Molecular aspects of leaf senescence

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Senescence is the last stage of leaf development and one type of programmed cell death that occurs in plants. The relationships among senescence programs that are induced by a variety of factors have been addressed at a molecular level in recent studies. Furthermore, an overlap between the pathogen-response and senescence programs is beginning to be characterized. The complexity of the senescence program is also evident in studies of senescence-specific gene regulation and the role of photosynthesis and plant hormones in senescence regulation. New molecular-genetic approaches are expected to be useful in unraveling the molecular mechanisms of the leaf senescence program.

Leaves are specialized photosynthetic organs and the plant invests much energy and nutrients in leaf production. After a photosynthetically productive period, the leaf's contribution of photosynthate to the plant diminishes and the leaf then enters its last stage of development: senescence. During senescence, nutrients such as nitrogen, phosphorus and metals that were invested in the leaf are reallocated to younger leaves and to growing seeds, or are stored for the next growing season[1]. Indeed, leaf senescence can be viewed as a recycling program at the organismal level.

Recently, much attention has been focused on leaf senescence as a form of programmed cell death (PCD), a genetically controlled system of self-destruction. At the cellular level, the senescence program unfolds in an orderly manner. Chloroplasts, which contain most of the protein in a leaf cell, are one of the first organelles to be targeted for breakdown. Other organelles, such as the peroxisome, also undergo biochemical changes as senescence proceeds[2]. The nucleus, which is needed for gene transcription, and the mitochondria, which are essential for providing energy, remain intact until the last stages of the senescence[3].

An easily observed event during senescence is the loss of chlorophyll, which typically starts at the leaf margins and progresses to the interior of the leaf blade (Fig. 1). Although this visible manifestation of senescence is the result of chlorophyll breakdown during chloroplast disassembly, many other catabolic events, such as protein, lipid and nucleic acid degradation also occur[3,4]. Consistent with the remobilization role of the senescence program, protein degradation is accompanied by an increase in levels of amino acids such as glutamine, which can be transported through the phloem[5].

Manipulating leaf senescence through breeding or genetic engineering might help to improve crop yields by keeping leaves photosynthetically active for longer[6]. For example, transgenic tobacco plants that show delayed leaf senescence because of the autoregulatory production of the senescence-inhibiting hormone cytokinin show increased seed production as well as increased biomass[7]. The senescence-specific expression of the maize gene *Knotted1* (which encodes a homeobox regulatory protein) in tobacco also causes a delayed-senescence phenotype similar to plants containing autoregulated cytokinin production; indeed, the expression of *Knotted1*-like genes causes increased cytokinin levels[8,9]. A delay in leaf senescence might also be desirable to increase shelf life of cut flowers and leaf crops such as lettuce.

### Molecular mechanisms of leaf senescence
Age-mediated and induced senescence
Leaf senescence can be triggered by internal age-dependent factors. A leaf has a characteristic life span of photosynthetic productivity, after which the senescence program is initiated[10]. Senescence can also occur prematurely if triggered by external

factors such as phytohormone application, shading, temperature and pathogen attack[3,10]. In fact, dark-induced senescence has repeatedly been used to induce uniform, rapid senescence[11]. An obvious question that arises is whether the molecular programs of senescence caused by different treatments are the same.

Over the past several years, many genes whose transcripts are upregulated during leaf senescence [often referred to as senescence-associated genes (SAGs)] have been identified[1,12]. Determining whether mRNAs or proteins are upregulated during senescence is complicated by the fact that total protein and RNA levels decline as senescence progresses[4,13]. Analyzing the expression of these SAGs in response to different senescence-inducing treatments has been used to address the extent of overlap between age-dependent leaf senescence and senescence induced by other factors.

