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REVIEW PAPER

Beyond the genetic code in leaf senescence

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Abstract

Leaf senescence is not only genetically programmed but also induced by exogenous stress to ensure completion of the plant life cycle, successful reproduction and environmental adaptability. Genetic reprogramming is a major aspect of leaf senescence, and the senescence signaling that follows is controlled by a complex regulatory network. Recent studies suggest that the activity of transcription factors together with epigenetic mechanisms ensures the robustness of this network, with the latter including chromatin remodeling, DNA modification, and RNA-mediated control of transcription factors and other senescence-associated genes. In this review, we provide an overview of the relevant epigenetic mechanisms and summarize recent findings of epigenetic regulators of plant leaf senescence involved in DNA methylation and histone modification along with the functions of small RNAs in this process.

Keywords: Epigenetic regulation, genetic reprogramming, histone modification, leaf senescence, senescence-associated genes, small RNAs.

Introduction

Plants convert CO_2 into carbohydrates through photosynthesis, and the leaf is a major site of photosynthetic metabolism. Photosynthesis is reduced during leaf senescence, and catabolic reactions are enhanced through the degradation of macromolecules, such as nucleic acids, proteins, and lipids, to yield reusable nutrients for developing organs, such as young leaves, reproductive organs, and seeds. The metabolic transition during leaf senescence can thus be viewed as a cost-effective mechanism responsive to both the genetic program and the environment. Senescence is genetically programmed and modulated in an age-dependent manner. The onset, progression, and termination of senescence are controlled by several layers of regulatory networks, including transcriptional and post-transcriptional regulation, protein modification, and hormone signaling (Woo *et al.*, 2013; Schippers, 2015).

Upon initiation of leaf senescence, genetic reprogramming resets the regulatory network of senescence-associated genes (SAGs). The nature of the initial signal that triggers leaf senescence remains unclear, but certain transcription factors (TFs) are known to act upstream of the senescence regulatory pathway to activate SAGs. The functional roles of individual TFs involved in leaf senescence were initially characterized through genetic studies, and subsequent transcriptome analyses provided further evidence of the functional complexity of TFs involved in leaf senescence (Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2002; Guo and Gan, 2006; Kim *et al.*, 2009; Breeze *et al.*, 2011; Woo *et al.*, 2013, 2016). Two TF families, NAC and WRKY, are well established as key regulators of the leaf senescence pathway (Hinderhofer and Zentgraf, 2001; Guo and Gan, 2006; Woo *et al.*, 2013).

Plants are exposed to biotic and abiotic stresses in the environment, and their stress response systems confer developmental flexibility to ensure successful completion of the reproductive phase. Leaf senescence is tightly linked to environmental adaptability, and the senescence pathway can be induced in response to external stimuli. There is some degree of overlap between the genetic programs that underlie genetically programmed senescence and exogenously induced senescence (Schippers *et al.*, 2015). ORESARA1 (ORE1), a key NAC TF that promotes leaf senescence in an age-dependent manner, is also implicated in salt-triggered senescence (Balazadeh *et al.*, 2010). Dark conditions and biotic stresses also activate age-dependent SAGs to induce the senescence program (Al-Daoud and Cameron, 2011; Sakuraba *et al.*, 2014; Fernández-Calvino *et al.*, 2016). Despite these examples of overlap in the signaling pathway between programmed and induced senescence, however, the shared and specific components between them remain unclear. To address this question, one study compared transcriptome data in response to 27 senescence-promoting stresses; the analysis showed that after long-term treatment, there was a high degree of overlap in the signaling pathway during leaf senescence progression, i.e. when senescence symptoms such as yellowing leaves are visible. In contrast, there was limited similarity between induced and programmed senescence at the early stages of stress treatment, indicating distinct initial onset signals (Guo and Gan, 2012).

Besides transcriptional regulation by TFs, epigenetic regulation is a key mechanism for modulating gene expression. Epigenetic regulation may occur at the level of RNA, DNA, or chromatin structure, thereby contributing to regulatory dynamics in signaling networks. In addition to *cis*- and *trans*-acting factors that induce SAGs at the transcriptional level, recent findings have shed light on the epigenetic regulation of plant senescence through non-coding RNAs, DNA modification, and histone modification (Ay *et al.*, 2014*a*). Here, we provide a general model and overview of the molecular basis of epigenetic and post-transcriptional regulation in plant leaf senescence.

