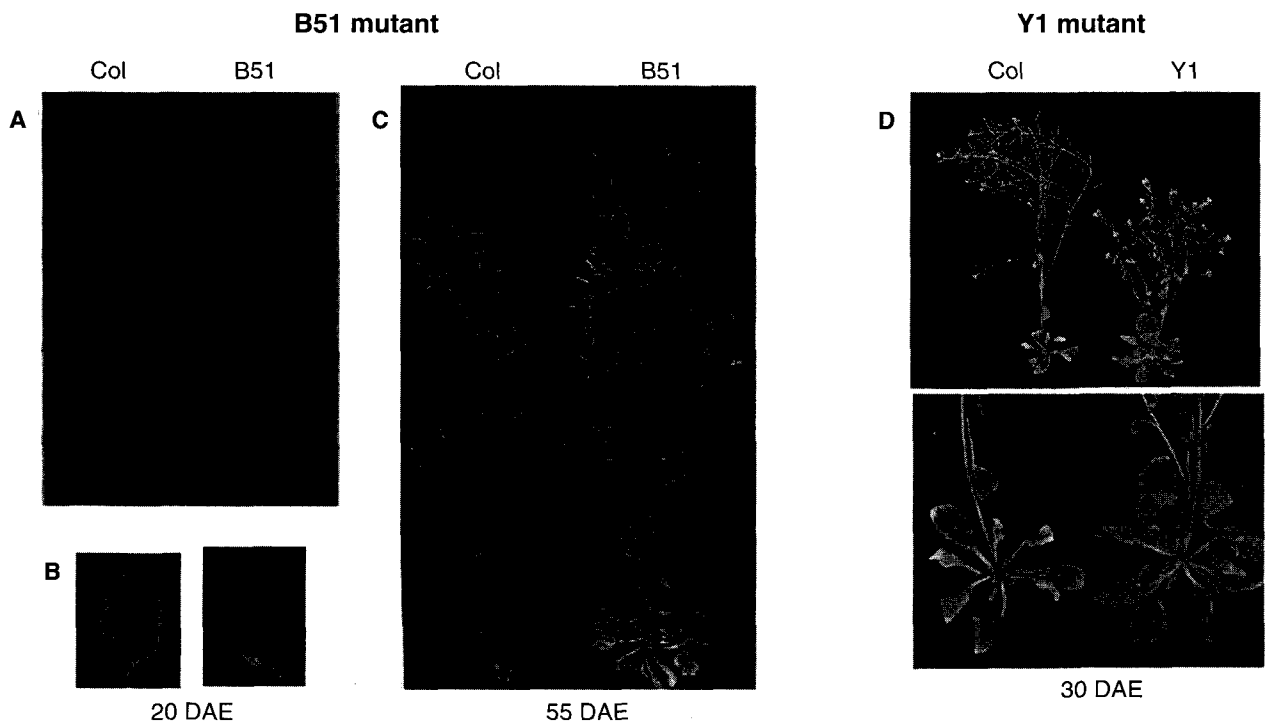

Figure 4. Phenotypes of mutant plants isolated from activation tagging pool. (A,B) Phenotype of a whole plant (A) and the fourth foliar leaf (B) of wild type and B51 mutant at 20 days after leaf emergence. (C) Phenotype of a whole plant of wild type and B51 mutant at 55 days after leaf emergence. (D) phenotype of a whole plant of wild type and Y1 mutant plants at 30 days after leaf emergence.

ing negative regulators of senescence (Figure 3). By screening approximately 5,000 individual activation tagging families at T2 generation, we isolated two delayed senescence mutants (Figure 4). These mutations showed dominant segregation pattern and cosegregated with the inserted T-DNA. Although we are in progress in cloning the responsible genes, the result show that activation tagging approach is a feasible approach in identifying genes that regulate senescence.

In screening of promoter trap lines, we are first screening the lines for senescence-associated expression of the reporter gene. Among over 2,000 lines we examined, we found 17 lines that showed senescence-associated GUS expression. We then examined the senescence phenotypes of these lines and found that two lines give delayed senescence phenotype (data not shown). This preliminary result also shows that this approach is feasible in identifying senescence-associated genes as well as the effect of the knock-out mutations in these genes.

Senescence-associated genes

Senescence is associated with up- or down-regulation of many genes that are involved in chlorophyll degradation, nucleic acid breakdown, protein degradation, and nitrogen and lipid remobilization [3, 7, 8].

Furthermore, for the senescence program to proceed, there should be genes that execute the degeneration process. Many of them would be involved in degradation process, including chlorophyll breakdown, and nitrogen and lipid remobilization.

To reveal the molecular physiology and to elucidate the molecular mechanism of leaf senescence, we are isolating and studying the regulation and function of the senescence-associated genes.

