Plant leaf senescence and death – regulation by multiple layers of control and implications for aging in general

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Summary

How do organisms, organs, tissues and cells change their fate when they age towards senescence and death? Plant leaves provide a unique window to explore this question because they show reproducible life history and are readily accessible for experimental assays. Throughout their lifespan, leaves undergo a series of developmental, physiological and metabolic transitions that culminate in senescence and death. Leaf senescence is an 'altruistic death' that allows for the degradation of the nutrients that are produced during the growth phase of the leaf and their redistribution to developing seeds or other parts of the plant, and thus is a strategy that has evolved to maximize the fitness of the plant. During the past decade, there has been significant progress towards understanding the key molecular principles of leaf senescence using genetic and molecular studies, as well as 'omics' analyses. It is now apparent that leaf senescence is a highly complex genetic program that is tightly controlled by multiple layers of regulation, including at the level of chromatin and transcription, as well as by post-transcriptional, translational and post-translational regulation. This Commentary discusses the latest understandings and insights into the underlying molecular mechanisms, and presents the perspectives necessary to enable our system-level understanding of leaf senescence, together with their possible implications for aging in general.

Key words: Aging, Leaf senescence, Chromatin-mediated regulation, Transcriptional regulation, Post-transcriptional regulation, Translational regulation, Post-translational regulation

Introduction

Virtually all organisms, after they age, end their life with senescence followed by death. How and why living organisms senesce and die are fundamental questions in biology. Senescence in plants is defined as the age-dependent programmed degradation and degeneration process of cells, organs or the entire organism, leading to death (Lim et al., 2007a). Senescence in plants occurs at various levels, most distinctively at the organ and organismal levels (Fig. 1). The outcome of plant senescence and death at level of the entire plant can be observed in rice, corn, soybean and wheat fields at their harvest times. By contrast, plant senescence at the organ level is manifested in the spectacular changes in leaf color and the subsequent death of autumn leaves. This type of senescence in plants is post-mitotic senescence (Box 1), whereas in animals, the term senescence is mostly used for mitotic senescence of cells, which refers to the loss of the capacity for undergo further cell division upon aging (Jeyapalan and Sedivy, 2008).

The leaf is the organ that characterizes plants as autotrophs and is perhaps the only practical source of food on earth because they fix carbon using light energy. Leaves initiate their life as leaf primodia. During their development and growth, they become photosynthetically competent and accumulate nutrients. Leaves then enter the senescence stage, followed by their death. Leaf senescence partly involves the process of 'wear and tear' during aging, but mostly is a tightly regulated process with a crucial biological purpose. During senescence, leaf cells undergo a dramatic transition in cellular metabolism and the degradation of cellular structures in an orderly manner; this is most distinctively observed in chloroplasts (Fig. 1B), where it is accompanied by the change of the leaf color owing to the breakdown of chlorophylls. In contrast, the mitochondria and nucleus remain intact until the final stages of leaf senescence. Other symptoms of senescence in plants include increases in lipid peroxidation and membrane leakiness, which are associated with the increase in reactive oxygen species (ROS) generation (Fig. 1C) (Thompson and Lake, 1987; Leshem, 1988). Metabolic changes during leaf senescence include the hydrolysis of macromolecules, such as proteins, lipids, nucleic acids and pigments that were accumulated during the growth phase (Watanabe et al., 2013). In annual plants, these hydrolyzed molecules are relocated to developing seeds and fruits. In trees, the nutrients that are degraded during leaf senescence are stored in stems or roots and are utilized later for the development of new leaves or flowers. The blooming of spring flowers occurs through the utilization of nutrients that have been relocated from senescing autumn leaves. Thus, senescence and death in leaves are active developmental strategies that crucially contribute to the fitness and survival of a plant.

Leaf senescence proceeds with age, but the process involves an intricate and comprehensive regulation of pathways that respond to the previous life stages of the leaf and to various endogenous and exogenous environmental factors in order to adjust its onset, progression and completion (Box 2) (Buchanan-Wollaston et al.,