Epigenetic regulation in plants

Epigenetic regulation causes changes in phenotype or expression without altering the underlying DNA sequences, primarily via DNA methylation, histone modification, and nucleosome positioning. These changes alter chromatin status, thereby leading to active or inactive gene expression. Nucleosomes are the main unit of chromatin and are composed of two copies of each histone protein, H2A, H2B, H3 and H4, with approximately 145–147 base pairs of DNA wrapped around the histone octamer (Luger *et al.*, 1997). The nucleosome structure can be condensed and decondensed, forming heterochromatin and euchromatin, respectively. DNA packaged around the histones determines the three-dimensional structure of chromatin, and as a result, gene transcription is under the control of DNA methylation and covalent histone modifications as well as chromatin remodeling factors and non-coding RNAs (Rice and Allis, 2001; Humbeck, 2013). The flexible structure of chromatin allows it to regulate gene transcription during distinct cellular processes. At the same time, chromatin modifications may regulate gene expression depending on the tissue, species, organelle, or age (Vanyushin and Ashapkin, 2011). Global changes in chromatin architecture and changes in gene transcription mediated by chromatin modifications play key roles throughout plant development, including leaf senescence.

DNA methylation

As a major epigenetic mark accompanied by the silencing of genes, DNA cytosine methylation has been implicated in numerous biological events, including gene and transposon silencing, imprinting, and X chromosome inactivation in eukaryotic cells (Law and Jacobsen, 2010). In the CG, CHG, and CHH (where H is A, T or C) DNA contexts, cytosine methylation is primarily mediated by DNA METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 3 (CMT3), and DOMAINS-REARRANGED METHYLTRANSFERASEs (DRMs), respectively, in plants. DNA methylation can be removed passively or actively. Active DNA demethylation involves proteins containing DNA glycosylase domains, such as REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), and DEMETER-LIKE proteins (DML2/3) (Gehring *et al.*, 2009). DNA methylation in plant genomes is more comprehensive and influences a wider range of sequences than in animal genomes (Vanyushin and Ashapkin, 2011), but how and whether DNA methylation changes as plants age remain open questions. In plants, gene activation is regulated by DNA methylation and demethylation at promoters or gene bodies. DNA methylation also contributes to the maintenance of genome stability through the silencing of transposable elements and other repetitive sequences that detrimentally affect genomes (Chan *et al.*, 2005; Groth *et al.*, 2016).

Histone modifications

The interaction between negatively charged DNA, proteins and histone N-terminal tails is altered by post-translational modifications such as methylation (me), acetylation (ac), ubiquitination, and phosphorylation at the N-terminal tails of core histone proteins (Strahl and Allis, 2000; Rice and Allis, 2001). Lysine, serine, threonine, and arginine residues at histone N-tails protruding from the histone octamer are modified by these different post-translational modifications, which ultimately affect chromatin structure and gene expression by altering the DNA–histone interaction and the accessibility of transcription factors (Strahl and Allis, 2000; Wu and Grunstein, 2000; Kim *et al.*, 2008*a*). For instance, at histone H3, acetylation and methylation occur at different lysine residues, including acetylation of lysine 9, 14, 18, 23, and 27 (H3K9ac, H3K14ac, H3K18ac, H3K23ac, and H3K27ac) and methylation of lysine 4, 9, 27, and 36 (H3K4me, H3K9me, H3K27me, and H3K36me) (Garcia *et al.*, 2007). Among the various histone modifications, H3K9ac and

H3K4me3 are associated with active gene transcription, whereas H3K9me2 and H3K27me3 marks are involved in gene repression (Jenuwein and Allis, 2001; Kouzarides, 2007). The findings from a number of studies have established that H3 and H4 histone modifications are correlated with the expression of certain genes related to development, senescence, flowering, and stress responses in plants (Alvarez-Venegas and Avramova, 2005; Ay *et al.*, 2009; Kim *et al.*, 2012; Janack *et al.*, 2016; Mengel *et al.*, 2017).

Chromatin remodeling

Enzyme activities that alter the accessibility of DNA under different conditions and in different tissues can affect DNA–histone interactions in two ways. First, chromatin remodelers may alter the interaction between DNA and the histone octamer non-covalently, requiring energy from ATP hydrolysis (Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011; Narlikar *et al.*, 2013). Second, chromatin modification enzymes can mediate covalent changes that add or remove residues from histones or DNA (Li *et al.*, 2007). Besides histone modifications and DNA methylation, ATP-dependent chromatin remodeling complexes can alter the positioning, occupancy, and composition of nucleosomes, thereby modulating the accessibility of the genome to regulatory proteins in a non-covalent manner (Jerzmanowski, 2007; Han *et al.*, 2015).

Chromatin remodeling factors play important roles in wide-ranging processes, including stem cell maintenance and differentiation, developmental stage transitions, and stress responses in plants and animals. Although much of the information about chromatin remodeling factors has come from studies in metazoans (Cairns, 2009; Hargreaves and Crabtree, 2011; Narlikar *et al.*, 2013), there are some experimental data from plants regarding the biochemical mechanism and components of chromatin remodeling complexes (Han *et al.*, 2015).