We initially cloned 5 senescence-induced genes by differential screening (Figure 5). The natures of the genes are shown in table 2. Some of these genes are revealing a part of senescence physiology [11, 13]. For example, the *SEN4* clone has a homology to endo-xyloglucan transferase (EXT) [16], which has two distinct activities, splitting of xyloglucan molecules and transglycosylation, and can

Table 2. The nature of senescence-associated genes isolated by differential screeinga.

Clones	putative function of the clones
SEN2	*Arabidopsis* CAT3
SEN3	polyubiquitin
SEN4	endoxyloglucan transferase
SEN5	unknown protein

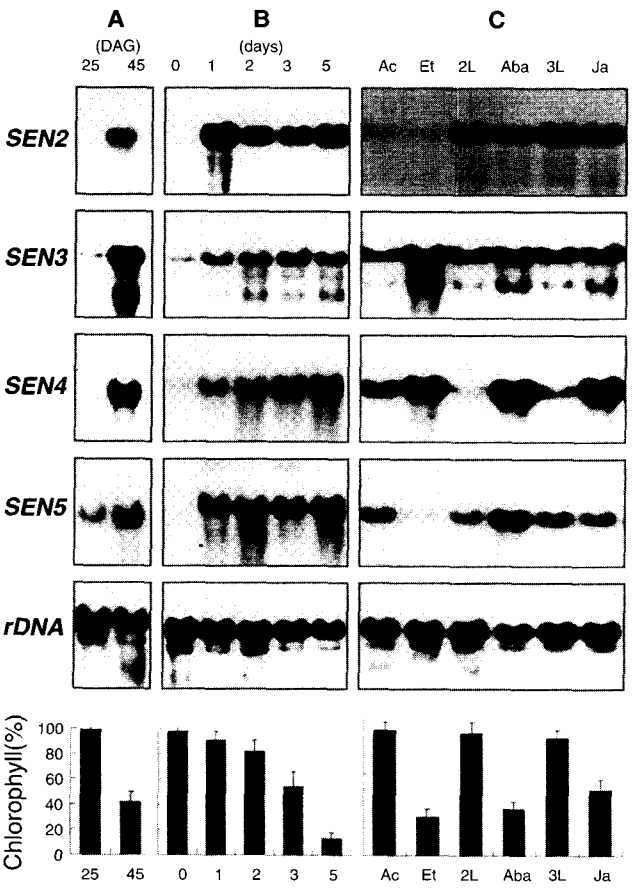

Figure 5. Expression pattern of the transcripts detected by SEN clones. A, Induction of SEN clones during age-dependent leaf senescence. The The third or fourth foliar leaves were harvested from a synchronously growing population at the indicated day after germination (DAG); B, Expression patterns of SEN clones during detachment/dark-induced senescence. The third or fourth foliar leaves at 18 DAG were detached and incubated in darkness for the indicated number of days; C, Expression patterns of SEN clones during phytohormone-induced senescence. The third or fourth foliar leaves at 18 DAG were detached and incubated in light only for 2 (2L) or 3 (3L) days in MES buffer (pH 5.8), in 0.1mM ABA (Aba) for 2days, in ethephon treatment, or in 50μM methyl jasmonate (Ja) for 3 days. The acid solution contained the hydrolytic compounds (HCL and H_3PO_4) of ethephon. A 30 μg portion of total cellular RNA was loaded in each lane. The amounts of loaded RNA in each lane were confirmed to be equal by rehybridization with ^{32}P-labeled 18S ribosomal RNA gene (rDNA) probe after the previous probes were stripped off. The Chl contents are presented as percent values relative to the Chl content of the leaves at 25 DAG (A), before dark treatment (B), at 0 day without hormone treatment(C). A sample for Chl measurement was extracted from two two leaves. Shown are average values of six independent samples for each time point. The vertical bars denote standard deviations.

modify a major hemicellulose of the cell wall. The *SEN3* clone is highly homologous to the polyubiquitin that has been shown to play a role in ubiquitin-dependent proteolysis [5]. The results suggest that ubiquitin-dependent degradation of proteins and cell wall degradation may occur during senescence.

We are also isolating more senescence-induced genes by PCR-select subtractive hybridization, since this approach allows partial normalization for enrichment of rare transcripts [4]. When we analyzed the subtracted clone, we found that 60% of the clones were senescence-upregulated and that 60% of senescence-upregulated clones were novel clones. The result shows that this approach is feasible in isolating novel senescence-associated genes.

Senescence in plants is affected not just by aging but also influenced by other factors such as hormones and stresses. When we examined the regulation of the *SEN* clones during senescence caused by several factors such as ethylene, abscisic acid, methl jasmonate, and dark-incubation, we found that they are differentially regulated (Figure 5B & 5C). This result leads us to a conclusion that the apparent senescence symptoms of *Arabidopsis* leaf appear similar regardless of the senescence-inducing factors, but the detailed molecular state of leaf cells during senescence induced by different senescence inducing factors are different (Figure 6).

