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Leaf Senescence: Systems and Dynamics Aspects

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Abstract

Leaf senescence is an important developmental process involving orderly disassembly of macromolecules for relocating nutrients from leaves to other organs and is critical for plants' fitness. Leaf senescence is the response of an intricate integration of various environmental signals and leaf age information and involves a complex and highly regulated process with the coordinated actions of multiple pathways. Impressive progress has been made in understanding how senescence signals are perceived and processed, how the orderly degeneration process is regulated, how the senescence program interacts with environmental signals, and how senescence regulatory genes contribute to plant productivity and fitness. Employment of systems approaches using omics-based technologies and characterization of key regulators have been fruitful in providing newly emerging regulatory mechanisms. This review mainly discusses recent advances in systems understanding of leaf senescence from a molecular network dynamics perspective. Genetic strategies for improving the productivity and quality of crops are also described.

Contents

INTRODUCTION

Meristematic tissue: tissue that has the ability to enlarge, stretch, and differentiate into other types of cells as it matures

Aging is by definition an addition of age, which leads to age-dependent deterioration or senescence of cells, tissues, organs, and organisms at later stages of life history in both animals and plants. However, the term senescence is used mostly in plants for deteriorative and degenerative physiological changes and in animals for mitotic or replicative senescence of cells. Plants show two types of senescence: mitotic or replicative senescence and postmitotic senescence (25). Mitotic senescence is exhibited in meristematic tissue following the arrest of cell division or replication activity. Postmitotic senescence occurs in plant organs such as leaves and floral petals after their cellular differentiation and maturation has completed and accompanies an active and programmed degenerative process (70). Senescence in plants occurs at various levels but most conspicuously at the

organ and organismal levels, as illustrated by the splendid autumn scenery of the color changes of leaf organs.

The leaf is the primary photosynthetic organ for energy harvesting and nutrient production at the growth and maturation stages. When a leaf enters the senescence stage, its cells undergo the sequential disorganization of cellular organelles and orderly changes in metabolism and gene expression (**Figure 1**). Major transitions during leaf senescence are the breakdown of chloroplasts and the replacement of carbon assimilation by the catabolism of chlorophyll and macromolecules. Increased catabolic activity is responsible for converting cellular materials accumulated during the leaf's growth phase into exportable nutrients. In annual plants, disassembled nutrients are supplied to developing seeds as a parental investment. In perennial plants, such as trees, nutrients are relocated to the stems or roots, which are used as storage resources for the development of new leaves or flowers in the next season. Thus, leaf senescence is a critical process for ensuring optimal production of offspring and better survival. The degeneration and remobilization processes, which occur concomitantly, are therefore likely to be orderly and highly coordinated (10, 68).

Leaf senescence spans the latter half of the leaf's life and thus constitutes a significant portion of its life history. Leaf senescence is basically controlled by developmental age. However, leaf senescence also proceeds by integrating various internal and external environmental signals into age information (10, 68) (**Figure 1**). These include nutritional and hormonal signals, water status, light regimes, and temperature change. Thus, the interconnection of highly intricate regulatory networks and multiple, crosstalking pathways should be operating during leaf senescence, and these networks should be dynamic to regulate and reflect the senescence state affected by various senescence signals (68).

In this review, we first briefly describe the molecular changes of leaf senescence and then discuss some recent results that explore new regulatory mechanisms identified by use of multi-omics technologies with computational biology and imaging tools and extensive molecular genetic analyses. We address the rapid identification of novel regulatory molecules and their regulatory networks, elucidation of the spatiotemporal dynamics of age-associated networks, and exploration of molecular mechanisms that integrate various environmental signals with senescence pathways (**Supplemental Table 1**). Furthermore, the current progress that translates basic research findings into practical applications for enhancing the productivity and quality of crops and/or prolonging the shelf life of horticulture products is overviewed.

Annual plant: a plant that performs its entire life cycle from germination to the production of seeds within a single growing season

Perennial plant: a plant that persists for more than two years

Multi-omics: biological analysis approach in which the data sets are multiple "omes", such as genomes, epigenomes, transcriptomes, proteomes, and microbiomes

Shelf life: period that a commodity remains effective and free from deterioration, without becoming unfit for use, consumption, or sale

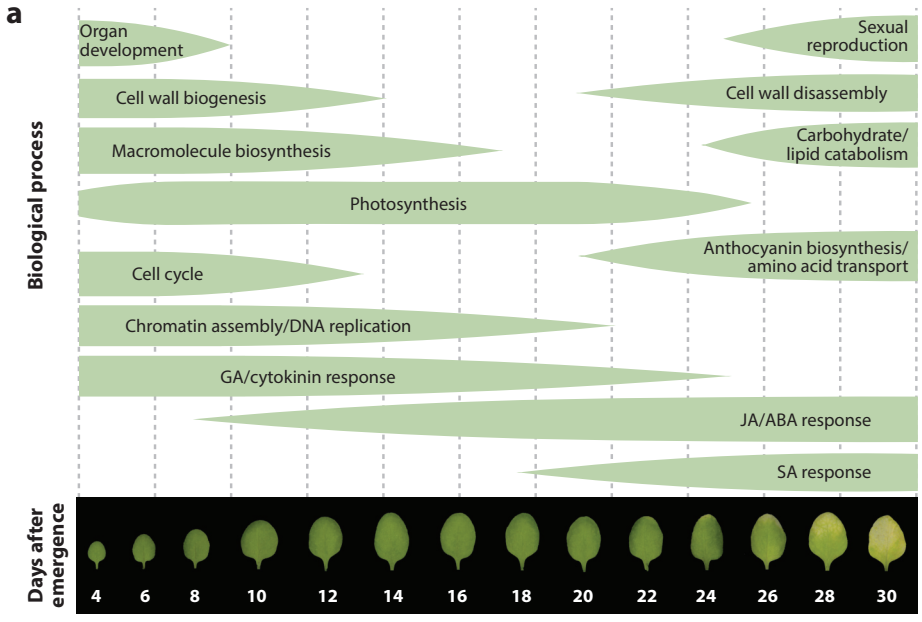
Supplemental Material >

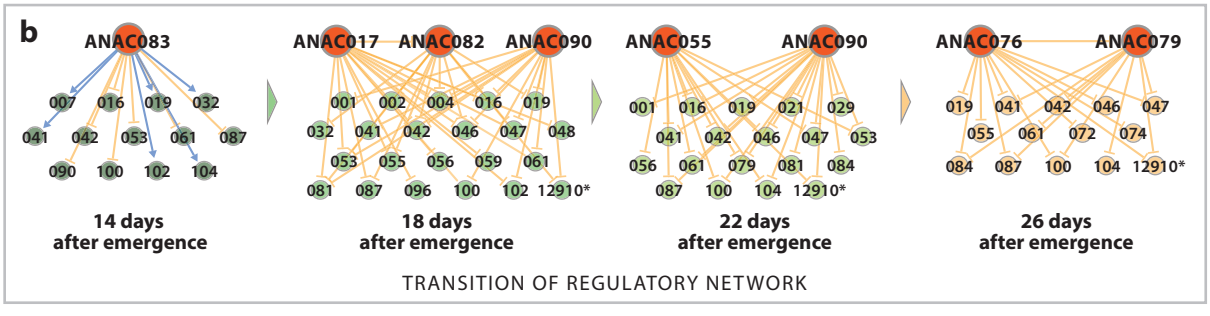
MULTIFACETED CHANGES IN CHLOROPLASTS AND MITOCHONDRIA DURING LEAF SENESCENCE

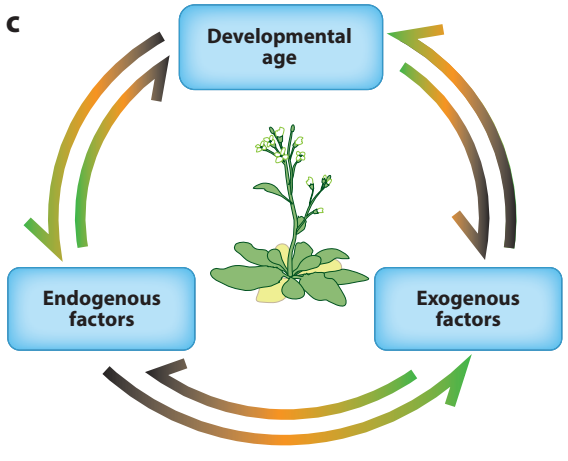
Leaf cells are subject to massive structural and biochemical changes in an orderly manner during senescence (68). One of the most distinctive characteristics of biochemical changes during leaf senescence is the dramatic metabolic transition from anabolism to catabolism, including the increased hydrolysis of macromolecules (**Figure 1**). The following section summarizes the state of knowledge about the cellular structural and biochemical changes during leaf senescence and describes the underlying molecular regulatory mechanisms, particularly with regard to chloroplasts and mitochondria.

Regulation of Chlorophyll Breakdown and Chloroplast Degeneration During Leaf Senescence

The first visible phenotypic change in leaf senescence is the color change of the leaf due to the preferential breakdown of chlorophyll concomitant with chloroplast disassembly. Chlorophyll







(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Overview of a leaf life history. (*a*) Leaves undergo developmental transitions throughout their life history. (*b*) Many molecular components during leaf senescence show complex interactions, and their functional and regulatory interaction networks change continuously with each progressive stage of senescence. One representative example is a temporal network dynamic involving NAC family transcription factors during leaf senescence. In each subnetwork, circles represent target NACs regulated by the corresponding hub NACs, and blue and orange lines indicate positive and negative regulations of target NACs by the hub NACs. Accordingly, leaf cells undergo dramatic shifts in physiology from biogenesis to the sequential degeneration of macromolecular and cellular structures, including the breakdown of the photosynthetic machinery in a coordinated manner. 12910* refers to At3g12910. Panel *b* adapted from Reference 49. (*c*) Leaf senescence, the last stage of leaf development, is tightly regulated by developmental age, but it is also influenced by various endogenous and exogenous factors that are integrated into age information. Abbreviations: ABA, abscisic acid; ANAC, ARABIDOPSIS NAM/ATAF/CUC; GA, gibberellin; JA, jasmonic acid; NAC, NAM/ATAF/CUC; SA, salicylic acid.

breakdown is governed by the pheophorbide *a* oxygenase (PAO)/phyllobilin pathway, which comprises a set of chlorophyll catabolic genes (*CCG*s) (40). This process is initiated by conversion from chlorophyll *b* to chlorophyll *a* by chlorophyll *b* reductase, which is encoded by *NONYELLOW COLORING1* (*NYC1*) and *NONYELLOW COLORING1–LIKE* (*NOL*), and 7-hydroxymethyl chlorophyll *a* reductase (HCAR). Magnesium is removed from chlorophyll *a* to be converted to pheophytin *a* by magnesium-dechetalase encoded by *NONYELLOWING*s*/STAYGREEN*s (*NYE*s*/SGR*s). Pheophytin *a* is then hydrolyzed by PHEOPHYTINASE (PPH) to produce pheophorbide *a* and phytol. Notably, the green color of chlorophyll catabolites is completely lost when the porphyrin ring of pheophorbide *a* is cleaved by PAO, resulting in oxidized red chlorophyll catabolite, which is subsequently catalyzed by red chlorophyll catabolite reductase to generate primary fluorescent chlorophyll catabolite (*p*FCC). Finally, *p*FCC is modified, transported into the vacuole, and isomerized to nonfluorescent products by acidic pH (40). Recent studies have taken advantage of the well-characterized *CCG*s to uncover the regulatory mechanisms that control chlorophyll breakdown (57).