Small RNAs

Small non-coding RNAs are a class of regulators important for many aspects of plant development, stress response, and metabolism. Plant small RNAs are typically divided into two major categories according to their biogenesis and mode of action (Axtell, 2013; Rogers and Chen, 2013). MicroRNAs (miRNAs), typically 21–22 nt long, are generated from hairpin-shaped RNA precursors by the RNaseIII activity of DICER-LIKE 1 (DCL1) in the nucleus. Mature miRNAs are transported into the cytosol and loaded onto the RNA-induced silencing complex (RISC), whereby miRNAs guide ARGONAUTE 1 (AGO1) to control the expression of cognate RNA targets by mRNA cleavage or translational repression at the post-transcriptional level. In addition to these 21-nt miRNAs, plants also generate 24-nt miRNAs from stem–loop structures via DCL3 activity. These miRNAs are loaded onto RISC complexes containing AGO4 rather than AGO1. They bind to the nascent transcripts generated from their own loci or target mRNAs and recruit the *de novo* DNA methylation machinery to adjacent DNA sequences for DNA methylation (Wu *et al.*, 2010). Small interfering RNAs (siRNAs) are produced from double-stranded RNA precursors by DCL3; 24-nt heterochromatic siRNAs (hc-siRNAs) mediate DNA methylation at transposons and repeat sequence loci to maintain chromosome integrity. Another class of plant siRNAs is the *trans*-acting siRNAs (tasiRNAs). tasiRNA precursors usually contain one or two binding sites for a miRNA trigger, which associates with AGO1 to guide the cleavage of the tasiRNA precursors. The 3′ fragments of the cleavage products are protected by SUPPRESSOR OF GENE SILENCING 3 (SGS3) and made double-stranded by RNA DEPENDENT RNA POLYMERASE 6 (RDR6). The double-stranded intermediates are further processed into a phased array of 21-nt siRNAs from positions adjoining the mRNA cleavage site. The mature tasiRNAs bind to RISC and direct cleavage of their target mRNAs in *trans*. Several miRNAs and tasiRNAs have been found to control plant senescence progression by tuning the expression of transcription factors or phytohormone response factors (Humbeck, 2013; Woo *et al.*, 2013); consistently, obvious senescence phenotypes result from changes in the abundance of these small RNAs or their target mRNAs. Genomic and bioinformatics studies have uncovered a broad spectrum of small RNAs related to plant senescence, and further genetic and molecular studies will help pinpoint their placement and roles in the plant senescence regulatory network.

Changes in DNA methylation during plant aging

A number of studies in recent years have examined the relationship between plant aging and DNA methylation (Dubrovina and Kiselev, 2016). For example, the functional relevance of MET1 activity for plant senescence was tested with a transgenic line with a *MET1* antisense gene under the *DME* promoter (*DME:MET1a/s*). Although DME promoter activity was only observed in proliferating cells, such as leaf primordia and lateral root primordia, the *DME:MET1a/s* line exhibited pleiotropic developmental defects, including delayed senescence, suggesting that MET1 DNA methylation may be involved in plant senescence (Kim *et al.*, 2008*b*). A more recent study showed that global DNA methylation is reduced during aging in Arabidopsis shoots using a methylation-sensitive DNA fragmentation assay. In addition, transcript levels of the methylation genes *CMT3* and *MET1* declined during the development and aging of Arabidopsis, whereas those of demethylation genes, including *ROS1*, *DME*, *DML2*, and *DML3*, increased (Ogneva *et al.*, 2016). Thus, altered abundance or activity of methylation/demethylation enzymes may contribute to the DNA methylation level. However, the specific effects of DNA methylation on leaf senescence are virtually unknown. Recently, the transcript levels of DNA methylation-related genes were quantified in Arabidopsis leaf, and both *MET1* and *ROS1* were down-regulated during senescence (Ay *et al.*, 2014*a*). However, DNA methylation levels

were not quantified, so no conclusions can be drawn regarding the correlation between DNA methylation status and the activity of these enzymes during leaf senescence.

Despite the emerging clues suggesting a functional link between DNA methylation and plant aging, there is no solid evidence of a DNA methylation-mediated leaf senescence mechanism. In particular, a fundamental question is whether the changes in DNA methylation are the cause of leaf senescence or a downstream result caused by the leaf senescence process. There is presently no conclusive evidence to answer this question, and new strategies are therefore required to address it. For example, developing an inducible system utilizing a genetic and/or pharmacological approach to control DNA methylation status in the leaf could help to answer this question. In addition, the regulation and activity of DNA methylation-regulating enzymes need to be more deeply investigated during leaf senescence. The downstream effects of DNA methylation in leaf senescence also need to be considered. One of the main functions of DNA methylation is to stabilize the genome through the repression of transposable elements (TEs). Active TEs can be mutagenic, and recent studies have revealed a relationship between TE activation/inactivation and effects on the expression of nearby genes and the stress response (Horváth *et al.*, 2017). Although several studies have investigated the expression patterns of TEs and DNA methylation-related enzymes during leaf senescence (Ay *et al.*, 2014*a*; Guo and Gan, 2012), the association between DNA methylation change and the levels of TEs and genes remains unclear. Comparative genome, methylome, and transcriptome analysis may be one strategy for predicting the correlation among functional activation/inactivation of TEs, gene expression, and DNA methylation in leaf senescence.