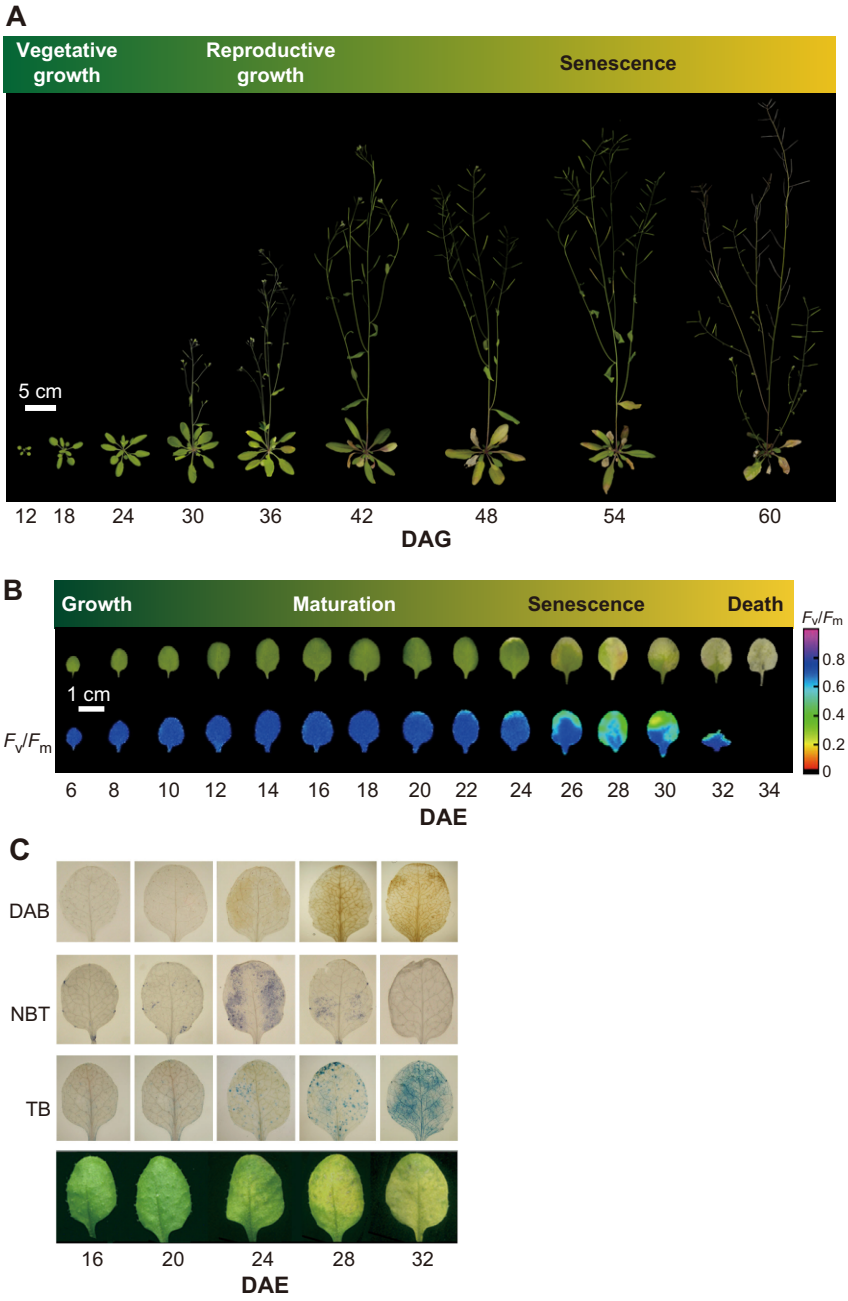


Fig. 1. Whole-plant senescence and leaf senescence in *Arabidopsis*. (A) Characteristics of whole-plant senescence in *Arabidopsis*. Representative wild-type *Arabidopsis* plants (Columbia ecotype) are shown from 12 days after germination (DAG) to 60 DAG at 6-day intervals. (B) Shown here are representative *Arabidopsis* fourth-rosette leaves from 6 days after emergence (DAE) to 34 DAE, shown at 2-day intervals (top row) together with their respective photochemical efficiency (F_v/F_m; bottom row). During senescence, leaves turn yellow and their photochemical efficiency gradually reduces, as indicated by the appearance of green and yellow in the leaves in the bottom row. (C) Levels of ROS and cell death increase with aging. Cell death is visualized here by Tryphan Blue (TB) staining at each time point. The presence of H_2O_2 and superoxide in *Arabidopsis* leaves are indicated by diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining, respectively.

2005; Lim et al., 2007a). A number of questions remain to be addressed regarding leaf senescence, such as what is its molecular nature and how are these molecular changes regulated in a time-dependent manner. Furthermore, how is the age of a leaf recognized and how is this information incorporated to make developmental decisions, such as leaf senescence and death? Another unresolved question is how the leaf senescence and death program interacts with endogenous and exogenous environments. Moreover, how have the molecular networks of the leaf senescence and death program evolved and what is the contribution of senescence genes to plant productivity and fitness? During the past decade, extensive genetic and molecular efforts have been undertaken to unravel the key molecular principles of leaf senescence and death, and have revealed the involvement of multi-layered regulatory modes.

In this Commentary, we present the latest understanding of leaf senescence from the perspective of chromatin-based, transcriptional, post-transcriptional, translational and post-translational regulation (Fig. 2; supplementary material Table S1). Although our focus is on leaf senescence in the model plant *Arabidopsis*, we also discuss the relevant findings in other organisms, including major crops.

Transcriptional regulation of plant leaf senescence

The onset, progression and completion of leaf senescence are regulated in a highly coordinated manner and involve an extensive reprogramming of gene expression. Temporal profiling of the transcriptome during *Arabidopsis* leaf senescence has revealed a global picture of senescence and its

Box 1. Glossary

Mitotic senescence: also known as proliferative senescence; demonstrated in the arrest of cell division in germ-line-like meristematic cells and the arrest of mitotic cell division at the early stages of fruit development; similar to replicative senescence in yeast and animal cells in culture.

Post-mitotic senescence: an active degenerative process; leads to the death of a cell that no longer undergoes mitotic cell division; occurs in some plant organs, such as leaves and floral petals; predominantly involves somatic organs.

NAC (NAM, ATAF1, 2, and CUC2) transcription factors: contain N-terminal NAC DNA-binding domains; comprise 117 members in *Arabidopsis*; are specific to plants and associated with diverse processes including the formation of secondary walls and biotic/abiotic stress responses, as well as leaf senescence.

WRKY transcription factors: characterized by their 60-amino-acid DNA-binding WRKY domain containing a zinc-finger motif; comprise 74 members in *Arabidopsis*; are specific to plants and involved in the regulation of various processes, such as biotic/abiotic stress responses, development and seed germination.

tasiRNAs (trans-acting small-interfering RNAs): a specialized class of endogenous small RNAs that originate from TAS-gene-derived transcripts; act in trans to regulate mRNAs at the post-transcriptional level, similar to miRNAs; are generated after the TAS transcripts are cleaved by an miRNA (in *Arabidopsis*, *miR173* triggers tasiRNA production from *TAS1* and *TAS2*, *miR390* targets *TAS3*, and *miR828* targets *TAS4*).

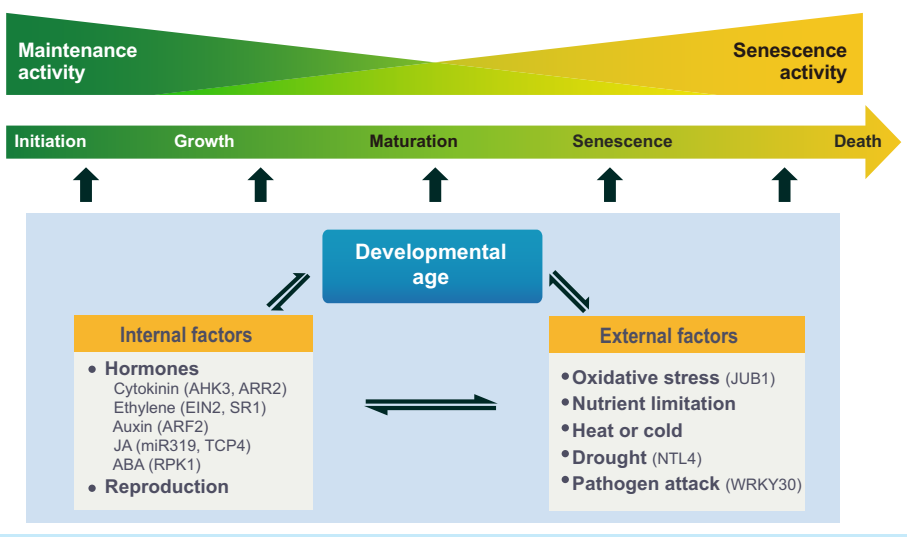
regulation that involves thousands of senescence-associated genes (SAGs), which is consistent with known biochemical and physiological data (Weaver et al., 1998; Miller et al., 1999; Hinderhofer and Zentgraf, 2001; Gepstein et al., 2003; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005; Pourtau et al., 2006; van der Graaff et al., 2006; Breeze et al., 2011). As leaf senescence is accompanied by genome-wide changes in gene expression, the dynamic activation of transcription factors is thought to be a key mechanism that controls the age-dependent expression of the thousands of SAGs. Two transcription factor families, NAC and WRKY, are the major transcription factors that regulate leaf senescence (Box 1). Intriguingly, these transcription factors are also induced by various stress responses, which is consistent with the notion that senescence is an integrated response of plants to endogenous developmental signals and environmental cues.