Particularly illuminating was the transcriptional regulation in chlorophyll breakdown that emerged from studies of *Arabidopsis* in recent years (**Figure 2**). Two *Arabidopsis* basic leucine zipper (bZIP) transcription factors (TFs), ABSCISIC ACID (ABA) INSENSITIVE 5 (ABI5) and ENHANCED EM LEVEL (EEL), directly activate the expression of major *CCG*s, including *NYC1* and *NYE1* during leaf senescence (94). Other bZIP TFs, ABA-RESPONSIVE ELEMENT BINDING FACTOR 2 (ABF2), ABF3, and ABF4, were also found to promote ABA-mediated chlorophyll degradation by directly activating the expression of *CCG*s, such as *NYC1*, *NYE1*, *NYE2*, and *PAO* through the ABA-signaling pathways (27). The NAM/ATAF/CUC (NAC) family TFs [ANAC016, 019, 046, and 055; ORESARA 1 (ORE1)/ANAC092; and RESPONSIVE TO DESICCATION 26 (RD26)/ANAC072], which are positive regulators of leaf senescence, directly bind to the promoters of a set of major *CCG*s (83, 88, 93, 94, 135). Moreover, ETHYLENE INSENSITIVE 3 (EIN3), a master positive regulator of ethylene signaling, promoted chlorophyll degradation by physically binding to *NYE1*, *NYC1*, and *PAO* promoters and activating their expression. The basic helix–loop–helix (bHLH) subgroup IIIe factors MYC2, 3, and 4 directly promote the expression of *NYE1*, *NYC1*, and *PAO* or indirectly promote expression by mediating the downstream NAC family TFs ANAC019, 055, and 072, which also activate *NYE1*, *NYE2*, and *NYC1* in jasmonic acid (JA) signaling pathways (135). PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) and PIF5 are known to directly regulate *NYC1* and/or *NYE1* expression (103, 130). More recently, SUPPRESSOR OF OVEREXPRESSION OF CO 1, a MADS box family TF, has been reported as a negative regulator of leaf senescence that directly inhibits the transcription of the *NYC1* and *PPH* genes (15).

Chloroplast degeneration is accompanied by the progressive loss of proteins and lipids as well as chlorophyll breakdown. Several senescence-associated stromal and vacuolar proteases accomplish

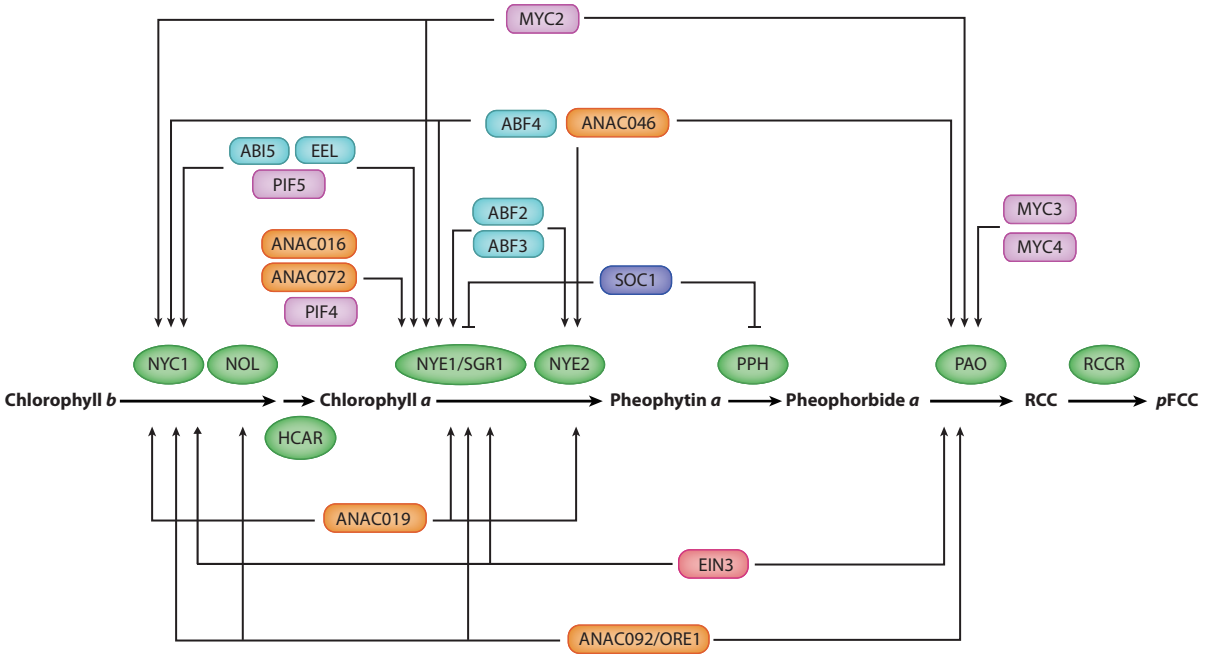


Figure 2

Transcriptional regulation of chlorophyll catabolic genes in the pheophorbide *a* oxygenase (PAO)/phyllobilin pathway. Chlorophyll catabolic genes are transcriptionally regulated by a variety of family transcription factors. *NONYELLOW COLORING 1* (*NYC1*), which encodes chlorophyll *b* reductase for conversion of chlorophyll *b* into chlorophyll *a*, is positively regulated by ABSCISIC ACID–RESPONSIVE ELEMENT BINDING FACTOR 4 (ABF4), ABSCISIC ACID INSENSITIVE 5 (ABI5), ABSCISIC ACID–RESPONSIVE NAM/ATAF/CUC 019/046/092 (ANAC019/046/092), ENHANCED EM LEVEL (EEL), ETHYLENE INSENSITIVE 3 (EIN3), MYC2, and PHYTOCHROME-INTERACTING FACTOR 5 (PIF5) transcription factors. Another chlorophyll *b* reductase gene, *NONYELLOW COLORING1-LIKE* (*NOL*), is known to be activated by ANAC092/ORESARA 1 (ORE1). NONYELLOWINGs (NYEs)/STAYGREENs (SGRs) possessing magnesium-dechelating activity are involved in converting chlorophyll *a* to pheophytin *a* by removing magnesium. ABF2/3/4, ABI5, ANAC016/019/046/072/092, EEL, EIN3, MYC2, and PIF4/5 transcription factors directly activate expression of the *NYE1/SGR1* gene, and ABF2/3/4 and ANAC019/046 activate expression of the *NYE2* gene. Notably, SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), a MADS box family transcription factor, directly inhibits the expression of *NYE1* and *PHEOPHYTINASE* (*PPH*). PPH hydrolyzes pheophytin *a* to produce pheophorbide *a* and phytol. Generation of RCC from pheophorbide *a* is mediated by PAO, whose expression is positively regulated by ANAC046/092, ABF4, EIN3, and MYC2/3/4. Red chlorophyll catabolite (RCC) is catalyzed by RCC reductase (RCCR) to generate primary fluorescent chlorophyll catabolite (*p*FCC).

Autophagy: an intracellular degradation system by which unnecessary or dysfunctional cytoplasmic components are delivered to and degraded in the lysosome

key roles in chloroplast protein degradation (39, 85). Lipid-degrading enzymes, such as phospholipase D, lytic acyl hydrolase, and lipoxygenase, also function in the hydrolysis of membrane lipids during leaf senescence (109, 110). For example, 13-lipoxygenase that accumulates in the plastid envelope has a role in selective chloroplast degeneration by attacking unsaturated membrane fatty acids, which in turn leads to the generation of holes for the mass release of stromal constituents (104). Furthermore, the importance of lytic vacuoles and autophagy is implicated in chloroplast degeneration and recycling (84).

The Morphological and Metabolic Changes of Mitochondria in Leaf Senescence

The number of mitochondria significantly decreases in senescing *Arabidopsis* and grapevine leaves (22, 44, 91). Meanwhile, mitochondria morphology is markedly altered from the elongated,

branched structures that are formed by interconnected mitochondria to enlarged, round-shaped structures during leaf senescence (91). In contrast, mitochondrial integrity and energy status are maintained until the final stages of leaf senescence in *Arabidopsis* (22). An integrated transcriptomic and metabolomic study revealed that mitochondria undergo metabolic alterations to orchestrate the selective catabolism of both amino acids and fatty acids for optimizing nitrogen redistribution as leaves senesce (22).

Transcriptional coordination between the nucleus and mitochondria appears less tight than that between the nucleus and chloroplasts during leaf senescence (118). Nevertheless, the necessity of nucleus–mitochondria communication in coordinately regulating gene expression for the proper initiation, execution, and completion of the leaf senescence process is undoubtable. A study of the mitochondrial ATP-dependent protease FtSH4 in *Arabidopsis* found that the mitochondrial reactive oxygen species (ROS) function as a communication signal with the nucleus, which alters the expression of WRKY TF genes and eventually controls leaf senescence (128). Additional studies on the detailed mechanisms that underlie the spatiotemporal dynamics of organellar communication will open up new areas of research to help understand how the organelles communicate to govern the various biochemical and molecular changes that occur during leaf senescence in response to continuously changing internal and external environments.

Reactive oxygen species (ROS): chemically reactive molecules and free radicals derived from oxygen; examples include peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen

THE FUNCTIONAL AND REGULATORY TRANSITIONS IN LEAF SENESCENCE

Orderly changes in physiology and biochemistry during leaf senescence are accompanied by changes in the expression of thousands of *senescence-associated genes* (*SAG*s). Recent studies indicate that the dynamic activation of TFs is a key contributor in the regulatory program through the control of *SAG* expression in leaf senescence. A process as complex as leaf senescence can be better understood in the context of molecular regulatory networks; thus, we highlight in this section the latest understanding of gene regulatory networks (GRNs) involving the NAC and WRKY TF families, the major TF families that control leaf senescence. Furthermore, this section describes the recent advent of omics technologies, which have brought remarkable advances to resolve the complicated processes of leaf senescence.