Histone modifications linked to leaf senescence

Histone acetylation

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) control histone acetylation and deacetylation (Kouzarides, 2007), and the functional balance between them is important for proper plant development. To identify epigenetic mechanisms that regulate developmental and stress-responsive processes related to leaf senescence, several research groups have utilized gain-of-function and loss-of-function mutants affecting histone modification enzymes. In a study investigating Arabidopsis histone deacetylase 1 (AtHD1 or AtHDA19), an anti-sense *AtHD1* transgenic line had dramatically reduced *AtHD1* transcript levels and pleiotropic developmental defects, including early senescence (Tian and Chen, 2001). HDA6, another histone deacetylase, was found to influence global histone acetylation levels in Arabidopsis (Wu *et al.*, 2008), based on the observation of higher H3 acetylation in the *HDA6* mutant *axe1-5* and in *HDA6* RNA-interference (*HDA6-RNAi*) plants compared with wild-type (WT). Interestingly, *axe1-5* and *HDA6-RNAi* plants exhibited delayed leaf senescence phenotypes based on chlorophyll content analysis and the photochemical efficiency of photosystem II (Wu *et al.*, 2008). Moreover, senescence marker genes, such as *SAG12* and *SEN4*, were down-regulated in *axe1-5* and *HDA6-RNAi*, suggesting that the delayed leaf senescence was due to the misregulation of SAGs. The putative histone acetyltransferase HOOKLESS1 (HLS1) plays a role in biotic and abiotic stress responses, and such factors are worth considering given the enhancement of leaf senescence under ABA and dark treatment. HLS1 directly interacts with the mediator subunit MED18 to access target loci to promote gene expression through H3 acetylation modification (Liao *et al.*, 2016). The histone acetyltransferase Elongator interacts with RNA polymerase II and possibly promotes transcription through histone acetylation. In tomato, the Elongator subunit 2-like gene *SlELP2L* was found to regulate leaf senescence, as suppression of *SlELP2L* led to accelerated leaf senescence accompanied by reduced transcript levels of *Rbcs-2*, a ribulose-1,5-biphosphate carboxylase-oxygenase (Rubisco) subunit gene and a potential indicator of the photosynthetic rate (Zhu *et al.*, 2015). The authors proposed several possible SlELP2L functional mechanisms including histone acetylation, ubiquitin–proteosome pathway function or DNA methylation, but further investigation is needed to identify the mechanism through which *SlELP2L* regulates leaf senescence.

Although altered leaf senescence phenotypes have been observed in the mutants of histone modification enzymes, it is not clear whether histone acetylation levels directly regulate the expression of senescence-related genes. Nevertheless, several studies have shown direct effects of histone modification enzymes on key SAGs in Arabidopsis and crop plants. For example, a new role of Arabidopsis histone deacetylase 9 (HDA9) in promoting the onset of leaf senescence was recently reported (Chen *et al.*, 2016). The study showed that HDA9 interacts with the SANT domain-containing protein POWERDRESS (PWR) and WRKY53 in a complex. Based on genome-wide profiling of HDA9 occupancy, PWR is necessary for the translocation of HDA9 into the nucleus and the direct association of HDA9 with the promoters of key negative regulators of senescence. In fact, H3K27ac levels were increased at HDA9 binding loci encoding SAGs (Chen *et al.*, 2016). In transgenic barley, RNAi-mediated knockdown of the *HvWHIRLY1* gene led to delayed leaf senescence under drought and high light intensity conditions (Janack *et al.*, 2016; Kucharewicz *et al.*, 2017). In particular, the transcript levels of *WRKY* and *NAC* family genes, which play important roles in senescence- and stress-associated signaling pathways, were reduced in the RNAi-mediated *WHIRLY1* knockdown line, suggesting that WHIRLY1 is an upstream regulator of drought stress-induced senescence. In drought-treated WT plants, H3K9ac enrichment was detected at the promoter and coding regions of the senescence-associated gene *HvS40*, along with WHIRLY1 binding at its promoter based on ChIP coupled with qRT-PCR. In plants with impaired WHIRLY1 accumulation, H3K9ac enrichment was not detected at this locus, suggesting that WHIRLY1 mediates epigenetic changes at senescence-associated genes (Janack *et al.*, 2016). The histone deacetylase OsSRT1, a rice homolog of SILENT INFORMATION REGULATOR 2 (SIR2), also regulates leaf senescence, based on the accelerated leaf senescence

and increased H3K9ac observed in an *OsSRT1* RNAi line. In addition to the altered expression of genes related to programmed cell death (PCD) and aging in the RNAi line, enriched H3K9ac was detected at the *SAG12* senescence marker gene (Huang *et al.*, 2007).