NAC transcription factors

Genome-wide transcriptome analyses have revealed that more than 30 NAC genes (Box 1) are significantly upregulated during leaf senescence in *Arabidopsis* (Jensen et al., 2010; Breeze et al., 2011; Kou et al., 2012). Functional analyses of mutants and/or transgenic plants with either impaired or overexpressed NAC genes show altered leaf senescence phenotypes, and the subsequent identification of their upstream regulatory factors and downstream targets have uncovered the gene regulatory networks that control leaf senescence (Guo and Gan, 2006; Zhang and Gan, 2012; Hickman et al., 2013). For example, NAC-LIKE, ACTIVATED BY AP3/PI (AtNAP/ANAC029), is a key senescence-regulating NAC transcription factor in *Arabidopsis*. Inducible overexpression of *NAP* in young leaves triggers precocious senescence, whereas a knockout mutant of *NAP* exhibits retarded leaf senescence (Guo and Gan, 2006). NAP is thought to regulate leaf senescence partially through its direct binding to the promoter of *SAG113*, a negative regulator of the

Box 2. Overview of the molecular basis of leaf growth and senescence

The self-maintenance activity of plant leaves is retained during growth and decreases at the senescence stage, whereas the senescence activity of leaves increases throughout their lifespan. Although the initiation of senescence cannot be clearly defined, the initiation and progression of leaf senescence depend on developmental age and are also influenced by various internal and external factors as illustrated in the figure. Internal factors include various phytohormones and reproduction. Cytokinin is the most effective senescence-retarding plant hormone, whereas ethylene, jasmonic acid (JA), abscisic acid (ABA) and salicylic acid are known to promote leaf senescence. Environmental factors that affect leaf senescence include oxidative stress, high or low temperature, drought, nutrient deficiency and pathogen infection.



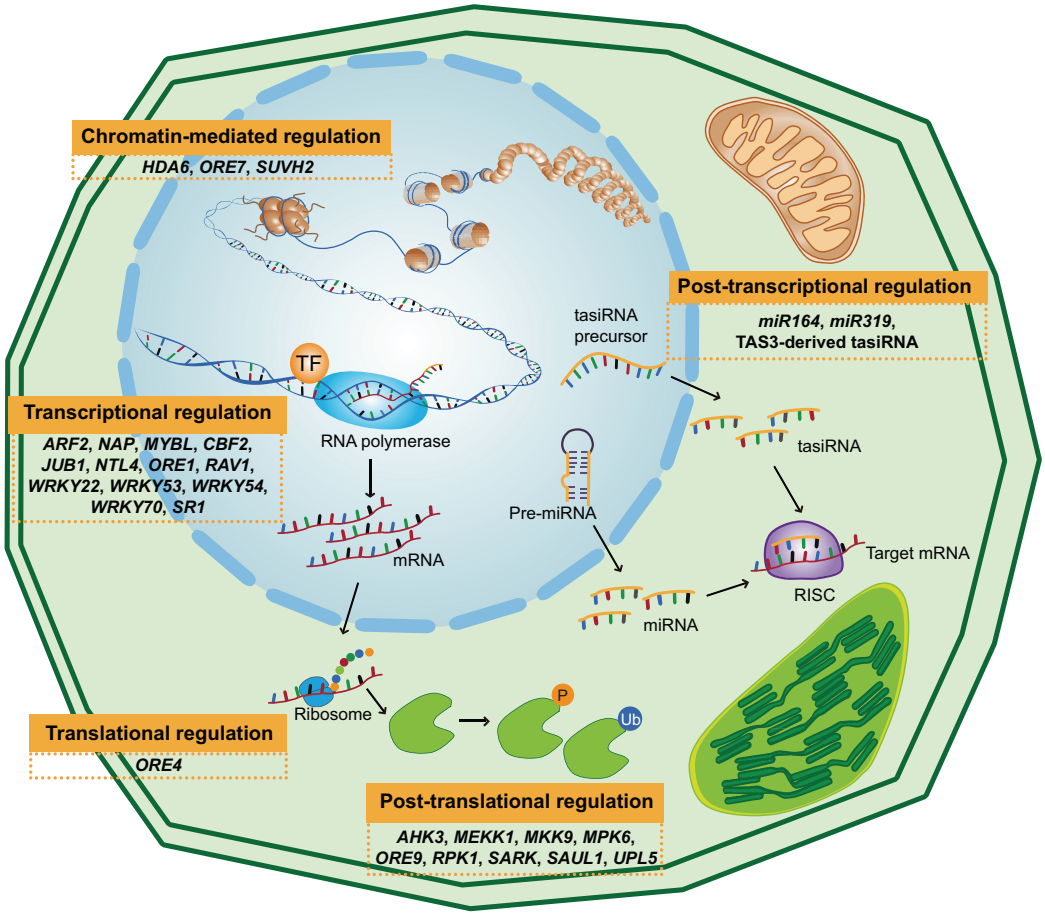


Fig. 2. Overview of the multiples layers of the regulation in leaf senescence. Multiple layers of regulation are involved in the control of leaf senescence, and illustrated here are chromatin-mediated, transcriptional, post-transcriptional, translational and post-translational modes of regulation. Chromatin-mediated regulation of leaf senescence includes histone modification and changes in chromatin architecture. Genome-wide changes in the expression of senescence-associated genes (SAGs) during leaf senescence involve the dynamic activation and/or suppression of diverse transcription factors (TFs). Post-transcriptional regulation of leaf senescence is primarily implemented by miRNAs and tasiRNAs. Post-translational modifications, such as phosphorylation and ubiquitylation, are also important for modulating leaf senescence. Key genes that control leaf senescence at each of the regulatory layer are indicated. Pre-miRNA, premature microRNA; RISC, RNA-induced silencing complex; Ub, ubiquitin.

abscisic acid (ABA) pathway that inhibits stomatal closure, which in turn triggers leaf senescence (Zhang and Gan, 2012).