The Temporal Dynamics and Functions of Gene Regulatory Networks Involving NAC Transcription Factors

Extensive efforts to define and organize the GRNs involving NAC TFs have elucidated how GRNs are dynamically regulated to effectively integrate multiple developmental and environmental signals during leaf senescence. For instance, ORE1 is a component of the trifurcate feed-forward loop for age-induced cell death involving EIN2 and *miRNA164* (*miR164*) (52). EIN3, which is a key TF in the EIN2-mediated ethylene-signaling cascade, induces the accumulation of the *ORE1* transcript by directly repressing *miR164* transcription (64). Additionally, five senescence-associated NAC TFs [NAC-LIKE, ACTIVATED BY AP3/PI (AtNAP)/ANAC029, ANAC019, ANAC047, ANAC055, and ORE1 SISTER 1 (ORS1)/ANAC059] have been identified as candidate downstream components of EIN2 (48). ORE1 and AtNAP, which are directly activated by EIN3, regulate both common [ANAC041 and 079 and VND-INTERACTING 2 (VNI2)] and different NAC TF targets during leaf senescence. The importance of a GRN involving ORE1 to interweave the light signaling pathway with the leaf senescence program was further inferred in the multiple feed-forward loops governed by PIF4, PIF5, EIN3, and ABI5 (94). Another coherent feed-forward loop involving ORE1 that includes both EIN3 and a few CCGs

provides a molecular basis by which EIN3 and its target, ORE1, accelerate ethylene-mediated chlorophyll degradation by directly activating *CCG* expression during leaf senescence in *Arabidopsis* (88). Thus, GRNs involving ORE1 function as integrators that coordinate various endogenous and environmental signals during leaf senescence.

In addition to ORE1, other NAC TFs have been implicated in leaf senescence GRNs, alongside other TF types, upon exposure to diverse stresses. One GRN involving MYB108 and its direct target, ANAC003, has a regulatory role in dark-stressed leaf senescence (21). Similarly, the GRNs that involve NAC TFs in *Arabidopsis* [ANAC032, 072, and 102, and *Arabidopsis* TRANSCRIPTION ACTIVATION FACTOR 1 (ATAF1), ATAF2, NAC WITH TRANSMEMBRANE MOTIF 1-LIKE 4 (NTL4), and VNI2] and rice (ONAC106, OsNAC2, and OsNAP) regulate leaf senescence triggered by diverse plant hormones or stresses through modulation of the expression of *SAG*s (61, 63, 65, 73, 74, 96, 106, 122).

Systematic analyses have provided valuable information regarding the distinct features and temporal transition of GRNs involving NAC TFs. A study on the GRNs of ANAC019, 055, and 072 using yeast one-hybrid, time-series gene expression data sets and microarray data analyses illustrated that the expression of each NAC TF gene upon different stress conditions is modulated by both differential and common upstream TFs (38). Another systematic study on 49 senescence-associated NACs presented time-dependent networks involving temporal transitions between the interactions of NAC TFs (49). These time-dependent networks identified a shift from positive to negative regulation among NAC TFs, which is primarily governed by ANAC017, 082, and 090 before leaf senescence begins. Intriguingly, the shared suppression of senescence-promoting processes by ANAC017, 082, and 090 included salicylic acid (SA) and ROS responses at presenescent stages. Thus, studies on the time-dependent change of networks reveal unique regulatory modules, which direct the timely induction of senescence-promoting processes.

The Dynamics and Functions of WRKY Gene Regulatory Networks Underlying Leaf Senescence

The role of GRN involving WRKY53 in leaf senescence is intensively studied. WRKY53, which is a positive regulator of leaf senescence, targets various *SAG*s including pathogen- and stress-related genes (77). The expression of *WRKY53* is directly suppressed by WHIRLY 1 (WHY1) (76) and is at least partially regulated by SUPPRESSOR OF VARIEGATION 3-9 HOMOLOG 2 (SUVH2)-mediated histone modification during leaf senescence (5) (**Figure 3**). Posttranslational modification also controls the levels of WRKY53; the HECT domain E3 UBIQUITIN PROTEIN LIGASE 5 (UPL5) ubiquitinates and induces the degradation of WRKY53 (79). The phosphorylation of WRKY53 by MAPK/ERK KINASE KINASE 1 (MEKK1) increases its ability to bind to targets, whereas interactions with EPITHIOSPECIFYING SENESCENCE REGULATOR (ESR) inhibit the DNA-binding activity of WRKY53 (78). A recent study on HISTONE DEACETYLASE 9 (HDA9) unveiled that the HDA9–POWERDRESS (PWR) complex is recruited to W-box-containing promoter regions in a WRKY53-dependent manner that in turn suppresses the expression of negative senescence regulators (19). Noticeably, WRKY57, which is one of the downstream targets of the PWR–HDA9–WRKY53 complex and which physically interacts with JASMONATE ZIM-DOMAIN 4 (JAZ4) and JAZ8, directly represses the expression of diverse *SAG*s in JA-induced leaf senescence (42). Together, these studies provide mechanistic insights into how a robust regulatory network involving WRKY53 is organized and functions to modulate leaf senescence. In addition to WRKY53, GRNs involving other WRKY TFs, including WRKY6, 22, 45, 54, 70, or 75, are thought to hold crucially important roles in leaf senescence (6, 14, 16, 32, 128). Further molecular genetic analyses that combine transcriptome, chromatin

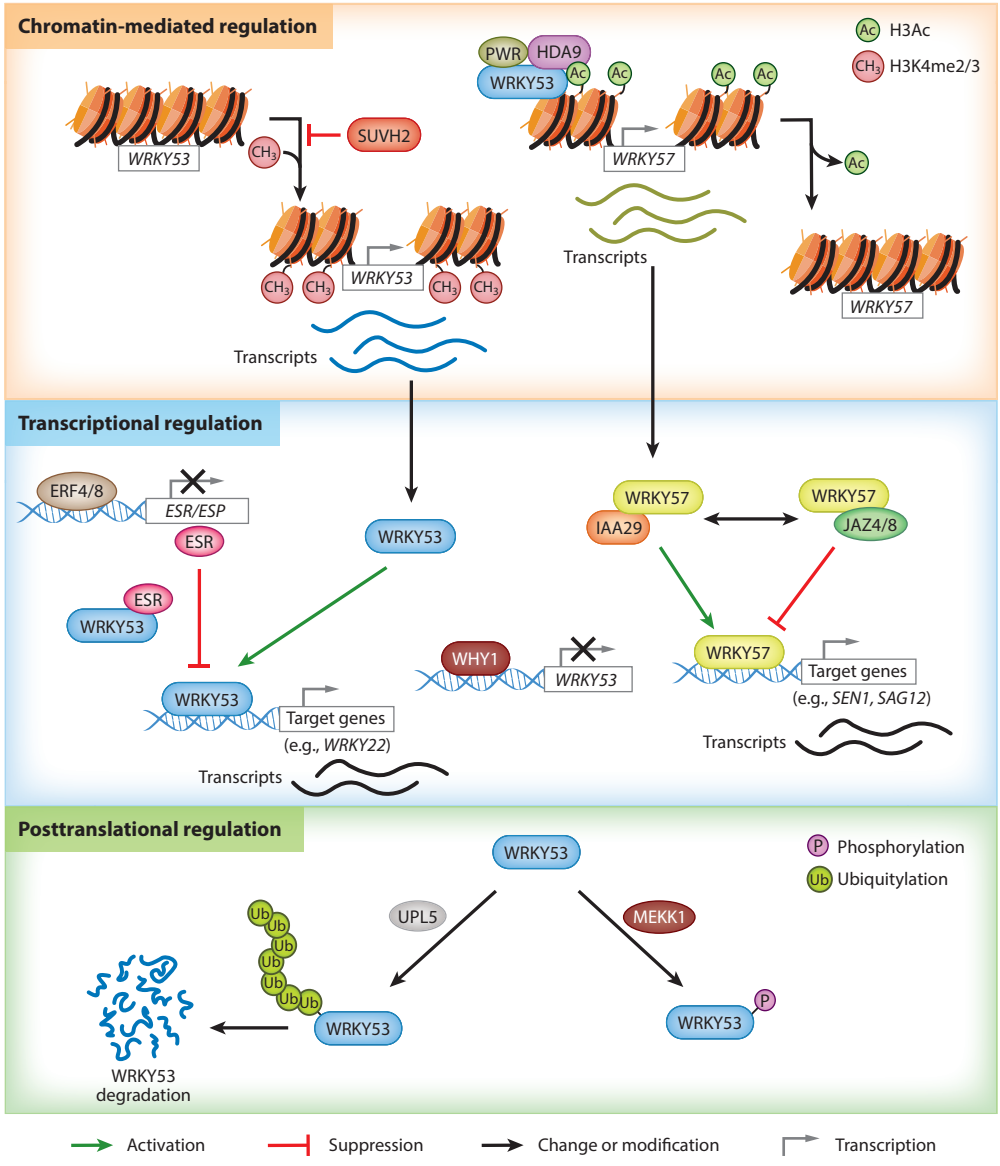


Figure 3

Multilayered regulatory mechanisms that involve WRKY53 to control leaf senescence. WRKY53 positively regulates leaf senescence through diverse means and is also subject to different modes of regulation. (*Top*) Upregulation of *WRKY53* expression during leaf senescence is at least partially regulated by SUPPRESSOR OF VARIEGATION 3-9 HOMOLOG 2 (SUVH2)-mediated histone methylation. WRKY53 recruits HISTONE DEACETYLASE 9 (HDA9) and POWERDRESS (PWR) to its target sites, which facilitates the removal of histone acetylation by HDA9 and suppresses *WRKY57* expression. (*Middle*) WRKY57's interaction with JASMONATE ZIM-DOMAIN 4 (JAZ4) and JAZ8 represses the expression of diverse *senescence-associated genes* (*SAG*s), and its interaction with indole-3-acetic acid 29 (IAA29) positively regulates jasmonic acid–induced leaf senescence. The expression of *WRKY53* is directly suppressed by WHIRLY 1 (WHY1) during transcriptional regulation. It is also known that the binding activity of WRKY53 to its target genes can be inhibited by interaction with EPITHIOSPECIFYING SENESCENCE REGULATOR (ESR), whose transcription is downregulated by ETHYLENE RESPONSE FACTOR 4 (ERF4) and ERF8. (*Bottom*) Two different posttranslational modifications control the levels and activities of WRKY53. For instance, UBIQUITIN PROTEIN LIGASE 5 (UPL5) ubiquitinates WRKY53, which induces its degradation, and MAPK/ERK KINASE KINASE 1 (MEKK1) phosphorylates WRKY53, which increases the binding to its targets.

immunoprecipitation sequencing (ChIP-seq), and systems biology analyses will contribute to unraveling the complex GRNs that control leaf senescence, including the identities of GRN components, how GRNs respond to different senescence-affecting factors, and how these GRNs are interlinked.