These results clearly indicate that gene regulation through histone acetylation and deacetylation is required for leaf senescence regulation. Considering that different HATs and HDACs have specific or redundant functions, it would be interesting to understand the mechanism by which a given HAT/HDAC recognizes its spatial and temporal target regions to induce leaf senescence progression.

Histone methylation

Like histone acetylation, histone methylation is another important epigenetic mark that regulates gene transcription in plants. The functional impact of histone methylation depends on which residues are methylated, the extent of methylation, and the chromatin context (Liu *et al.*, 2010). Mono-, di-, or trimethylated lysine residues of histone H3 may be involved in either the suppression or the enhancement of gene expression (Zhang *et al.*, 2009). For example, high levels of H3K4me3 are generally associated with enhanced gene expression and active genes, based on findings in yeast (Pokholok *et al.*, 2005), rice (Li *et al.*, 2008), Arabidopsis (Kim *et al.*, 2008*a*; Zhang *et al.*, 2009), and human (Barski *et al.*, 2007). In contrast, H3K9me2, H3K9me3, and H3K27me3 are associated with the silencing of gene expression (Jackson *et al.*, 2002; Turck *et al.*, 2007; Zhang *et al.*, 2007; Bernatavichute *et al.*, 2008). The identification of histone demethylases, such as lysine-specific demethylase 1 (LSD1) (Shi *et al.*, 2004; Metzger *et al.*, 2005) and JmjC domain-containing proteins (Tsukada *et al.*, 2006; Whetstine *et al.*, 2006; Yamane *et al.*, 2006), revealed the reversible nature of histone methylation marks.

In a genome-wide analysis of histone methylation changes associated with leaf senescence in Arabidopsis (Brusslan *et al.*, 2012), H3K4me3 was found to be increased on up-regulated genes and decreased on down-regulated genes in old leaves compared with young leaves during senescence. Up-regulated genes also had reduced H3K27me3 levels during senescence. However, the transcriptionally active H3K4me3 mark was not required for the regulation of all senescence up-regulated genes: around 50% of senescence up-regulated genes were depleted of H3K4me3 in both mature and senescent leaves, and the senescence-associated genes *SAG12* and *At1g73220* were dramatically activated without the H3K4me3 mark (Brusslan *et al.*, 2012). A follow-up study further monitored two active marks, H3K4me3 and H3K9ac, and used a more defined time scale during leaf senescence. H3K4me3 was relatively dominant compared with H3K9ac, and a subset of differentially expressed genes during leaf senescence was significantly correlated with the level of H3K4me3 (Brusslan *et al.*, 2015).

Heterochromatic decondensation has been observed in senescent leaves, particularly for heterochromatic repressive marks H3K9me2 and H3K27me2 (Ay *et al.*, 2009). Furthermore, upon induction of the leaf senescence regulator *WRKY53*, H3K4me2 and H3K4me3 levels were found to be markedly increased at the 5′-end and coding regions of the *WRKY53* locus. However, overexpression of the histone methyltransferase gene *SU(VAR)3–9 HOMOLOG 2* (*SUVH2*), which inhibits heterochromatin decondensation, repressed the transcription of *WRKY53* and the senescence-associated genes *SIRK*, *SAG101*, *A083*, *SAG12*, and *SAG24*, resulting in delayed leaf senescence (Ay *et al.*, 2009). In a more recent study, about 50% of all examined senescence-related regulatory factors (SRRFs) were disrupted when *SUVH2* was overexpressed, further suggesting that SUVH2-mediated chromatin modification is involved in the regulation of leaf senescence (Ay *et al.*, 2014*b*). Future experimental data related to senescence-associated histone modifications and altered chromatin status at candidate genes will help address whether senescence-specific chromatin structure changes are significant and/or widespread and whether heterochromatization in *SUVH2*-overexpressing plants inhibits the expression of senescence-specific genes (Ay *et al.*, 2014*b*).

Taken together, these findings show that histone modifications and global changes in chromatin structure regulate senescence-associated gene transcription in plants.