The mRNA levels of 11 SAGs were analyzed in response to several senescence-inducing treatments, including phytohormones[14]. The treatment that showed the greatest overlap with age-mediated senescence-specific gene expression was detachment of leaves followed by dark incubation. This treatment induced the expression of all but one of the genes that are expressed in age-mediated leaf senescence. However, this treatment also induced genes that are not related to age-mediated senescence. In whole plants subjected to darkness, only about half of the age-related SAGs were induced. In detached leaves incubated in the light, all age-related SAGs
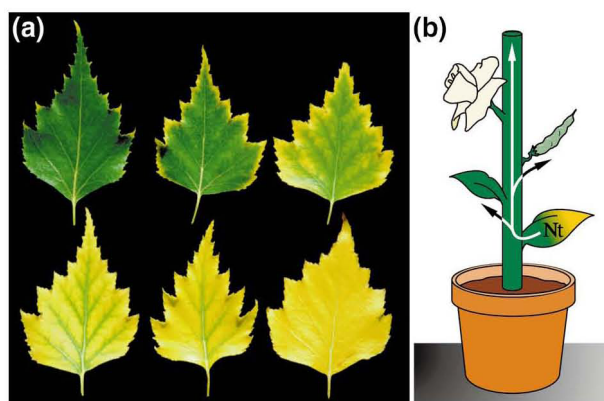
Fig. 1. (a) Senescing leaves can be recognized by their characteristic loss of chlorophyll. Often, the last areas of a leaf that senesce are close to veins, presumably because these are needed for nutrient export. The top-left leaf is just starting to senesce; the bottom-right leaf is in the most-advanced stage of senescence. (b) As a leaf senesces, nutrients (Nt) such as nitrogen, phosphorus and metals are reallocated to other parts of the plant such as developing seeds and leaves.

Table 1. Examples of defense-related genes that are induced in leaf senescence					
Gene	**Comments**	**mRNA induced by**		**Species**	**Ref.**
		SA	**Senescence**		
ND	Proteins with the same biochemical characteristics as PR1a and 1b were detected in senescing leaves	ND	ND	Tobacco	38
Ypr10	Ribonuclease-like *Phaseolus vulgaris* promoter fused to GUS is induced during senescence and by SA in tobacco	+	+	*Phaseolus vulgaris*	39
CBP-20	In PR-4 group Protein CBP-20 accumulates in response to SA and senescence	ND	+	Tobacco	40
LSC94	PR-1-a-like	+	+	*Brassica napus*	41
LSC222	Chitinase	+	+	*Brassica napus*	41
ND	Proteins imunologically related to β-1,3-glucanase and P4 PR-proteins of citrus and tomato are induced during leaf senescence. Chitinase activity was shown to increase during senescence	ND	ND	*Petroselinum crispum*	42
LSC212	Similar to radish (*Raphanus sativus*) anti-fungal PR-protein	ND	+	*Brassica napus*	43
LSC54	Metallothionein *Brassica* promoter fused to GUS shows induction in response to pathogens	+	+	*Brassica napus*	22,41
ELI3-2(SAG25)	Cinnamyl alcohol dehydrogenase	+	+	*Arabidopsis*	19
NIT2(SAG27)	Nitrilase (synthesizes auxin)	+	+	*Arabidopsis*	19
AtOSM34	Osmotin-like, PR-5 class	+	+	*Arabidopsis*	19,44
SAG26	Cinnamyl alcohol dehydrogenase	+	+	*Arabidopsis*	19
SAG29	Similar to *MtN3*, a gene expressed in early *Rhizobium*-induced root nodule	+	+	*Arabidopsis*	19
SENU4	PR protein P6	ND	+	Tomato	45
HIN1		−	+	Tobacco	23

Abbreviations: ND, not determined; SA, salicylic acid; PR protein, pathogensis-related protein; minus symbol = not induced by SA or senescence; plus symbol = induced by SA or senescence.

were induced, but this treatment was a weaker inducer than detachment followed by darkness. Ethylene induced chlorophyll loss and the expression of SAGs but the induction was not as strong as detachment with dark treatment. In abscisic-acid-treated leaves, only half of the age-related SAGs were induced.

In similar work, the expression of four cDNA clones was analyzed during age-mediated senescence and during senescence induced by detachment with dark or light treatments[15]. The four genes were induced in all cases. In further studies, treatments with phytohormones (ethylene, abscisic acid or methyl jasmonate) led to chlorophyll loss, but the four genes studied showed differences in expression. As noted in this study[15], treatments that cause chlorophyll loss do not necessarily induce the organized process of nutrient salvage that is characteristic of the age-induced-senescence program.

Although different treatments produce chlorophyll loss, the underlying molecular programs might not be identical and broader effects on gene expression than the induction of SAGs are often observed. At the molecular level, age-mediated leaf senescence is most similar, but not identical, to senescence induced by darkness in detached leaves and to ethylene treatment. However, these studies have not addressed differences among treatments at the protein level.