ORESARA1 (ORE1, ANAC092) is another NAC transcription factor that is a positive regulator of leaf senescence in *Arabidopsis* (Kim et al., 2009; Balazadeh et al., 2010). Expression of *ORE1* is induced during leaf aging by ETHYLENE INSENSITIVE 2 (EIN2), but is negatively regulated by the microRNA164 (miR164; discussed below). ORE1 exerts its regulatory function by controlling the expression of 170 genes, including that of 78 known SAGs (Balazadeh et al., 2010). BIFUNCTIONAL NUCLEASE1 (BFN1) is a direct target of ORE1 (Matallana-Ramirez et al., 2013), suggesting that ORE1 regulates the degradation of nucleic acids by activating the expression of *BFN1* during leaf senescence (Fig. 3). Recently, the characterization of ORE1-interacting proteins led to the postulation of a new regulatory mechanism of leaf senescence that is mediated by ORE1 (Rauf et al., 2013). That study found that ORE1 interacts with the G2-like transcription factors GLK1 and GLK2, which are important for chloroplast development and maintenance. During early leaf development, GLKs are highly expressed, which results in the activation of target genes such as photosynthesis-related genes. As leaves get older, the expression of *ORE1* is increased, which in turn, sequesters GLKs and results in their reduced transcriptional activity. Therefore, ORE1 appears to control leaf senescence by triggering the expression of many SAGs and by modulating the activity of other transcription factors through protein–protein interaction.

JUNGBRUNNEN1 (JUB1, ANAC042) is an H_2O_2-induced NAC transcription factor that activates the expression of *DREB2A* and of several ROS-responsive genes (Wu et al., 2012). Transgenic *Arabidopsis* plants overexpressing *JUB1* exhibit delayed leaf senescence and are tolerant to various abiotic stresses. In contrast, *jub1* knockdown plants exhibit precocious senescence and reduced abiotic stress tolerance. These data indicate that JUB1 negatively regulates leaf senescence by lowering cellular H_2O_2 levels, thereby diminishing the effect of positive regulators in leaf senescence. In addition, a drought-responsive NAC transcription factor, NAC WITH TRANSMEMBRANE MOTIF 1-LIKE 4 (NTL4), plays a role in coupling ROS metabolism to drought-induced leaf senescence (Lee et al., 2012). NTL4 binds directly to the promoters of genes that

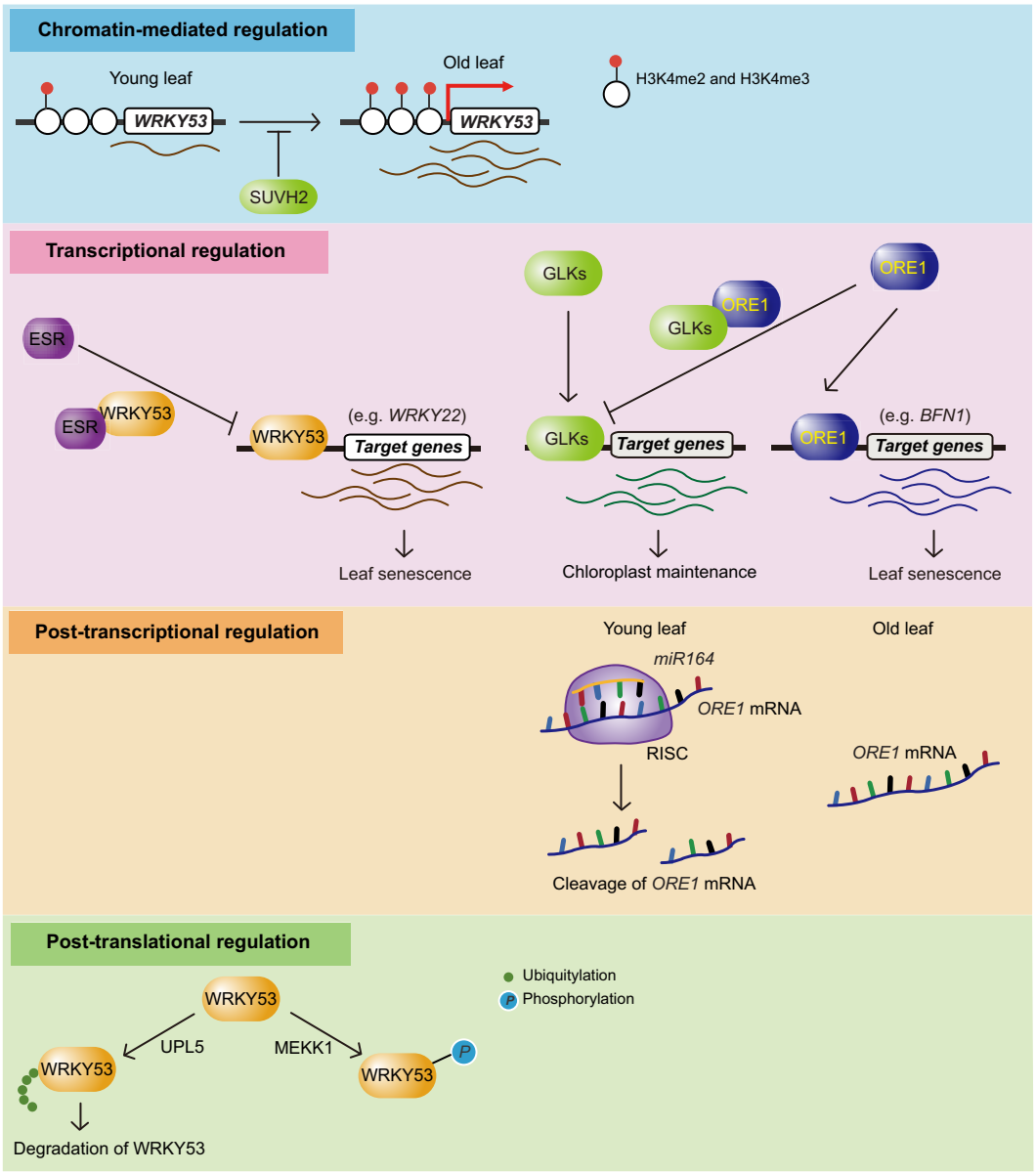


Fig. 3. Multiple layers of regulation that involve WRKY53 and ORE1 to control leaf senescence. Two well-known positive regulators of senescence are WRKY53 and ORE1; they control leaf senescence through diverse means and are also subject to different modes of regulation. Upregulation of *WRKY53* expression during leaf aging is partly dependent on an increase of active histone marks modulated by SUVH2 (chromatin-mediated regulation). In addition, different post-translational modifications also control the levels of WRKY53. For instance, phosphorylation of WRKY53 by MEKK1 increases its binding to its targets, and ubiquitylation of WRKY53 by UPL5 appears to degrade WRKY53 (post-translational regulation). As a transcription factor, WRKY53 regulates the expression of many SAGs and stress-related genes (transcriptional regulation). It is also known that WRKY53 is inactivated by interacting with ESR. Expression of *ORE1* is regulated in an age-dependent manner by EIN2 and miR164. In the young *Arabidopsis* leaves, miR164 suppress *ORE1*, but in their old stage, EIN2 suppresses miR164 and thereby induces *ORE1* expression, which leads to age-dependent senescence and cell death of leaves (post-transcriptional regulation). ORE1 controls the expression of many SAGs including *BFN1* (transcriptional regulation). ORE1 also modulates the activity of GLKs through protein–protein interactions.

encode enzymes involved in ROS biosynthesis during drought-induced leaf senescence to promote ROS production. Collectively, these findings indicate that NAC transcription factors integrate different internal and environmental signals with the developmental age and contribute to execute the senescence program.