Chromatin remodeling factors required for leaf senescence

The role of SWI/SNF chromatin remodelers in leaf senescence remains unclear, but some of these enzymes are known to act in this process. For example, the role of the SWI2/SNF2 chromatin remodeling protein DRD1 in regulating leaf senescence was recently investigated (Cho *et al.*, 2016). DDM1 and DRD1 are two members of the same SWI2/SNF2 family, and the *ddm1-2* and *drd1-6* mutants both harbor a mutation in the helicase domain. *ddm1-2* and *drd1-6* mutants exhibit delayed leaf senescence and a prolonged lifespan. After 5 days of dark-induced senescence in Arabidopsis, a significant inhibition of senescence-associated genes was observed in *drd1-6*. Because DRD1 was previously shown to be involved in RNA-directed DNA methylation (Kanno *et al.*, 2004), the authors analysed epigenetic regulation with quantitative expression analysis of 180 bp centromeric (CEN) and transcriptionally silent information (TSI) repeats in WT and *drd1-6* plants during dark-induced senescence. Although expression levels were strongly enhanced in both WT and mutant plants, CEN and TSI repeats were more highly expressed in WT than in *drd1-6*, suggesting that DNA methylation-mediated transcriptional gene silencing of CEN and TSI repeats through DRD1 activity may be linked to dark-induced senescence (Cho *et al.*, 2016). DDM1 synergistically regulates DNA methylation with DRD1 in an RdDM-independent pathway (Zemach *et al.*, 2013), suggesting that DDM1-mediated leaf senescence regulation might be distinct from that of DRD1. Based on these findings, the authors proposed that the ATP-helicase domain of SWI2/SNF2 chromatin remodelers is important for the regulation of leaf senescence (Cho *et al.*, 2016).

Another chromatin remodeling factor involved in the regulation of developmental processes in plants is the AT-hook protein ORESARA 7 (ORE7). The *ORE7*/*ESC* gene, which

encodes a protein with an AT-hook DNA-binding motif, was found to modify chromatin structure during interphase, potentially affecting the regulation of leaf longevity (Lim *et al.*, 2007*b*). Specifically, *ORE7* transcription had a dosage-dependent impact on the initiation of leaf senescence and chromatin organization (Lim *et al.*, 2007*b*). Both overexpression lines and an activation-tagged *ORE7* mutant displayed a delayed leaf senescence phenotype (Lim *et al.*, 2007*a*,*b*). In the activation-tagged *ORE7* mutant, 368 genes were characterized as senescence-associated, indicating that chromatin remodeling through *ORE7* function is important for the regulation of leaf senescence.

miRNA-mediated leaf senescence regulation

The NAC protein family member ORE1 positively regulates age-induced cell death in plants, and its expression is negatively regulated by miR164 (Kim *et al.*, 2009). ETHYLENE INSENSITIVE 2 (EIN2) is an important ethylene signaling pathway component and promotes senescence progression by repressing miR164 expression. *EIN2* expression increases upon leaf aging, resulting in *ORE1* repression by miR164 at early stages and *ORE1* activation at later stages. EIN2 also directly activates *ORE1* expression independently of miR164, forming a trifurcate feed-forward pathway to ensure the fine-tuning of plant senescence and cell death (Kim *et al.*, 2009). miR319 represses the expression of several members of the *TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP)* family, and miR319 overexpression or *TCP4* mutations result in delayed leaf senescence in Arabidopsis (Schommer *et al.*, 2008). An important gene involved in the jasmonic acid (JA) synthesis pathway, *LIPOXYGENASE 2* (*LOX2*), was identified as a downstream target of TCP4. Application of exogenous methyl jasmonate (MeJA) to miR319-overexpressing plants restored the delayed leaf senescence phenotype, demonstrating that miR319 regulates plant senescence progression by repressing JA biosynthesis.

Plant GROWTH-REGULATING FACTOR (GRF) transcription factors control cell proliferation and organ size and are negatively regulated by miR396 (Debernardi *et al.*, 2012). Specifically, miR396 antagonizes the expression pattern of *GRFs* and restricts their function to certain organ areas (Rodriguez *et al.*, 2010). Transformation of a miR396-resistant version of *GRF3* into Arabidopsis extended leaf longevity, and miR396 overexpression greatly induced the expression of senescence marker genes *SEN1* and *SEN4* (Debernardi *et al.*, 2014), implying a role of miR396 in senescence control. Plant tasiRNAs are a group of secondary siRNAs. Their biogenesis is initiated with cleavage directed by miRNA triggers, followed by phased cleavage by DCL4 that yields 21-nt small RNAs (Yoshikawa *et al.*, 2005). TAS3 production is triggered by miR390, and it represses the expression of the auxin response genes *ARF2*, *ARF3*, and *ARF4* (Marin *et al.*, 2010). Among them, *ARF2* is involved in senescence progression, and the *arf2* mutant has a delayed leaf senescence phenotype (Ellis *et al.*, 2005; Lim *et al.*, 2010). Thus, TAS3 may inhibit plant senescence via *ARF2* repression.