Defense responses and leaf senescence
Many genes have been identified as being induced during leaf senescence in different species. Some of these encode products that are similar to the pathogenesis-related proteins (PR proteins) but are not always bona fide PR proteins[16], and therefore we shall refer to these genes as defense-related (DR) genes (Table 1). PR proteins are associated with the hypersensitive response (HR; a disease-resistance response that results from incompatible pathogen interactions) and

with systemic acquired resistance (SAR) defense programs[17,18]. Therefore, one explanation for the induction of DR genes in senescent leaves is that opportunistic infections commonly accompany senescence and DR-gene expression is a normal pathogenesis response that usually accompanies senescence. However, DR genes are still induced during leaf senescence in *Arabidopsis* plants grown in sterile conditions[19]. This indicates that the expression of DR genes might be an integral part of the senescence program rather than a direct response to pathogen infection.

Some of the DR genes identified as SAGs are known to be induced by salicylic acid (Table 1), which is thought to be a signal molecule for SAR (Ref. 20). Thus, salicylic acid could also be involved in a signal-transduction cascade that leads to the induction of SAGs during senescence. To test this hypothesis, the expression of certain DR genes was examined during leaf senescence in transgenic plants unable to accumulate salicylic acid or to mount the SAR response owing to the expression of the *nahG* gene[19]. The five genes studied still showed senescence-associated expression in these plants, suggesting that salicylic acid accumulation is not essential for the senescence-associated induction of these genes.

Both leaf senescence and the HR are forms of PCD. However, leaf senescence occurs over a relatively long period and leads to nutrient salvage; during HR, by contrast, pathogen attack triggers rapid cell death that results in the formation of a region of dead cells around the infection site[21]. Although senescence and HR have distinct roles, it is possible that, at the molecular level, both cell-death pathways share certain steps.

To address this question, the expression of a reporter gene transcriptionally fused to the promoter of the *Brassica napus* senescence-associated gene *LSC54* was examined in transgenic

Arabidopsis plants[22]. Plants were challenged with both compatible and incompatible isolates of the fungus *Peronospora parasitica* and of the bacterial pathogen *Pseudomonas syringae*. During the HR induced by incompatible isolates, early, localized reporter-gene expression was detected at the site of infection but no detectable expression was observed with compatible isolates. However, reporter-gene expression was slowly induced in response to infection by compatible isolates, accompanying the appearance of the senescence-like symptoms – cotyledon yellowing in the case of *Peronospora* and water-soaked lesions with the bacterial necrotroph *Pseudomonas*. These results suggest that there is some overlap between the genetic pathways for HR, senescence and pathogen-induced necrotic-like cell death, at least in the expression of *LSC54*.

Another study compared the expression of two early markers of the HR, *HIN1* and *HSR203J*, and the leaf-senescence marker gene *SAG12* during both HR and leaf senescence in tobacco[23]. Although *HSR203J* was not induced during senescence, *HIN1* mRNA accumulated during late stages of leaf senescence, indicating some degree of overlap between the cell-death programs of HR and senescence. *SAG12* expression was analyzed in transgenic tobacco plants containing the *Arabidopsis SAG12* promoter fused to the reporter gene *GUS*. Low levels of *SAG12* promoter activity were detected during late stages of tobacco-mosaic-virus infection. However, histochemical staining showed that GUS was active only in cells surrounding the HR lesions, and these often exhibited chlorotic symptoms. Similar results were obtained with *Pseudomonas*-induced HR. Therefore, the pattern of *SAG12* expression differs both temporally and spatially from molecular markers of HR.

In summary, there appears to be some overlap between the leaf-senescence and pathogen-defense programs, but the extent and significance of this overlap remains to be determined. Perhaps there is a common factor, such as a stress or a physiological response (e.g. increased ethylene production), that induces the expression of certain genes during both senescence and defense responses. As discussed below, a decline in photosynthesis might be an inducer of senescence. In cells undergoing HR or other defense responses, a decline in photosynthesis might, in turn, initiate the senescence program late in the defense response. In addition, the senescence program might have incorporated features of the pathogen-defense response to protect the senescing leaf against opportunistic pathogens. Thus, there might be common regulators of both programs and mutants affected in pathogen response might also be affected in senescence, and vice versa; the *Arabidopsis acd1* mutant is a possible example[24].