The importance of NAC transcription factors as central regulators of leaf senescence has also been demonstrated in major crops. NAM-B1, an NAC transcription factor in ancestral wild wheat (*Triticum turgidum* L. ssp. *dicoccoides*), promotes senescence and facilitates nutrient remobilization from leaves to grains, thereby improving the protein, zinc, and iron content of the grain (Uauy et al., 2006; Waters et al., 2009). Two *Brassica napus* NAC transcription factors (NAC2 and NAC5) (Zhong et al., 2012) and a bamboo NAC transcription factor (NAC1) (Chen et al., 2012) have also been shown to regulate leaf senescence when they are expressed in *Arabidopsis* plants.

WRKY transcription factors

The involvement of the WRKY transcription factor family (Box 1) in regulating leaf senescence has also been intensively studied. WRKY53 has a positive role in leaf senescence by targeting various SAGs, including pathogen-related genes, stress-related genes and transcription factor genes (Fig. 3) (Miao et al., 2004). Interestingly, the DNA-binding activity of WRKY53 is inhibited by its interaction with EPITHIOSPECIFYING SENESCENCE REGULATOR (ESR) (Fig. 3), which functions as a negative regulator of leaf senescence (Miao and Zentgraf, 2007). WRKY54 and WRKY70 also have roles in leaf senescence. Although the leaves of single mutants of *WRKY54* and *WRKY70* show either no noticeable or only a weak premature senescence phenotype, respectively, the leaves of the *wrky54 wrky70* double mutant exhibit a clear early-senescence phenotype (Besseau et al., 2012), indicating that WRKY54 and WRKY70 have cooperative and partly redundant functions in controlling leaf senescence. WRKY53, WRKY54 and WRKY70 all interact independently with WRKY30, which has been suggested to be involved in ROS signaling (Scarpeci et al., 2013). Therefore, the negative senescence regulators WRKY54 and WRKY70 might regulate senescence in concert with the positive senescence factor WRKY53, possibly through interactions with WRKY30. Moreover, WRKY22, a downstream target of WRKY53, is a positive regulator of dark-induced leaf senescence (Zhou et al., 2011); ectopic expression of *WRKY22* accelerates leaf senescence, whereas *wrky22*-knockout plants exhibit delayed leaf senescence. Taken together, these observations imply that the combinatorial interactions of WRKYs integrate both positive and negative signals that result in the complex transcriptional networks necessary for fine-tuning the regulation of leaf senescence at the transcriptional level.

Other transcription factors

The functions of other transcription factors in the regulation of leaf senescence have also been studied. In *Arabidopsis*, the Related to ABI3/VP1 (RAV) transcription factor family member RAV1 has been proposed to positively regulate leaf senescence (Woo et al., 2010). In addition, the ectopic expression of *MYBL*, an *Arabidopsis* R-R-type MYB-like transcription factor, causes precocious leaf senescence, indicating that MYBL is a positive regulator of leaf senescence (Zhang et al., 2011). Furthermore, C-REPEAT/DEHYDRATION RESPONSIVE ELEMENT BINDING FACTOR 2 (CBF2) appears to be a negative regulator of leaf senescence, as its overexpression delays leaf senescence (Sharabi-Schwager et al., 2010). Interestingly, several transcription factors that are associated with hormone signaling have been found to be regulators of leaf senescence. For example, AUXIN RESPONSE FACTOR 2 (ARF2) has an important role in modulating auxin-mediated leaf senescence (Lim et al., 2010), and SIGNAL RESPONSIVE 1 (SR1), a calmodulin-binding transcription factor, regulates ethylene-induced senescence by directly binding to the *EIN3* promoter, a positive transcription factor in the ethylene signaling pathway (Nie et al., 2012).

Gene regulatory networks

Recent high-throughput and computational analyses have identified a number of gene regulatory networks that are involved in leaf senescence. For example, a network that is controlled by *ORE1* during salt-stress-induced leaf senescence has been delineated by using microarray-based expression profiling of inducible *ORE1*-overexpressing transgenic whole plants (Balazadeh et al., 2010). Similarly, Breeze et al. proposed a gene regulatory network model on the basis of temporal expression profiling of SAGs during *Arabidopsis* leaf senescence (Breeze et al., 2011). Their network model predicts the effects of ORE1 on the expression of a number of its known downstream target genes, as well as on several stress-related transcription factors. Information on co-regulated pathways and promoter motif analysis could further expand these transcriptional network models. For instance, a gene regulatory network model that involves ANAC019, ANAC055 and ANAC072, all with known roles in leaf senescence and stress responses, has been generated from high-throughput yeast one-hybrid assays and timecourse gene expression data (Hickman et al., 2013) and allowed the identification of potential upstream regulatory genes, as well as predicted new downstream genes. Further approaches that combine chromatin immunoprecipitation sequencing (ChIP-seq), gene expression profiling, and computational analyses will contribute to elucidate the complex gene networks that regulate the signaling pathways of different senescence-affecting factors and how these pathways are interlinked.

Chromatin-mediated regulation

In eukaryotes, the remodeling of the chromatin structure regulates the accessibility of the transcriptional machinery to DNA and so controls gene expression. Chromatin structure is modified by the binding of histones to DNA and histone post-translational modifications, including acetylation, methylation and phosphorylation, as well as by DNA methylation, which affects the ability of proteins to bind to the chromatin (Peterson and Laniel, 2004). A growing body of evidence indicates that regulation of the chromatin structure through histone modification and chromatin-remodeling enzymes is a key mechanism in the control of leaf senescence. Here, we will mainly focus on the regulation of SAGs as mediators of leaf senescence by chromatin-mediated mechanisms.