Noncoding RNAs (ncRNAs): functional RNA molecules that do not encode proteins and that regulate gene expression at the transcriptional and posttranscriptional levels

Degradome sequencing: next-generation sequencing of the 5′ ends of RNA degradation products, which can reveal many known and novel plant miRNA (small interfering RNA) targets

Omics Approaches for Temporal Profiling of Molecular Processes in Leaf Senescence

The advent of transcriptomic technologies has equipped us to address the fundamental principles of leaf senescence on a genome-wide scale in diverse plant species. Transcriptomic studies, particularly in *Arabidopsis*, have provided multidimensional insights into the functional and regulatory aspects of leaf senescence. Initial attempts to identify differentially expressed genes during developmental leaf senescence in *Arabidopsis* using a DNA microarray technique for genome-wide profiling have led to the identification of a distinct chronology of metabolic processes and signaling pathways during leaf senescence (7, 126). For example, the sequential downregulation of the genes involved in anabolic processes, including amino acid biosynthesis, chlorophyll biosynthesis, carbon fixation, and photosynthesis, and the sequential upregulation of the genes involved in catabolic processes, including autophagy, caspase activity, cell wall degradation, and lipid catabolism, occur during developmental leaf senescence. The genes involved in hormone signaling pathways, including cytokinin, ABA, and ethylene, are also coordinately altered. Furthermore, the importance of the temporal regulation of NAC and WRKY family TFs for cascades of biological changes is highlighted in the progression of leaf senescence. Furthermore, a comprehensive, multiple time-course analysis of *Arabidopsis* leaf transcriptomes using RNA sequencing (RNA-seq) has enabled a much deeper understanding of the regulatory features that underlie the leaf senescence process, such as temporal coordination of transcriptomes in senescing leaves that is tighter than in growing ones and importance of the interorganellar coordination between nuclear and chloroplast transcriptomes (118).

Comparative analyses of diverse leaf senescence transcriptomes provide a deeper understanding of the molecular mechanisms of potential crosstalk among various senescence-promoting factors (2, 11, 33, 113). Convergent regulatory mechanisms execute leaf senescence processes derived from the different initial response pathways of each senescence-promoting factor. Moreover, such analyses could facilitate the dissection of complicated molecular programs underlying leaf senescence. A recent comparative transcriptome analysis in an ethylene-insensitive mutant and a constitutive cytokinin response mutant during dark-induced leaf senescence has revealed that ethylene promotes leaf senescence through the transcriptional activation of the genes involved in stress-related responses, whereas cytokinin suppresses leaf senescence by maintaining cellular and translational activities (51).

Another noticeable attempt to delineate the landscape of epigenetic regulation during leaf senescence is the genome-wide identification and characterization of small noncoding RNAs (ncRNAs). Genome-wide analyses of the changes in microRNA (miRNA) during leaf development and senescence with degradome sequencing revealed that the miRNA–target gene regulatory networks that underlie developmental leaf senescence are involved in nutrient mobilization and cell structural integrity (107). A systemic study of Argonaute 1–enriched small RNAs (smRNAs) on the expression of *SAG*s in *Arabidopsis* and rice identified conserved smRNAs and target genes in both species, which further indicates their conserved roles in the regulation of leaf senescence (87). A recent comprehensive smRNA-seq analysis throughout the lifespan of the *Arabidopsis* leaf revealed the temporal dynamics and roles of small and long ncRNAs during leaf senescence (118). An abundance of approximately 30% of smRNAs dynamically alters as leaves

age. An investigation of the age-dependent regulatory networks involving smRNAs and their potential targets further revealed that miRNAs and 21-nucleotide smRNAs regulate distinct processes to coordinate transcriptional programs during leaf senescence. Liu et al. (71) identified 168 circular RNAs (circRNAs), including 35 circRNAs that were differentially expressed during leaf senescence. Construction of the circRNA–miRNA–messenger RNA (mRNA) network during leaf senescence uncovered that circRNAs are involved in the chlorophyll metabolism and JA signal transduction through *circ-AT5G43822–miR400–PAO* and *circ-AT3G61420–miR863-3p–JAZ1* networks, respectively; this suggests the participation of circRNAs as new posttranscriptional regulators for controlling biological processes during leaf senescence. Although epigenomic investigations of leaf senescence have provided molecular evidence for the importance of diverse epigenetic mechanisms that underlie the leaf senescence process, future challenges to characterize a repertoire of small ncRNAs—such as *trans*-acting, small-interfering RNAs and small ncRNAs that originate from ribosomal RNAs (rRNAs), transfer RNAs, or small nuclear RNAs—and long ncRNAs will advance our understanding of the ncRNA-involved regulatory mechanisms that modulate leaf senescence.

In addition, recent genome-wide analyses of histone modification changes have indicated that epigenetic regulation is another key regulatory mechanism of leaf senescence. For example, combinatorial analyses of ChIP-seq data for two histone modifications and transcriptome data manifested a significant correlation between temporal changes in gene expression and an active histone mark, trimethylation on histone H3 lysine 4 (H3K4me3), in a subset of genes with altered expression patterns during *Arabidopsis* leaf senescence. This included *WRKY75*, which encodes one of the key players of leaf senescence (8, 9). Such studies have provided insights into the important regulatory role of histone modification in gene expression during leaf senescence.

To date, proteomic and metabolomic approaches to leaf senescence research still lag behind DNA- and RNA-based high-throughput techniques, although they provide a global physiochemical view of cellular status and its regulation during leaf senescence. Further investigations that combine proteomic approaches and the immunoprecipitation of protein complexes are required to unmask novel regulatory aspects of leaf senescence programs.

MULTILAYERED REGULATION OF STRESS-INDUCED LEAF SENESCENCE

Leaf senescence proceeds with age and involves intricate regulatory programs that respond to various external factors and internal factors, including all of the leaf's developmental processes (**Figure 1**). This section highlights key findings that illustrate the complexity of the regulatory programs of stress-induced leaf senescence, which are finely regulated at multiple chromatin, transcriptional, posttranscriptional, and posttranslational regulatory levels.

Chromatin-Mediated Regulation of Stress-Induced Leaf Senescence

The control of chromatin conformation through histone modification and chromatin-remodeling enzymes is an emerging key regulatory mechanism of leaf senescence that can be triggered by either biotic or abiotic stresses. A few recent reports have provided further evidence of the pivotal roles of histone modification in the regulation of stress-induced leaf senescence. A deficient mutant of the *Arabidopsis HDA9* gene exhibits delayed dark-induced leaf senescence phenotypes (19). WRKY53 recruits HDA9 and PWR to its target sites, which facilities the removal of histone 3 acetylation (H3Ac) by HDA9 and suppresses the expression of key negative regulators of senescence, such as *AUTOPHAGY 9* (*ATG9*) and *WRKY57* (**Figure 3**). A loss-of-function

mutant of an *Arabidopsis* histone acetyltransferase gene, *HOOKLESS 1* (*HLS1*), leads to enhanced leaf senescence phenotypes by dark, pathogen, or ABA treatment (66). HLS1 enhances the expression of *WRKY33* and *ABI5*—which, respectively, are a well-known pathogen and an ABA-signaling regulator—through modulating H3Ac on chromatin. The significance of the chromatin-mediated regulation in dark-induced leaf senescence has also been highlighted by characterizing the mutations of chromatin-modifier genes, such as *DEFECTIVE IN RNA–DIRECTED DNA METHYLATION 1* (*DRD1*) and *DECREASED DNA METHYLATION 1* (*DDM1*) (20). Further studies are needed to uncover both how diverse chromatin modifiers differentially respond to various internal and external factors to effectively regulate leaf senescence and how TFs and chromatin modifiers cooperate to regulate gene expression during stress-induced leaf senescence.

Transcriptional Regulation of Stress-Induced Leaf Senescence

This section summarizes the progress made toward the identification and characterization of TFs that have a role in the regulation of stress-induced leaf senescence and describes how such present knowledge lays a foundation for addressing important challenges in unveiling the TF-mediated regulatory mechanisms of stress-induced leaf senescence.

The NAC TF family is the most intensively characterized TF family involved in stress-induced leaf senescence in diverse plants. Studies of *Arabidopsis* have provided evidence that NAC TFs mediate the regulation of leaf senescence by coupling stress-related signaling with senescence-related transcriptional modules. For example, ANAC016, a positive regulator of dark, salt, and oxidative stress–induced leaf senescence, directly binds to promoters of *AtNAP* and *ORS1* and enhances their transcript levels (54). Moreover, both ANAC016 and AtNAP directly bind to the promoter of *ABF2* that encodes a TF that is important in the ABA signaling pathway and represses its transcription (95). Recently, *SGR1/NYE1*, which is a major *CCG*, was identified as another direct downstream target of ANAC016 (93). Another NAC TF that functions as a positive regulator of dark and oxidative stress–induced leaf senescence, ATAF1, activates *ORE1* transcription and represses *GOLDEN 2–LIKE 1* (*GLK1*) transcription by directly binding to their promoters (28). Additionally, ANAC032 is a positive regulator of dark, oxidative, osmotic, and salt stress–induced leaf senescence by modulating the expression of *SAG*s, such as *NYE1*, *SAG113*, and *SAG201* (73). RD26, a positive-regulator of dark-induced leaf senescence (63), directly enhances the expression of diverse repertoires of downstream genes, including *CHLOROPLAST VESICULATION*, which is involved in chloroplast protein degradation; *LYSINE KETOGLUTARATE REDUCTASE/SACCHAROPINE DEHYDROGENASE*, which is involved in lysine catabolism; and *ALPHA-AMYLASE1*, *SUGAR-PORTER FAMILY PROTEIN 1*, and *SWEET15*, which are involved in carbohydrate metabolism and transport (43).

The involvement of the APETALA 2 (AP2)/ETHYLENE RESPONSE FACTOR (ERF) TF family in regulating stress-induced leaf senescence has also been thoroughly studied. AtERF4 and AtERF8 have positive roles in dark-induced leaf senescence via direct downregulation of *ESR*, which negatively regulates WRKY53 (56) (**Figure 3**). In addition, an AP2/ERF family TF in mulberry, MnDREB4A, is known as a negative regulator of leaf senescence triggered by diverse stresses, including drought and salt stresses (72). Overall, the intensive studies over the past several years have clearly illuminated the significance of TF-mediated regulation as a key regulatory mechanism underlying stress-induced leaf senescence. Further systematic studies designed to reveal the characteristics and dynamics of protein–protein and/or protein–DNA interactome networks involving numerous TFs will be necessary for more comprehensive insights into global TF-mediated regulatory mechanisms of stress-induced leaf senescence.