Senescence regulatory elements

Studies on different SAGs reveal a diverse range of gene-activation patterns during senescence[14], indicating that plant senescence involves multiple regulatory pathways. Therefore it is not surprising that no conserved regulatory elements were found in the promoters of different SAGs of a given species. However, a sequence comparison of the promoter regions of one senescence-specific gene (*SAG12*) in two different species revealed that the promoter region contains a conserved senescence-response element[25,26]. This promoter region does not contain consensus sequences for known DNA-binding proteins, suggesting that the regulation of developmental senescence involves a new or divergent class of transcription factors. Dissecting the molecular characteristics of senescence-regulatory elements will probably involve the difficult task of understanding multiple and novel regulatory pathways, but this effort will be necessary to understand the molecular regulation of plant senescence.

Photosynthesis and senescence

One of the conspicuous changes that occurs during senescence is a rapid decline in photosynthesis[27], and it has been proposed that the decline of photosynthetic activity below a certain threshold level acts as a senescence-inducing signal[3,10]. Although this model has not been proved, the following recent evidence is consistent with the theory that declining photosynthetic activity plays a role in initiating senescence.

It is well known that elevated sugar levels repress the expression of photosynthesis-associated genes, presumably via an end-product negative-feedback system. It has been shown recently that hexokinases (which catalyze hexose phosphorylation as the first step of hexose metabolism) are involved in sugar sensing in higher plants[28,29]. Studies using hexokinase overexpressors and antisense expressors show that increased hexokinase levels in plants simulate a rise in sugar levels, whereas decreased levels simulate lowered sugar levels; that is, hexokinase overexpression is associated with reduced photosynthetic activity, and reduced hexokinase expression causes reduced sugar inhibition of photosynthesis-associated genes[28,29]. One notable phenotype of hexokinase overexpression is accelerated senescence, suggesting that reduced photosynthetic activity causes accelerated senescence. Recently, a glucose-insensitive *Arabidopsis* mutant (*gin2*) has been shown to have a lesion in one of the hexokinases (J. Sheen, pers. commun.). Because *gin2* shows delayed senescence as well as reduced glucose sensitivity, the sugar levels sensed by hexokinases might have direct or indirect effects on the induction of senescence.

Given the possible interplay between photosynthesis and the initiation of senescence, it would be interesting to identify the signaling molecules that relate photosynthesis to senescence. Because sugars are primary products of photosynthesis, sugar levels could be part of the signaling system. Studies using a gene that is regulated specifically by senescence (*SAG12*) have shown that exogenously supplied sugars can repress gene expression in senescent *Arabidopsis* leaves[25]. Moreover, the expression of this gene can be induced rapidly by sugar deprivation in cultured *Arabidopsis* cells (Y-S. Noh and R.M. Amasino, unpublished). These results suggest that the senescence pathway represented by *SAG12* expression can be activated by low sugar levels. Furthermore, transgenic tomato plants showing accelerated leaf senescence as a result of overexpression of *Arabidopsis* hexokinase, had lower levels of glucose and fructose in their leaves than wild-type plant leaves[29]. However, studies in tobacco plants showed that the glucose and fructose, but not the sucrose, levels increase as the leaves progress through senescence[30]. These increased sugar levels during tobacco-leaf senescence are unexpected in light of the hexokinase-overexpression and *SAG12*-expression studies. Therefore, there is mounting evidence that sugar levels can influence senescence, but more studies are required to determine causal relationships and variation among species.

Mitochondria play an important role in the regulation of animal PCD by sensing the health of their cell. Metabolic perturbations contribute to a loss of mitochondrial membrane integrity releasing elicitors that switch on the cell-death processes[31]. As has been mentioned, plant mitochondria remain functional until the final stages of leaf senescence, making it unlikely that mitochondria act in the same way to initiate the cell death associated with leaf senescence[32]. Perhaps the chloroplast has a regulatory role in leaf senescence similar to the role of mitochondria in animal PCD. Aging or stress that contribute to a loss of photosynthetic output or membrane integrity in the chloroplast might produce signals that initiate the senescence program.