A recent elegant study that combines ChIP-seq and gene expression analysis revealed genome-wide changes in histone methylation that are associated with leaf senescence in *Arabidopsis* (Brusslan et al., 2012). That study found that genes with increased levels of histone H3 trimethyl lysine 4 (H3K4me3), a mark of actively transcribed chromatin, are upregulated in old leaves during leaf senescence, whereas genes that show decreased levels of this histone mark in old leaves are downregulated compared with that in young leaves. With regard to histone H3 trimethyl lysine 27 (H3K27me3), an inactive histone mark, they found that genes that lose this mark in old leaves are upregulated during leaf senescence. This study demonstrates the importance of histone methylation in the regulation of gene expression during leaf senescence. Further evidence for a link between histone methylation and regulation of leaf senescence comes from an analysis of the histone methyltransferase SU(VAR)3-9 HOMOLOG 2 (SUVH2) (Ay et al., 2009). Overexpression of *SUVH2* results in inhibition of the transcriptional activation of *WRKY53* and its targets, leading to delayed leaf senescence. Furthermore, senescence-specific increases in the active histone marks H3 dimethyl lysine 4 (H3K4me2) and H3K4me3 in the promoter region of *WRKY53* are reduced in *SUVH2*-overexpressing transgenic plants. Moreover, a significant increase in the inactive histone marks H3K27me2 and H3K27me3 at the 5′ end of *WRKY53* is found in *SUVH2*-overexpressing plants, but not in the wild type.

HISTONE DEACETYLASE 3 (HDA6), an RPD3-type histone deacetylase, is another well-known factor that regulates leaf senescence (Wu et al., 2008). HDA6 affects the global levels of histone acetylation and is involved in many aspects of plant development, including the response to jasmonic acid (JA), leaf senescence and flowering time. A loss-of-function mutation of *HDA6* causes delayed leaf senescence phenotypes, including downregulation of the expression of several SAGs, although it is not clear yet whether HDA6 directly affects the expression of these SAGs. Another example for regulation of leaf senescence at the chromatin level is ORE7 (also called ESCAROLA), which is an AT-hook DNA-binding protein (Lim et al., 2007a; Lim et al., 2007b). Overexpression of *ORE7* results in delayed leaf senescence and changes in the organization of chromatin during interphase.

As for plants, animals, including humans, experience alterations in chromatin organization and associated gene expression changes as they age (Gonzalo, 2010). Several lines of evidence indicate that there is a global hypomethylation of the genome during aging, concomitant with an increased methylation of specific gene promoter regions (Calvanese et al., 2009). The global decrease in DNA methylation is caused by a progressive decrease in the efficacy of DNA methyltransferase I to act on heterochromatic regions with aging (Fraga and Esteller, 2007). Changes in the pattern of histone methylation are another key factor in chromatin-mediated regulation of gene expression during aging. For example, the *Drosophila* histone demethylase (HDM) Kdm4A, a homolog of the human JMJD2 family, controls genes that are required for lifespan (Lorbeck et al., 2010); its disruption in *Drosophila* results in a reduction in the lifespan of the male and the downregulation of the expression of *Hsp22*, which is associated with longevity. Another example for the role of histone methylation in controlling lifespan comes from the ASH-2 trithorax complex, which trimethylates H3K4 in *Caenorhabditis elegans* (Greer et al., 2010). Deficiencies in members of the ASH-2 complex, comprising ASH-2, WDR-5 and the histone methyltransferase SET-2, extend the lifespan in the parental generation and regulate the lifespan of descendants over several generations (Greer et al., 2011). Thus, it is becoming evident that dynamic chromatin modifications are important in altering gene expression patterns during aging, and, hence, control leaf senescence. Furthermore, chromatin-mediated gene regulation appears to be an evolutionarily conserved mechanism for controlling aging, senescence and lifespan in plants and in animals.

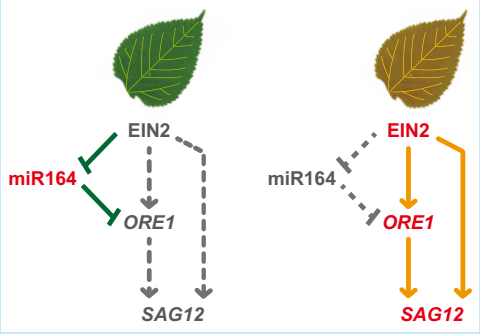
Post-transcriptional regulation

Once transcribed, mRNAs undergo post-transcriptional regulation, notably the regulation of mRNA stability. Recently, non-coding RNAs (ncRNAs), such as small-interfering RNAs (siRNAs) and miRNAs, have been reported to be important regulators that control mRNAs at the post-transcriptional level. In animals, the importance of miRNAs in regulating cellular senescence and aging processes is well known. In *C. elegans*, lin-4 (the first miRNA to be identified) regulates lifespan (Boehm and Slack, 2005). Furthermore, miR-71 in *C. elegans* and miR-17-92 in mammals are regulators of cellular senescence and aging (de Lencastre et al., 2010; Grillari et al., 2010).

In plants, an increasing number of reports describe the contributions of small ncRNAs, including miRNA and trans-acting siRNA (tasiRNA; Box 1), to leaf senescence. Notably, miR164 and its target *ORE1* control leaf senescence in *Arabidopsis*

Box 3. Trifurcate feed-forward pathway for age-dependent senescence and cell death

The trifurcate feed-forward pathway for age-dependent cell death involves EIN2, ORE1 (a plant-specific NAC-family transcription factor, also known as ANAC092) and miR164 (Kim et al., 2009). ORE1 is a transcription factor that has a positive role in cell death and its expression is induced in an age-dependent manner by EIN2. *ORE1* is negatively regulated by miR164 at earlier stages of leaf life, which is alleviated at their later stages because of the age-dependent downregulation of miR164 expression by EIN2. Mathematical modeling suggests that the trifurcate loop is a robust biological mechanism that ensures death upon aging (Kim et al., 2009). The modeling further shows that a transient induction of EIN2 does not lead to death owing to the presence of the miR164 pathway, thereby demonstrating a new paradigm of transitions between regulatory networks in age-dependent leaf senescence and cell death process. The figure is adapted from Kim et al. (Kim et al., 2009)



(Kim et al., 2009). As mentioned above, the expression of this positive regulator of leaf senescence is negatively regulated by miR164 (Fig. 3). The gradual decrease in miR164 expression with leaf aging, through the activation of EIN2, ultimately results in the upregulation of *ORE1* expression. This finding has led to the postulation of a trifurcate feed-forward pathway (Box 3) for age-dependent leaf senescence comprising *EIN2*, *ORE1* and miR164. In addition to miR164, miR319, through its targets, the TCP (TEOSINTE BRANCHED/CYCLOIDEA/PCF) transcription factors, which appear to coordinate leaf growth and senescence, regulates leaf senescence, partly by modulating JA biosynthesis. miR319-overexpressing plants exhibit delayed leaf senescence, whereas overexpression of *TCP4*, a *miR319* target, causes premature leaf senescence (Schommer et al., 2008). Taken together, these reports implicate miRNAs as important leaf senescence regulators in plants, as well as in animals.