To identify the function of these genes in senescence, we are taking a systematic antisense approach for many of these genes. Our initial results show that two of the clones we tested delayed senescence phenotype (Figure 7). Thus, with this approach, we should be able to find more of the regulatory genes in senescence very soon.

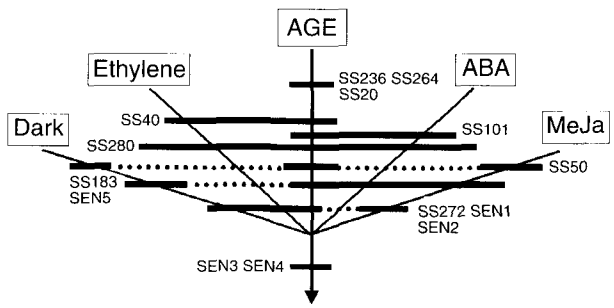

Figure 6. Hierarchic diagram of gene expressions of the senescence-associated clones.

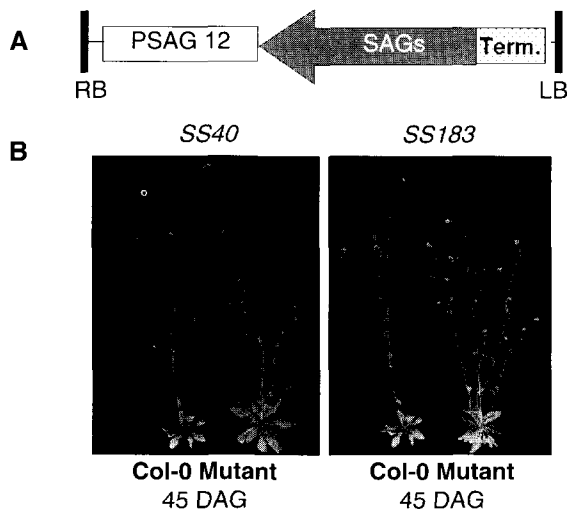

Figure 7. Functional analysis of the SAG clones by antisense approach. A, The T-DNA region of the antisense SAG cDNA constructs. RB; right border, PSAG 12; the promoter of the Arabidopsis SAG12 gene, Term; transcription terminal sequence, LB; left border; B, Phenotypes of wild-type and two antisense transgenic plants (SS40 and SS183) at 45 day after germination (DAG).

Conclusions

From these studies, we learned senescence is fully amenable to molecular and genetic analysis as a firm genetic phenomenon. We are seeing that senescence field is not in the twilight zone anymore and is, like many other biological processes, amenable to molecular and genetic analyses.

Senescence is an integrated response of a cell, tissue, organ, or an organism to age, developmental status, and environment. Furthermore, since senescence is the other side of cellular proliferation and growth, many of genes that affect plant growth and development would also affect senescence. In this sense, it is rather surprising that so little is known on genes that alter senescence. One reason for this would be that many of the genes that alter senescence but are also involved in other biological processes function earlier than senescence stage. Phenotypic effects of the genes that occur before senescence stage can mask observation of their effect on senescence. This limitation may be overcome by employing an approach to examine their influences on senescence using senescence-specific promoters in transgenic plants. We expect that many regulatory genes involved in cellular processes other than senescence will prove to affect senescence through this type of approach. These genes are not considered as regulators specific for senescence. However they will help to under-

stand the mechanism of senescence and will also be useful in engineering of plant senescence for application purposes.

It is unlikely that an organism contains a full set of genes just to perform senescence. Rather it is more reasonable to think that an organism utilize many of genes involved in other processes such as germination and stress responses for senescence program through differential regulation of the genes. Thus, the number of genes specifically involved in senescence may be limited. At the same time, it is certainly possible that there exist genes that are specifically involved in senescence. This is easily inferred from the presence of genes induced specifically during senescence and from identification of genetic mutations that affect senescence with a minimal effect on other developmental processes.

It is exciting that the regulatory mechanism of plant senescence can be examined by taking advantage of *Arabidopsis* as a model system. It is especially exciting that genetic analysis of senescence is feasible in this model plant. Genetic analysis and molecular cloning of genes that alter senescence in *Arabidopsis thaliana* should provide a wealth of information regarding regulation of plant senescence.