Senescence regulatory genes

A picture is emerging of the conditions that initiate leaf senescence and of the promoter elements required for SAG expression. However, little is known of the regulatory genes that coordinate senescence at the molecular level. Studies of mutants from several species have revealed genes that regulate some aspects of leaf senescence. In soybean and the grass *Festuca pratensis*, mutants have been identified that remain green long after wild-type leaves have yellowed[33]. Often, this so called

'stay-green' phenotype results from a block to chlorophyll degradation. However, these mutants undergo most of the other biochemical changes associated with senescence, indicating that the mutation does not affect the regulation of senescence as a whole.
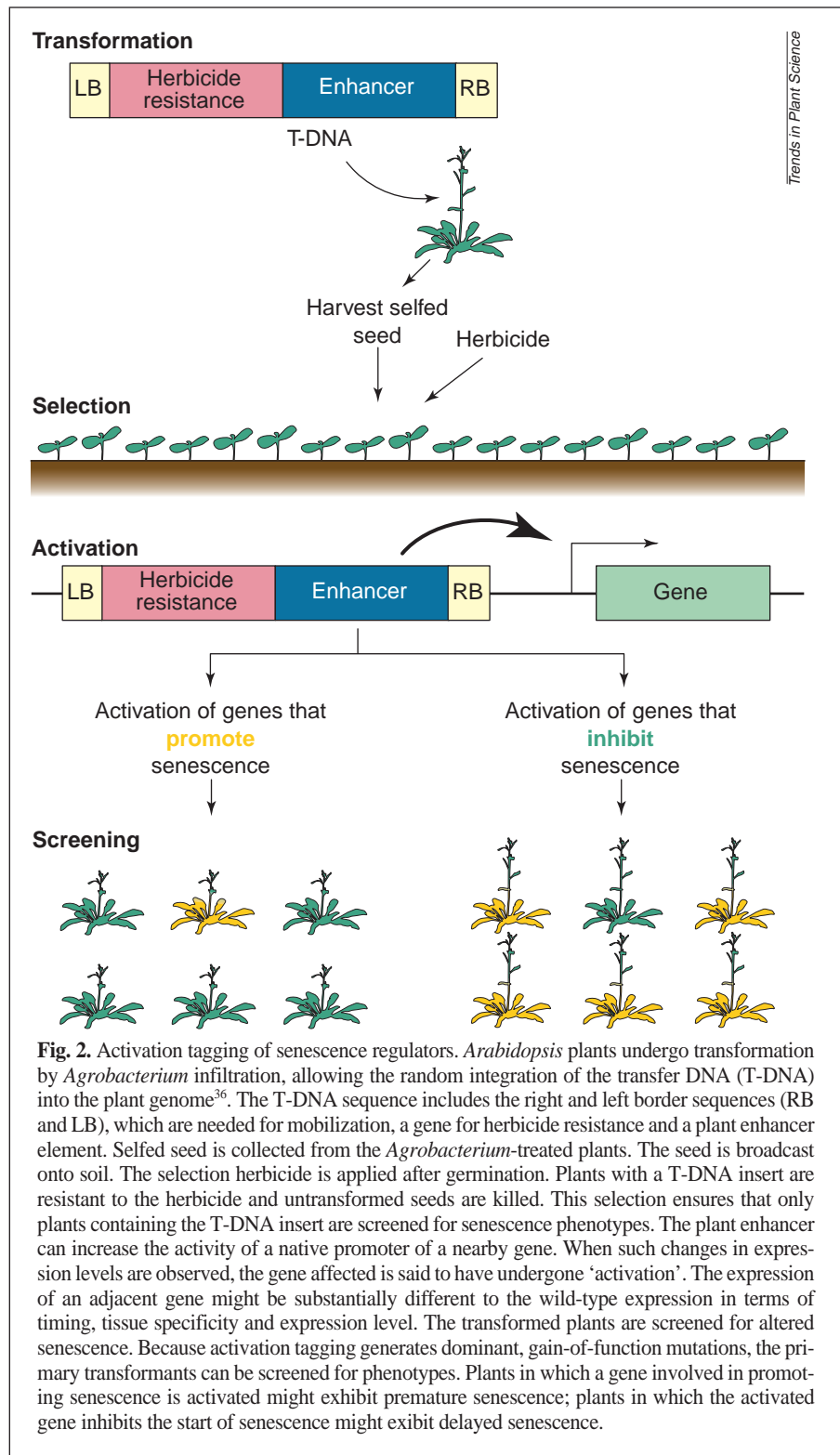
In *Arabidopsis*, several mutants with delayed leaf senescence have been identified[34,35]. Some of these mutants have revealed an overlap between ethylene signaling and leaf senescence. The ethylene-receptor mutant *etr1* was shown to have delayed senescence[35]. In addition, two ethylene-insensitive mutations in the gene *EIN2* were identified in a screen for mutants with delayed senescence[34], as well as two potentially senescence-specific mutants, *ore1* and *ore9* (Ref. 34). In both *ore1* and *ore9* mutants, the senescence syndrome appears to progress normally but is initiated several days later than in wild-type plants[34].