Another interesting group of post-transcriptional regulators of leaf senescence are the tasiRNAs, a plant-specific class of endogenous small RNAs. For example, miR390 triggers the production of the tasiRNAs that are generated from *TAS3*; one of their targets is *ARF2*, a positive regulator of leaf senescence (Lim et al., 2010; Marin et al., 2010). This observation suggests that miR390 promotes leaf senescence by controlling the level of the *TAS3*-modulated mRNA *ARF2*.

On the basis of these above studies, it is clear that post-transcriptional regulation appears to play a much larger role in diverse biological processes than previously thought and the regulation of leaf senescence is likely to involve additional

types of post-transcriptional mechanisms, including alternative splicing, mRNA editing, nonsense-mediated decay, long ncRNAs and natural antisense transcripts.

Translational regulation

Translational regulation, including translation initiation and elongation, is known to further modulate gene expression in a wide range of biological scenarios. However, only a little is known about the regulation of leaf senescence at the translational level, and elucidating its role will be an important challenge for unveiling the mechanisms underlying leaf senescence.

The *Arabidopsis* mutant *ore4*, which possesses a knockdown mutation of the *PLASTID RIBOSOMAL SMALL SUBUNIT PROTEIN 17* (*PRPS17*) gene, exhibits delayed leaf senescence phenotypes (Woo et al., 2002). Because PRPS17 is a component of the plastid ribosome, the reduced expression of *PRPS17* in the *ore4* mutant is expected to result in reduced translation rate in the chloroplast. Similarly, several recent studies in *C. elegans* and *Drosophila* have revealed a link between protein synthesis and aging, and have shown that reduced mRNA translation extends lifespan (Arquier et al., 2005; Hansen et al., 2007; Syntichaki et al., 2007). Further characterization of PRPS17 might provide important insights into the relationship between translational regulation of plastid genes and leaf senescence.

An interesting recent study found that the synthesis of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) subunits is controlled at the translational level during rice leaf senescence (Suzuki and Makino, 2013). Rubisco in higher plants is composed of eight small subunits, which are encoded by the RBCS family of nuclear genes, and of eight large subunits, encoded by RBCL plastid genes (Dean et al., 1989). RBCS subunits are translated in the cytoplasm and transported into the plastid, where they are assembled with the RBCL subunits. As these two Rubisco components are encoded in different gene compartments, but need to be assembled stoichiometrically to form the holoenzyme, their expression must be tightly coordinated. The mRNA levels of RBCS subunits gradually decrease during leaf aging, but the decline in the mRNA levels of RBCLs is slower than that in RBCS. Interestingly, the difference in the amount of mRNA is compensated for through a modulation of the translation rate of the two mRNA species, implying that there is a mechanism that coordinates the expression of the Rubisco subunits at the translational level. Further extensive protein profiling approaches need to be undertaken to elucidate the mechanisms that underlie the translational regulation of leaf senescence.

Post-translational regulation

Different post-translational modifications, such as phosphorylation, glycosylation, ubiquitylation, methylation and acetylation affect the conformation, activity, stability and localization of proteins. Therefore, it is not surprising that post-translational regulation is emerging as an additional level of control of leaf senescence, as discussed below.

Protein phosphorylation

Genome-wide expression analyses implicate protein phosphorylation in the regulation of leaf senescence; the expression of numerous protein kinases and phosphatases is altered during aging of *Arabidopsis* (Breeze et al., 2011), and several key kinases and phosphatases have an important role in modulating leaf senescence, such as the members of the mitogen-activated protein kinase (MAPK) signaling cascade, MAP KINASE KINASE 9 (MKK9) and MAP KINASE 6 (MPK6). MKK9 directly phosphorylates and activates MPK6 (Zhou et al., 2009) and the expression of *MKK9* increases during leaf senescence in *Arabidopsis* (Zhou et al., 2009). The *mkk9* loss-of-function *Arabidopsis* mutant exhibits delayed leaf senescence, whereas overexpression of *MKK9* leads to precocious leaf senescence. The *mpk6* loss-of-function mutant also shows delayed leaf senescence. Notably, the loss-of-function mutation of *MPK6* partially suppresses the premature senescence phenotype obtained by overexpression of *MKK9*, indicating that MKK9 functions upstream of MPK6 to promote leaf senescence, although it also might have additional target(s). The above-mentioned transcription factor WRKY53 is also a target of the MAPK pathway; it is phosphorylated by MEKK1 (Fig. 3), which increases its ability to bind to DNA at its target promoters (Miao et al., 2007). The roles of the MAPK cascade that involves MEKK1–MKK1/2–MPK4 in biotic stress signaling have been also well established (Pitzschke et al., 2009). Thus, MEKK1 appears to coordinate biotic stress response with the induction of senescence through WRKY53.

Another interesting example for the role of protein phosphorylation in the regulation of leaf senescence is *Arabidopsis* histidine kinase 3 (AHK3), a cytokinin receptor with histidine kinase activity. Cytokinins are potent senescence-retarding hormones. However, the molecular mechanism of cytokinin-mediated delay of plant senescence long remained unknown until a mutant (*ore12-1*) with a gain-of-function mutation in the extracellular domain of *AHK3* was isolated; this gain-of-function mutant and transgenic overexpression of *AHK3* delayed leaf senescence (Kim et al., 2006). ARR2, a type-B *Arabidopsis* response regulator, is phosphorylated by AHK3 in a cytokinin-dependent manner (Kim et al., 2006). Furthermore, whereas overexpression of *ARR2* leads to delayed leaf senescence, overexpression of ARR2 in which the AHK3 phosphorylation site is mutated does not, suggesting that ARR2 phosphorylation by AHK3 controls leaf senescence.

Recently, two receptor-like protein kinases were identified as important regulators of leaf senescence, probably through modulating plant hormone signaling (Lee et al., 2011). RECEPTOR PROTEIN KINASE 1 (RPK1) is an ABA-inducible membrane-bound receptor kinase and has an important regulatory role in ABA-mediated and age-dependent leaf senescence. Loss-of-function mutations in *RPK1* lead to a significant delay in ABA-induced and age-dependent leaf senescence (Lee et al., 2011). However, the exact mechanism by which RPK1 receives and transduces senescence signals to its targets to regulate leaf senescence requires further investigation. Soybean (*Glycine max*) SENESCENCE-ASSOCIATED RECEPTOR-LIKE KINASE (GmSARK) and its *Arabidopsis* homolog (AtSARK) were shown to be positive regulators of leaf senescence (Xu et al.,2011). The authors found that GmSARK-overexpressing *Arabidopsis* plants exhibit altered responses to multiple hormones, such as ethylene, auxin and cytokinin. Moreover, the overexpression of GmSARK or its *Arabidopsis* homolog AtSARK results in premature leaf senescence, which could be suppressed by the inhibition of auxin transport or ethylene signaling. These findings imply that *SARK* might regulate leaf senescence, as well as hormone responses, by affecting the phosphorylation status of their target proteins.