Many mature miRNAs derive from genes belonging to the *MIR* family. Although mature miRNAs from different family members may differ by a few nucleotide mismatches, they potentially recognize the same target sequence. Each *MIR* gene is transcribed by Pol II from its own promoter (Lee *et al.*, 2004), underscoring how the expression of miRNAs can be spatiotemporally dynamic and specific. For example, three *MIR164* genes (*MIR164a*, *MIR164b*, and *MIR164c*) encode miR164, and miR164 is known to act in the two distinct developmental processes of leaf shaping and leaf senescence by repressing *CUP-SHAPED COTYLEDON1* (*CUC1*)/*CUC2* and *ORE1*, respectively. Thus, the correlation between the spatiotemporal expression of a miRNA and its target may govern the functional dynamics of a given miRNA. The spatiotemporal expression patterns of leaf senescence-regulating miRNAs and their correlation to targets therefore warrant investigation. Furthermore, spatial or temporal restrictions between miRNAs and their targets should also be considered along with how plants circumvent or respond to these restrictions.

Small RNAs differentially expressed during the leaf lifespan

Genomic studies have allowed large-scale identification of small RNAs potentially involved in the regulation of different developmental processes and stress responses. Analysis of small RNAs from the growth-to-maturation (G-to-M) stage and the maturation-to-senescence (M-to-S) stage of leaf development suggested that plants mobilize different spectrums of small RNAs at these two stages to regulate distinct biological processes (Woo *et al.*, 2016). At the M-to-S stage, many small RNAs involved in the ABA response, disaccharide metabolism, and lipid metabolism pathways were dramatically decreased, indicating that plants utilize small RNAs as a general strategy to coordinate senescence progression. Because AGO1 is the major effector of miRNA activity in plants, AGO1-associated small RNAs can be used as a provisional indicator of active miRNAs in certain cells. Among these miRNAs in the study, 64 miRNAs were verified as targeting 42 SAGs, suggesting a possible role of miRNAs as modulators in the leaf senescence network (Qin *et al.*, 2016). In another study, senescence-regulating miRNAs and their targets were identified by deep sequencing of small RNAs and cleaved targets. The majority of them were known to be involved in nutrient remobilization and cell structure integrity, indicating that miRNAs might play a role in catabolic recycling during leaf senescence (Thatcher *et al.*, 2015). By comparing the small RNA profiles of early and late senescence lines of both maize and rice, several new miRNA species in addition to well-known species were found to be differentially expressed between the early and late lines (Xu *et al.*, 2014; Wu *et al.*, 2016). Interestingly, the studies in rice and maize identified common miRNA species, including miR159, miR160, miR167, and miR172, indicating conserved functionality of these miRNAs in plant senescence progression. Forty-four small RNAs related to dark-induced plant senescence were similarly identified in an analysis of small RNA

data from dark-treated and control Arabidopsis (Huo *et al.*, 2015). Genome-wide data have provided valuable clues about the roles of small RNAs in plant senescence, but further genetic and molecular studies are needed to fully illustrate the network by which small RNAs regulate leaf senescence.

Concluding remarks

Wide-ranging biological processes are epigenetically regulated in plants. Plant senescence, whether age-dependent or stress-induced, involves DNA methylation, histone modification, small RNAs and nucleosome remodeling, underscoring how multiple layers of epigenetic mechanisms are integrated to modulate the genetic reprogramming that occurs during senescence (Fig. 1). In addition, the interplay between genetic and epigenetic regulation is also required, insofar as it ensures robust control of the senescence pathway. A good example is the regulation and function of *WRKY53*, which acts at the early stage of leaf senescence to promote the process (Miao *et al.*, 2004). At the onset of leaf senescence, WRKY53 levels are tightly linked to histone methylation, as its expression, active/repressive histone methylation status, and the leaf senescence phenotype are known to be strongly correlated (Ay *et al.*, 2009). Through its specific binding domain in target genes, active WRKY53 binds to the promoter regions of various SAGs to genetically regulate their expression (Miao *et al.*, 2004). Interestingly, WRKY53 may also act epigenetically in a complex with HDA9 to alter histone acetylation levels at these target loci (Chen *et al.*, 2016) (Fig. 1). Despite our limited understanding of how extensively genetic and epigenetic regulatory mechanisms are coordinated in leaf senescence, this question represents a major consideration for future studies.