Posttranscriptional Regulation of Stress-Induced Leaf Senescence

On the basis of recent genome-wide analyses of ncRNAs during leaf senescence (87, 107, 118) and the well-characterized miRNA-mediated regulatory networks involving *miR164* or *miR319* (52, 98), the contributions of posttranscriptional regulation to leaf senescence are evident. This section discusses regulations mediated by RNA splicing and processing, where advances in posttranscriptional regulatory mechanisms of stress-induced leaf senescence are recently emerging. Discovery of the roles of *Arabidopsis* minor spliceosomal protein U11–48K as a positive regulator in dark-induced leaf senescence is the first example of the involvement of RNA splicing in the regulation of stress-induced leaf senescence (120). U11-48K is one of the ribonucleoproteins unique to the minor spliceosome that catalyzes the splicing of minor U12-type introns. The positive correlation between the severity of abnormal developmental phenotypes and the degree of impairment in *U12* intron–splicing in the *u11–48k* mutants further indicates that correct splicing of *U12* introns is necessary for normal plant growth and development, including dark stress–induced leaf senescence. A more recent study on the sugarcane MYB TF ScMYB2 provides elegant evidence supporting that differential expression of alternatively spliced transcripts might be an important posttranscriptional regulatory mechanism in the control of drought stress as well as leaf senescence (31). Likewise, the importance of RNA processing in stress-induced leaf senescence was initially demonstrated in a study of the chloroplast RNA splicing and ribosome maturation family member subfamily 4 (CFM4) in *Arabidopsis*. The *cfm4* mutant exhibited delayed leaf senescence phenotypes and abnormal patterns of chloroplast rRNA processing (60), indicating the importance of rRNA processing in stress response, including dark-induced leaf senescence. The potato (*Solanum tuberosum*) RNA-binding protein *StUBA2a/b*, when overexpressed in *Arabidopsis*, causes early leaf senescence phenotypes under dark-stress conditions, which is accompanied by altered expression of SA biosynthetic and signaling genes as well as autophagy-associated genes (81). Despite the importance of posttranscriptional regulation in biological processes, there are still relatively few examples for stress-induced leaf senescence. Thus, the next key step will be to investigate the roles of posttranscriptional regulatory mechanisms such as mRNA processing (5′-capping and 3′-end processing), mRNA modification, and mRNA export machineries in stress-induced leaf senescence.

Posttranslational Regulation of Stress-Induced Leaf Senescence

Another regulatory layer of stress-induced leaf senescence involves diverse posttranslational modifications, which can influence the conformation, activity, stability, and localization of proteins. One of the most extensively characterized posttranslational modification mechanisms in the regulation of stress-induced leaf senescence is protein phosphorylation and dephosphorylation through kinases and phosphatases. For example, an S-domain receptor-like kinase, OsSIK2, is a negative regulator of leaf senescence, which is triggered by diverse environmental stresses such as darkness, drought, and salt (17). Another rice receptor-like cytoplasmic kinase, *Oryza sativa* BILATERAL BLADE SENESCENCE 1 (OsBBS1)/OsRLCK109, is shown to function as a negative regulator of dark- or salt-induced leaf senescence (125).

Recent studies on the *SNF1-RELATED KINASE* (*SnRK*) genes in *Arabidopsis* have further revealed the importance of protein phosphorylation–mediated regulation of stress-induced leaf senescence. ABA-activated SnRK2s phosphorylate ABFs and RELATED TO ABA INSENSITIVE 3/VP1 (RAV1) TFs, which subsequently activates the expression of *SAG*s including *ORE1*. By contrast, another *Arabidopsis* SnRK, SnRK1/*Arabidopsis* SNF1 KINASE HOMOLOG 10 (AKIN10) is a negative regulator of dark-induced leaf senescence (45). SnRK1/AKIN10 directly interacts, phosphorylates, and destabilizes EIN3, which suppresses expression of EIN3

target genes including *ORE1*. Additional evidence on the importance of reversible protein phosphorylation in stress-induced leaf senescence is supported by the characterization of the *Arabidopsis SENESCENCE-SUPPRESSED PROTEIN PHOSPHATASE* (*SSPP*) gene. SSPP negatively regulates dark-induced leaf senescence through direct dephosphorylation of SENESCENCE-ASSOCIATED RECEPTOR-LIKE KINASE (SARK), a positive regulator of leaf senescence (119).

Protein ubiquitylation is another well-defined posttranslational modification in the regulation of stress-induced leaf senescence. Two plant U-box (PUB) E3 ubiquitin ligases, PUB12 and 13, are involved in defense response and dark stress–induced leaf senescence (134). Similarly, PUB44/SENESCENCE-ASSOCIATED E3 UBIQUITIN LIGASE 1 (SAUL1)/NOT ORE-SARA 1 (NORE1) have also been reported to mediate signals from temperature- and humidity-dependent defense programs and leaf senescence (23, 59, 115). A recent study demonstrated that the ubiquitin receptor DA1 and the E3 ubiquitin ligase BIG BROTHER (BB) in *Arabidopsis*, both of which are known to restrict leaf size, also have roles as positive regulators of dark-induced leaf senescence (114). Further systemic studies to identify novel posttranslational modifiers that function in this process and to understand the regulatory networks involving upstream regulators, downstream targets, and interaction partners will help to unravel the posttranslational regulatory mechanisms of stress-induced leaf senescence.

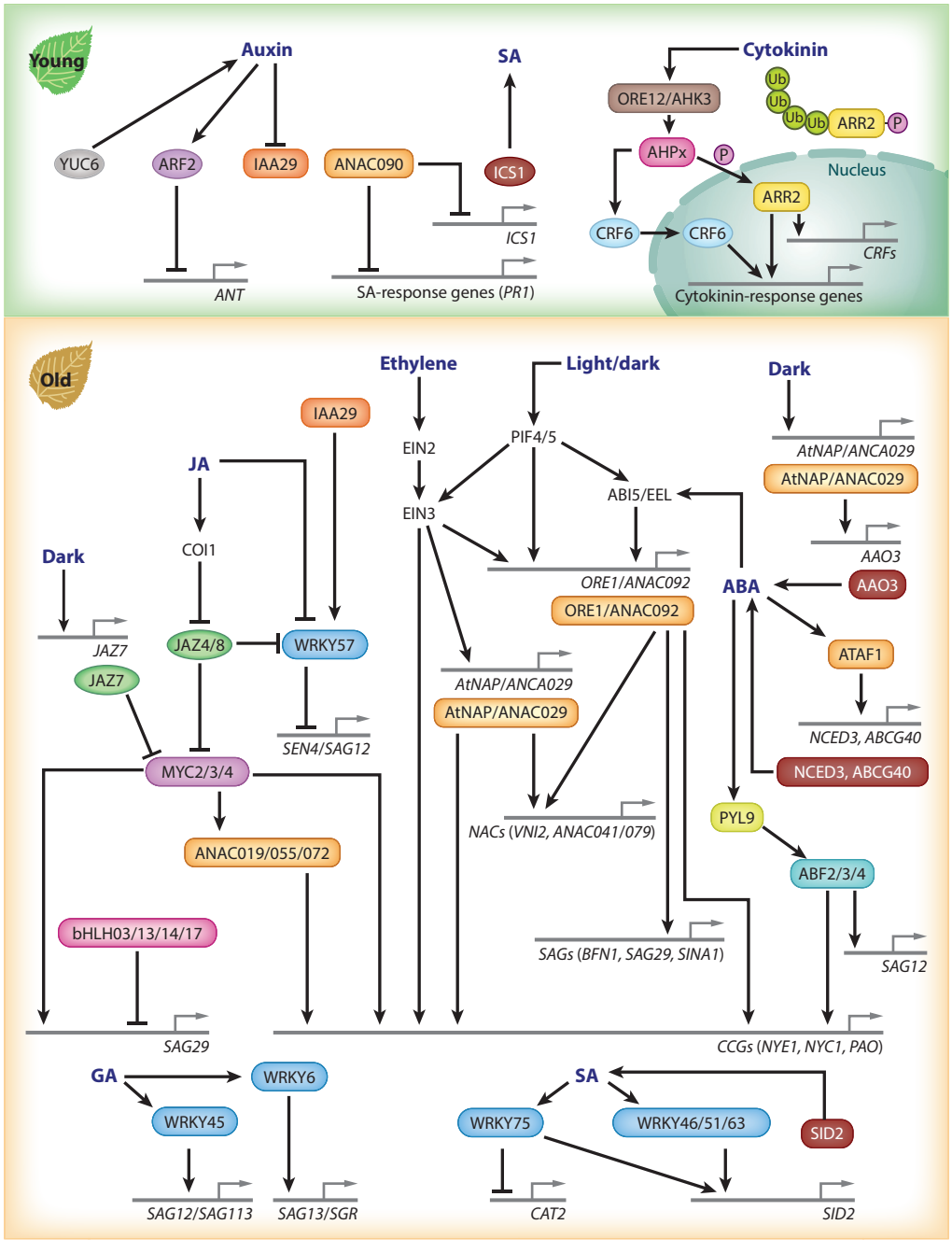
CROSSTALK BETWEEN HORMONE SIGNALING AND LEAF SENESCENCE

Leaf senescence of higher plants involves genetically and environmentally regulated processes and intimate crosstalk with hormonal interactions (26, 68). Indeed, global gene expression analysis and characterization of genetic mutants have revealed that all classical plant hormones can potentially affect leaf senescence at all stages of leaf development. Recently, regulatory molecules, including TFs that are associated with hormone signaling, have been identified as key regulators of leaf senescence (**Figure 4**).

JA, a lipid-derived plant hormone ubiquitous in the plant kingdom, regulates diverse plant defense responses and various development processes (41). The effects of JA on leaf senescence have long been known, based on findings that the endogenous JA level increases in senescent leaves and that exogenous application of JAs induces leaf senescence in a variety of plant species. Consistent with the increased JA level during leaf senescence, expression of genes involved in the JA biosynthesis pathways *LIPOXYGENASE 1* (*LOX1*), *LOX3*, *LOX4*, *ALLENE OXIDE SYNTHASE* (*AOS*), and *ALLENE OXIDE CYCLASE 1* (*AOC1*) is increased in senescing leaves (37). The mutant of CORONATINE INSENSITIVE 1 (COI1), a component of a receptor for JA, abolishes JA-induced leaf senescence, indicating that key components of JA signaling pathway also have roles in leaf senescence (37).