Prospects for future research

The traditional genetic approach might not be sufficient to identify many of the key regulators of leaf senescence. As described, the start of senescence can be affected by many factors, possibly through redundant pathways. Thus, the loss of a regulatory element in one pathway might not significantly alter the senescence program when redundant pathways would still be functional. This means that a genetic approach that mainly generates single loss-of-function mutations might not generate mutants with dramatically altered senescence.

A broad way to search for genes that affect senescence is to use activation tagging[36] (Fig. 2). An activation-tagging vector consists of a DNA-insertion element (T-DNA) with a strong plant promoter or enhancer. Large populations of *Arabidopsis* plants can be generated with a T-DNA integrated randomly into the genome. The promoter or enhancer contained in the T-DNA can cause nearby genes to be expressed at higher levels than would occur normally. These activated genes usually behave as dominant, gain-of-function mutations. In the study of senescence, such a dominant event might be particularly valuable. Although the loss of a senescence regulator might go unnoticed, the activation of a senescence regulator might be apparent. For example, a gene whose product promotes senescence might cause premature senes-

Fig. 2. Activation tagging of senescence regulators. *Arabidopsis* plants undergo transformation by *Agrobacterium* infiltration, allowing the random integration of the transfer DNA (T-DNA) into the plant genome[36]. The T-DNA sequence includes the right and left border sequences (RB and LB), which are needed for mobilization, a gene for herbicide resistance and a plant enhancer element. Selfed seed is collected from the *Agrobacterium*-treated plants. The seed is broadcast onto soil. The selection herbicide is applied after germination. Plants with a T-DNA insert are resistant to the herbicide and untransformed seeds are killed. This selection ensures that only plants containing the T-DNA insert are screened for senescence phenotypes. The plant enhancer can increase the activity of a native promoter of a nearby gene. When such changes in expression levels are observed, the gene affected is said to have undergone 'activation'. The expression of an adjacent gene might be substantially different to the wild-type expression in terms of timing, tissue specificity and expression level. The transformed plants are screened for altered senescence. Because activation tagging generates dominant, gain-of-function mutations, the primary transformants can be screened for phenotypes. Plants in which a gene involved in promoting senescence is activated might exhibit premature senescence; plants in which the activated gene inhibits the start of senescence might exibit delayed senescence.

cence when activated. Similarly, an inhibitor of senescence that is activated might delay senescence in a dominant fashion. Any potential regulator identified by activation tagging will require careful characterization to ensure that its effect on senescence is authentic and not an artefact of its being expressed at higher-than-normal levels by the presence of the enhancer or promoter[36].

Another technical advance that will help to elucidate gene function during senescence is the ability to find insertion mutations in genes. Known as reverse genetics, this process involves PCR-based identification of T-DNA or transposon insertions in known genes[37]. Because most work in senescence has focused on identifying SAGs, a key task is now to identify the functions of these SAGs. Thus, plants with mutations in known SAGs can be isolated and these mutants then screened for phenotypes. A major advantage of this approach is the ability to assemble double, triple and higher-order combinations of mutants and to screen these for phenotypes. Using conventional phenotype-driven genetic approaches, single and (rarely) double mutants are usually

identified because the probability of triple or higher-order mutations randomly occurring in the same plant is extremely low. With a reverse-genetic approach, however, one can readily assemble multiple mutants of SAGs or other genes suspected to play a role in senescence and examine the effect of the multiple-mutant state on the senescence program. By studying the altered senescence regulation, catabolic processes and metabolite export in these mutants, we will begin to gain a molecular understanding of the events that underlie the changing colors of senescent leaves.

Acknowledgements

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