There are a number of mutations that are known to show premature senescence. Early senescence symptoms may result from any perturbation of homeostasis of cells and thus genes for most of early senescence mutations may not directly be involved in control of senescence. Regulation of senescence may be viewed as a matter of balance between two antagonistic self-maintenance and senescence gene activities. A program for down-regulation of genes for self-maintenance may also be important for initiation and progression of senescence. Thus, it is certainly possible that there exist genes that repress activation of senescence program and mutations in these negative regulators of senescence may cause earlier senescence. In contrast to the early senescence mutants, many of genes for delayed senescence mutations with minimal abnormalities in other developmental processes are likely regulatory genes directly and specifically involved in controlling senescence.

Genes that alter senescence may be isolated through map-based cloning and from the T-DNA or transposon insertional mutant lines. A reverse genetic approach would be also possible. For example, *in planta* function of senescence-associated genes may be identified by isolating an insertional line in the gene of interest or by making transgenic plants that over-express or express antisense transcripts to down-regulate expression of the gene preferably using senescence-specific promoters. The candidate genes obviously include the genes for transcriptional factors and signal transduction components that are induced during senescence. It should be also possible to isolate transcription factors that regulate senescence-associated gene such as *SAG12* using yeast one hybrid system. Senescence may be controlled not just by differential regulation but also by regulating activities of regulatory components. A proteomic approach that idenfies differential modification of regulatory components during senescence may identify a novel regulator of senescence.

It should be noted that judgement on whether a gene alters senescence should not rely on a simple visual observation of chlorophyll loss or yellowing. To be quali-

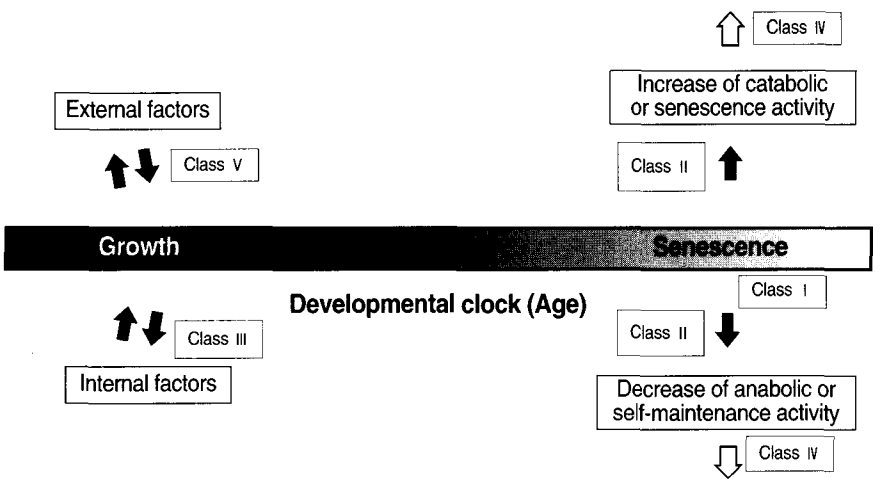

Figure 8. The five classes of the genes that alter senescence. Class I, genes controlling developmental aging process; Class II, regulatory genes that up-regulate expression of senescence-associated genes or down-regulate catabolic or self-maintenance genes; Class III, genes controlling other endogenous biological processes in plant but also affect senescence; Class IV, down-stream genes that are involved in executing the senescence process such as genes for cellular disintegration and nutrient recovery; Class V, genes that acts in response to environmental factors.

fied as an up-stream regulatory gene for senescence, the effect of the gene must be assayed with multiple physiological or molecular senescence. The useful and convenient senescence markers would include the photosynthetic efficiency, senescence-associated enzyme activities, change of protein levels, and ion leakage, and gene expression, etc. Since senescence is associated with a complicated and differential up- or down-regulation of genes depending on the endogenous and environmental factors that affects senescence, a more complete picture on the role of a gene that alter senescence may be achieved through use of a DNA chip.

In summay, we provide our conceptual view on the genes that are involved in senescence as follows.

Although senescence occurs in an age-dependent manner in many species [1,6], the initiation and progression of senescence can be modulated by a variety of environmental factors such as temperature, mineral deficiency, drought conditions, and pathogen infection. It is also known that internal factors such as plant growth regulators, reproduction, and cellular differentiation influence senescence [14, 15]. Accordingly, genes involved in senescence may be conceptually categorized into five classes. These include genes controlling the developmental aging process (**class I**), regulatory genes that up-regulate expression of senescence-induced genes or down-regulate catabolic or self-maintenance genes (**class II**), genes controlling other endogenous biological processes in plant but also affecting senescence (**class III**), down-stream genes that are involved in executing the senescence process such as genes for cellular disintegration and nutrient recovery (**class IV**), and genes acting in response to environmental factors (**class V**). Senescence, like other biological process, may conceptually involve initiation and progression stages. The genes in the class I, II III, and V would be involved in initiation and/or progression. The genes in the class IV would be mostly involved in progression (Figure 8).

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