JAZ4 and JAZ8, key repressors in JA signaling pathway (108), were found to physically interact with WRKY57 to negatively regulate JA-induced leaf senescence (42). WRKY57 further interacted with the INDOLE-3-ACETIC ACID INDUCIBLE 29 (IAA29) protein, a repressor of auxin signaling pathways, which positively regulates JA-induced leaf senescence, suggesting that JA and auxin antagonistically regulate JA-induced leaf senescence through WRKY57. Another JAZ protein, JAZ7, controlled dark-induced leaf senescence (124).

Recently, MYC2, 3, and 4, targets of JAZ repressors, were identified as functioning redundantly to activate JA-induced leaf senescence by binding to and activating the promoter of *SAG29* (86). Interestingly, bHLH subgroup IIId factors, bHLH03, 13, 14, and 17, also bind to the *SAG29* promoter and repress its expression to attenuate MYC2/3/4-activated JA-induced leaf



(Caption appears on following page)

Figure 4 (*Figure appears on preceding page*)

Crosstalk between hormone signaling and leaf senescence: effects of hormones on leaf senescence. (*Top*) In young leaves, auxin and cytokinins act as major antisenescing hormones. Auxin suppresses the expression of *SAG*s through the ARF2–ANT pathways. ARR2 and CRF6 are downstream components in the pathway by which AHK3 and TCS mediate enhanced leaf longevity in response to cytokinin. (*Bottom*) In old leaves, JA, ethylene, ABA, SA, and GA signaling pathways initiate and promote leaf senescence. MYC2/3/4, targets of JAZ repressors in JA signaling pathways, activate *CCG*s and *SAG*s in dark-triggered and JA-triggered leaf senescence. ANAC019/055/072, downstream NAC TF genes of MYC2/3/4, also activate *CCG*s for chlorophyll degradation during leaf senescence. ORE1/ANAC092 involves feed-forward loops integrating ethylene (EIN2 and EIN3) and ABA (ATAF1 and ABI5/EEL) into the developmental aging program. ORE1 and AtNAP/ANAC029 activate *NAC*s, *CCG*s, and *SAG*s to promote leaf senescence. The key regulators of ABA signaling pathways ABF2/3/4 also directly activate *CCG*s and *SAG*s through the PYLs–PP2C–SnRK2 regulatory module for ABA-triggered leaf senescence. Besides NAC TFs, WRKY TFs act as positive regulators of leaf senescence. WRKY46/51/63/75 promote leaf senescence through the production of endogenous SA, whereas WRKY6/45 involve GA-triggered leaf senescence by activating *CCG*s and *SAG*s. WRKY75 further represses the expression of *CAT2*, indicating a tripartite amplification loop involving WRKY75, SA, and ROS. Abbreviations: AAO, ABSCISIC ALDEHYDE OXIDASE; ABA, abscisic acid; ABC, ATP-BINDING CASSETTE; ABF, ABSCISIC ACID–RESPONSIVE ELEMENT BINDING FACTOR; ABI, ABSCISIC ACID INSENSITIVE; AHK, *ARABIDOPSIS* HISTIDINE KINASE; ANAC, ABSCISIC ACID–RESPONSIVE NAM/ATAF/CUC; ANT, AINTEGUMENTA; ARF, AUXIN RESPONSE FACTOR; ARR, *ARABIDOPSIS* RESPONSE REGULATOR; ATAF, *ARABIDOPSIS* TRANSCRIPTION ACTIVATION FACTOR; AtNAP, NAC-LIKE, ACTIVATED BY AP3/PI; *BFN*, *BIFUNCTIONAL NUCLEASE*; bHLH, basic helix–loop–helix; *CAT*, *CATALASE*; CCG, chlorophyll catabolic gene; COI, CORONATINE INSENSITIVE; CRF, CYTOKININ RESPONSE FACTOR; EEL, ENHANCED EM LEVEL; EIN, ETHYLENE INSENSITIVE; GA, gibberellin; IAA, indole-3-acetic acid; JA, jasmonic acid; JAZ, JASMONATE ZIM-DOMAIN; NAC, NAM/ATAF/CUC; NCED, NINE-*CIS*-EPOXYCAROTENOID DIOXYGENASE; *NYC*, *NONYELLOW COLORING*; *NYE*, *NONYELLOWING*; ORE, ORESARA; PIF, PHYTOCHROME-INTERACTING FACTOR; PP2C, PROTEIN PHOSPHATASE 2C; PYL, PYRABACTIN RESISTANCE 1–LIKE; ROS, reactive oxygen species; SA, salicylic acid; *SAG*, *senescence-associated gene*; *SEN*, *SENESCENCE*; *SGR*, *STAYGREEN*; SID, SALICYLIC ACID INDUCTION–DEFICIENT 2; *SINA*, *SEVEN IN ABSENTIA*; SnRK, SNF1-RELATED PROTEIN KINASE; TCS, two-component system; TF, transcription factor; *VNI*, *VND-INTERACTING*.

senescence. This antagonistic regulation by activators and repressors would mediate JA-induced leaf senescence at levels suitable for plant survival in fluctuating environmental conditions. Additionally, MYC2/3/4 proteins were found to regulate JA-induced chlorophyll degradation directly by binding to the promoters of major *CCG*s. Furthermore, JA signaling downstream of the MYCs ANAC019/055/072 also regulates the expression of a similar set of *CCG*s (135), indicating the hierarchical and coordinated regulation of JA-induced chlorophyll degradation by two TF family genes.

ABA is one of the most effective plant hormones in terms of promoting leaf senescence. Exogenous application of ABA induces senescence-associated mRNAs and promotes leaf senescence (58), indicating the presence of a link between ABA signaling and leaf senescence. Moreover, a variety of biotic and abiotic stresses elevate ABA level and activate ABA-signaling pathways leading to senescence (92).

Recent studies on ABA-signaling pathways revealed that three essential core components—PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTOR (RCAR), clade A type 2C PROTEIN PHOSPHATASES (PP2Cs), and subclass III SnRK2s—mediate ABA-promoted leaf yellowing and senescence. A large-scale screening of transgenic plants overexpressing the *PYL* family showed that PYL9 promotes drought resistance and leaf senescence by inhibiting PP2Cs and activating SnRK2s (133). Intriguingly, under drought conditions, leaf senescence apparently helps to generate a greater osmotic potential gradient, which causes water to preferentially flow to developing tissues for plant survival. This observation is particularly notable in that leaf senescence is a critical strategy to survive under extreme drought conditions and in that a tight link between drought survival and leaf senescence exists through ABA signaling.

Osmotic potential: a measure of the tendency of a solution to withdraw water from pure water by osmosis across a differentially permeable membrane

Recently, ABF2/3/4 were found to activate the expression of *SAG29* (and possibly other *SAG*s, such as *SAG12*) and *CCG*s through the core system (PYLs–PP2C–SnRK2) of ABA-signaling

pathways. This suggests roles for ABF2/3/4 in the regulation of ABA-triggered leaf senescence and chlorophyll degradation (27). Several NAC TFs are also involved in chlorophyll degradation via induction of the ABA biosynthetic genes. AtNAP increases ABA levels and induces the expression of genes involved in chlorophyll degradation (121). Notably, the rice NAC TF OsNAC2 promotes leaf senescence by inducing ABA biosynthetic genes and downregulating ABA catabolic genes. OsNAC2 also directly regulates the chlorophyll degradation genes *OsSGR* and *OsNYC3* (74). Collectively, the enhanced levels of ABA through the induction of ABA biosynthetic genes support the role of ABA pathways in leaf yellowing and senescence.

SA has long been known to promote natural leaf senescence (68, 90). The endogenous SA gradually increases as a leaf ages, which induces the expression of several *SAG*s during leaf senescence. More than 70% of the WRKY gene family members belonging to different groups are responsive to pathogen infection and SA treatment, and a large portion of them are implicated as regulators of senescence in *Arabidopsis*. WRKY53 and WRKY70 have roles as positive and negative regulators of senescence (6, 77), respectively, and mutant studies on WRKY54, the closest homolog of WRKY70, indicated the functional redundancy or cooperative role of WRKY54 and WRKY70 in leaf senescence (6). Furthermore, WRKY46, WRKY51, WKRY63, and WKRY75 promote SA production by directly inducing the transcription of *SALICYLIC ACID INDUCTION–DEFICIENT 2* (*SID2*) (128). WRKY75 further repressed ROS scavenging by repressing *CATALASE 2* (*CAT2*) (32), indicating that a tripartite amplification loop involving WRKY75, SA, and ROS has a major role in promoting leaf senescence.

ANAC090 was recently identified as a negative regulator of SA-mediated leaf senescence (49); loss of *ANAC090* leads to increased SA levels and accelerated leaf senescence. In general, SA and ROS mutually promote each other's accumulation during leaf senescence; however, *anac090* mutants show no significant difference in the levels of superoxide and H_2O_2 from the wild type. Crosstalk between SA and ROS responses can maintain the levels of antioxidants sufficiently to protect the senescing leaf cells from premature death, ensuring the slow degeneration of cells during leaf senescence.

The gaseous phytohormone ethylene is a widely acknowledged positive regulator of leaf senescence. Exogenous ethylene treatment accelerates leaf senescence, and the ethylene biosynthetic genes *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID* (*ACC*) *SYNTHASE* (*ACS*) and *ACC OXIDASE* (*ACO*) are upregulated in senescing leaves, consistent with an increased ethylene level during leaf senescence (113). Leaf senescence progression is fine-tuned by regulating the stability of key proteins in ethylene-signaling pathways to balance growth and senescence during leaf development. For example, expression of *ACS7* is upregulated during both natural and dark-induced leaf senescence, and high accumulation of ACS7 protein leads to precocious leaf senescence (105). Intriguingly, degradation of ACS7 depends on the first 14 N-terminal amino acid residues, and the degradation of ACS7 is negatively regulated by leaf senescence signaling, allowing ethylene to reach an appropriate level for leaf development. In addition, the protein levels of EIN3 are also modulated by an evolutionarily conserved cellular energy sensor, SnRK1/AKIN10 (45). SnRK1/AKIN10 blocks the photosystem II (PSII) electron transport chain leading to intracellular sugar starvation/energy deprivation. Thus, SnRK1/AKIN10 activity enhances cell viability and delays organ senescence under energy restricted conditions.