Protein ubiquitylation

Ubiquitin-mediated post-translational modification is being recognized as a key regulatory step in diverse physiological processes, including cell cycle progression, environmental stress responses and hormone signaling (Komander and Rape, 2012). In various animal systems, the activity of the proteasome, which in most cases degrades ubiquitylated target proteins, decreases during aging, and these changes might be causally related to aging and age-associated diseases (Löw, 2011). Post-translational modifications by ubiquitin and ubiquitin-like proteins also appear to be important for the regulation of leaf senescence. Several ubiquitin-dependent degradation pathways are associated with leaf senescence, and in plants, the E3 Ub-ligases are responsible for polyubiquitylation of proteins and their subsequent selective degradation by the 26S proteasomes.

The *Arabidopsis ore9* mutant was originally isolated from a forward genetic screen for mutants with delayed leaf senescence phenotypes (Oh et al., 1997; Woo et al., 2001). Subsequent work showed that ORE9, also referred to as MORE AXILLARY GROWTH 2 (MAX2), is also involved in photomorphogenesis (Shen et al., 2007), branching (Stirnberg et al., 2002), as well as signaling by strigolactone (Gomez-Roldan et al., 2008; Umehara et al., 2008) and karrikin (Nelson et al., 2011). ORE9 is an F-box protein and forms an S-phase kinase-associated protein 1 (SKP1)/Cullin/F-box protein (SCF) E3 ligase complex, SCF^{ORE9}, which might target specific substrates, including key negative regulators of leaf senescence. A more direct evidence for the control of leaf senescence by ubiquitin-mediated protein degradation has been shown for the HECT domain E3 ubiquitin-protein ligase UPL5, which binds to WRKY53 (Miao and Zentgraf, 2010). Here, inducible overexpression of *UPL5* results in decreased levels of WRKY53, implying that UPL5 is involved in WRKY53 degradation, most probably through its ubiquitin-ligase activity. The senescence phenotype of *UPL5*-knockout plants is similar to that of plants overexpressing WRKY53, further supporting the notion that UPL5 regulates leaf senescence through the degradation of WRKY53, which is a positive regulator of leaf senescence. Another ubiquitin-ligase involved in leaf senescence is SENESCENCE-ASSOCIATED UBIQUITIN LIGASE 1 (SAUL1), a plant U-box-armadillo E3 ubiquitin-ligase (Raab et al., 2009). SAUL1 is a negative regulator of plant senescence, as *saul1* loss-of-function mutants display early senescence under low-light conditions. In addition, the expression of key senescence regulators, such as *ORE1*, *WRKY53* and *WRKY6* is altered in *saul1* mutants (Vogelmann et al., 2012). Taken together, these studies support the importance of protein ubiquitylation in the control of leaf senescence. Future challenges include determining the targets for ubiquitylation and their molecular function within the context of leaf senescence control. We anticipate that ubiquitin-mediated regulation is far more important for leaf senescence than the few available reports discussed here might suggest.

Conclusions and future perspectives

Evidently, plant leaves are a unique and readily amenable genetic system to understand processes that are associated with aging, senescence and death. Furthermore, leaf senescence provides another unique opportunity to investigate the process of ordered degradation, whereas many biological questions address biogenesis and assembly processes. It is now obvious that leaf senescence is regulated by a tightly controlled genetic program that involves regulatory mechanisms acting at multiple levels. Nevertheless, we currently only have an incomplete picture of molecular processes underlying leaf senescence.

Considering that leaf senescence is a highly complex process that involves the collective functions of multiple genes and signaling pathways that integrate age information and various endogenous and exogenous signals throughout the leaf lifespan, it is not surprising that leaf senescence is controlled with multiple layers of regulation. Furthermore, senescence is not a single state, but involves continuous time-dependent transitions of cellular physiology and metabolism. Thus, senescence needs to be understood from a temporally dynamic perspective. Senescence is also expected to be a spatially coordinated process that includes cell–cell and organ–organ interactions. Therefore, fully understanding leaf senescence might require a paradigm shift in analyzing and describing the process. In this regard, the use of multiple 'omics' approaches, such as analysis of the total RNA transcriptome, proteome, metabolome and phenome at multiple time points, coupled with computational modeling, will be a crucial next step towards a better understanding of leaf senescence and death. Other system-level data sets, such as protein and genetic interactions or protein localization maps, could be also integrated into these approaches.

We would like to emphasize the importance of analyzing the leaf phenome to obtain spatio-temporal information for morphological and physiological transitions to better understand the senescence processes. To date, leaf senescence has mostly been studied only at senesced and aged states. However, plants undergo a series of developmental and age-associated transitions throughout their lifecycle. Thus, senescence and subsequent death is an integral part of the plant lifecycle and inevitably linked to any previous stages. Hence, this process should be understood from the perspectives of the life strategy in the plant.

The molecular and genetic components that regulate leaf senescence are being actively investigated as discussed here. However, cellular processes are executed by molecular networks that encompass complex interactions between DNA, RNA, proteins and metabolites. Therefore, further efforts are needed to understand leaf senescence and death from the perspective of transitions of molecular networks, of network modules and their interactions, rather than from an individual component-based perspective, as exemplified here in the age-dependent transition of network modules that ORE1 is involved in.

It should also be noted that leaf cells collectively undergo senescence and death when they age. However, individual cells within senescing leaf are in highly heterogeneous states. This raises the question of how this heterogeneity in dying cells is coordinated with the senescence of the entire leaf. Leaf senescence is also affected by the overall developmental and physiological states of the entire plant. It is an important to understand how the senescence and death are systemically integrated within the entire plant. We also do not yet know how the regulatory components or networks of leaf senescence are integrated with plant productivity, including seed setting. The mechanism that controls the relocation of nutrients from senescent leaves is another important aspect that has not been explored.

Furthermore, leaf senescence is an evolutionarily acquired developmental strategy and, therefore, we would expect that plants that have evolved in different environments would have different senescence physiology and regulatory modes. Accordingly, it will be highly informative to perform

comparative studies of various *Arabidopsis* ecotypes and of different plants with a distinct senescence strategy. This will allow us to define the metabolic and physiological processes of senescence, their regulatory principles, and the evolution of senescence and death program in terms of spatial and temporal transition of the molecular, cellular and organ networks. Comparative studies of various organisms, including at the molecular and system level, are also required to understand biological processes such as aging, senescence and death.

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