The functional relevance of epigenetic regulation is well established, but many questions remain unanswered. For example, how broadly do individual epigenetic regulators and/or mechanisms apply, and how are they integrated with the genetic regulatory network to enhance regulatory complexity? Chronological transcriptome analysis has shown that small non-coding RNAs (ncRNAs) are differentially expressed during the leaf lifespan (Woo *et al.*, 2016), indicating that other diverse small RNAs besides known miRNAs may also regulate senescence. If so, what are their targets, and where are they positioned in the regulatory network? To identify small RNAs involved in leaf senescence along with their targets, age-dependent small RNAs and mRNAs need to be defined from large transcriptome datasets and correlated with

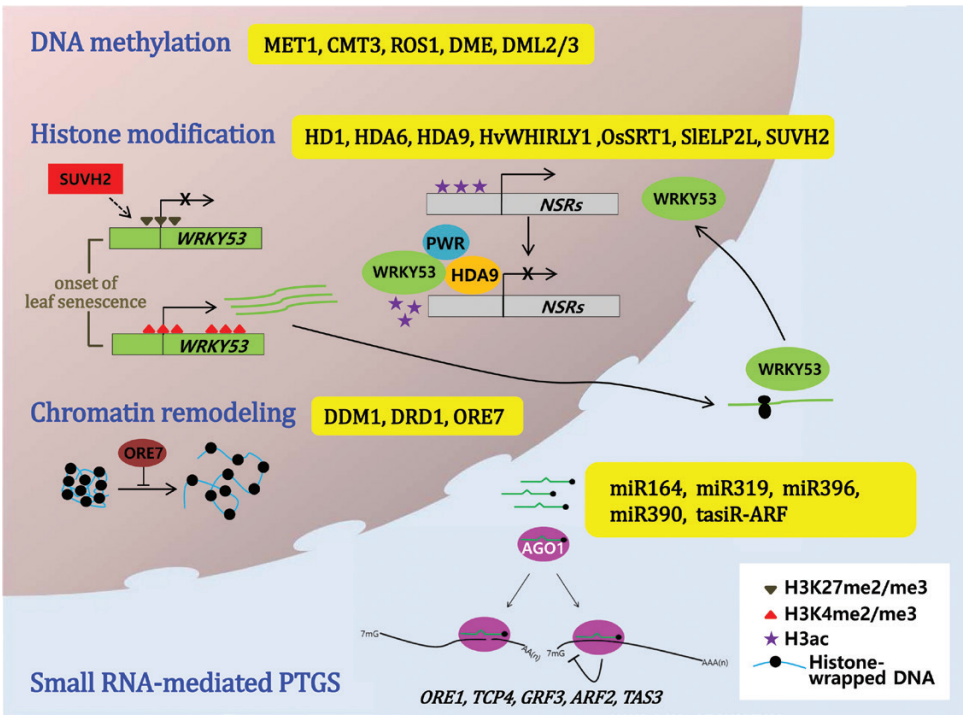

Fig. 1. Epigenetic and small RNA-mediated mechanisms and related factors regulating plant leaf senescence. Plant leaf senescence is regulated at multiple levels by epigenetic mechanisms and small RNA activity. First, DNA methylation levels change during leaf senescence; several DNA methylation factors (MET1, CMT3, ROS1, DME, DML2/3) may be involved in this process based on their altered expression levels, but the detailed mechanism is not yet known. Second, several histone modification enzymes have been implicated in leaf senescence regulation in Arabidopsis and crop plants. As an example of histone modification-mediated genetic reprogramming, the regulation and function of *WRKY53* are diagrammed. *WRKY53* transcription is balanced by repressive (H3K27me2/me3 regulated by SUVH2) and active (H3K4me2/me3) histone marks during leaf senescence progression. The functional WRKY protein recruits the HDA9 complex to negative senescence regulators (NSRs) to repress their expression by removing H3ac marks, thereby promoting leaf senescence. Third, chromatin structure affects leaf senescence. ORE7 negatively regulates leaf senescence by repressing chromatin decondensation. DDM1 and DRD1, members of the SWI/SNF family of chromatin remodelers, putatively function as positive regulators of leaf senescence, but the detailed mechanism is unknown. Lastly, small RNAs such as miRNAs and tasiRNAs guide the AGO1 complex to negatively regulate SAGs by mRNA cleavage or translational inhibition. PTGS, post-transcriptional gene silencing.

expression analysis, degradome data, or computational prediction. Moreover, are other types of regulatory ncRNAs involved in leaf senescence? Long ncRNA (lncRNA) has been characterized as a functional molecule with known roles in cellular senescence in animals (Montes and Lund, 2016). There are lncRNAs that are differentially expressed during the leaf lifespan (Woo *et al.*, 2016), suggesting that they might also have a role in leaf senescence. Senescence phenotypes could be assessed to determine the functional relevance of such lncRNAs in the leaf senescence pathway using overexpression or knockdown lines. The corresponding targets and functional mechanisms could be subsequently investigated based on the known regulatory mechanisms of lncRNAs in plants and animals or new strategies. Future studies will shed light on genetically and epigenetically coordinated complexity and help uncover whether and how certain regulatory mechanisms are particularly well suited for age-related development and reprogramming events.

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