The roles of gibberellins (GAs), a large group of tetracyclic diterpenoids, in leaf senescence have been elusive, particularly due to their involvement in diverse developmental processes (1). However, studies suggest that GAs are also involved in the senescence process. Leaf senescence is retarded in the *ga1–3* mutant, where GA biosynthesis is blocked and DELLA proteins GA INSENSITIVE (GAI), REPRESSOR OF GA1-3 (RGA), RGA-LIKE 1 (RGL1), RGL2, and RGL3—the negative regulators of GA signaling pathways—abnormally accumulate (18). In

contrast, despite the deficiency in GA biosynthesis, leaf senescence occurred earlier in the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant (abbreviated as *Q-DELLA/ga1-3*), suggesting that DELLA repression retards natural leaf senescence. Recently, WRKY45 was found to function in the GA-mediated signaling pathway to positively regulate age-triggered leaf senescence (16). Both the delayed leaf senescence phenotype in the loss-of-function mutant *wrky45* and the accelerated leaf senescence phenotype in *WRKY45*-overexpressing plants establish WRKY45 as a positive regulator of leaf senescence. WRKY45 interacts with the DELLA protein RGL1, and this interaction impairs the transcriptional activities of WRKY45 on target genes, including *SAG12* and *SAG113*. Similarly, the DELLA protein RGA negatively regulates dark-induced senescence and chlorophyll degradation through interaction with WRKY6 (131). The interaction results in impaired transcriptional activation by WRKY6 of the downstream target genes, including *SAG13* and *SGR*. Under darkness, *WRKY6* expression increases, but the expression of *DELLA*s decreases, and together these attenuate the repression of RGA on WRKY6 transcriptional activity. Together, the GA–WRKY link observed in these studies provides new insight into the transcriptional regulation of GA-promoted leaf senescence in *Arabidopsis*.

Two-component system (TCS): a stimulus-response coupling mechanism to sense and respond to different environments; a membrane-bound sensor kinase and a DNA-binding response regulator

It is well known that the exogenous application of active cytokinins and an increase in the content of endogenous cytokinins can delay senescence (68). The senescence-delaying effect of cytokinin depends upon the canonical two-component system (TCS) in *Arabidopsis*. Perception of cytokinin by the receptor kinase *ARABIDOPSIS* HISTIDINE KINASE 3 (AHK3) and subsequent phosphorylation of the type B response regulator *ARABIDOPSIS* RESPONSE REGULATOR 2 (ARR2) are required for increased leaf longevity (50). CYTOKININ RESPONSE FACTORs (CRFs), a subset of the plant-specific AP2/ERF domain–containing TF family, have been shown to act as a side branch of the canonical TCS pathway. CRF6 was reported to act as a negative regulator of dark-induced senescence (137). A recent study further demonstrated that expression of *CRF6* is induced by oxidative stress as well as by cytokinin and that CRF6 functions as a negative regulator to repress cytokinin-associated genes during oxidative stress (136), which indicates that CRF6 attenuates the cytokinin signaling to allow an improved stress response.

Early studies indicate that auxins have a role in the suppression of leaf senescence (82, 101). An increased level of auxin in *YUCCA 6* (*YUC6*)-overexpressing plants delayed leaf senescence in natural and dark-induced senescence conditions by reducing *SAG12* expression (53). The thiol-reductase activity of YUC6 further mediates a delay in leaf senescence via the activation of genes involved in redox signaling and auxin redistribution (13). AUXIN RESPONSE FACTOR 2 (ARF2), a repressor of auxin signaling, was found to function in the auxin-mediated regulation of leaf longevity by suppressing the expression of *SAG*s (69). Recently, AINTEGUMENTA (ANT), a member of the AP2/ERF TF family, was identified as a downstream regulator of ARF2 (24). The loss-of-function *ant-1* mutant shows premature leaf senescence, whereas overexpression of *ANT* leads to a delay in leaf senescence. Moreover, *ant-1* mutant represses the delayed leaf senescence phenotype in *arf2-5* mutant plants. Taken together, these findings indicate that ANT is involved in the regulation of leaf senescence downstream of ARF2.

OTHER EMERGING REGULATORY MECHANISMS OF LEAF SENESCENCE

This section highlights other regulatory mechanisms of leaf senescence.

Light Signaling and Leaf Senescence

Light is arguably the most significant environmental factor and light signaling pathways have an important role in regulating leaf senescence. Phytochromes are photoreceptors that signal red

and far-red light information. In *Arabidopsis*, red light negatively regulates leaf senescence, while far-red light positively regulates it (67). Phytochrome signaling is mediated through various PIFs (12). Positive roles of PIF1, 3, 4, and 5 in the control of age- and dark-induced leaf senescence have been well demonstrated (94, 103, 130). Upon prolonged darkness, increased PIF activities directly enhance the expression of multiple targets of senescence regulatory components, and this in turn triggers leaf senescence (94). PIF4 and PIF5 regulate dark-induced leaf senescence, at least partially through direct activation of *ABI5*, *EEL*, and *EIN3*. It was further revealed that *ORE1* is a direct target of PIF4, PIF5, and their three targets, ABI5, EEL, and EIN3, indicating that ORE1 functions as a mediator to integrate phytochrome B–mediated light signaling to promote leaf senescence in light-deprived conditions. Moreover, PIF4 directly activates the expression of the chlorophyll-degeneration regulatory gene *NYE1* and also directly represses *GLK2*, a G2-LIKE TF gene that is important for maintenance of chloroplast activity (103, 130). This finding is in line with the previous observation that ORE1 sequesters GLK1 and GLK2 through protein–protein interaction, which leads to a reduction in the transcriptional activity of GLKs as leaves get older (89). It is thus now obvious that the PIF proteins are core components that transduce photoreceptor-mediated light signaling to key leaf senescence regulators. A recent report reveals that phytochrome A and phytochrome B antagonistically regulate far-red-light-enhanced leaf senescence, which is mediated by WRKY6 (67). Further studies are certainly required to elucidate the detailed molecular mechanisms of how light signaling pathways are interconnected with other internal and external senescence-regulating factors.

Circadian clock oscillator: a biochemical oscillator that cycles with a stable phase and is synchronized with solar time

Evening complex (EC): a critical component of the core oscillator in regulating circadian outputs; mutation of any EC component leads to circadian arrhythmia

Circadian Rhythm, Aging Clock, and Leaf Senescence

Temporal regulation of development is critical for organisms' fitness, so mechanisms measuring the passage of time and executing developmental transitions at the appropriate time should exist. However, there is still no clear understanding of how plants recognize the passage of time.

The circadian clock is a part of the endogenous time-keeping mechanisms that allow organisms to anticipate and prepare for daily and seasonal changes in their surrounding environments (75) and is a good candidate for temporal coordination. Indeed, there is accumulating evidence supporting that the circadian clock is linked with leaf senescence through cross-regulatory networks. Kim et al. (47) showed that the circadian clock is affected by leaf age. The circadian period gets shorter in older leaves, which are regulated through the circadian clock oscillator TIMING OF CAB EXPRESSION 1 (TOC1). This finding provides the first glimpse into understanding how age-dependent changes in the circadian clock are incorporated into age-dependent developmental decisions.

The *Arabidopsis* circadian evening complex (EC), composed of EARLY FLOWERING 3 (ELF3), ELF4, and LUX ARRHYTHMO (LUX), was shown to negatively regulate JA-induced leaf senescence (132). All mutations in the EC components showed accelerated natural leaf senescence and more pronounced JA-induced leaf senescence, whereas plants with an overexpression of *ELF3* displayed the delayed senescence symptom when leaf senescence was artificially induced by JA treatment. The EC component LUX directly binds to the promoter of the *MYC2* TF gene, which encodes a key activator of JA-induced leaf senescence, thereby leading to repression of *MYC2* expression. Furthermore, the *myc2 myc3 myc4* triple mutation abolished the accelerated JA-induced leaf senescence seen in EC mutants, confirming that a core component of the circadian clock gates JA signaling via MYC TFs to regulate leaf senescence.

CIRCADIAN CLOCK–ASSOCIATED 1 (CCA1)-mediated control of leaf senescence was also evident (102, 117). Mutation of *CCA1* causes early leaf senescence, suggesting that there is a negative role for CCA in the regulation of leaf senescence. Intriguingly, CCA1 represses the

positive senescence regulator *ORE1* and activates the chloroplast maintenance gene *GLK2* by directly binding to the promoters of both genes. It is thus conceivable that CCA regulates the expression of *ORE1* and *GLK2* to inhibit leaf senescence at the juvenile stage, but with aging, the declined expression of *CCA1* and *GLK2* attenuates the inhibition of leaf senescence, and the accumulation of ORE1 promotes senescence initiation.

Nitrogen remobilization efficiency: the proportion of nitrogen in the crop or crop component at anthesis that is not present at harvest

It is interesting that ORE1 is also under circadian regulation. This implies that ORE1 is an integrator of age-dependent senescence and the circadian clock. A recent report revealed that PSEUDO-RESPONSE REGULATOR 9 (PRR9), a component of the circadian clock, regulates leaf senescence by directly enhancing expression of *ORE1* and also by repressing *miR164* expression, setting a feed-forward loop (46). Delineation of the interaction networks between the circadian clock and leaf senescence systems will contribute to future understanding of how plant developmental processes are controlled in aging.

Age Gating: Function of a Senescence Regulatory Gene

The circadian system shows circadian gating: The same degree of input shows a different degree of output depending on the time of day (29, 55). The *RECEPTOR PROTEIN KINASE 1* (*RPK1*) gene leads to very different phenotypic effects on leaf development: *Arabidopsis* leaves show enhanced senescence when *RPK1* is induced in old leaves, whereas the leaves show arrested growth with no sign of senescence when *RPK1* is induced in young leaves (58). Thus, the *RPK1* gene leads to drastically different phenotypes depending on leaf age, indicating that an age gating of gene function may exist in plants.

AGRONOMIC IMPLICATION OF LEAF SENESCENCE RESEARCH

Recent advances in physiological and molecular understandings of leaf senescence have led to various strategies of manipulating leaf senescence for agricultural improvement. Here, we discuss two major approaches: One is to identify genes or develop strategies for the improvement of nitrogen remobilization efficiency, and the other is to manipulate key senescence regulatory genes.

Development of Strategies for Efficient Nitrogen Utilization

Nitrogen is the element predominantly remobilized during leaf senescence. Recent evidence shows that autophagy is also an essential process for nitrogen remobilization from leaves to seeds (4, 36). A nitrogen-15 tracing study of autophagy mutants defective in autophagosome expansion and enclosure—*atg5*, *atg9*, and *atg18* in *Arabidopsis*—revealed that the autophagy machinery participates in the control of up to 60% of nitrogen remobilization to seeds under a low-nitrate condition and up to 20% under a high-nitrate condition (30). Due to their decreased nitrogen-remobilization capacity, these *atg* mutants display lower seed production regardless of plant nutrition and overaccumulate nitrogen compounds, such as soluble proteins, ammonium, and peptides identified as degradation products of the Rubisco in rosette leaves, during senescence. The role of autophagy in Rubisco degradation and nitrogen remobilization was also confirmed in the rice *Osatg7* mutant and the maize *atg12* mutant (62, 116). Conversely, *ATG5*- and *ATG7*-overexpressing *Arabidopsis* exhibit delayed senescence and enhanced growth, seed set, and seed oil content (80), providing further mechanistic insight into genetic stimulation of autophagy for the improvement of plant productivity.

Additional investigation of the complex regulatory mechanisms of autophagy is still needed, especially in view of utilizing autophagy for agricultural purposes. It should be noted that autophagy rather than just the proteolysis process itself might control protein degradation as a

shuttle between the substrates and the protease. Thus, the identification of autophagy cargos and the respective proteases that contribute to nitrogen use efficiency will also be of immense importance.

Chloroplast degradation during leaf senescence generates nutrients, such as sugars, lipids, and amino acids for remobilization. It is also well known that glutamine and asparagine, which have two nitrogen atoms per molecule, are preferred molecules to transport nitrogen exported in the phloem sap of plants. Overexpression of the cytosolic *GLUTAMINE SYNTHETASE 1* (*GS1*), which functions in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine, has been successful in numerous cases with the goal of improving crops' nitrogen use efficiency (47). However, the outcome has generally been inconsistent, possibly due to deregulation of GS1 activity via metabolic imbalances (10, 92). Thus, spatially and temporally regulated *GS* overexpression strategies should be considered.

In contrast to the numerous studies on the transgenic modification of GS1 (111), very few studies have been carried out on the effects of plastidic GS2. Wheat varieties with the *TaGS2-2Ab* allele have a higher grain nitrogen concentration. Intriguingly, transgenic expression of *TaGS2–2Ab* under the control of its own promoter in wheat resulted in higher grain yield. It should be also noted that while *GS* genes certainly have an important role in nitrogen remobilization during senescence, other enzymes, such as proline dehydrogenase and glutamate dehydrogenases, could also participate in nitrogen remobilization by providing an ammonium substrate.

It is highly desirable to transport the most nutrient possible from senescing leaves to seeds. Amino acid and peptide transporters that are up- or downregulated during leaf senescence could be potential candidates. *Arabidopsis* AMINO ACID PERMEASE 8 (AAP8) was shown to be expressed in the phloem of source leaves, suggesting its function in phloem loading. In the *aap8* mutant, decreased amino acid phloem loading and partitioning to sinks were observed, resulting in decreased silique and seed numbers (97). A potential role of AAPs in phloem loading and nitrogen remobilization was also supported from studies in pea, where overexpression of the endogenous AAP1 transporter in the leaf phloem led to increased source-to-sink allocation of amino acids and improved seed yield (127).

Transcriptome analyses during leaf senescence have been performed with many crop plants. However, most of the genes predicted to be involved in efficient nitrogen usage are functionally uncharacterized. The CRISPR/Cas9-based genetic editing system and high-throughput screening with a noninvasive phenotyping system in the mutants, transgenic lines, or varieties are likely to facilitate the identification of key molecules and the manipulation of those genes. As a complementary approach, analyzing the metabolic flux analysis during leaf senescence is likely to yield further insights into the molecular mechanisms of nitrogen remobilization.

Nitrogen use efficiency: the ratio between the amount of nitrogen removed from the field by crops and the amount of nitrogen fertilizer applied

Phloem loading: the process of loading carbon into the phloem sieve tubes at the source for transport to different sinks in plants

CRISPR/Cas9: a simple yet powerful tool for editing genomes that easily alters DNA sequences and modifies gene function

Manipulation of Key Regulatory Genes Associated with the External and Internal Factors that Affect Leaf Senescence

Manipulation of leaf senescence exploiting key regulatory genes is primarily based on altering the signaling or level of endogenous hormones and changing the expression of key TFs that control *SAG*s.

Hormone-mediated strategy. Successful modulation of endogenous cytokinin levels was obtained using an autoregulated senescence inhibitor system in which the isopentenyl transferase coding gene, *IPT*, is fused to the *SAG12* promoter of *Arabidopsis*. This strategy was successfully applied in many plant species including important agronomic crops, such as rice, wheat, and lettuce, and resulted in a higher seed set and in the accumulation of more biomass (34). It should be

noted that prolonged leaf longevity may prevent effective nutrient recycling. This may be in good agreement with the fact that, in many crop plants, stay-green cultivars usually show higher grain yield, but nitrogen remobilization in these cultivars is lower than expected. A balance between delayed leaf senescence and nutrient translocation to sink tissues is probably needed for increasing yield in crops (34).

Ethylene is a representative hormone promoting leaf senescence. Research on the genetics of ethylene biosynthesis has been successfully translated from *Arabidopsis* to crops and from the laboratory to the field. For example, in maize, downregulation of *ACS6* shows prolonged leaf longevity when exposed to drought stress (123) and results in a significant increase in grain yield when exposed to drought stress in the field (35, 99, 123). AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (ARGOS) proteins are negative regulators of ethylene response. Interestingly, CRISPR/Cas9-engineered variants of maize with increased *ZmARGOS8* expression levels show a higher grain yield under drought stress conditions (99, 100).

Given the wide variety of ethylene-mediated developmental processes, disease and stress responses, and interaction with other hormone responses, genetic alteration of upstream members of the ethylene-signaling pathway often results in pleiotropic phenotypes. Thus, downstream players, such as the ETHYLENE RESPONSE FACTORs, could be better candidates to target. The overexpression of *HIGHER YIELD RICE*, a rice *ERF*, has increased shoot biomass and grain yield under normal and drought conditions (3). Unraveling the highly complex regulatory networks in which ERFs are involved will enable new advances in the targeted engineering of ethylene-mediated responses.

Transcription factor–mediated strategy. Senescence-manipulating technology involving some key senescence-specific TFs, such as AtNAP, appears very promising for crop improvement.

Attempts have been made to suppress the expression of the *AtNAP* homologous genes in a variety of plant species (34). Analysis of the *OsNAP*-repressed transgenic lines shows a 24% increase in rice grain yield. Similarly, suppression of the maize ortholog of *AtNAP*, *ZmNAP*, by RNA interference (RNAi) silencing showed delayed leaf senescence and a 15–30% increase in thousand grain weight (129). RNAi silencing of three NAP orthologs in wheat flag leaves caused a 24-day delay in the senescence of flag leaves (112). The drastic delay of senescence in wheat, however, did not lead to any increase in yield. Moreover, the RNAi plants showed a reduction in grain protein, zinc, and iron content (112). Nutrient remobilization from senescing leaves critically contributes to grain yield and quality, so an extended delay of leaf senescence may not be desirable in some crops.

It is expected that different plant species will have different senescence physiology. Thus, knowledge obtained from a model species may not be transferrable to other plant species. Comparative study of the functions of the homologous regulatory genes of senescence among plant species that have adapted in different environments may also reveal important aspects of the senescence program.

SUMMARY POINTS

1. Complex transcriptional regulation involving diverse transcription factors (TFs), including bZIP (ABI5 and EEL), NAC (ANAC019 and ORE1), and MYC (MYC2, 3, and 4) family TFs, have uncovered several key regulatory mechanisms that control chlorophyll breakdown during leaf senescence.

2. Temporal dynamics and functions of gene regulatory networks involving the NAC and WRKY TF families reveal important roles for these families in the regulation of leaf senescence. Notable findings include the identification of key regulatory network modules involved in ethylene-mediated chlorophyll degradation and NAC TF hub molecules responsible for time-dependent NAC network transitions.
3. A range of transcriptomic, epigenomic, proteomic, and metabolomic studies have improved our current understanding of the functional and regulatory transitions in leaf senescence.
4. Senescing leaves have tighter temporal coordination of transcriptomes than growing leaves.
5. The regulatory programs of leaf senescence are finely regulated at multiple chromatin, transcriptional, posttranscriptional, translational, and posttranslational levels by integrating internal and external signals into the leaf senescence program.
6. The circadian clock is emerging as a key regulator of leaf senescence.
7. Autophagy is an important process for nitrogen recycling and remobilization during senescence. Manipulation of the autophagy pathway is a promising strategy for the improvement of crop yields.
8. Senescence-manipulating technology utilizing the P_{SAG}-*IPT* autoregulatory senescence inhibition system, ethylene pathways, and AtNAP, one of the key senescence regulatory TFs, was successful at improving yields and the length of postharvest storage in many crop plants, showing great potential for commercialization.

FUTURE ISSUES

1. Most of the previous studies on leaf senescence have been focused on the late stages of aging. However, leaf senescence, as an integral part of plant development, is affected not just by the current internal and external conditions, but by all of the earlier developmental stages. Thus, it may be better understood from a life history perspective.
2. Leaf senescence involves crucial time-dependent physiological changes and environmental responses. Thus, the complex process of senescence might be better understood in the context of molecular network dynamics. Multilayered interaction-based analyses of senescence, including the dynamics of protein–protein, protein–DNA, protein–RNA, and RNA–RNA complexes in a time-dependent manner or spatial networks that involve mitochondria, chloroplasts, vacuoles, cytoplasm, and nuclei, are a crucial next step.
3. Integration of multi-omics data will decode spatiotemporal, age-associated networks. Critically missing are proteomic data. In addition, the use of plant phenomics will be required to investigate physiological changes along the entire lifespan of a leaf and to elucidate age-associated changes in morphology, physiology, and molecular behaviors in a comprehensive manner. Integrative analyses of multi-omics data sets will enable the identification of key determinants in the developmental transition to the senescence stage by mapping the modules whose temporal changes are mostly correlated with those of physiological, molecular, and metabolic characteristics.

4. Senescence is regulated by age. How age information is perceived and how it is processed are fundamental questions for enhancing our understanding of leaf senescence. Studies on the interaction of age information with the circadian clock are a first step toward answering this question.
5. *Arabidopsis* leaves show a vast variety of leaf senescence phenotypes. Evolutionary mechanisms associated with reproduction may be pursued utilizing these ecotypes. High-throughput phenomic analysis of various physiological and developmental traits from *Arabidopsis* accessions will facilitate the evaluation of each trait with respect to its importance for fitness.
6. The mechanisms observed in *Arabidopsis* need to be compared with those in crop plants and further compared with those in animals.
7. Genetic resources and knowledge for manipulating leaf senescence have been rapidly expanding. Because leaf senescence involves the spatial and temporal regulation of complex networks, a finer approach that includes the spatial and temporal modification of gene expression will be needed. Approaches such as the CRISPR/Cas9 system for genome editing should help toward this goal.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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